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Supplementary Materials for

RRM2 enhances MYCN-driven neuroblastoma formation and acts as a synergistic target with CHK1 inhibition

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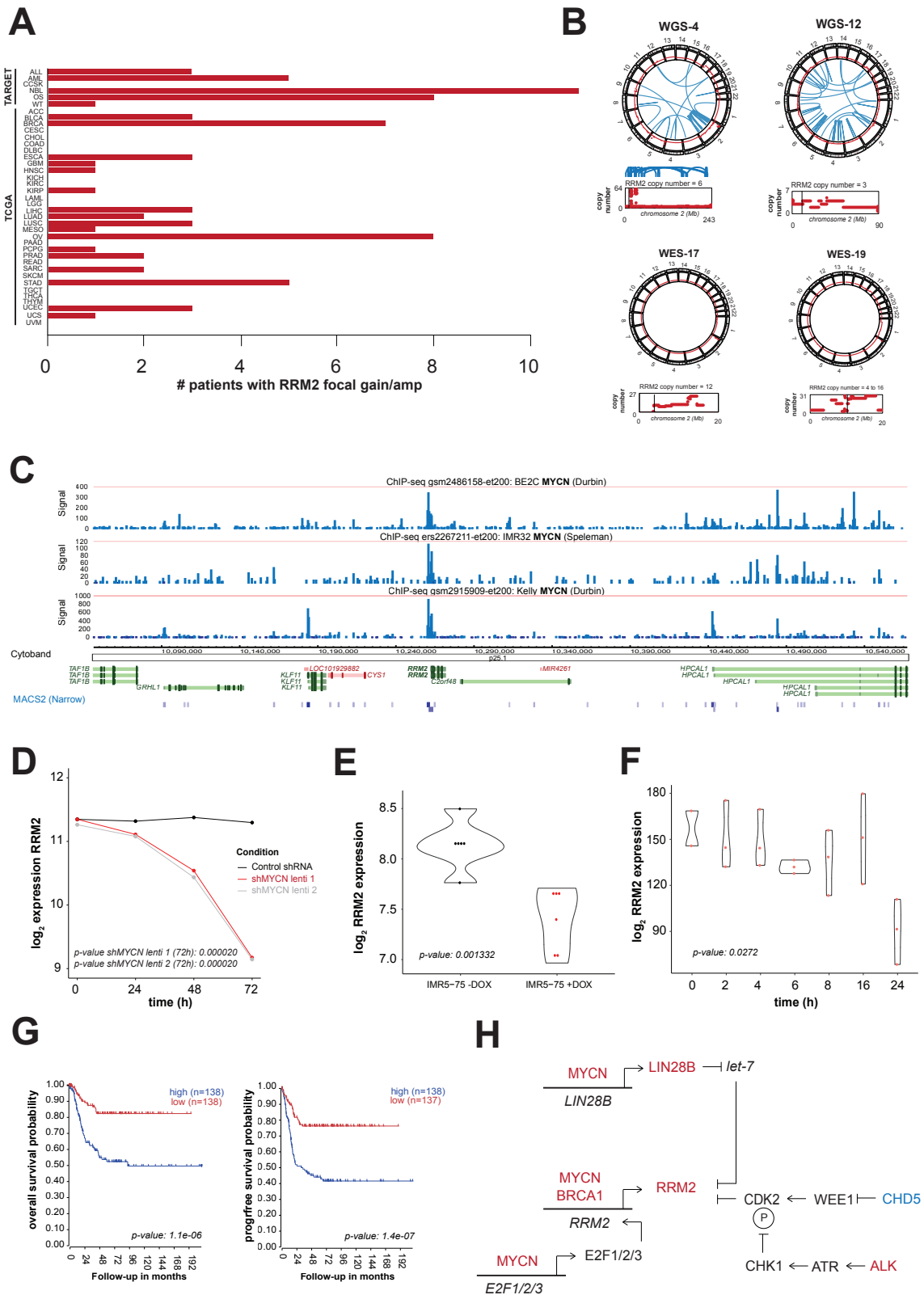
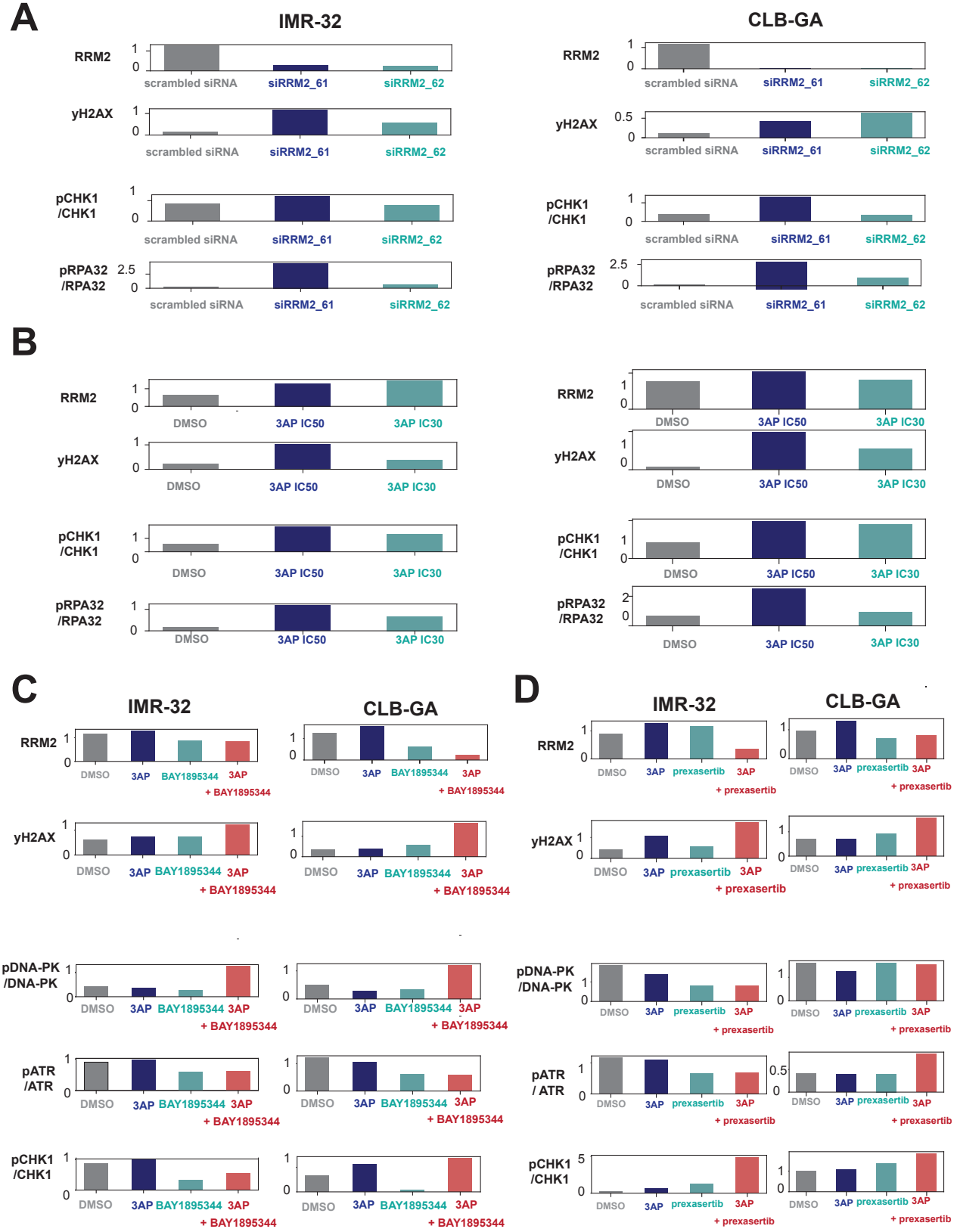
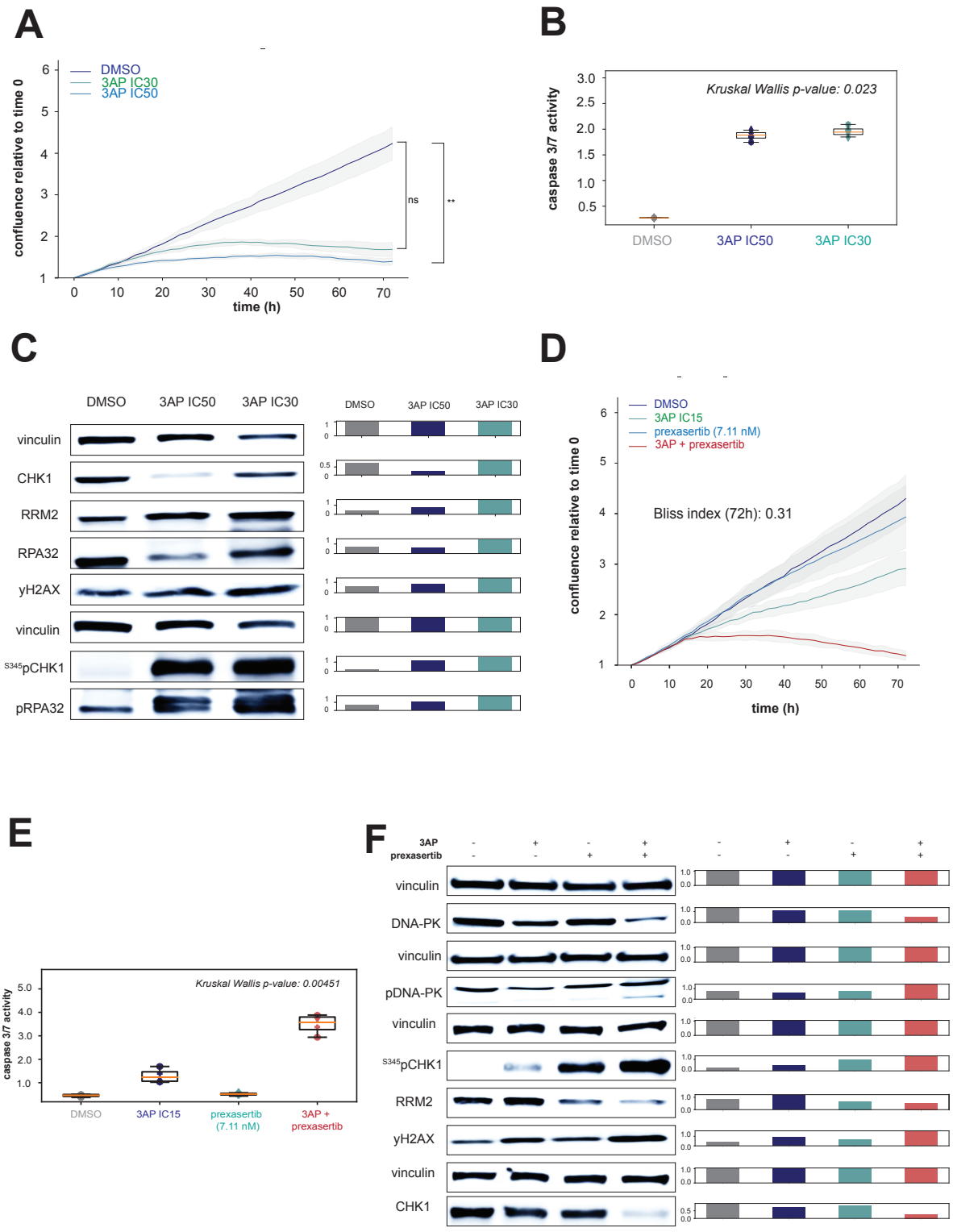

