

Supplementary materials for:

Verteporfin-induced proteotoxicity impairs cell homeostasis and survival in neuroblastoma subtypes independent of YAP/TAZ expression

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Supplementary Materials and Methods

Human Protein-kinase profile

The Proteome Profiler Human Phospho-Kinase Array Kit (ARY003B; R&D Systems Inc.) was used to investigate the protein kinase phosphorylation profiling of GI-ME-N cells treated for 24 h with 5 μ M VPF. The cells were lysed and prepared for analysis according to the manufacturer's protocol. A total of 400 μ g protein lysate were used per array set. The immunodetection of target proteins was done using the WesternBright Sirius kit (Advansta) and an ImageQuant LAS 400 mini reader (GE Healthcare). The signal intensity measurement for quantitative analysis was done using the ImageJ software version 1.50e.

Supplementary Table

Suppl. Table 1: Origin and characteristics of *MYCN*-non-amplified and *MYCN*-amplified metastatic neuroblastoma cell lines used in this study (*atcc.org*)

Cell line	Characteristics
GI-ME-N	▶ Origin: bone marrow metastasis of a 2 years old female ▶ 6 months chemotherapy (poor response, very aggressive)
SK-N-AS	▶ Origin: bone marrow metastasis biopsy – 6 years old female ▶ Histology: poorly differentiated embryonal neuroblastoma
SH-EP	▶ Subclone of SH-SY5Y cell line (enriched for the “S”-subtype cells) ▶ Origin: bone marrow metastasis biopsy – 4 years old female
SH-SY5Y	▶ thrice-cloned sub-line of SK-N-SH cell line ▶ Origin: bone marrow metastasis biopsy – 4 years old female ▶ Patient underwent repeated rounds of radiation and chemotherapy
BE(2)-M17	▶ a twice-sub-cloned cell line derived from the SK-N-BE(2) cell line ▶ Origin: bone marrow metastasis biopsy – 22 months old male ▶ Patient had repeated courses of chemotherapy and radiotherapy
IMR-32	▶ Origin: abdominal metastasis biopsy – 13 months old male ▶ Histology – neuroblastoma with rare areas of organoid differentiation

Suppl. Table 2: List of antibodies used in this study and the assay-specific applied concentrations

Antibody	Company	Catalogue #	WB	IF
Atg16L1	Cell Signaling Technology	8089	1000	100
BECLIN-1	Cell Signaling Technology	3495	1000	-
CDC42	Cell Signaling Technology	2466	1000	-
CDK2	Cell Signaling Technology	2546	1000	-
CDK4	Cell Signaling Technology	12790	1000	-
CDK6	Cell Signaling Technology	3136	2000	-
c-Jun	Cell Signaling Technology	9165	1000	-
Cleaved Caspase-3	Cell Signaling Technology	9664	1000	400
Cleaved PARP	Cell Signaling Technology	5625	1000	400
C-MYC	Cell Signaling Technology	13987	1000	500
COX IV	Cell Signaling Technology	4850	1000	-
Cyclin D1	Cell Signaling Technology	2978	1000	-
Cyclin D3	Cell Signaling Technology	2936	1000	-
DCX	Santa Cruz Biotechnology	sc-8066	500	200
FAK	Cell Signaling Technology	13009	1000	-
FOXD3	Cell Signaling Technology	2019	1000	-
GAPDH	Santa Cruz Biotechnology	sc-59540	5000	-

