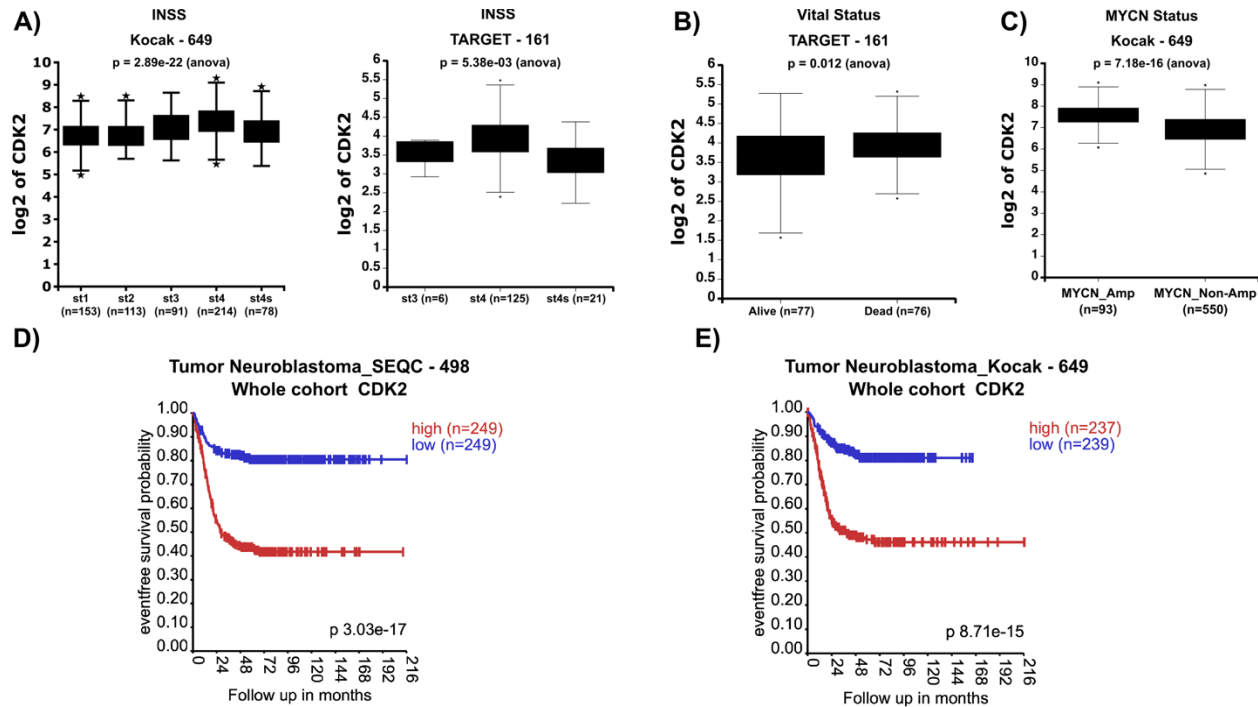


Supplementary Table S2: sequences of qPCR primers used in this study.

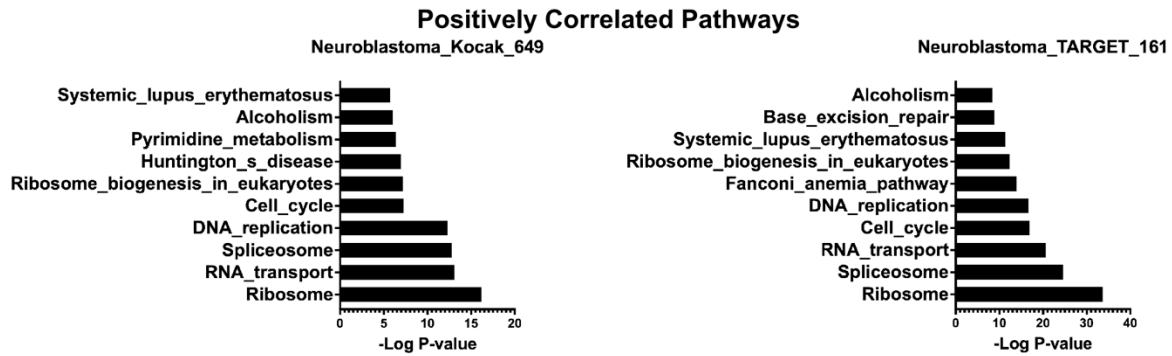
Primer	Sequence 5'-3'
GAP43-For	GAGCAGCCAAGCTGAAGAGAAC
GAP43-Rev	GCCATTTCTTAGAGTTCAGGCATG
MEGF8-For	CGGAAAATGGCTTCAACCAGCAG
MEGF8-Rev	CTCGTGGTAGACAGCAGAGTGA
SLIT2-For	CAGAGCTTCAGCAACATGACCC
SLIT2-Rev	GAAAGCACCTTCAGGCACAACAG
STMN2-For	CCAGAAGAAACTGGAGGCTGCA
STMN2-Rev	GCTTTTCCTCCGCCATCTTGCT
SHANK3-For	AGGATCACACCCGCCGAGATTA
SHANK3-Rev	CTACAGACTTGGTCCGTGGAATC
CDK2-For	ATGGATGCCTCTGCTCTCACTG
CDK2-Rev	CCCGATGAGAATGGCAGAAAGC
MYCN-For	ACCACAAGGCCCTCAGTACCTC
MYCN-Rev	TGACAGCCTTGGTGTTGGAGGA
GAPDH-For	TCGGAGTCAACGGATTTGGT
GAPDH-Rev	TGAAGGGGTCATTGATGGCA

Supplementary Figures

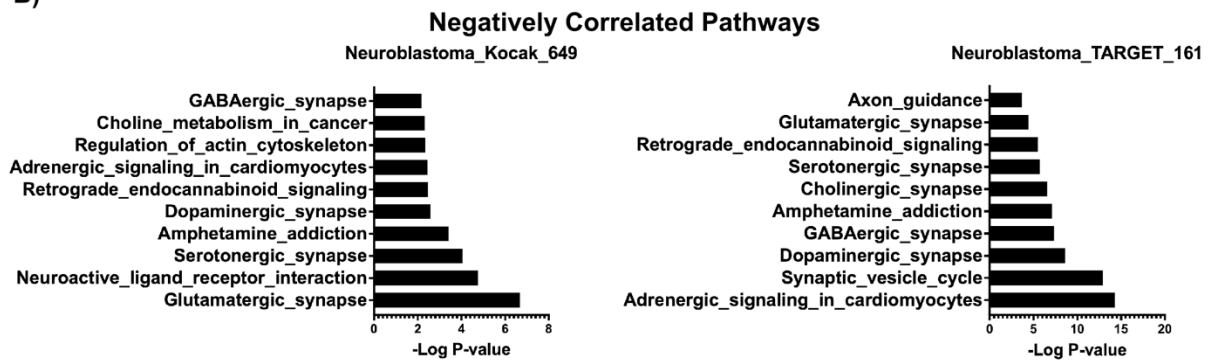


Supplementary Figure 1. High CDK2 expression levels correlate with high-risk neuroblastoma, MYCN-amplification, and poor prognosis. A) Log2 of CDK2 mRNA levels in different NB stages as defined by The International Neuroblastoma Staging System (INSS) in the Kocak-649 and TARGET-161 cohort studies. B) Log2 of CDK2 mRNA levels in NB tumors of alive and dead patients in the TARGET-161 cohort. C) Log2 of CDK2 mRNA levels in the *MYCN*-amplified vs. *MYCN*-non-amplified NB patient subgroups in the Kocak-649 cohort. (D-E) Kaplan–Meier-analysis of event-free survival datasets based on CDK2 expression of NB patients in the SEQC-498 (D) and KOKAK-649 (E). Statistical significance was calculated using R2-Genomics built-in one-way ANOVA for correlation of CDK2 mRNA levels with clinical parameters, and Bonferroni correction of raw *p*-values for survival analysis.

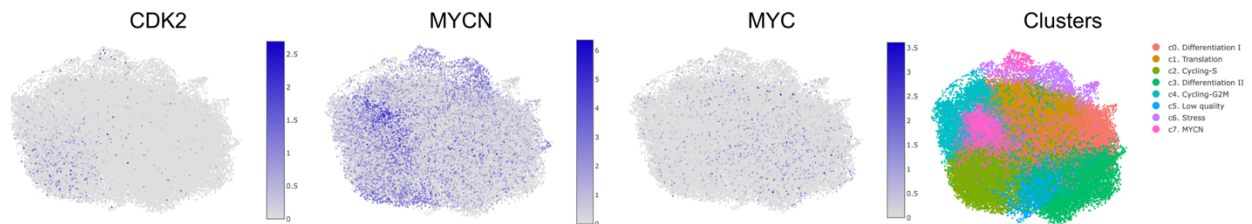
A)



B)

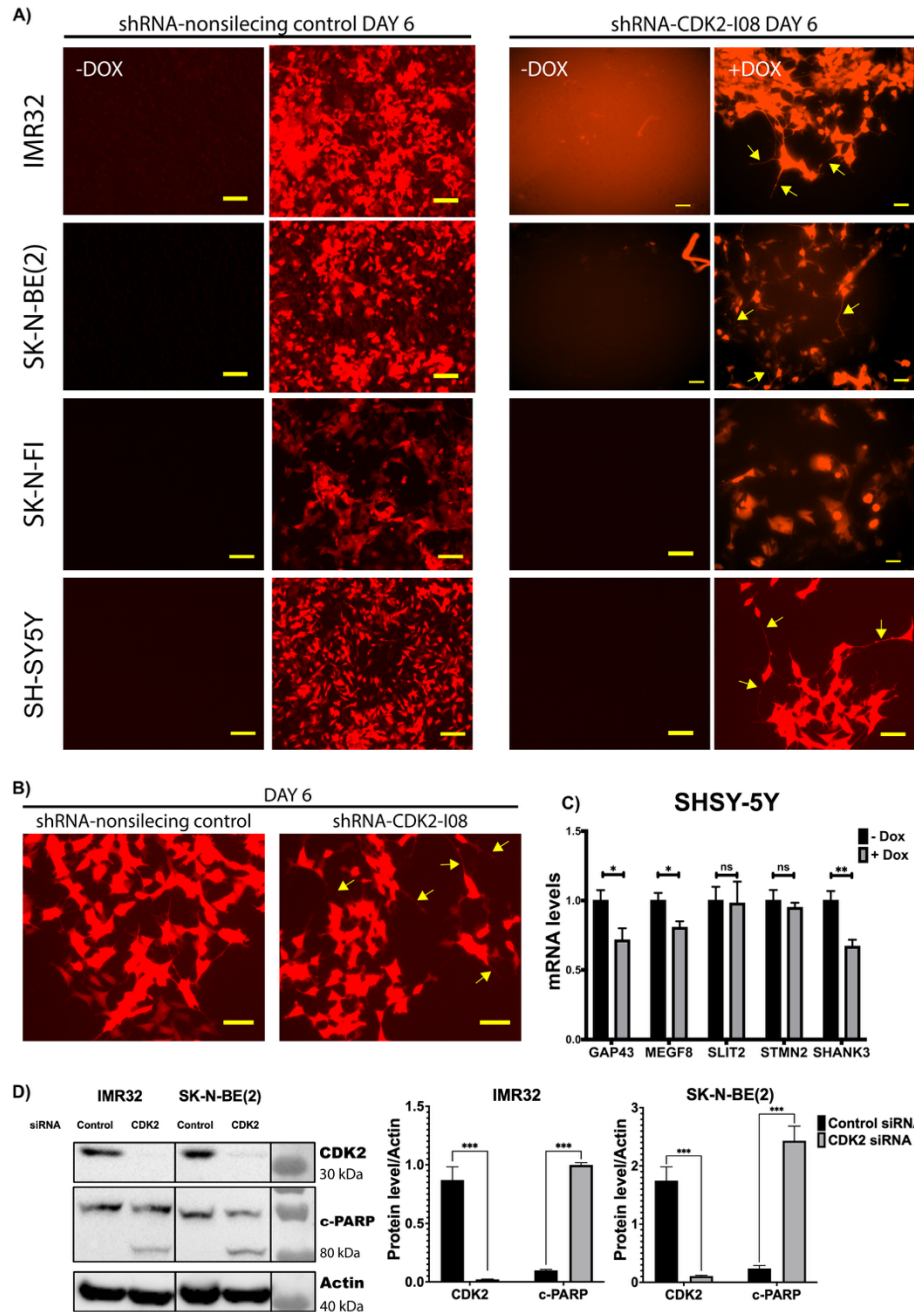


C)



Supplementary Figure 2. CDK2 expression correlates with undifferentiated phenotype in NB. A- B) Kyoto Encyclopedia of Genes and Genomes (KEGG) of genes whose expression positively (A) and negatively (B) correlated with CDK2 in the KocakK-649 and TARGET-161 NB cohort studies. Statistical significance was calculated using R2-Genomics built-in one-way ANOVA. C) Analysis of CDK2, MYCN, and MYC expression level in single cell RNA-seq data available at NBAtlas: A harmonized single-cell transcriptomic reference atlas of human neuroblastoma (<https://single->

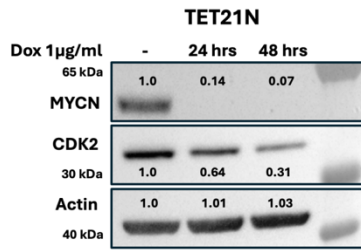
cell.be/nbatlas/) in the NB tumor subset. The last panel represent NB tumor cell clusters as defined by gene expression signature.