A
TARGET
ALL
AML
CCSK
NBL
OS
WT
TCGA
ACC
BLCA
BRCA
CESC
CHOL
COAD
DLBC
ESCA
GBM
HNSC
KICH
KIRC
KIRP
LAML
LGG
LIHC
LUAD
LUSC
MESO
OV
PAAD
PCPG
PRAD
READ
SARC
SKCM
STAD
TGCT
THCA
THYM
UCEC
UCS
UVM
0
2
4
6
8
10
patients with RRM2 focal gain/amp
B
WGS-4
WGS-12
WES-17
WES-19
RRM2 copy number = 6
RRM2 copy number = 3
RRM2 copy number = 12
RRM2 copy number = 4 to 16
copy number
chromosome 2 (Mb)
C
ChIP-seq gsm2486158-et200: BE2C MYCN (Durbin)
ChIP-seq ers2267211-et200: IMR32 MYCN (Speleman)
ChIP-seq gsm2915909-et200: Kelly MYCN (Durbin)
Signal
Cytoband
MACS2 (Narrow)
D
log2 expression RRM2
time (h)
Condition
Control shRNA
shMYCN lenti 1
shMYCN lenti 2
p-value shMYCN lenti 1 (72h): 0.000020
p-value shMYCN lenti 2 (72h): 0.000020
E
log2 RRM2 expression
p-value: 0.001332
IMR5−75 -DOX
IMR5−75 +DOX
F
log2 RRM2 expression
p-value: 0.0272
time (h)
G
overall survival probability
high (n=138)
low (n=138)
p-value: 1.1e-06
Follow-up in months
progrfree survival probability
high (n=138)
low (n=137)
p-value: 1.4e-07
Follow-up in months
H
MYCN
LIN28B
let-7
LIN28B
MYCN
BRCA1
RRM2
RRM2
CDK2
WEE1
CHD5
P
CHK1
ATR
ALK
MYCN
E2F1/2/3
E2F1/2/3