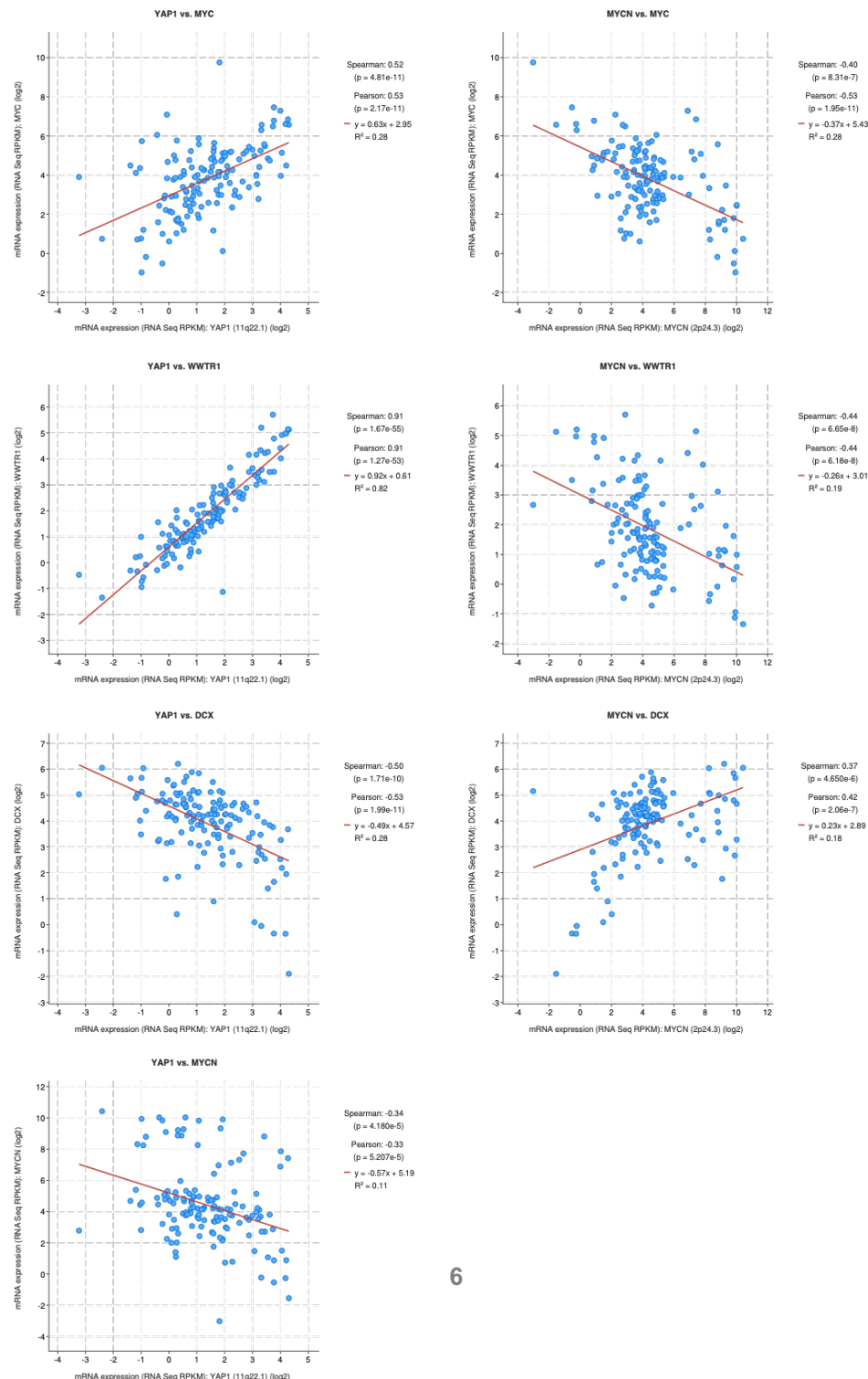
Antibody	Company	Catalogue #	WB	IF
GM130	Cell Signaling Technology	12480	1000	-
ILK1	Cell Signaling Technology	3856	1000	-
<u>Integrin α4</u>	Cell Signaling Technology	8440	1000	-
<u>Integrin β1</u>	Cell Signaling Technology	9699	1000	-
<u>Integrin β3</u>	Cell Signaling Technology	13166	1000	-
<u>Integrin β4</u>	Cell Signaling Technology	4707	1000	-
Ki67p	Leica	NCL-Ki67p	1000	1000
LC3A/B	Cell Signaling Technology	12741	1000	100
N-Cadherin	Cell Signaling Technology	13116	1000	-
NCAM	Santa Cruz Biotechnology	sc-106	200	200
NF2	Santa Cruz Biotechnology	sc-332	200	-
N-MYC	Santa Cruz Biotechnology	sc-53993	500	400
N-MYC	Cell Signaling Technology	51705	1000	-
p-Akt (Ser473)	Cell Signaling Technology	4060	500	-
Pan-TEAD	Cell Signaling Technology	13295	1000	-
PAX3	Cell Signaling Technology	12412	1000	-
P-c-Jun	Cell Signaling Technology	3270	1000	500
PERIPHERIN	Santa Cruz Biotechnology	sc-7604	200	-
p-ERK 1/2 (p44/42)	Cell Signaling Technology	4695	1000	-
pFAK (Y397)	Abcam	ab39967	600	150
P-HSP27	Cell Signaling Technology	9709	1000	200
P-p38 MAPK	Cell Signaling Technology	4511	500	-
p-p44/42	Cell Signaling Technology	4370	500	200
P-SAPK/JNK	Cell Signaling Technology	4668	1000	-
<u>Rac1/2/3</u>	Cell Signaling Technology	2465	1000	-
SOX10	Cell Signaling Technology	89356	1000	-
SOX9	Cell Signaling Technology	82630	1000	-
β -CATENIN	Cell Signaling Technology	8480	1000	100
STAT3	Cell Signaling Technology	9139	1000	-
TAZ	Cell Signaling Technology	70148	1000	-
TAZ	Cell Signaling Technology	4883	700	-
TH	Pel-Freez Biologicals	40101	500	-
TUJ1	BioLegend	PRb-435P	5000	1000
TUJ1	Covance	MMs-435P	1000	1000
VIMENTIN	BD Pharmingen™	550513	1000	500
YAP	Cell Signaling Technology	14074	1000	100
YAP/TAZ	Santa Cruz Biotechnology	sc-101199	200	100
CD44	eBiosciences	17-0441-82	-	1:50
CD29	eBiosciences	11-0299-42	-	1:50

Suppl. Table 3: Guide RNA sequences for CRISPR/Cas9 -mediated knock-out of YAP and TAZ

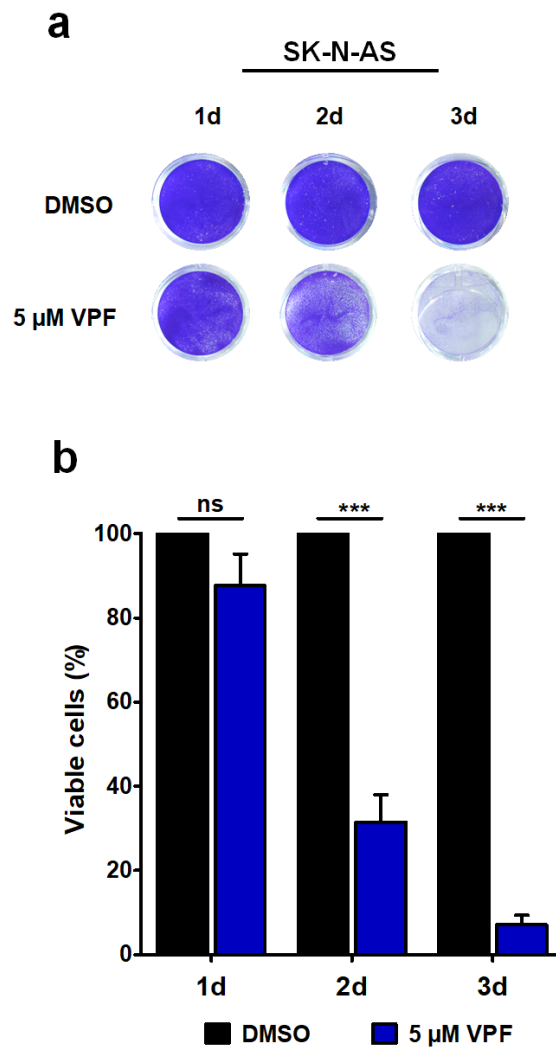
Target	Santa Cruz Biotechnology #	gRNAs sequence
YAP	sc-400040-NIC	gRNA1: GGCGACCCAGGCGGCGCCGC gRNA2: GCCGGTTGCCCCGGGTCCGGA
TAZ	sc-400320-NIC	gRNA1: TCCAGCACCGACTCGTCGGG gRNA2: CGCGAGTGCGAGCCCGAATC