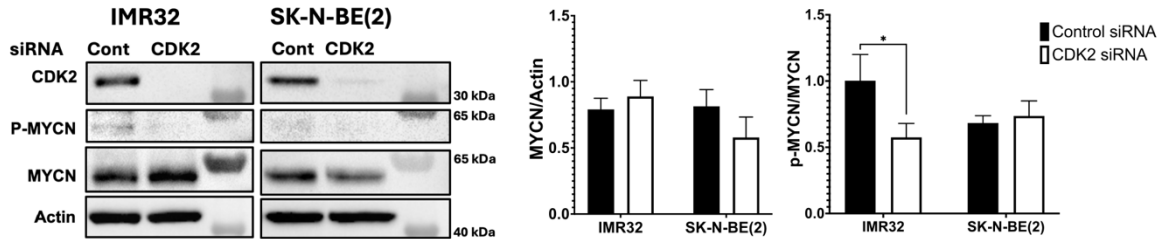
Supplementary Figure 3. CDK2-KD induces neuronal differentiation in NB cell lines. A) monitoring RFP signal and neurite outgrowth in response to 6-day treatment with 1 mg/ml doxycycline in IMR32, SK-N-BE(2), SK-N-FI, and SH-SY5Y cell lines expressing shRNA-control or shRNA-CDK2-I08. B) RFP and cell morphology of SH-SY5Y cell line expressing shRNA-control or shRNA-CDK2-I08 following 6-day treatment with 1 mg/ml doxycycline. Scale

bars represent 50 μ m. Arrow heads indicate neurite outgrowth. C) qRT-qPCR analysis of neuronal differentiation markers in shRNA-CDK2-I08 expressing SH-SY5Y cell line treated with 1 mg/ml doxycycline for 6 days. GAPDH was used as a housekeeping gene for qRT-qPCR. Mock H₂O treatment was used as control. D) Western blot analysis and quantification of CDK2 and cleaved-PARP in IMR32 and SK-N-BE(2) cell lines transfected with control-or CDK2-siRNA for 72 hours. Error bars represent the standard deviation of three independent biological experiments. p-value was calculated using two-tailed, unpaired Student t-test in GraphPad prism. * p-value $\leq$ 0.05; ** p-value $\leq$ 0.01; *** p-value $\leq$ 0.001. Actin was used as loading control for western blots.

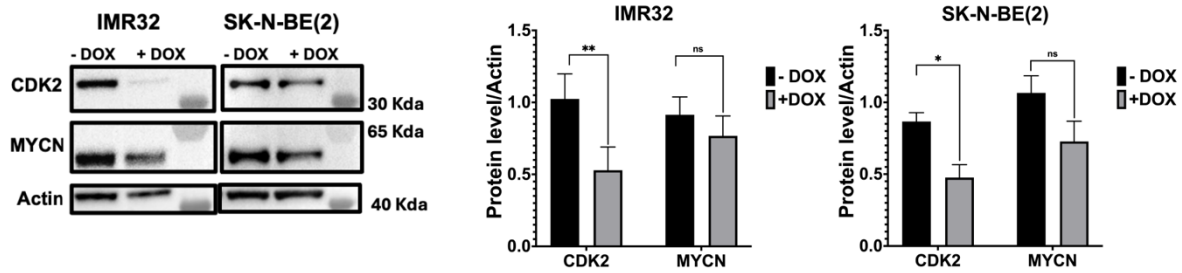
A)



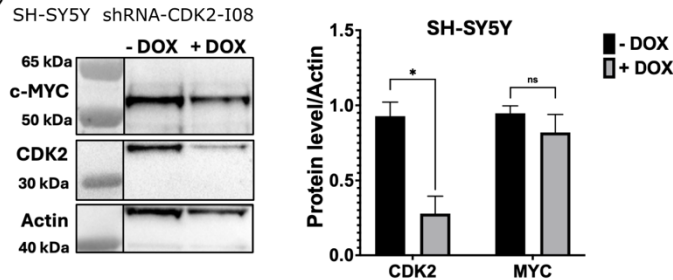
B)



C)

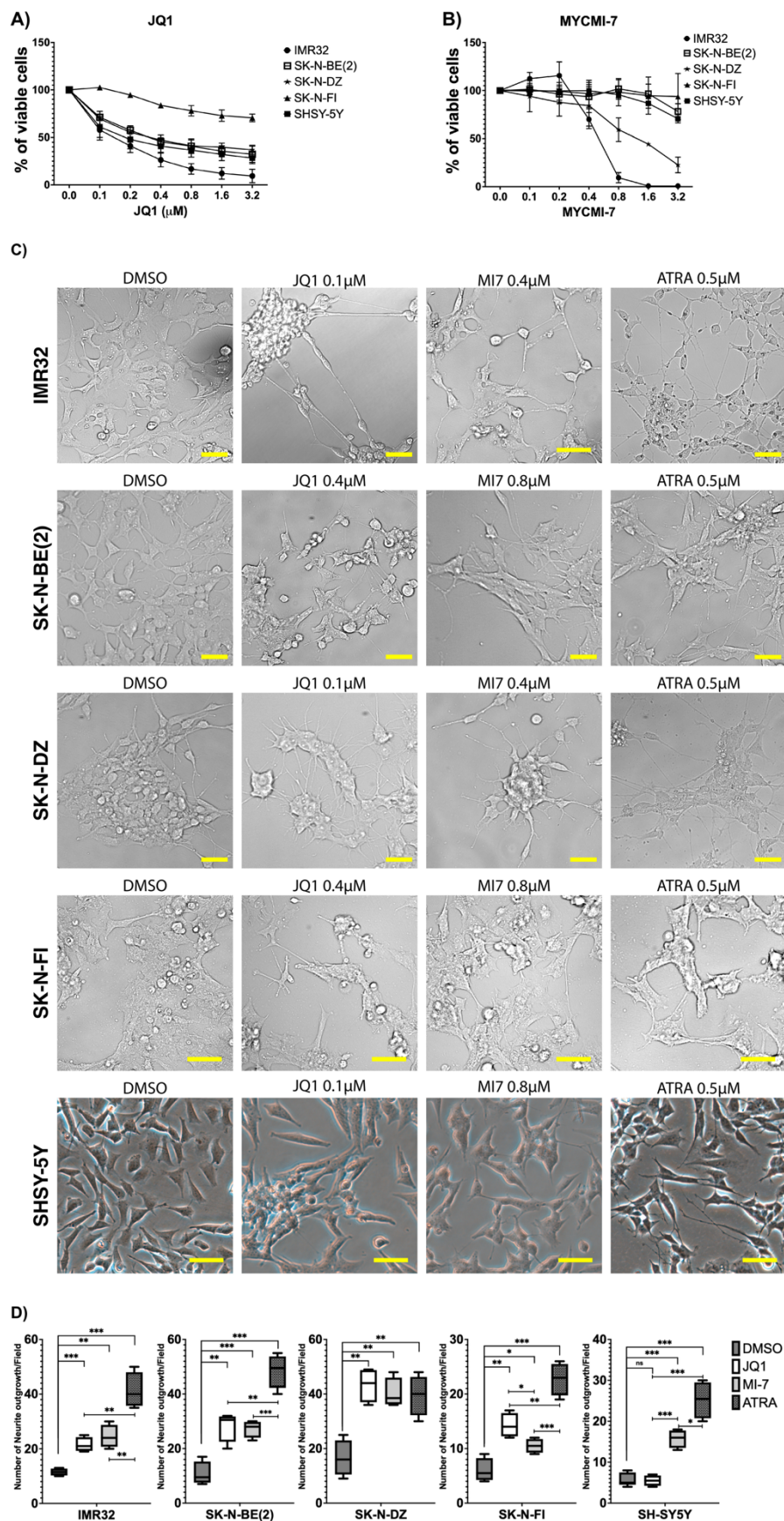


D)

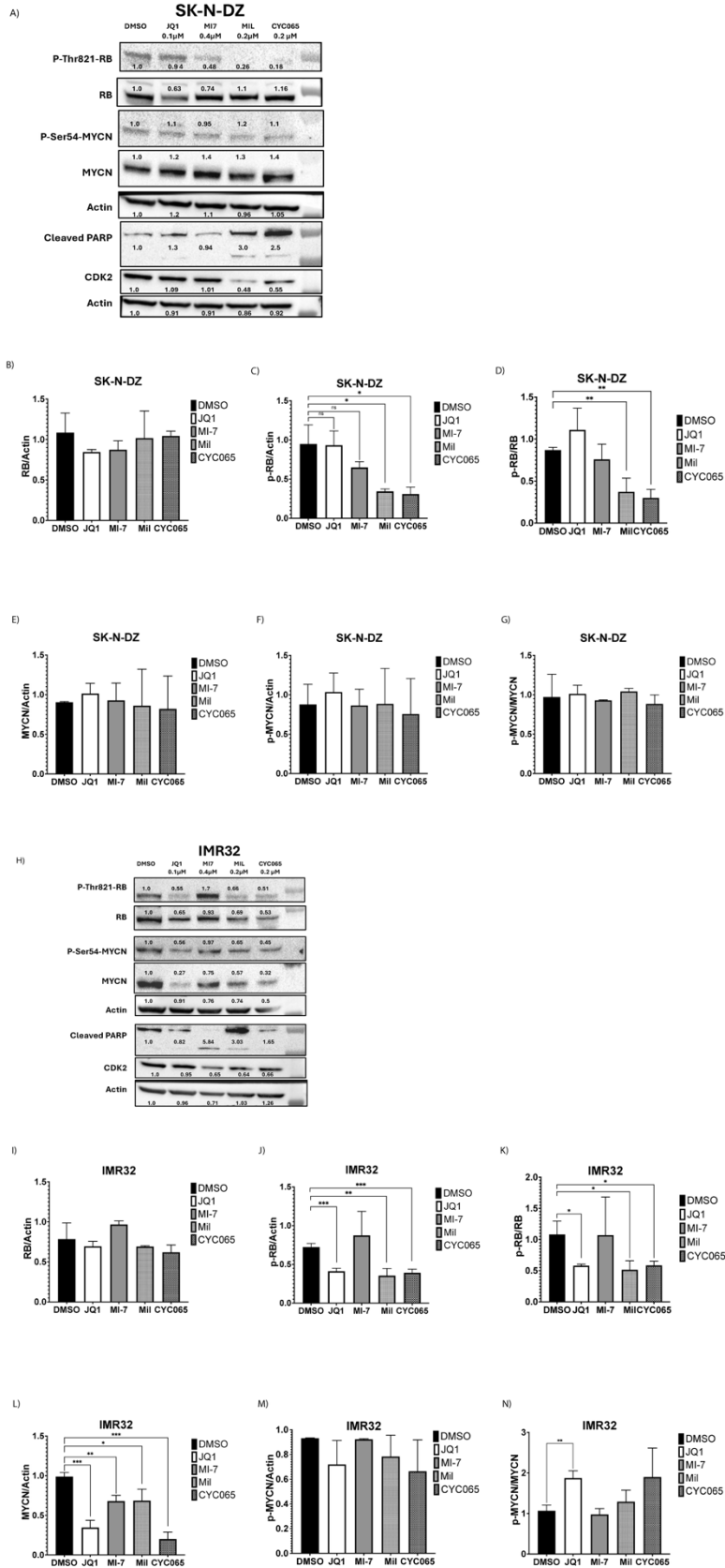


Supplementary Figure 4. CDK2 is a MYCN target gene in neuroblastoma and modulates the MYC pathway. A) Western blot analysis and quantification of CDK2 and MYCN protein levels in TET21N cell line in response to 24 and 48 hrs 1 mg/ml doxycycline treatment. B) Analysis of CDK2, MYCN, MYCN Ser54-phosphorylation levels in IMR32 and SK-N-BE(2) cell lines following 48 hrs transfection with control- or CDK2-siRNA. The bars measure total MYCN/Actin