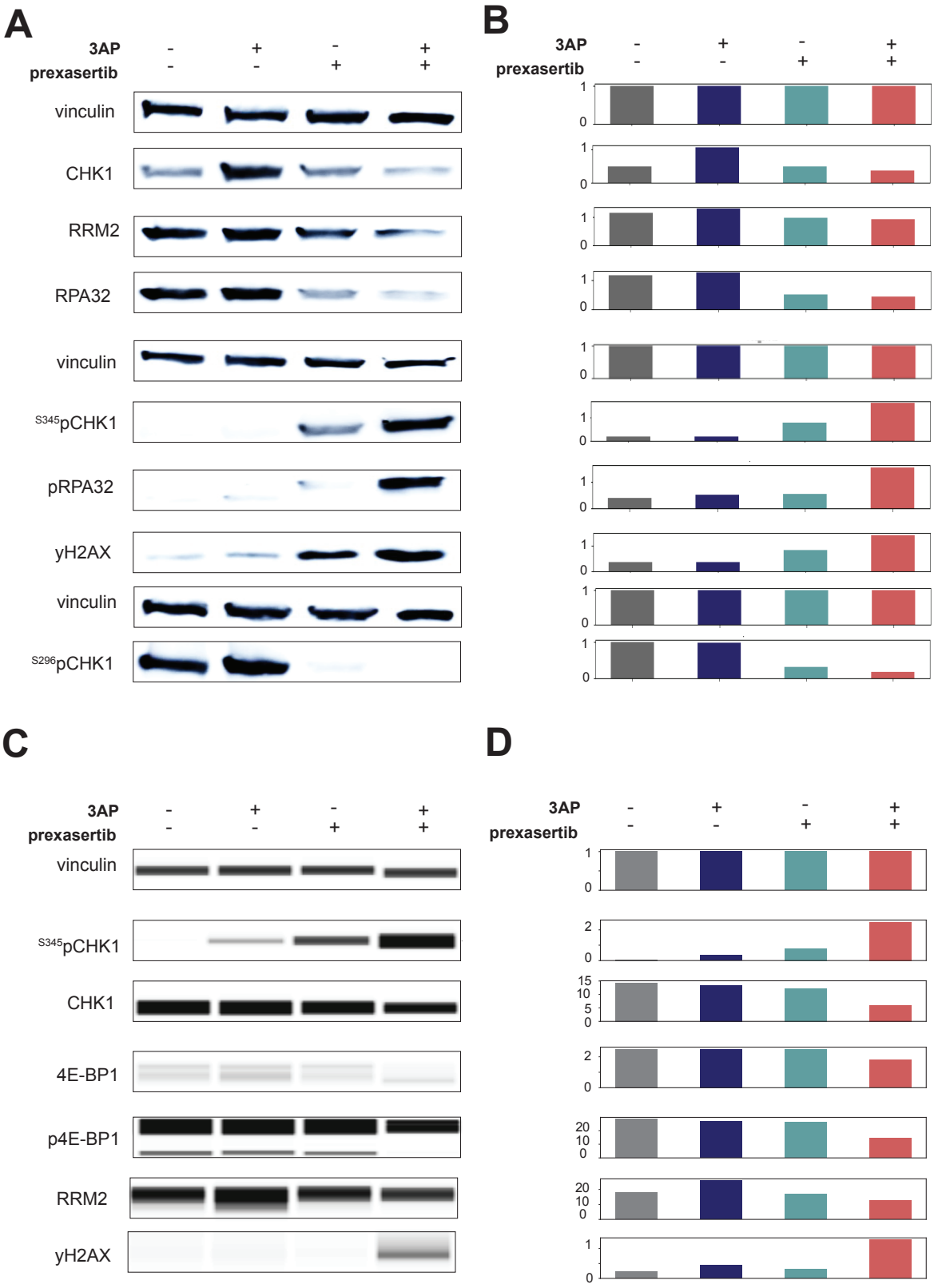
Supp. Fig. 1: High *RRM2* expression is a poor prognostic factor in neuroblastoma. A. Bar plot indicating the number of neuroblastoma patients in the TCGA or TARGET cohorts with a focal *RRM2* gain or amplification; **B.** Additional whole genome or whole exome sequencing arrayCGH data of another cohort of 419 neuroblastoma cases uncovers the identification of 60 cases with RRM2 gain or amplification (*top*), with detailed CNV profiles of some complex amplicons in which RRM2 is involved, including WGS-12 with noted chromotrypsis of 2p involving the *RRM2* locus (*bottom*); **C.** Publicly available MYCN ChIP-seq data shows MYCN binding at the promotor region of the *RRM2* locus (hgserver2.amc.nl); **D.** *RRM2* expression is downregulated upon shRNA mediated MYCN knockdown in IMR32 neuroblastoma cells; **E.** *RRM2* expression is downregulated following doxycyclin inducible MYCN downregulation in IMR5-75 neuroblastoma cells; **F.** R*RM2* overexpression is reduced over time upon inducible MYCN downregulation in SHEP21N neuroblastoma cells; **G.** Overall and event-free patient survival for neuroblastoma cases with high versus low *RRM2* expression in 2 other independent patient cohorts (respectively Kocak (n=649) and SEQC (n=498) cohorts, hgserver2.amc.nl); **H.** Scheme depicting RRM2 at the nexus of a copy number driven regulatory network in neuroblastoma (*red*: gene is gained or amplified in neuroblastoma, *blue*: gene displays copy number loss in neuroblastoma).