Supplementary Figures

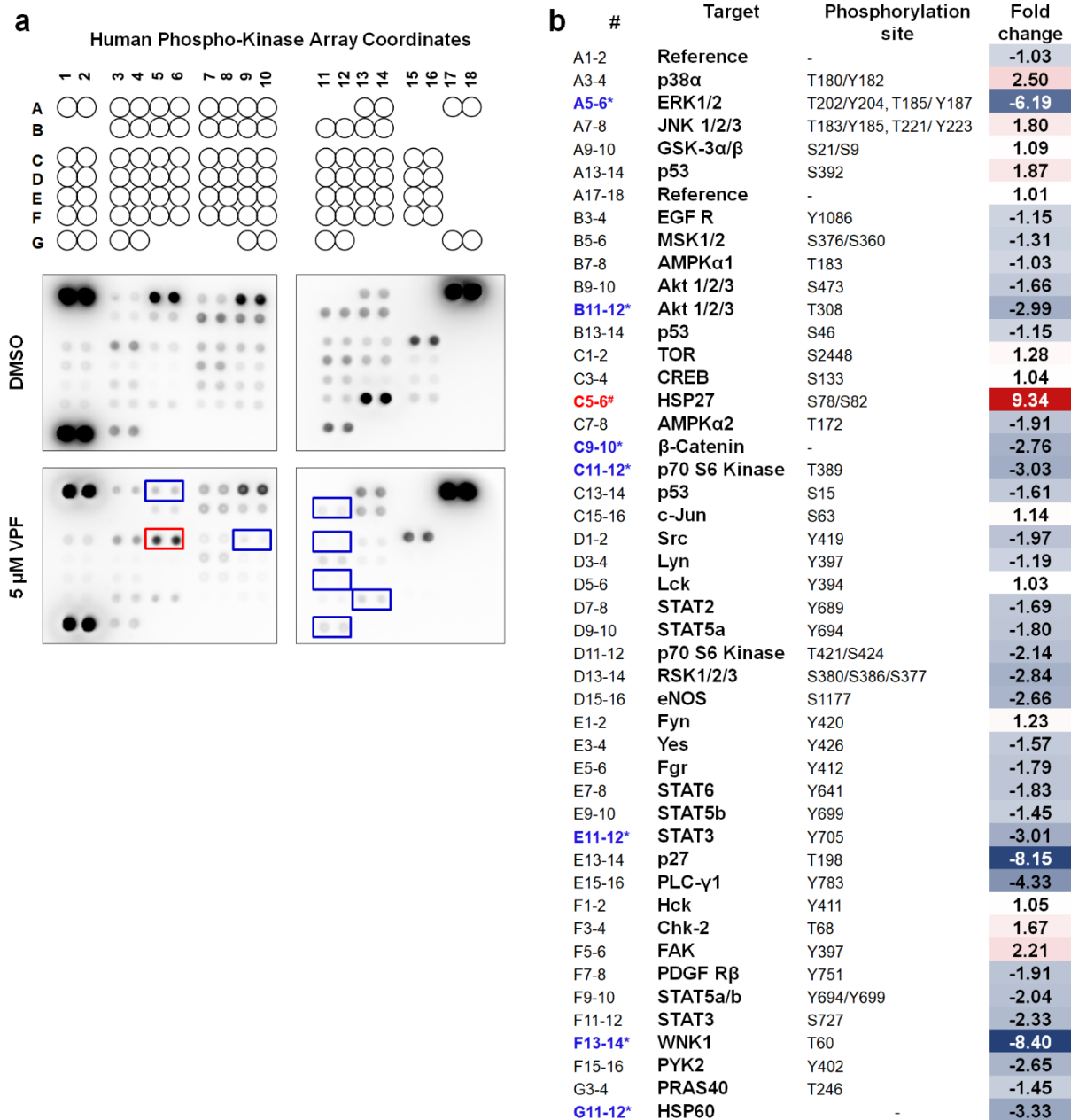
Supplementary Figure 1: *YAP1* expression positively correlates with *MYC* expression and negatively correlates with *MYCN* expression. Scatter plots illustrating the correlation between *YAP1*-*MYC*-*MYCN*-*WWTR1*-*DCX* gene expression in 143 bulk RNA sequenced NB patient samples. The results presented are in whole based upon data generated by the Therapeutically Applicable Research to Generate Effective Treatments (<https://ocg.cancer.gov/programs/target>) initiative, phs000467, available at <https://portal.gdc.cancer.gov/projects>. The figures were generated using the cbiportal.org platform.



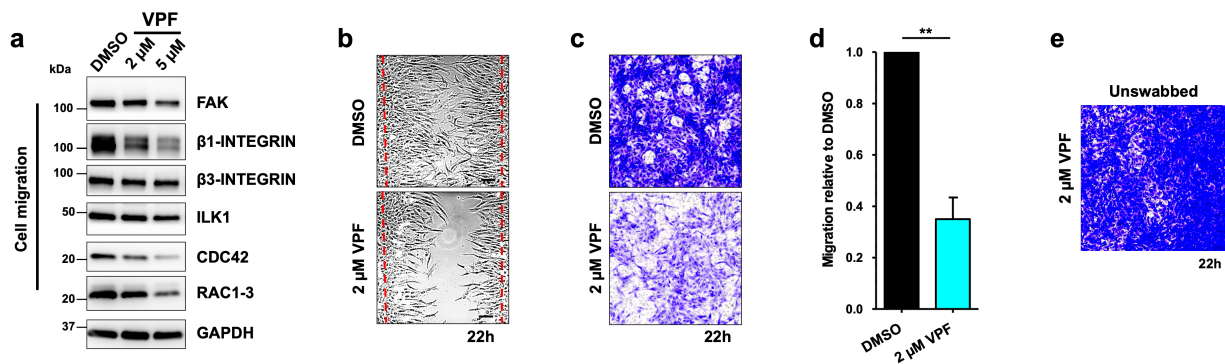
Supplementary Figure 2: Verteporfin efficiently targets YAP-positive SK-N-AS NB cells. (a) Representative images of crystal violet staining of viable SK-N-AS cells following 1 to 3 days of 5 μ M verteporfin (VPF) treatment and (b) the corresponding quantification of viable cells. Data is represented as average $\pm$ SEM (n=4), Two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test was used to assess the statistical significance (ns = not significant; ***p $\leq$ 0.001).