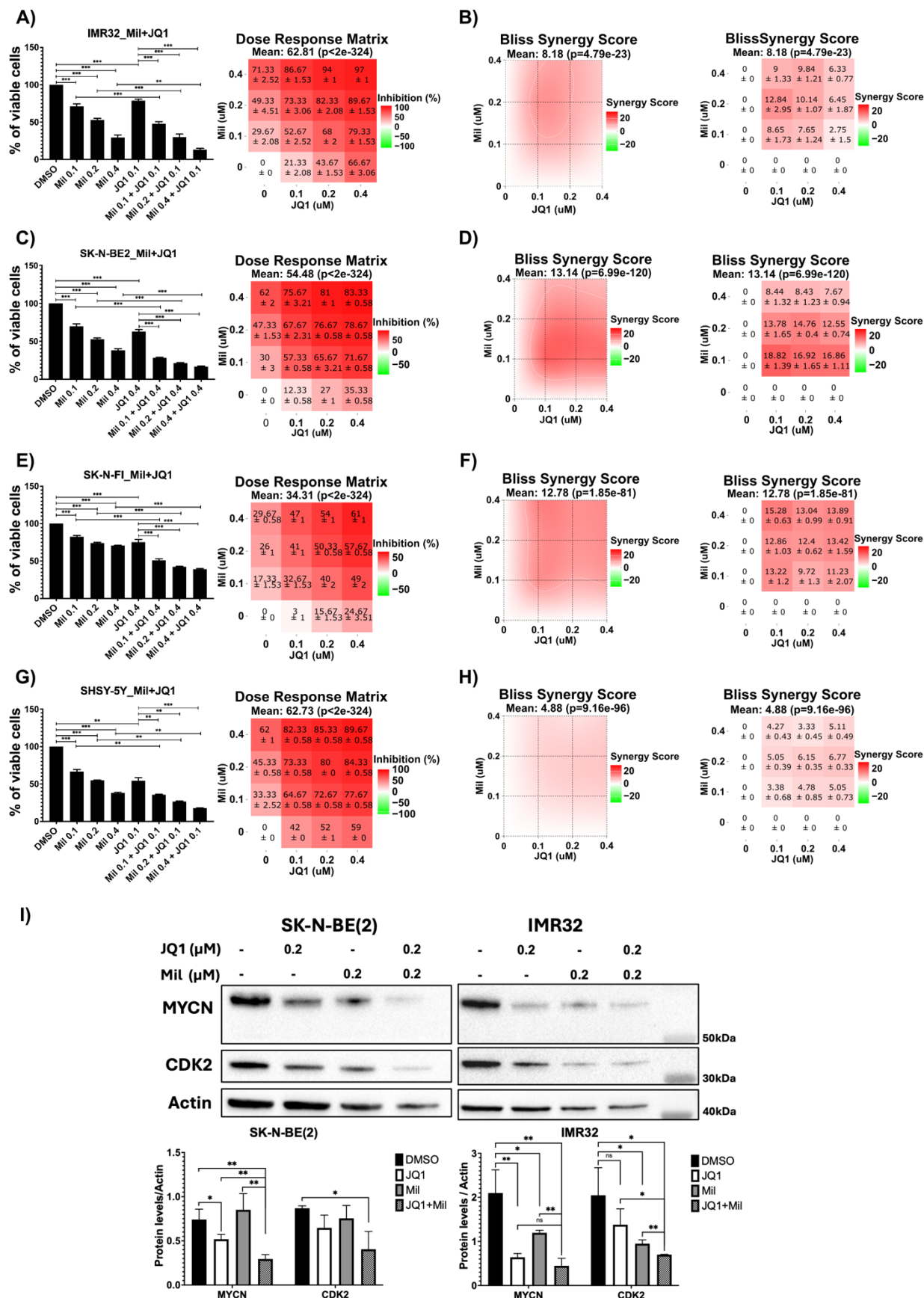
levels and the ratio of MYCN phosphorylation at Ser54/MYCN. Error bars represent the standard deviation of at least three independent biological experiments. C) Western blot analysis and quantification of CDK2 and MYCN protein levels in the *MYCN*-amplified cell lines IMR32 and SK-N-BE(2) stably transduced with CDK2 shRNA-I08 treated with H₂O or 1 mg/ml doxycycline for 3 days. D) Western blot analysis and quantification of CDK2 and MYCN protein levels in the *MYCN*-non-amplified cell line SH-SY5Y stably transduced with CDK2 shRNA-I08 treated with H₂O or 1 mg/ml doxycycline for 3 days. Error bars represent the standard deviation of three independent biological experiments. p-value was calculated using two-tailed, unpaired Student t-test in GraphPad prism. * p-value ≤ 0.05 ; ** p-value ≤ 0.01 ; *** p-value ≤ 0.001 . Actin was used as loading control for western blots.



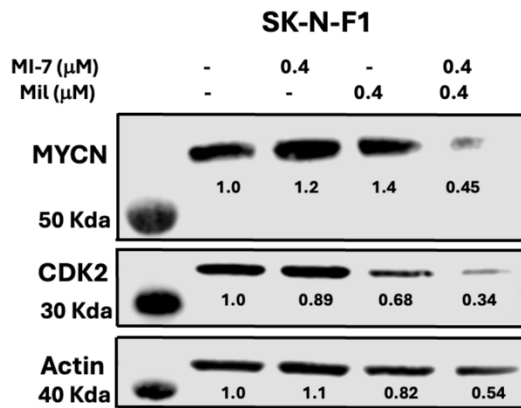
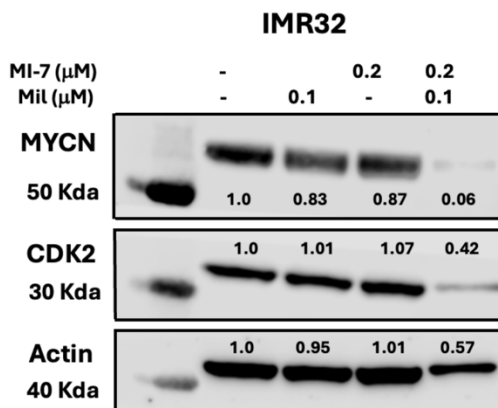
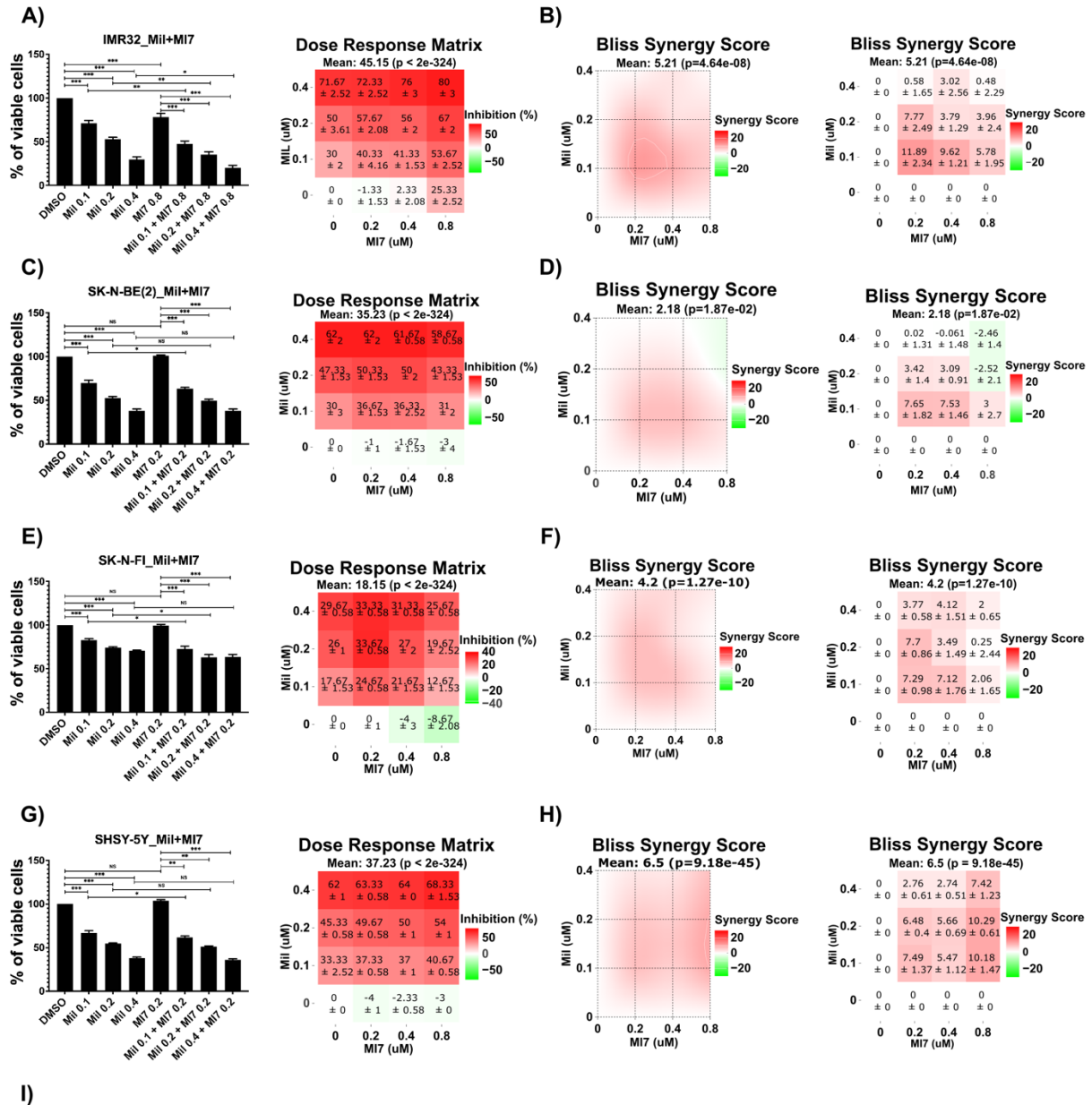
Supplementary Figure 5. MYC/N inhibition induces neuronal differentiation in NB cell lines. A- B) Cell viability analysis of human NB cell lines used in this study in response to 72 hours treatment with range of JQ1 (A) and MYCMI-7 (B) concentrations. DMSO was used as control treatment. Error bars represent the standard deviation of three independent biological experiments. C) Bright field images of IMR32, SK-N-BE(2), SK-N-DZ, SK-N-FI and SH-SY5Y cell lines following 6-day treatment with DMSO and indicated concentrations of JQ1, MYCMI-7, and ATRA. Please note that DMSO and ATRA images are the same used in Figure 4D since all treatments and analyses belonged to the same experiment. Scale bars represent 50 μ m. D) Quantification of neurite outgrowth in all cell lines as indicated in Figure S4D, which was manually counted using ImageJ software. Bars represent the median number of neurite extensions per field of three independent biological experiments. The p-value was calculated using two-tailed, unpaired Student t-test in GraphPad Prism. * p-value $\leq$ 0.05; ** p-value $\leq$ 0.01; *** p-value $\leq$ 0.001.