A
IMR-32
CLB-GA
RRM2
yH2AX
pCHK1 /CHK1
pRPA32 /RPA32
scrambled siRNA
siRRM2_61
siRRM2_62
B
DMSO
3AP IC50
3AP IC30
C
IMR-32
CLB-GA
DMSO
3AP
BAY1895344
3AP + BAY1895344
pDNA-PK /DNA-PK
pATR /ATR
pCHK1 /CHK1
D
IMR-32
CLB-GA
prexasertib
3AP + prexasertib

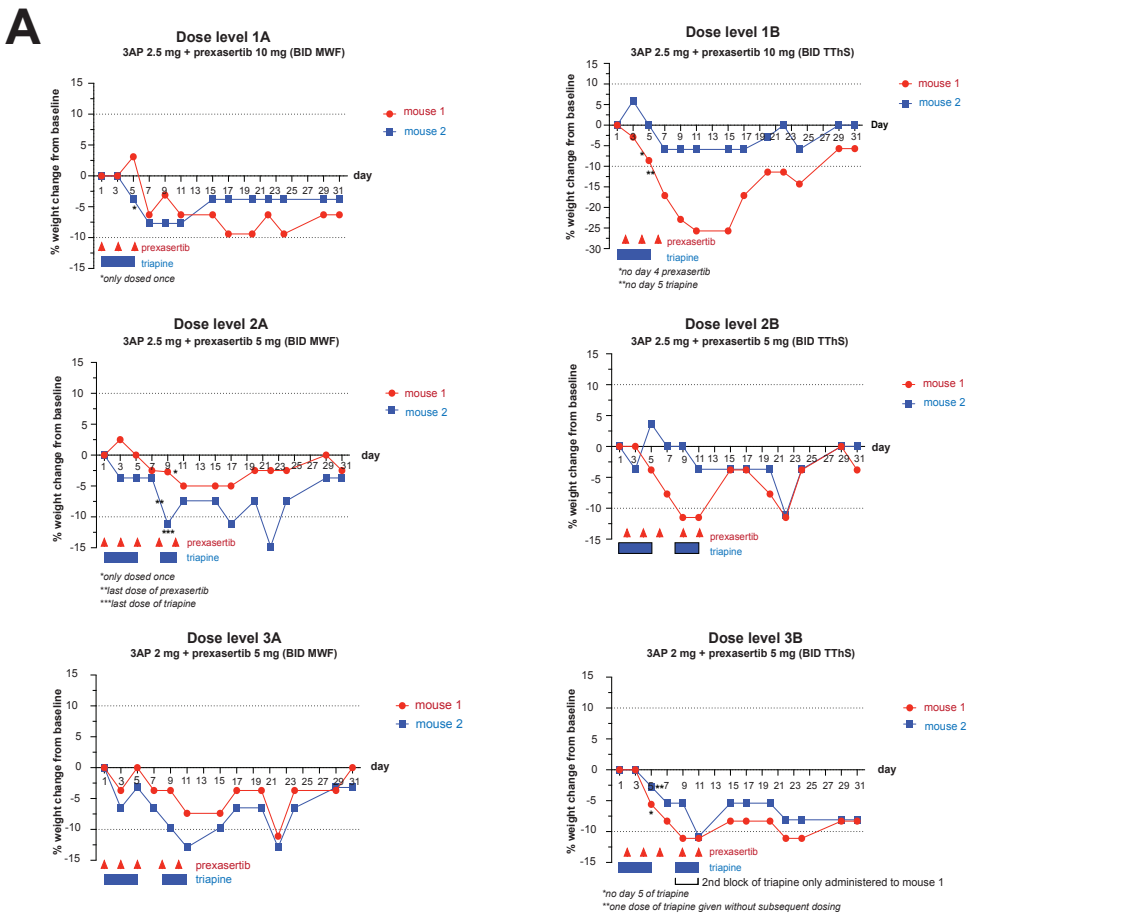
Supp. Fig. 2: Quantification of immunoblotting images throughout the manuscript. A. Quantification of immunoblotting for RRM2, yH2AX, CHK1/pCHK1 and RPA32/pRPA32 following transient knockdown of RRM2 by two independent siRNAs in IMR-32 and CLB-GA neuroblastoma cells; **B.** Quantification of immunoblotting for RRM2, yH2AX, CHK1/pCHK1 and RPA32/pRPA32 following exposure to 3AP for 48h at respective IC30 and IC50 concentrations for IMR-32 and CLB-GA cells; **C.** Quantification of immunoblotting for RRM2, yH2AX, DNA-PK/pDNA-PK, ATR/pATR and CHK1/pCHK1 in IMR-32 and CLB-GA neuroblastoma cells following combined 3AP-BAY1895344 treatment in comparison to single agent exposure or control treatment (DMSO); **D.** Quantification of immunoblotting for RRM2, yH2AX, DNA-PK/pDNA-PK, ATR/pATR and CHK1/pCHK1 in IMR-32 and CLB-GA neuroblastoma cells following combined 3AP-prexasertib treatment in comparison to single agent exposure or control treatment (DMSO);All images were quantified by ImageJ and bar plots were normalized relative to vinculin levels.