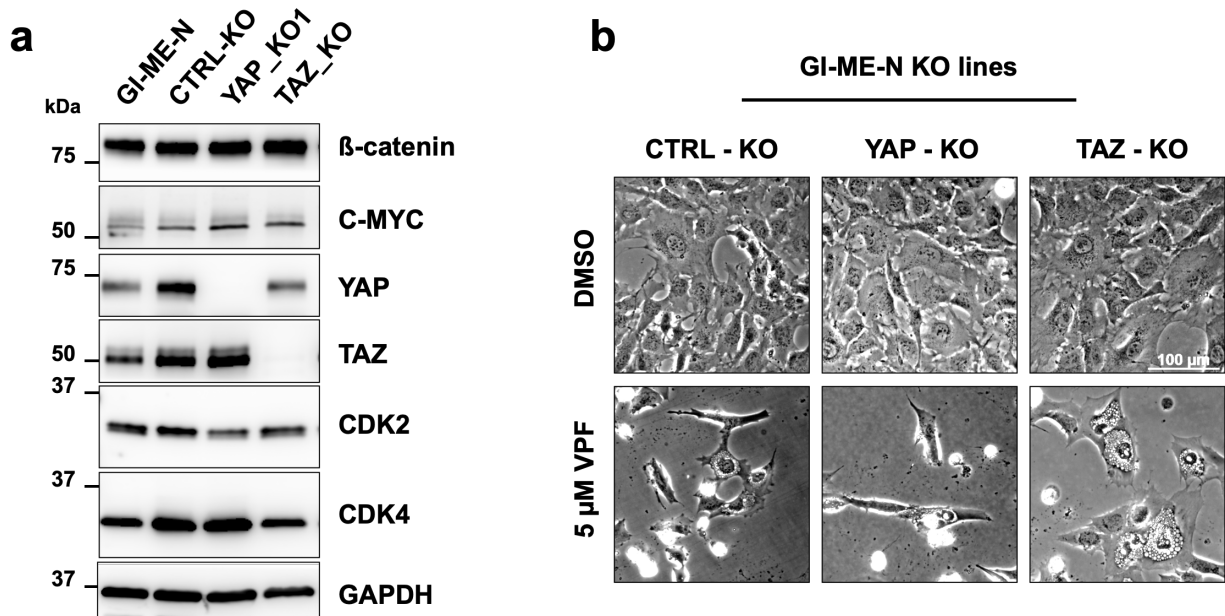
Supplementary Figure 3: Verteporfin impacts the phosphorylation profile of multiple kinases, particularly those regulating cell homeostasis and cell stress. (a) Representative blots of a Human Phospho-Kinase Array of GI-ME-N cells treated for 24 h with 5 μ M VPF or DMSO (vehicle control) highlighting noticeable changes in the phosphorylation profile of HSP27, pERK1/2, p27, WNK1, PLC- γ 1, HSP60, AKT, STAT members, p70 (n=2). **(b)** Table showing the fold change in the phosphorylation profile of indicated targets following 24h treatment of GI-ME-N cells with DMSO or 5 μ M VPF. * = blue rectangular, # = red rectangular



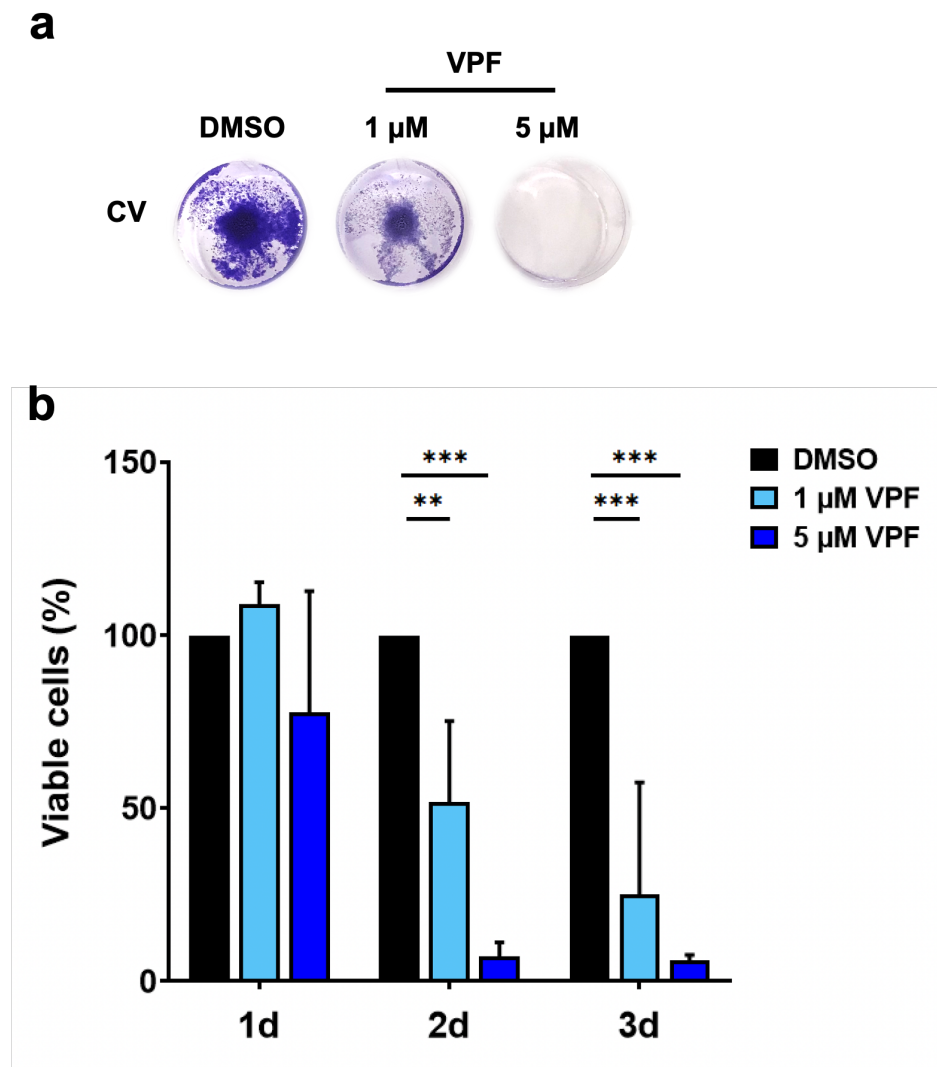
Supplementary Figure 4: Verteporfin impairs cell migration in GI-ME-N cells. (a) Western blot analysis of GI-ME-N cells treated for 24h with either 2 or 5 μ M VPF shows downregulation of pro-migratory factors FAK, β 1-integrin, CDC42, RAC1-3, compared to the DMSO control. Representative phase contrast images of an in vitro scratch assay (b) and a transwell migration assay (c) at 22h post 2 μ M VPF treatment of GI-ME-N cells. Both migration assays show a reduction in the migratory potential of GI-ME-N cells upon treatment with VPF. (d) Quantification of migrating GI-ME-N cell treated with 2 μ M VPF, assessed by transwell migration assay and quantified using crystal violet staining. Bars represent average $\pm$ SEM (n = 4), statistical significance was determined using a one sample t-test: **p $\leq$ 0.01. Scale bar: 100 μ m. (e) Bright-field image of GI-ME-N cells remaining on the upper part of an unswabbed transwell membrane at 22h following 2 μ M VPF treatment, confirming that the observed reduction in migration is not caused by impairment in cell survival.