Supplementary Figure 6. Western blot analysis and quantification of phosphorylated-Thr821-RB, total RB, phosphorylated-Ser54-MYCN, total MYCN, cleaved PARP, and CDK2 protein levels in SK-N-DZ (A) and IMR32 (H) cell lines following 72 hours treatment with indicated concentrations of JQ1, MYCMI-7, Milciclib, and CYC065. Actin was used as loading control. B-D) Quantification of RB, p-Thr821-RB, and p-RB/RB ratio in SK-N-DZ. E-G) Quantification of MYCN, p-Ser45-MYCN, and p-MYCN/MYCN ratio in SK-N-DZ. I-K) Quantification of RB, p-Thr821-RB, and p-RB/RB ratio in IMR32. L-N) Quantification of MYCN, p-Ser45-MYCN, and p-MYCN/MYCN ratio in IMR32. Error bars represent the standard deviation of three independent biological experiments. p-value was calculated using two-tailed, unpaired Student t-test in GraphPad prism. * p-value ≤ 0.05 ; ** p-value ≤ 0.01 ; *** p-value ≤ 0.001 .



Supplementary Figure 7. Combinations of Milciclib and JQ1 demonstrate potent inhibitory effects on NB cell lines. A-H) analysis of cell viability, dose-response, and drug synergy score of Milciclib and JQ1 combinations in IMR32 (A-B), SK-N-BE(2) (C-D), SK-N-FI (E-F), SH-SY5Y (G-H) cell lines. (I) Western blot analysis and quantification of MYCN and CDK2 protein levels in SK-N-BE(2) and IMR32 cell lines treated for 72 hrs with indicated concentration of Milciclib and JQ1 in single and combination treatment. Actin was used as loading control. Error bars represent the standard deviation of three independent biological experiments. The p-value was calculated using two-tailed, unpaired Student t-test in GraphPad Prism. * p-value ≤ 0.05 ; ** p-value ≤ 0.01 ; *** p-value ≤ 0.001 . Synergy score of the different drug combinations was calculated using the Bliss synergy models. Synergy score of less than -10 indicates antagonism; from -10 to 10 indicates additive effects; while synergy score larger than 10 suggests synergistic action.

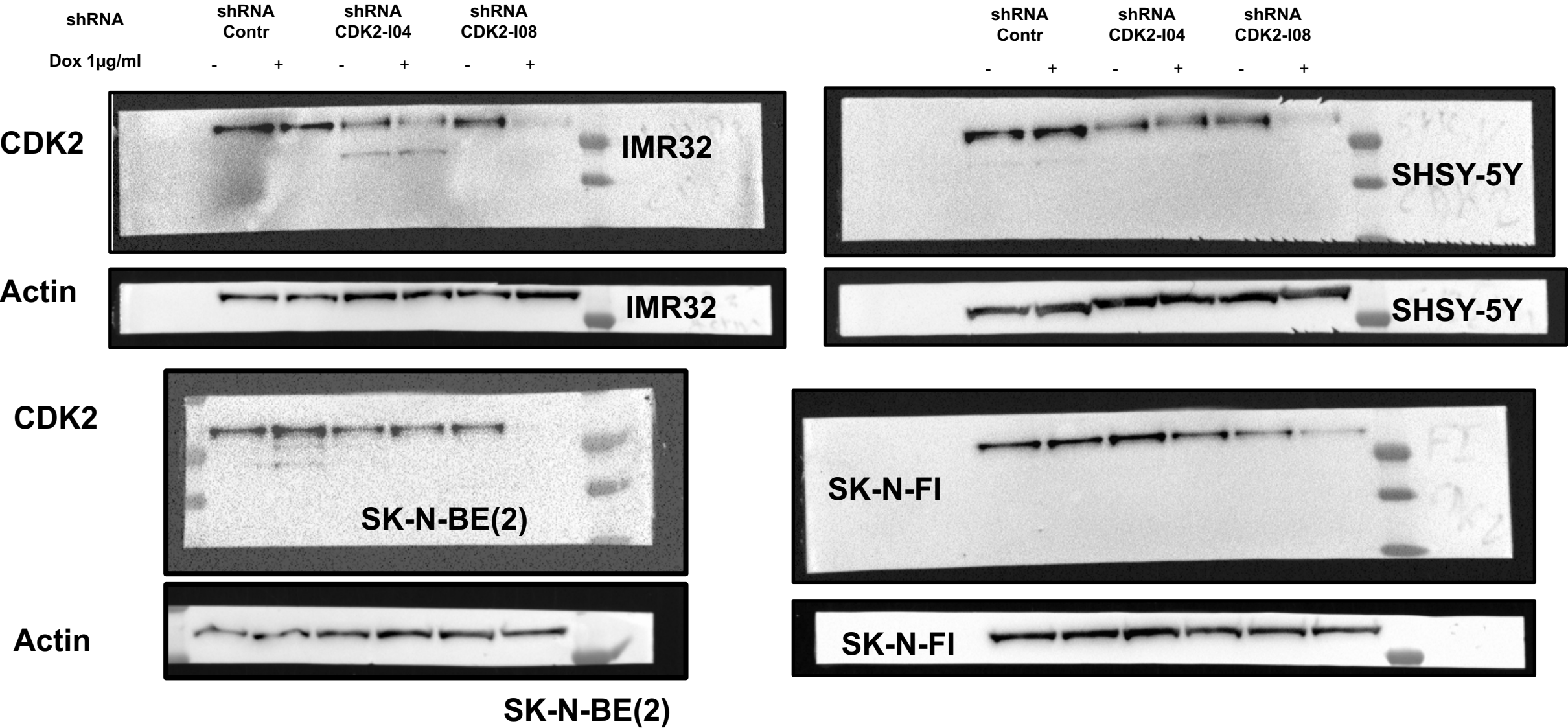


Supplementary Figure 8. Combinations of Milciclib and MYCMI-7 demonstrate additive effects in NB cell lines irrespective of *MYCN*-amplification status. Analysis of cell viability, dose response matrix, bliss synergy scores of NB cell lines IMR32 (A-B), SK-N-BE(2) (C-D), SK-N-FI (E-F), and SHSY-5Y (G-H) treated for 72 hours with indicated concentrations of Milciclib and MYCMI-7. I) Western blot analysis and quantification of MYCN and CDK2 protein levels in IMR32 and SK-N-F1 cell lines following 72 hrs treatment with indicated concentrations of Milciclib and MYCMI-7 in single and combination settings. Actin was used as loading control. Error bars represent the standard deviation of three independent biological experiments. The p-value was calculated using two-tailed, unpaired Student t-test in GraphPad Prism. * p-value ≤ 0.05 ; ** p-value ≤ 0.01 ; *** p-value ≤ 0.001 . Synergy score of the different drug combinations was calculated using the Bliss synergy models. Synergy score of less than -10 indicates antagonism; from -10 to 10 indicates additive effects; while synergy score larger than 10 suggests synergistic action.

Gel type		Tris-Glycine						Tris-Acetate*		Bis-Tris*					
Gel concentration		4-20%	8-16%	10-20%	8%	10%	12%	15%	3-8%	7%	4-12%	10%		12%	
Running buffer		Tris-Glycine						Tris-Acetate		MOPS	MES	MOPS	MES	MOPS	MES
		Apparent Molecular Weights, kDa													
% lenght of gel ↓	10				180		180	180	180						
	20	180	180	130	180	130	130	130	150	140	140	140	140	140	140
	30	130	130	100	130	100	100	100	120	115	115	115	115	115	115
	40	100	100	70	100	70	70	70	80	80	80	80	80	80	80
	50	70	70	55	70	55	55	55	85	65	65	65	65	65	65
	60	55	55	40	55	40	40	40	120	50	50	50	50	50	50
	70	40	40	35	40	35	35	35	85	40	40	40	40	40	40
	80	35	35	25	35	25	25	25	65	30	30	30	30	30	30
	90	25	25	15	25	15	15	15	50	25	25	25	25	25	25
	100	15	15	10	15	10	10	10	40	15	15	15	15	15	15

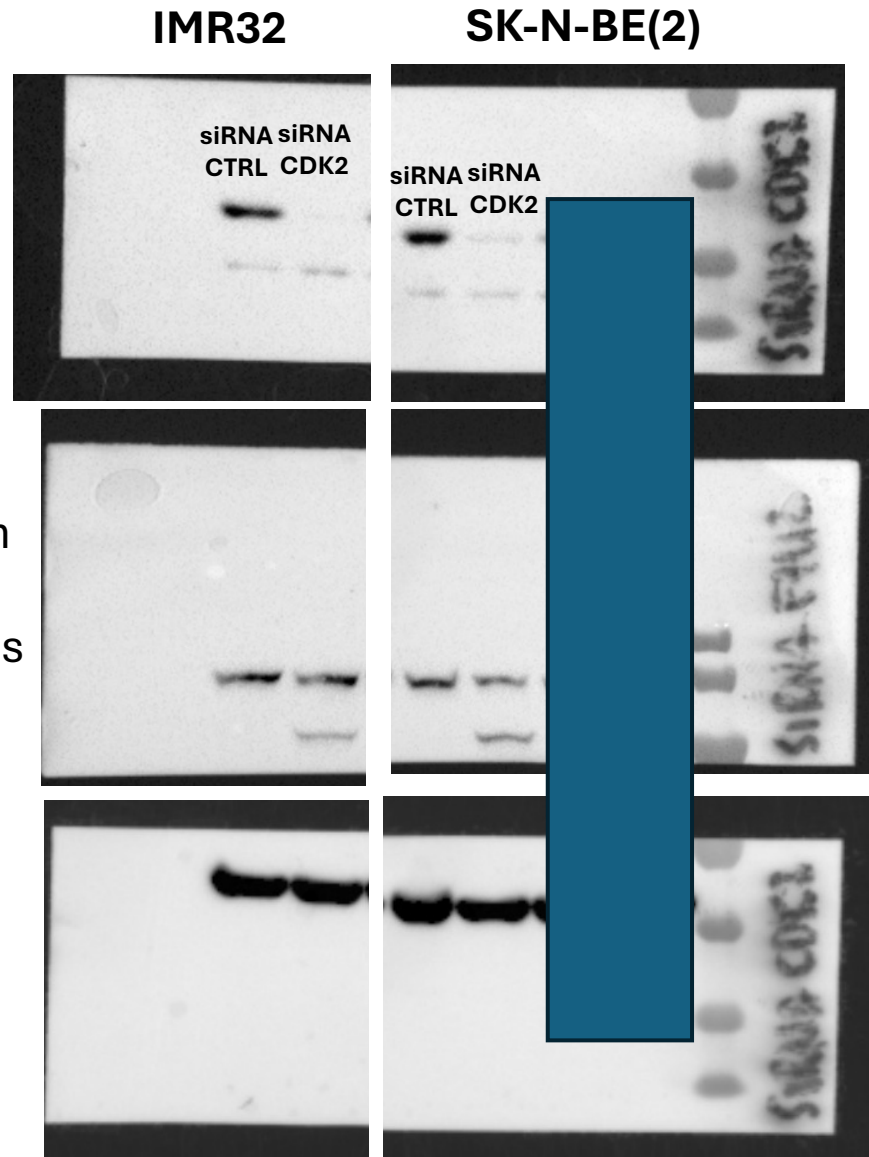
- All westerns were ran using 4-12% Bis-Tris gels in MOPS buffer. PageRuler™ Prestained Protein Ladder , 10 to 180 kDa (Thermo Fisher Catalog#: 26616) was used as Marker. The PageRuler migration pattern is highlighted in red.
- Please note that all membranes were cut around the molecular weight of the analyzed proteins before blocking and incubation with primary antibodies.
- Western blots are arranged according to their listing/appearance order in the manuscript.