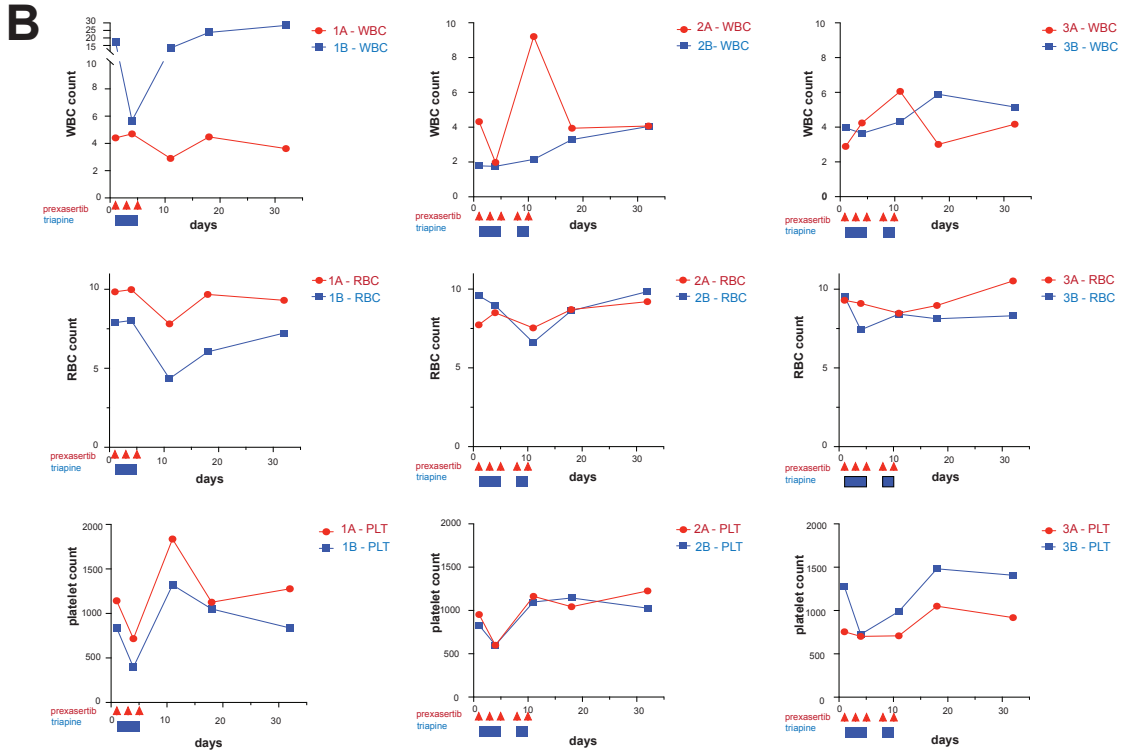

A
DMSO
3AP IC30
3AP IC50
confluence relative to time 0
time (h)
ns
**
B
caspase 3/7 activity
Kruskal Wallis p-value: 0.023
DMSO
3AP IC50
3AP IC30
C
DMSO
3AP IC50
3AP IC30
vinculin
CHK1
RRM2
RPA32
γH2AX
vinculin
S345pCHK1
pRPA32
D
DMSO
3AP IC15
prexasertib (7.11 nM)
3AP + prexasertib
Bliss index (72h): 0.31
confluence relative to time 0
time (h)
E
caspase 3/7 activity
Kruskal Wallis p-value: 0.00451
DMSO
3AP IC15
prexasertib (7.11 nM)
3AP + prexasertib
F
3AP
prexasertib
vinculin
DNA-PK
vinculin
pDNA-PK
vinculin
S345pCHK1
RRM2
γH2AX
vinculin
CHK1

Supp. Fig. 3: Combined RRM2-CHK1 pharmacological inhibition acts synergistically in the p53 mutant neuroblastoma cell line SK-N-BE(2)-C: (A) Cell confluence of SK-N-BE(2)-C cells is reduced following 3AP single drug treatment, both with IC30 and IC50 concentrations; **(B)** 3AP exposure significantly induces cell death as measured by the Caspase glo assay (Promega); **(C)** Immunoblotting following 3AP treatment of SK-N-BE(2)-C cells with 3AP shows upregulated ^{S345}pCHK1 and pRPA32 levels. In addition total CHK1 levels are downregulated following 3AP exposure at IC50 concentration; **(D)** Exposure of SKN-BE(2)-C cells to 3AP-prexasertib combination treatment shows a synergistic negative effect on cell confluence as measured by Incucyte live cell imaging; **(E)** Combined 3AP-prexasertib treatment of SK-N-BE(2)-C cells significantly induces cell death compared to control or (low dose) single treatments; **(F)** Immunoblotting following combined 3AP-prexasertib treatment shows upregulation of ^{S345}pCHK1 and yH2AX levels, concomitant with a reduction of RRM2 and total CHK1 protein levels.