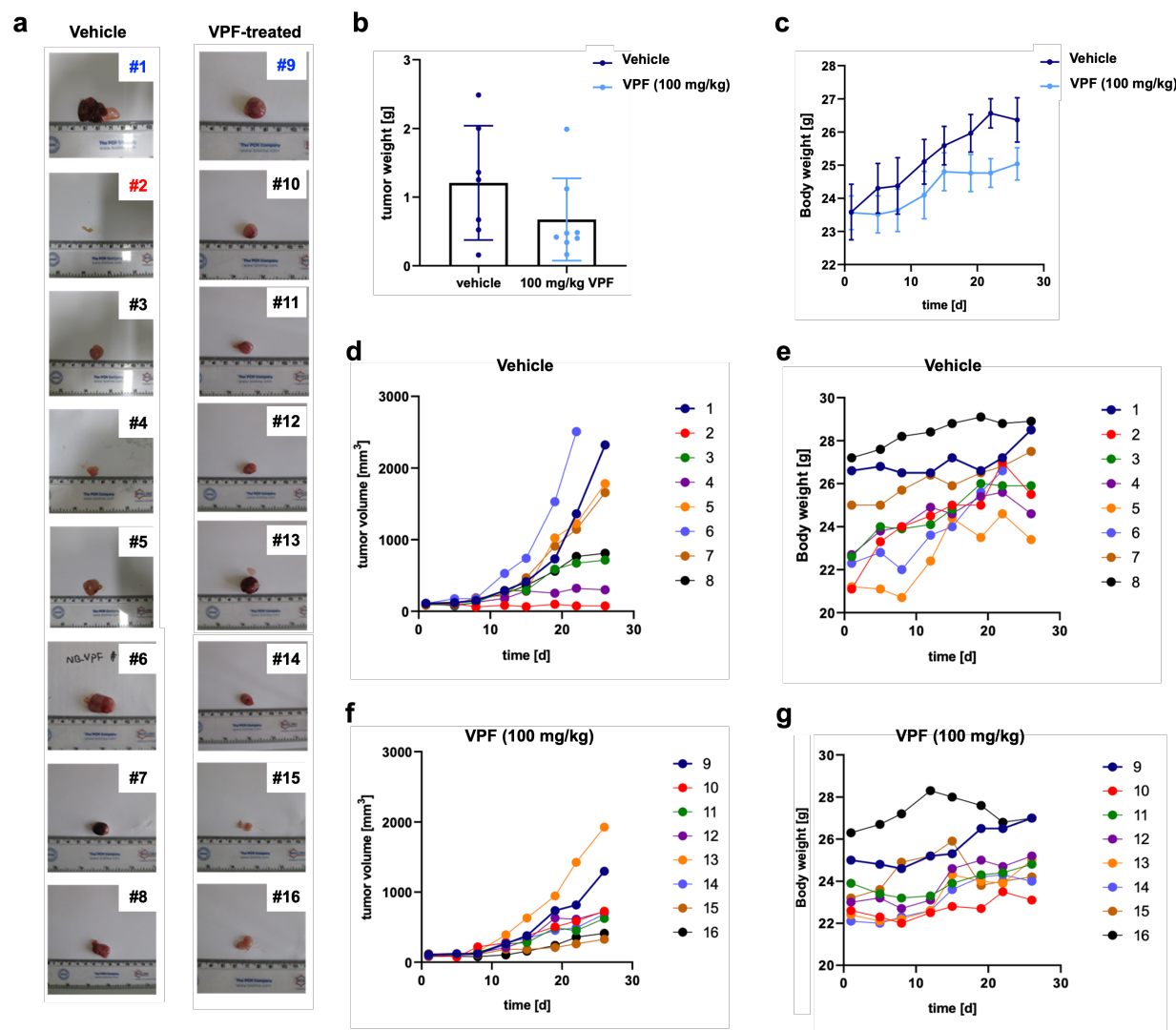
Supplementary Figure 5: YAP/TAZ expression is not required for verteporfin-induced cell death in neuroblastoma cells. (a) Immunoblot characterization of CRIPSR/Cas9 nickase-generated YAP and TAZ knock-out (KO) GI-ME-N NB lines. (b) Phase-contrast images of YAP, TAZ and control (CTRL) GI-ME-N KO lines treated for 48h with 5 μ M VPF illustrating no change in GI-ME-N cell sensitivity even after KO of YAP or TAZ. Scale bar: 100 μ m.