Uncropped western blots related to Figure 3A

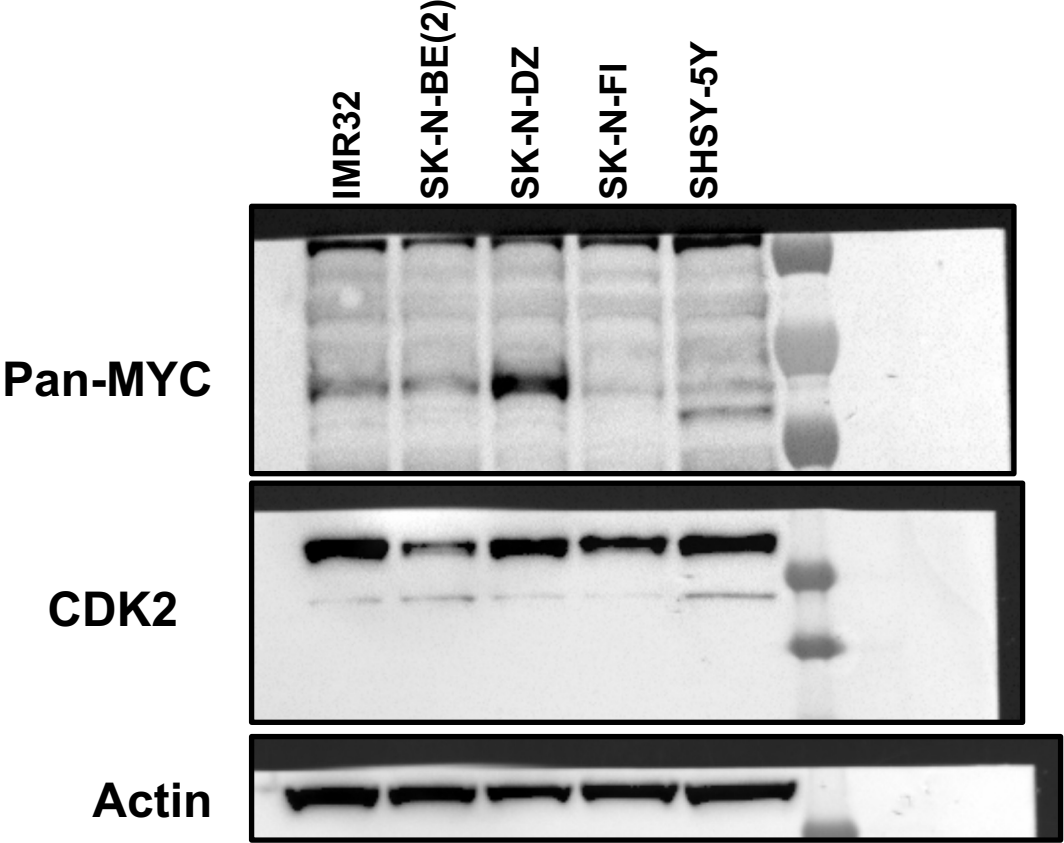
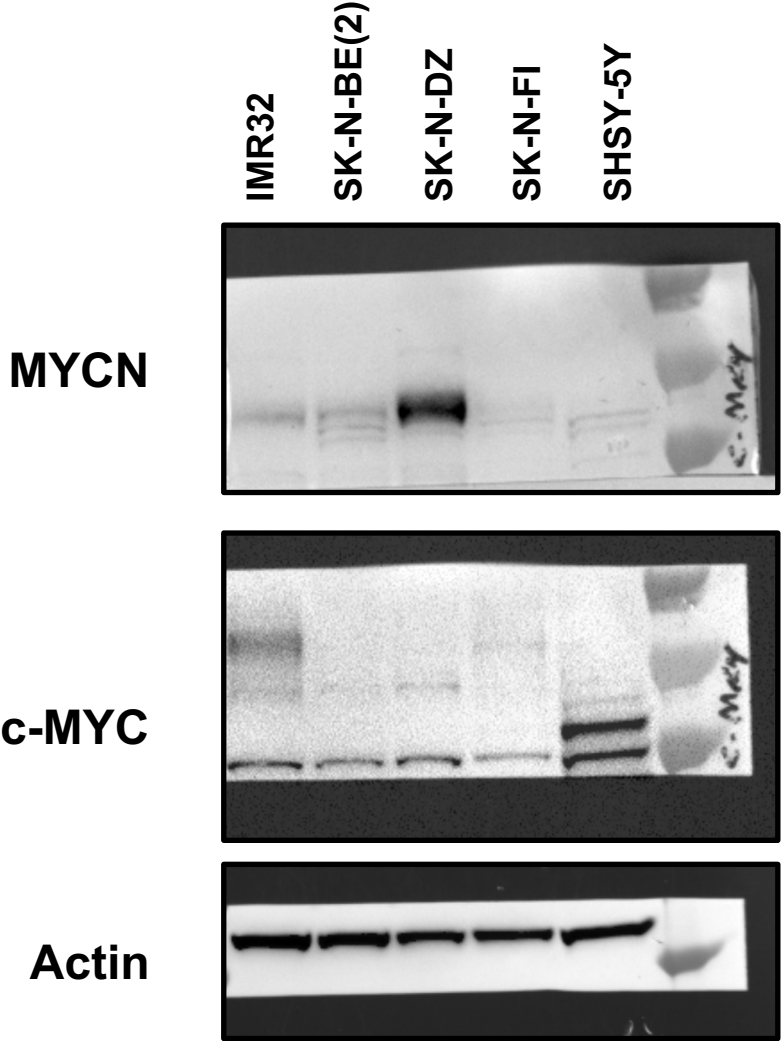


Uncropped western blots related to Figure S3D

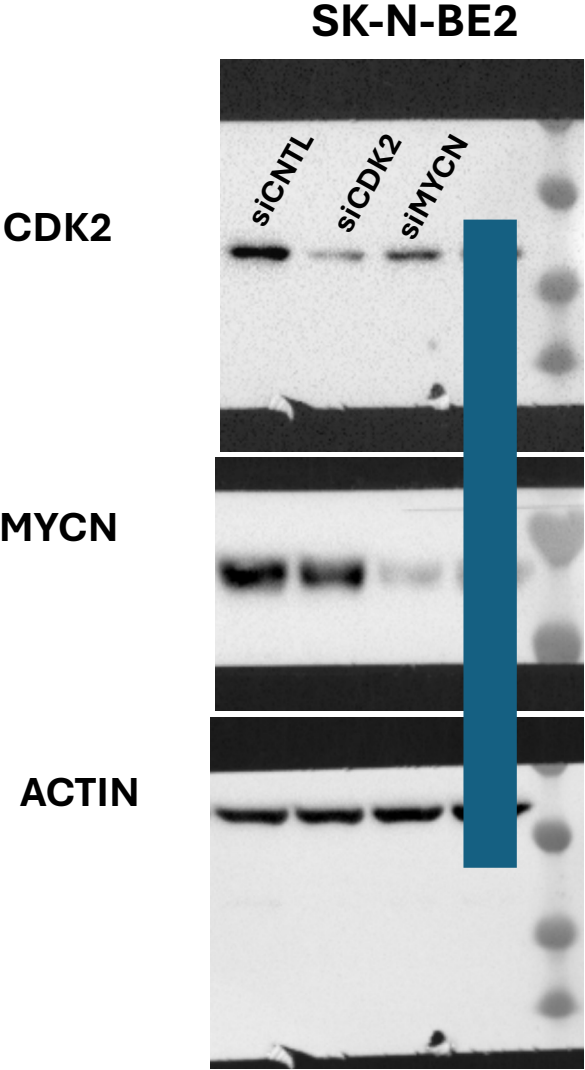
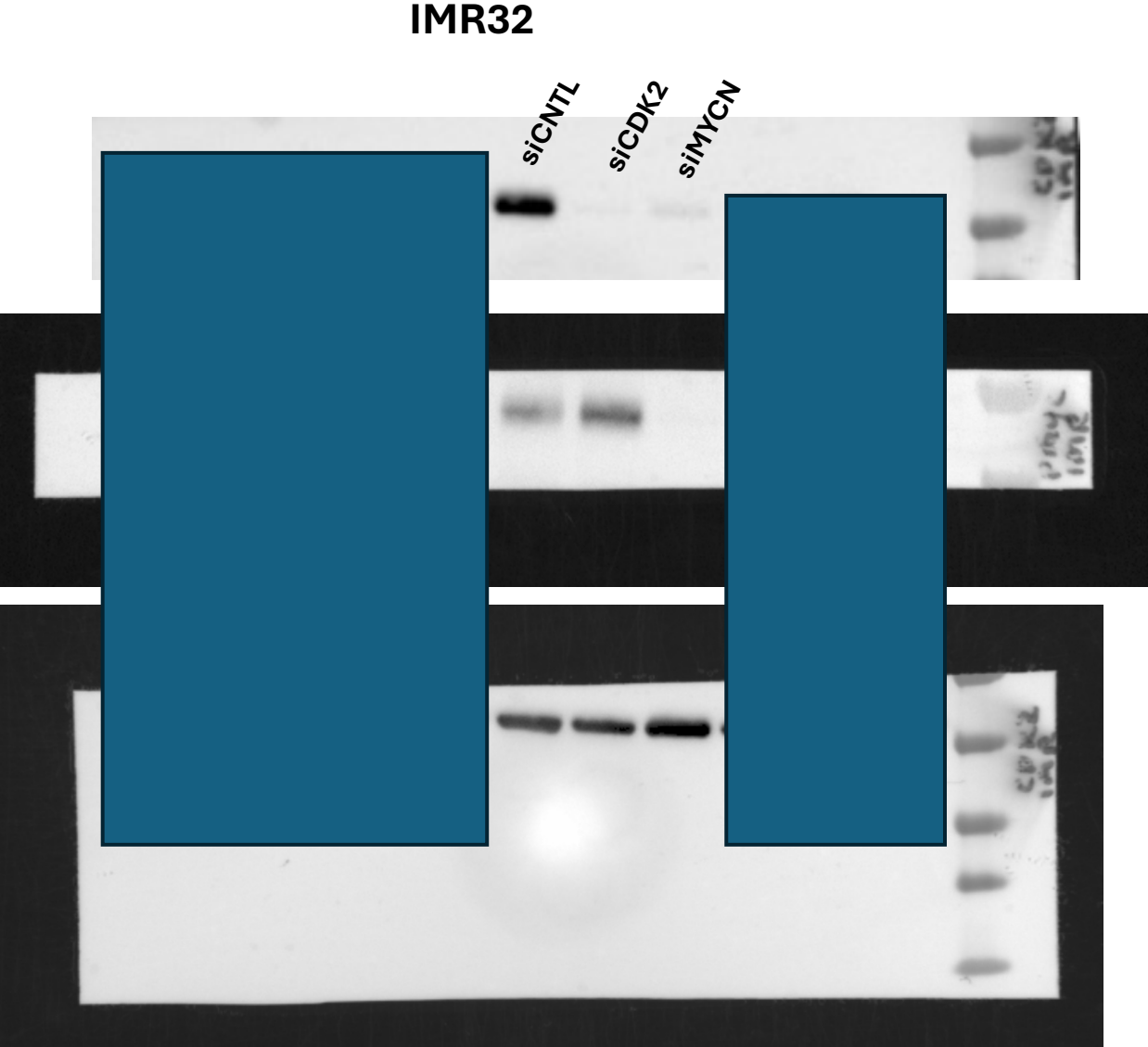
Same
membrane for
all blots, but in
between
irrelevant lanes
are cropped
out