A
3AP
prexasertib
vinculin
CHK1
RRM2
RPA32
vinculin
S345pCHK1
pRPA32
yH2AX
vinculin
S296pCHK1
B
3AP
prexasertib
C
3AP
prexasertib
vinculin
S345pCHK1
CHK1
4E-BP1
p4E-BP1
RRM2
yH2AX
D
3AP
prexasertib

Supp Fig. 4: **Combined 3AP-prexasertib treatment reduced pCHK1 autophosphorylation and p4E-BP1 levels. (A)** IMR32 cells treated with 3AP-prexasertib combination display reduced ^{S296}pCHK1 autophosphorylation concomitant with reduced ^{S345}pCHK1 and reduced total CHK1 as well as RRM2 protein levels compared to control or single drug treatment; **(B)** Quantification of the immunoblotting relative to vinculin as presented in **(A)**; **(C)** Reduced p4E-BP1 levels (activation) upon 3AP-prexasertib combination treatment is marked by the disappearing lower p4E-BP1 band; (**D**) Quantification of the immunoblotting relative to vinculin as presented in **(C)**


A
Dose level 1A
3AP 2.5 mg + prexasertib 10 mg (BID MWF)
% weight change from baseline
day
mouse 1
mouse 2
prexasertib
triapine
*only dosed once
Dose level 1B
3AP 2.5 mg + prexasertib 10 mg (BID TThS)
Day
*no day 4 prexasertib
**no day 5 triapine
Dose level 2A
3AP 2.5 mg + prexasertib 5 mg (BID MWF)
*only dosed once
**last dose of prexasertib
***last dose of triapine
Dose level 2B
3AP 2.5 mg + prexasertib 5 mg (BID TThS)
Dose level 3A
3AP 2 mg + prexasertib 5 mg (BID MWF)
Dose level 3B
3AP 2 mg + prexasertib 5 mg (BID TThS)
2nd block of triapine only administered to mouse 1
*no day 5 of triapine
**one dose of triapine given without subsequent dosing


B
WBC count
1A - WBC
1B - WBC
2A - WBC
2B- WBC
3A - WBC
3B - WBC
RBC count
1A - RBC
1B - RBC
2A - RBC
2B - RBC
3A - RBC
3B - RBC
platelet count
1A - PLT
1B - PLT
2A - PLT
2B - PLT
3A - PLT
3B - PLT
days
prexasertib
triapine

Supp. Fig. 5: Evaluation of the impact of combined 3AP-prexasertib treatment in NGS mice on weight of the mice and hematopoietic toxicity. (A) No mortality was observed for any of the dose levels tested. Significant weight loss was observed in treated animals at the highest dose level 1 (i.e., triapine 2.5 mg/kg + prexasertib 10 mg/kg) and treatment was only tolerated for 1 week due to severe weight loss and poor clinical appearance. In contrast, animals treated at dose level 2 and dose level 3 had few to no dosing holidays and were tolerated for nearly 2 treatment cycles; (**B**) Serial blood count measurements were performed and show treatment-associated anemia and thrombocytopenia, but with consequent recovery.

Table S1.

$-\log_{10}$ (adj.p)	$\log_2$(FC)	protein names
2.85	-3.90	RPS29
3.34	-2.82	HMGB2
2.94	-2.08	QIL1
2.41	-1.97	HEXIM1
1.43	-1.91	KRR1
1.33	-1.89	ZC3H4
4.40	-1.85	ZNF593
1.28	-1.83	CSNK2B
4.12	-1.79	RBM42
2.45	-1.65	MRPS2
1.54	-1.60	MLLT1
1.50	-1.58	PPIB
1.02	-1.37	EMG1
1.33	-1.34	TMPO
1.91	-1.28	DDX39B
1.61	-1.23	KPNA1
1.30	-1.04	TSR1
1.10	-1.02	RFC4
1.59	-0.98	PPIH
1.13	-0.97	SRRT
1.97	-0.96	WBP11
1.18	-0.86	EHMT1
1.47	-0.85	NME4
1.08	-0.78	ASH2L
3.37	-0.76	MBD3
4.88	-0.72	PHAX
1.52	-0.68	PA2G4
2.71	-0.63	DEK
2.14	-0.63	VARS
2.65	-0.57	CTCF
2.24	-0.56	NOL4
1.23	-0.54	CIRBP
1.27	-0.54	MORF4L2
1.32	-0.52	MORF4L1
2.94	-0.52	CDK2AP1;CDK2AP2