Supplementary Figure 6: Verteporfin efficiently targets YAP-negative IMR-32 neuroblastoma cells. (a) Representative images of crystal violet staining of viable IMR-32 cells following 1 to 3 days with 1 μ M or 5 μ M verteporfin (VPF) treatment and (b) the corresponding quantification of viable cells. Data is represented as average $\pm$ SEM (n = 4). Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used to assess the statistical significance (**p $\leq$ 0.01; ***p $\leq$ 0.001).



Supplementary Figure 7: Verteporfin reduces SH-SY5Y *in vivo* tumor growth. (a) Images of tumors isolated from vehicle control vs VPF-treated mice. In blue are marked images included in main Fig. 6, in red is marked xenograft #2 from the vehicle group that failed to engraft, therefore was excluded from the comparative statistical analysis. Graphical representation of tumor weight (b) and body weight (c) in control - vehicle treated group (dark blue) and 100 mg/kg VPF-treated group (light blue). Data represented as average $\pm$ SEM (n = 7* control group, n = 8 VPF-treated). Individual measurements of tumor volume and bodyweight of vehicle-treated mice (d, e) or 100 mg/kg VPF-treated mice (f, g), respectively. Measurements were taken twice per week.



Supplementary Figure 8: Uncropped western blot images. Black rectangular shapes are indicative of the area included in the main and supplementary figures.