Uncropped western blots related to Figure 4A

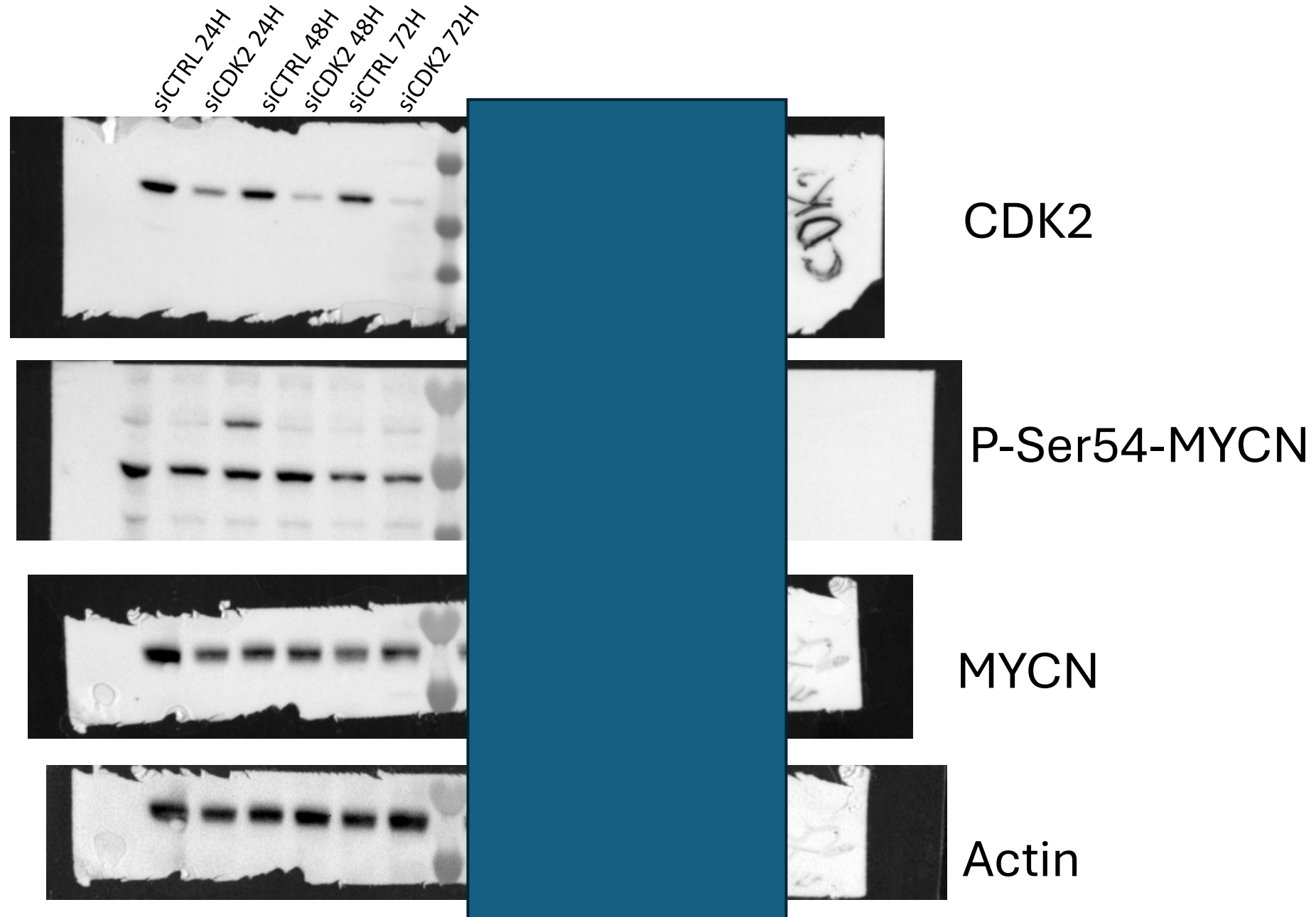


Uncropped western blots related to Figure 6C

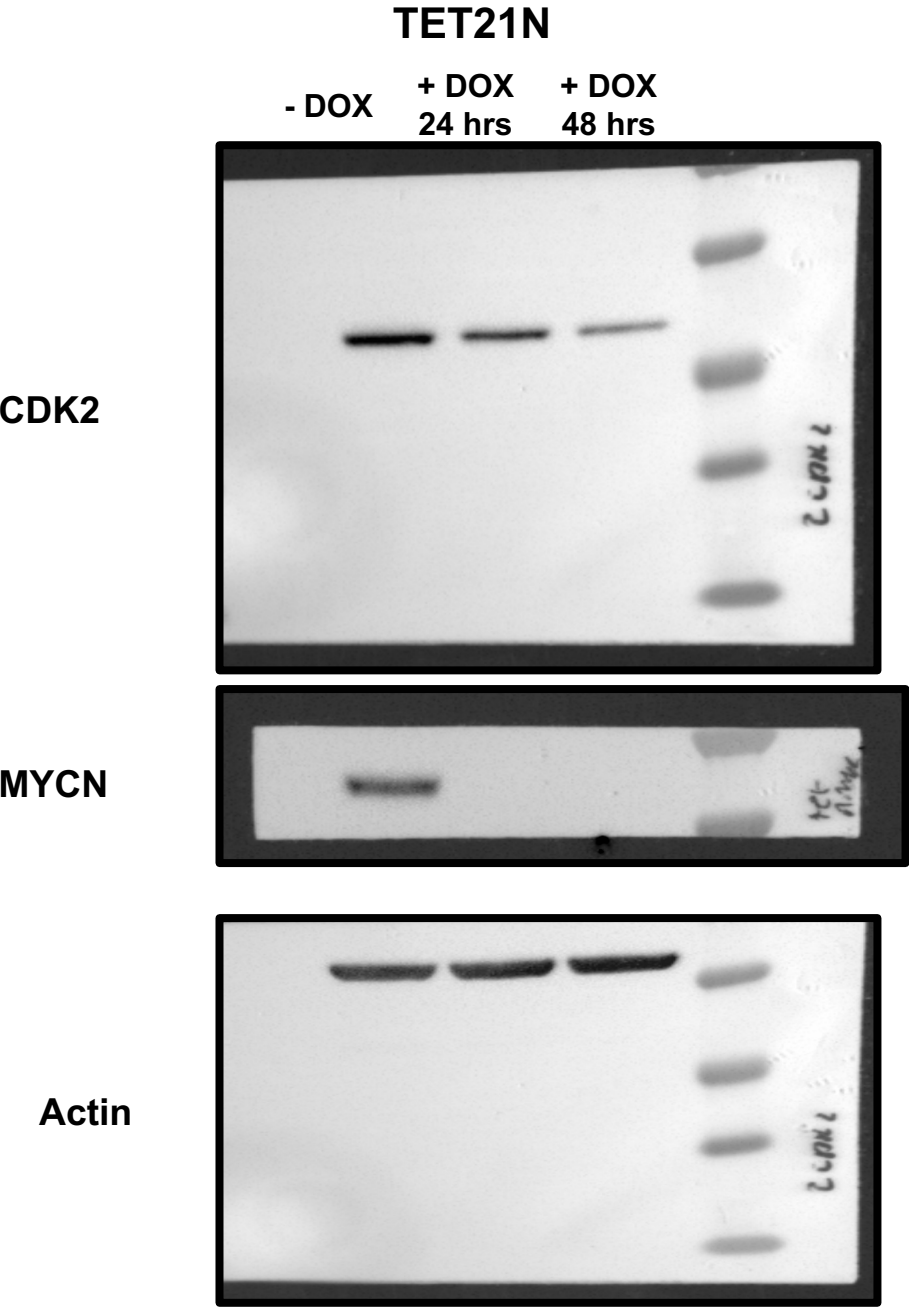


Same membrane
for all blots, but in
irrelevant lanes
are cropped out

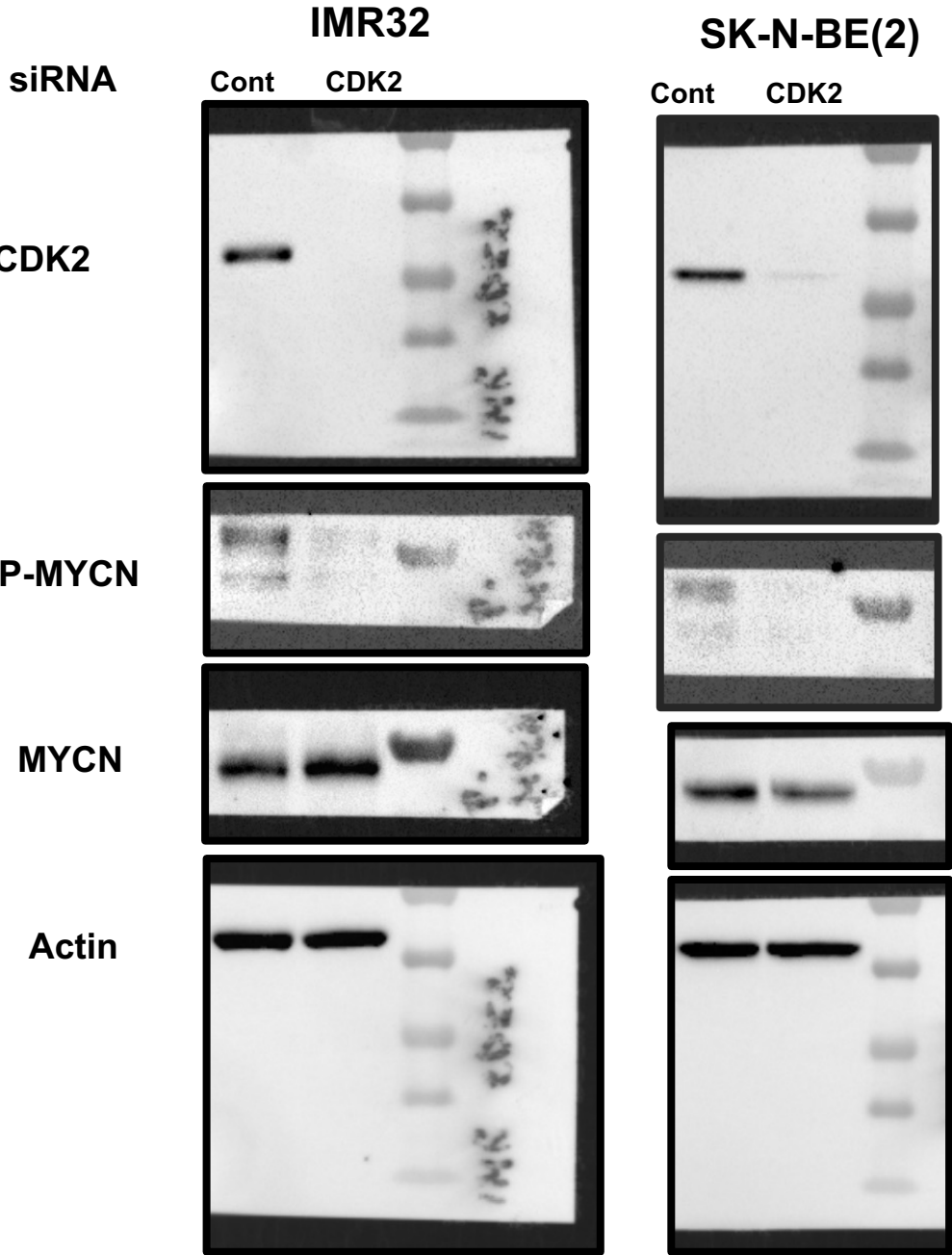
Uncropped western blots related to Figure 6E



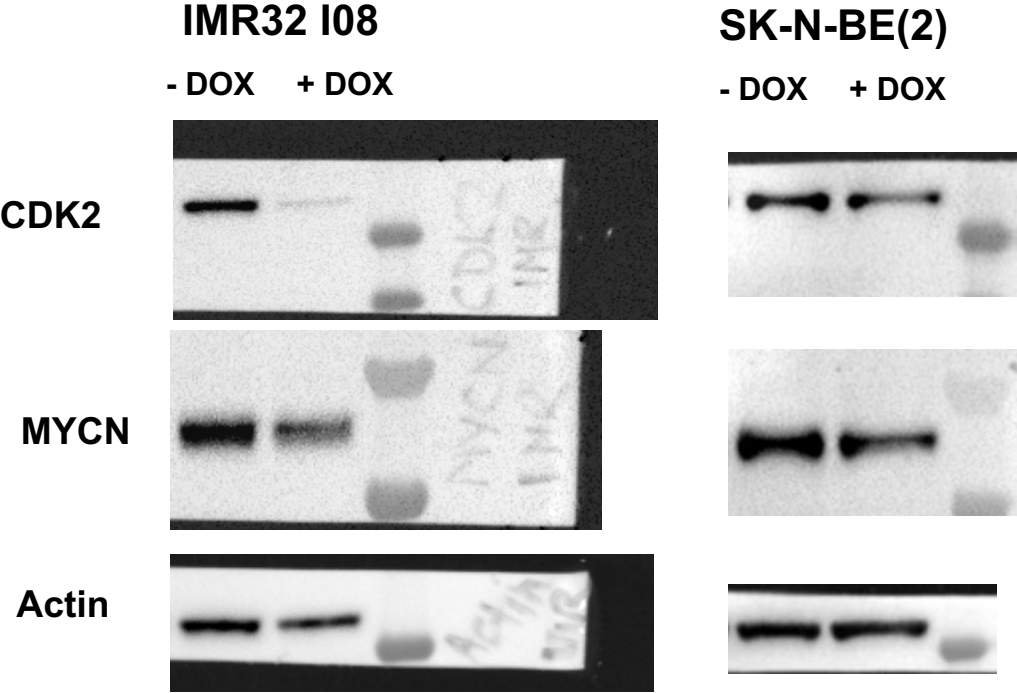
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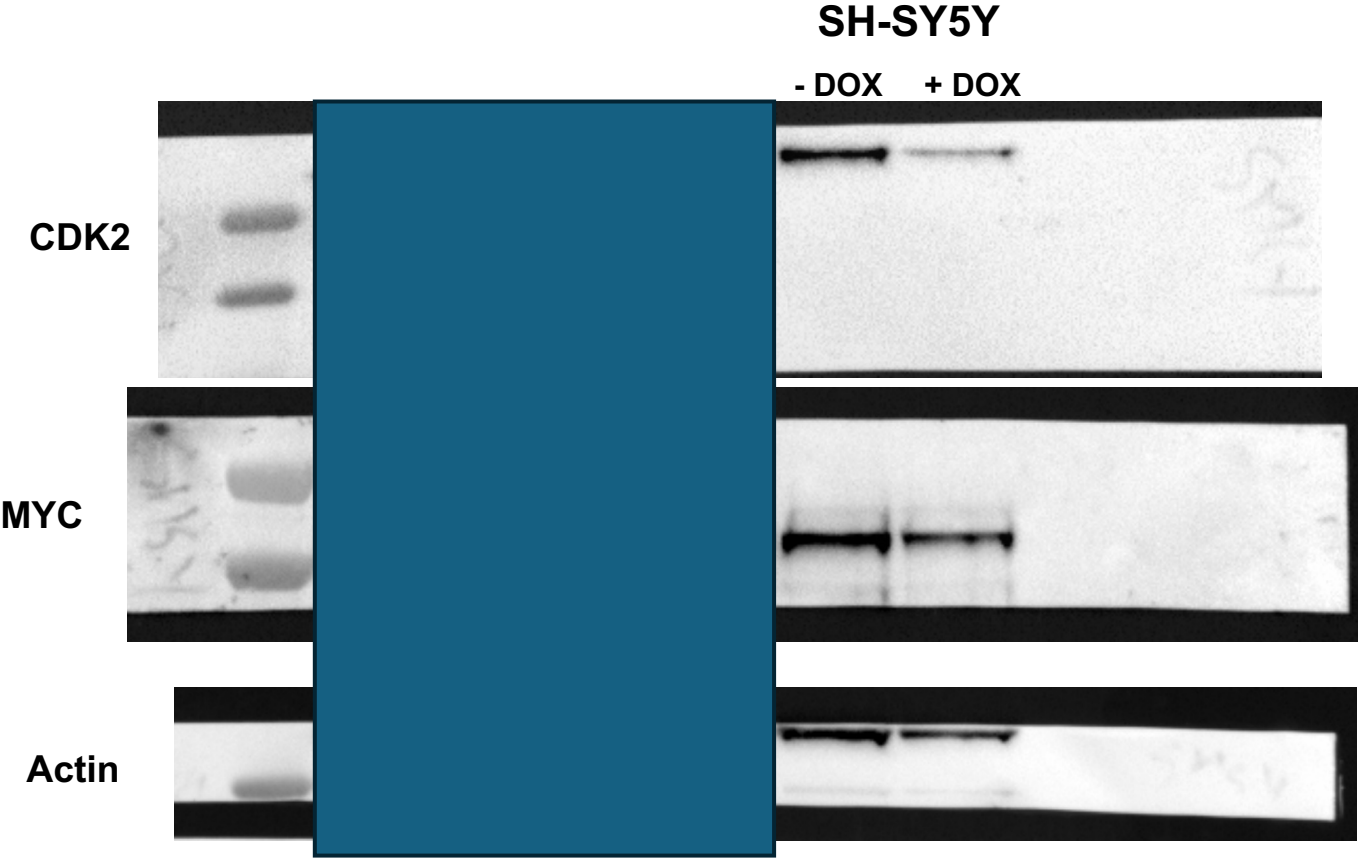
Uncropped western blots related to Figure 4SB

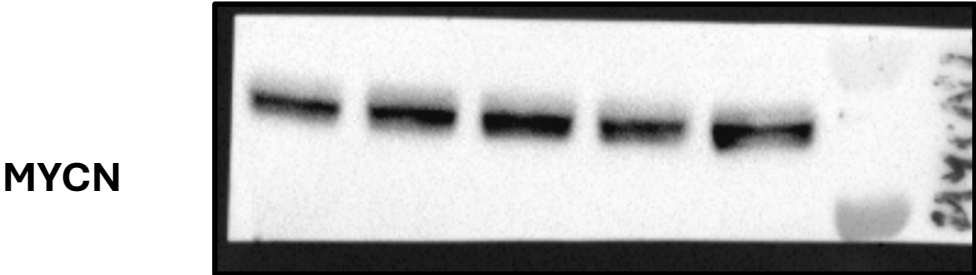
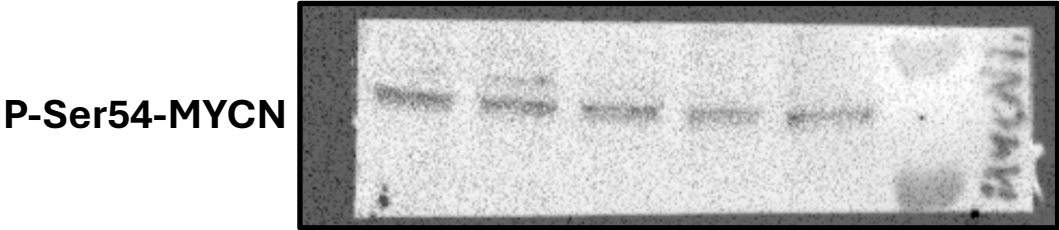
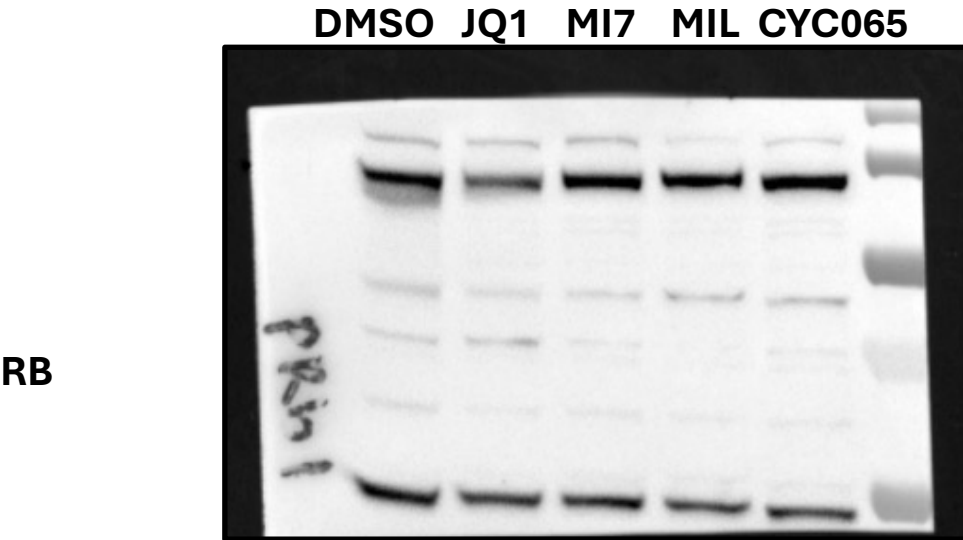


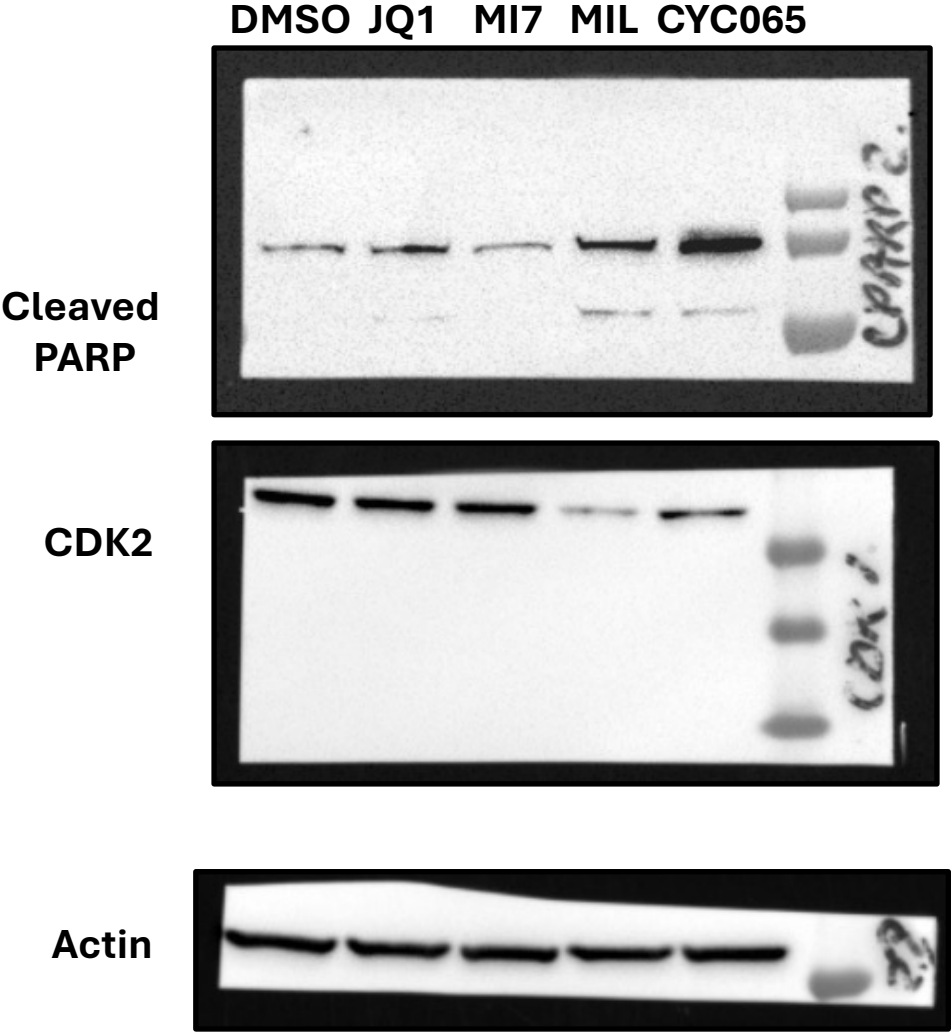
Uncropped western blots related to Figure 4SC



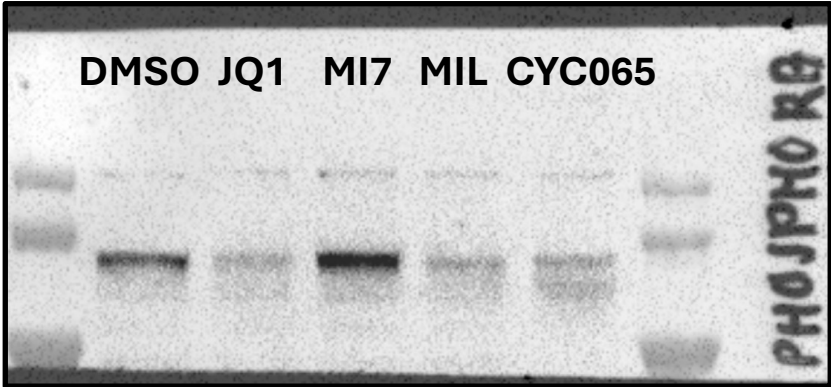
Uncropped western blots related to Figure 4SD