Figure 1 western blot images

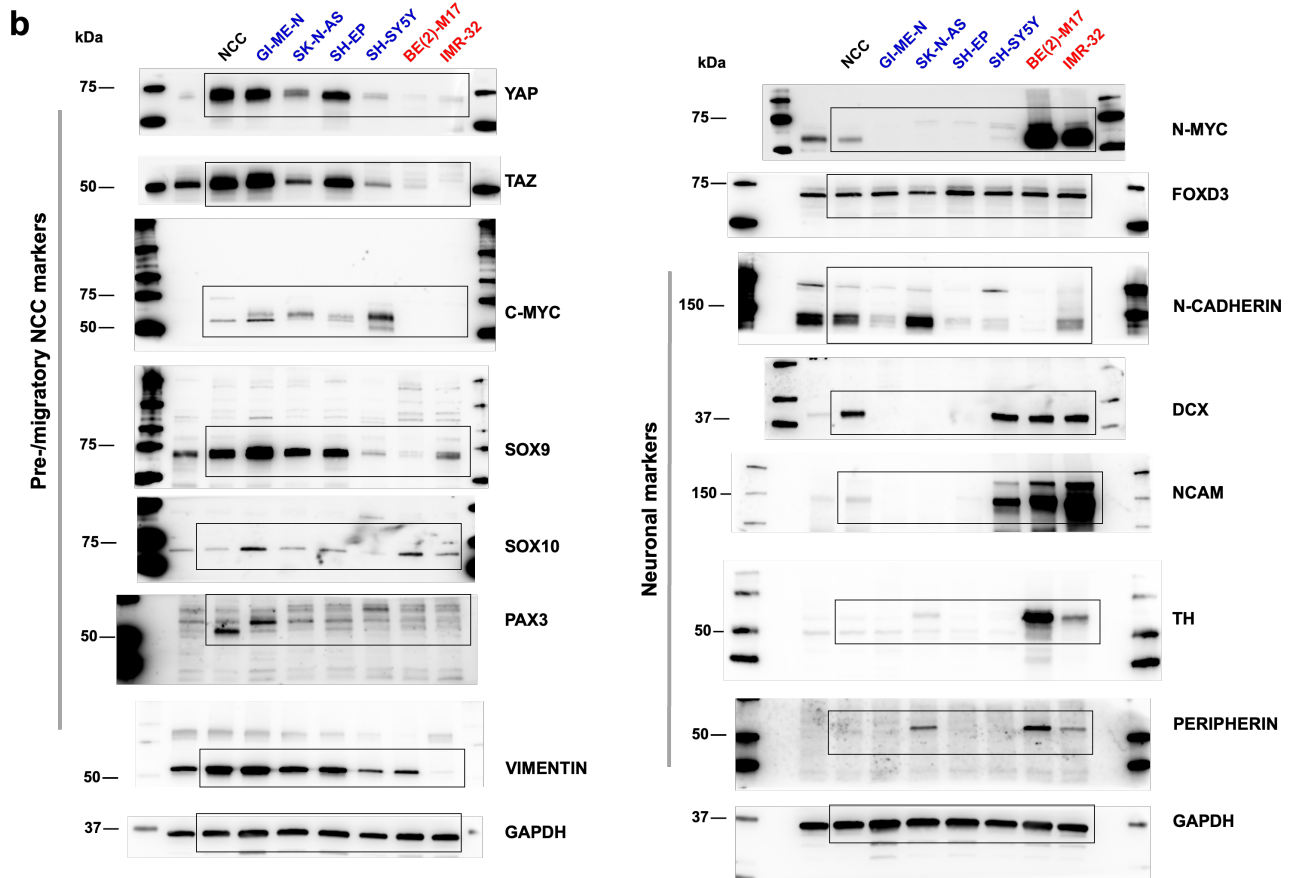


Figure 3 western blot images

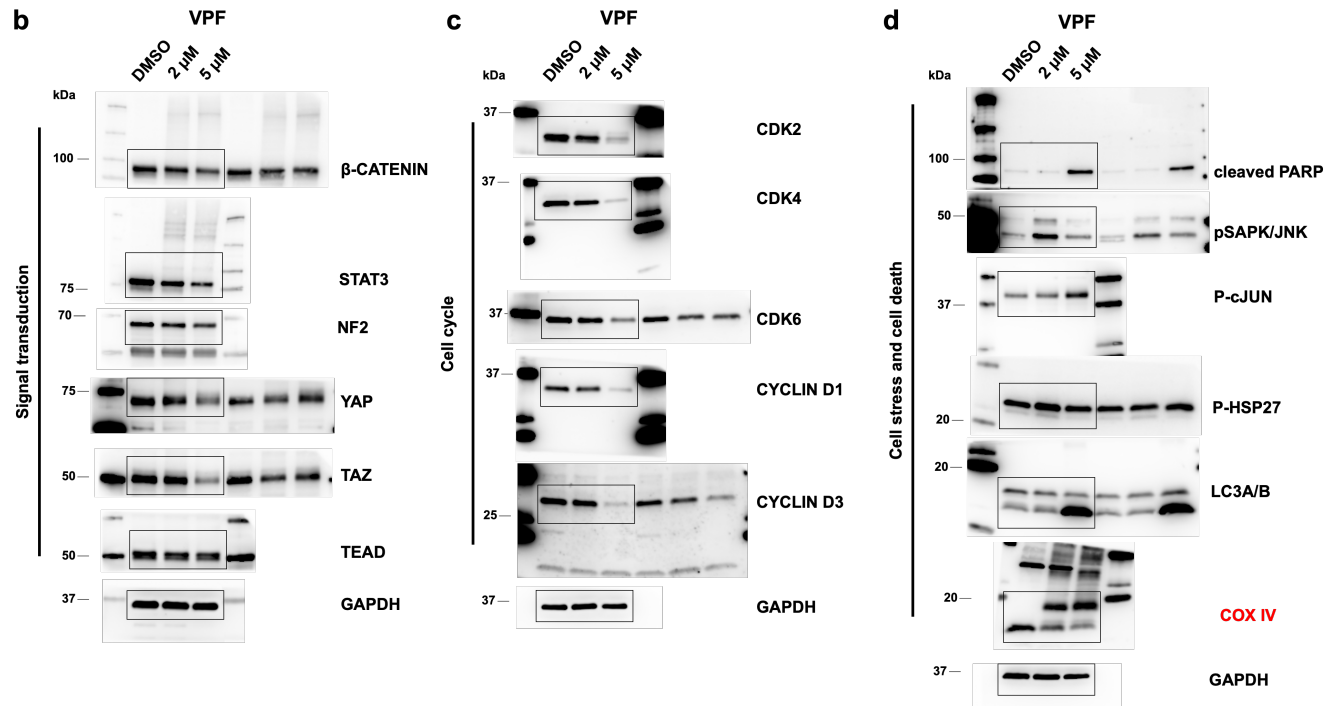


Figure 4 western blot images

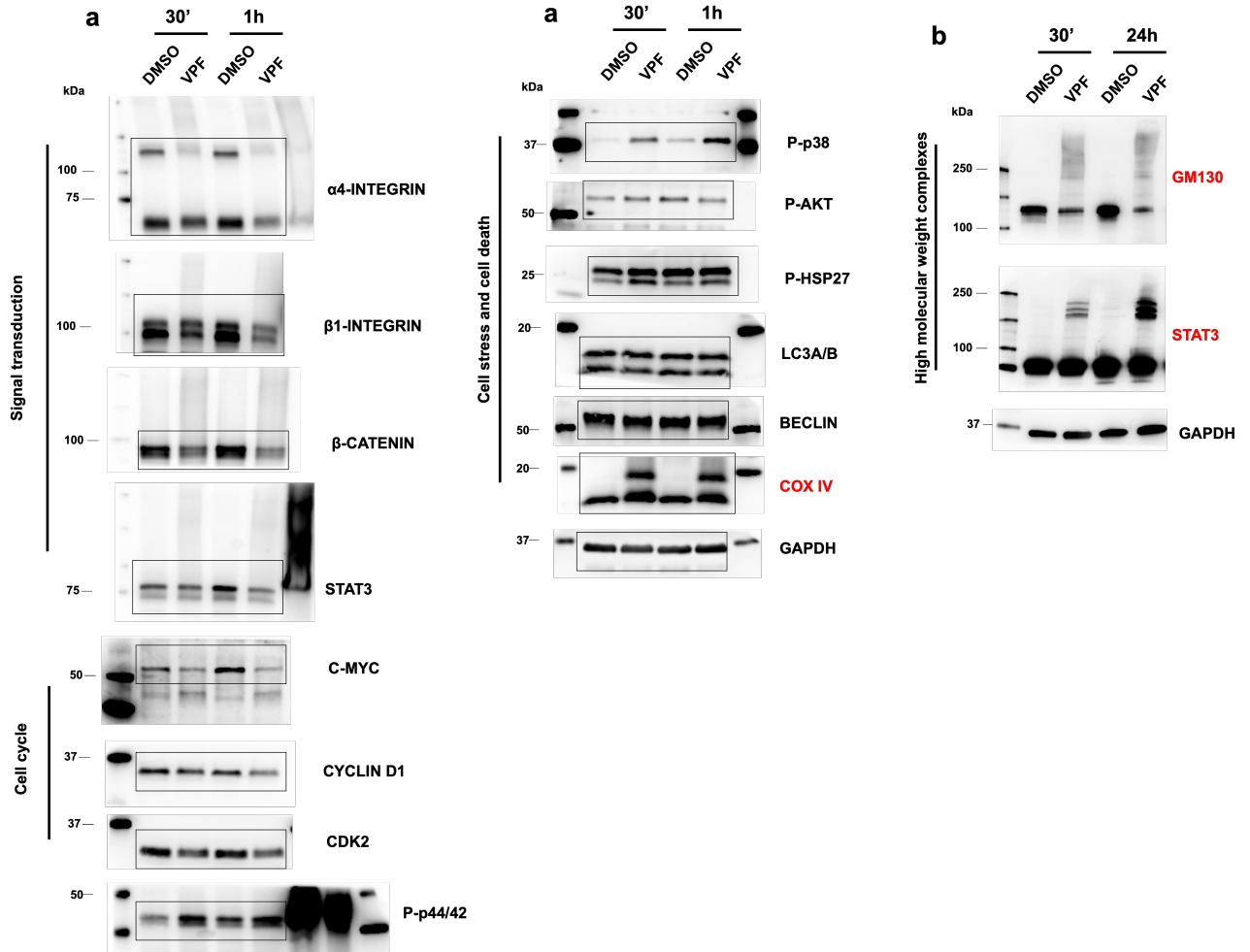