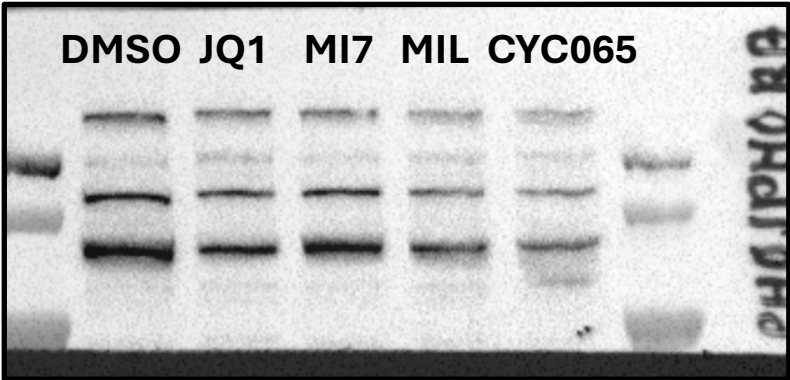




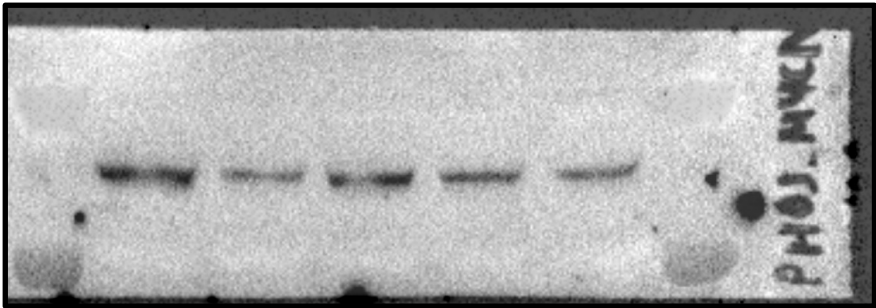
P-Thr821-RB



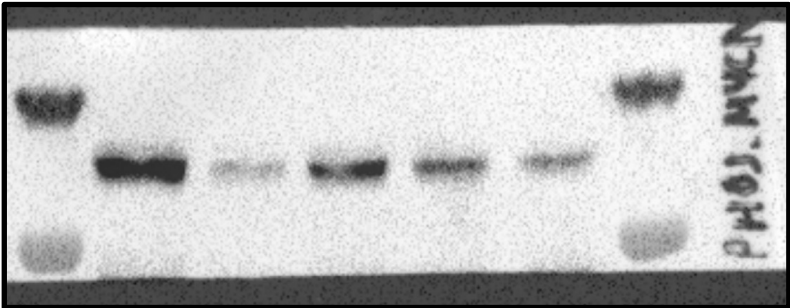
RB



P-Ser54-MYCN



MYCN

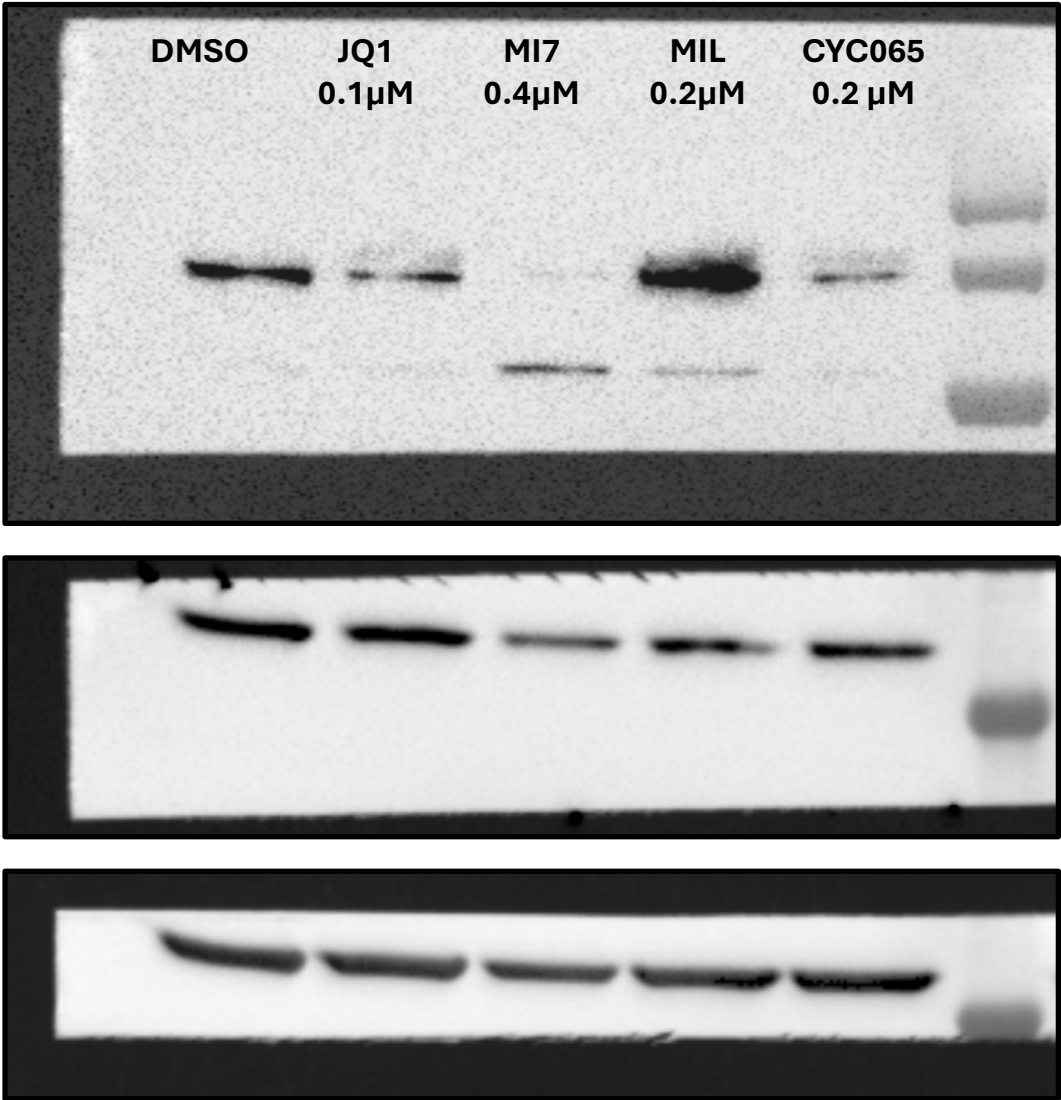


Actin



Uncropped western blots related to Figure S6H

IMR32

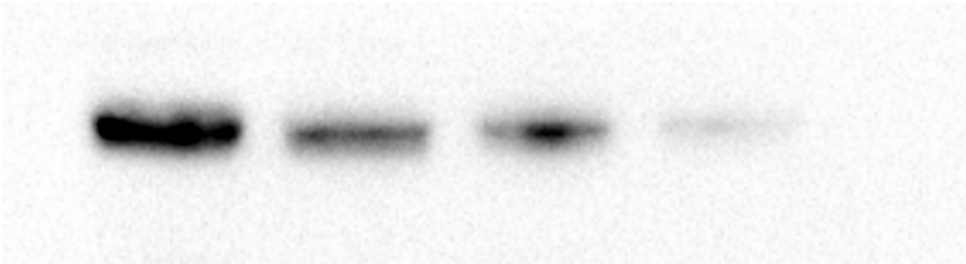


Uncropped western blots related to Figure S7I

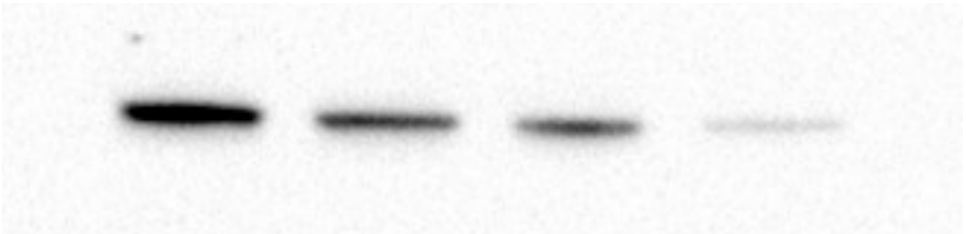
SK-N-BE(2)

-	0.2	-	0.2
-	-	0.2	0.2

MYCN



CDK2

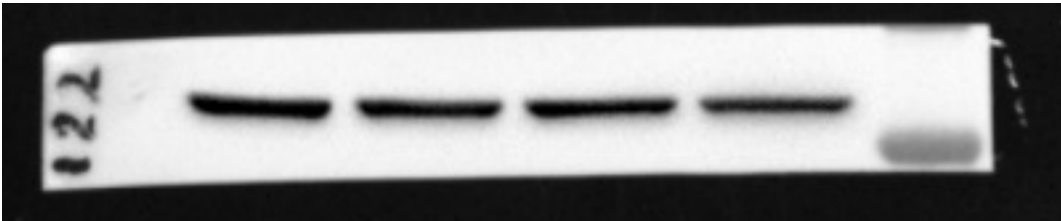
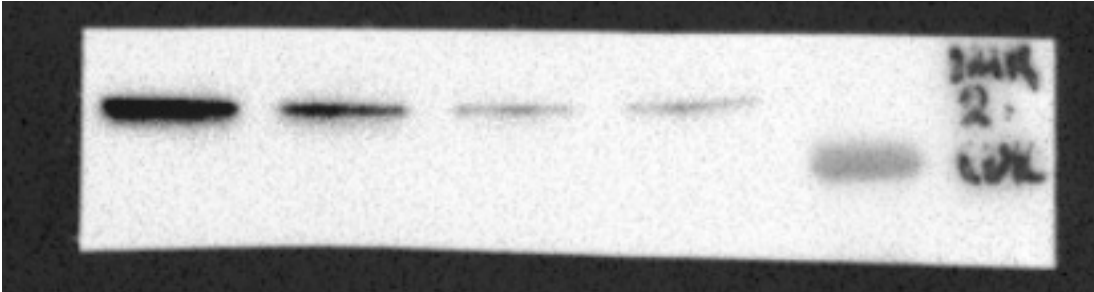
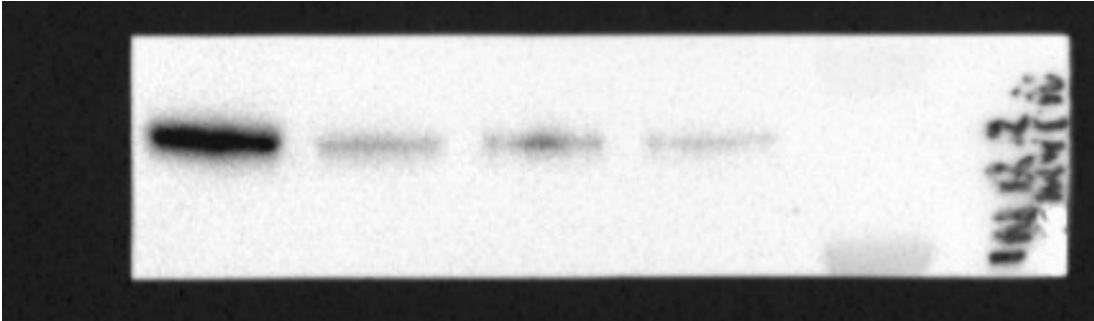


Actin



IMR32

JQ1 (μM)	-	0.1	-	0.1
Mil (μM)	-	-	0.1	0.1



Uncropped western blots related to Figure S8I