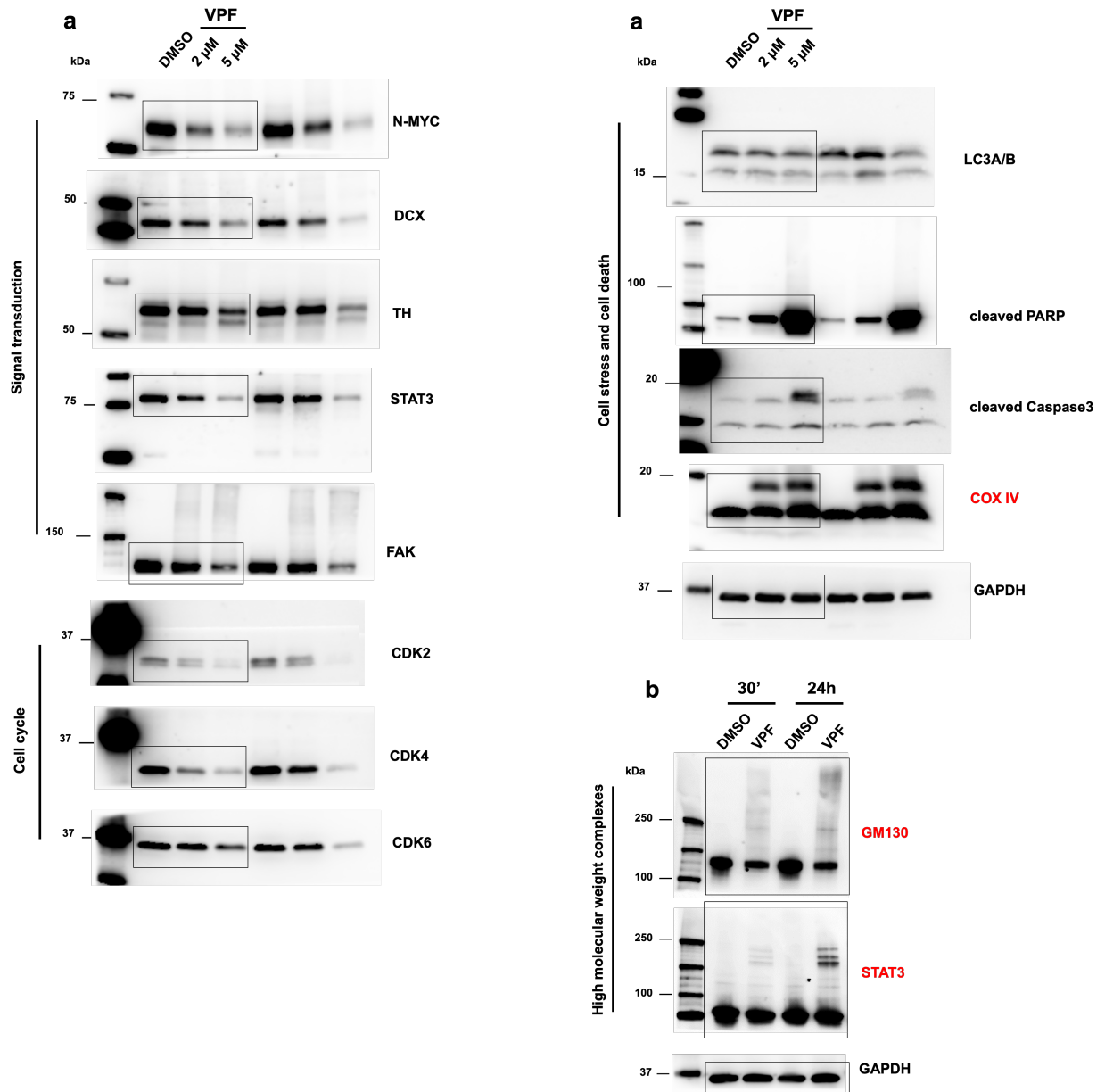
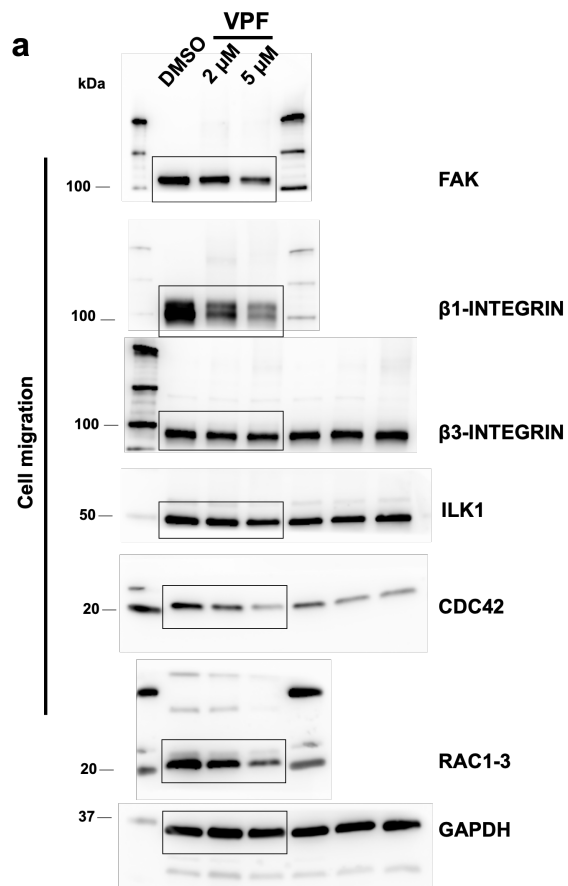


Figure 5 western blot images



Supplementary Figure 4 western blot images



Supplementary Figure 5 western blot images