2.91	-0.52	TOP1
2.43	-0.52	PAF1
2.32	-0.48	SRP9
3.92	-0.48	MTDH
3.49	-0.48	RBBP5
4.23	-0.48	BOD1L1
2.42	-0.47	SART3
1.70	-0.47	C3orf17
2.05	-0.47	CTR9
2.47	-0.46	HDGFRP2
2.21	-0.46	SNRPD2
2.54	-0.46	ZC3H11A
2.45	-0.46	XRCC6
1.42	-0.45	DPY30
2.12	-0.44	PHF6
5.70	-0.44	CHD4
3.15	-0.43	NOLC1
2.35	-0.43	PNISR
2.12	-0.42	XRCC5
1.88	-0.42	RAD50
1.96	-0.42	EEF1G
1.57	-0.41	HNRNPAB
2.86	-0.41	MTA2
2.24	-0.41	HDAC1
3.35	-0.41	CDC73
3.47	-0.41	ADNP
3.36	-0.39	PRPF31
1.91	-0.38	RBBP7
1.67	-0.37	ANAPC7
1.76	-0.37	SF3A2
3.50	-0.36	MRTO4
2.94	-0.36	NUSAP1
1.83	-0.36	LEO1
2.52	-0.35	RPS15
3.33	-0.35	HCFC1
3.73	-0.35	SRP14
1.99	-0.35	ZBTB33
1.93	-0.35	HTATSF1
1.74	-0.34	PIP5K1A;PIPSL

4.06	-0.34	MTA1
2.29	-0.34	RFC2
2.36	-0.34	TOX4
2.52	-0.34	NCBP1
2.48	-0.34	MKI67
1.81	-0.34	GATAD2B
1.82	-0.34	DDX5
2.05	-0.33	TRMT1L
2.34	-0.33	SART1
2.21	-0.33	SIN3B
2.00	-0.33	DDX23
2.67	-0.32	TBL2
2.01	-0.32	KIAA0020
2.21	-0.31	NOP16
2.82	-0.31	SNRPA1
3.10	-0.31	GRWD1
1.93	-0.31	SUDS3
1.96	-0.31	KPNA2
1.84	-0.30	RFC1
3.19	-0.30	SNRPB2
2.12	-0.30	SF3A3
1.89	-0.29	RFC3
2.36	-0.29	PRPF3
2.67	-0.29	RPS7
2.46	-0.28	ZC3H18
4.49	-0.28	BRD4
2.29	-0.28	TPR
2.84	-0.28	WDR43
2.23	-0.27	ALYREF
2.57	-0.27	BPTF
2.50	-0.26	SMARCB1
2.54	-0.25	WDR18
2.81	-0.25	CDC23
2.51	-0.24	CDK9
2.88	-0.24	ZCCHC8
4.75	-0.24	SF3A1
3.67	-0.24	OGT
2.89	-0.23	RPL9
4.50	-0.22	DIDO1

Supp. Table 1: **Overview of all significantly enriched putative RRM2 upstream regulators using CasID.**

Table S2.

Cell line	Origin	MYCN status	RRM2 status
IMR-32	Versteeg	Amp	N-Ampl
CLB-GA	Combaret	N-Ampl	N-Ampl
SK-N-AS	ATCC	N-Ampl	N-Ampl
UKF-NB-3	Speleman	Amp	No-information
SK-N-FI	Versteeg	N-Ampl	N-Ampl
SK-N-BE2(c)	Lunec	Amp	N-Ampl
SH-EP	Helen	N-Ampl	N-Ampl
NB-1	JHSF	Amp	N-Ampl
SH-SY5Y	Schulte	N-Ampl	Ampl
N206	Versteeg	Amp	Ampl
NIH3T3	Verfaillie	-	-

Supp. Table 2: Cell lines origin (Amp: amplified; N-Ampl: Not-amplified).

Table S3.

Products Tissue Culture	Obtained
RPMI 1640 medium	Life Technologies Europe
Pen Strep	ThermoFisher
L- Glutamine	ThermoFisher
N-2,100X	ThermoFisher
B-27,50X	ThermoFisher
DMEM F-12 Glutamax	Life Technologies Europe
DMEM/F-12 (1:1)	Life Technologies Europe
DMEM	Life Technologies Europe
B-mercaptoethanol	Sigma-Aldrich
MEM non-essential amino acids Solution (100X)	Life Technologies Europe
hygromycin B	Life Technologies Europe
CellTiter-Glo® reagent	Promega
384-well plates	Corning
96- well plates	Corning
T75 flask	VWR
T25 flask	VWR
PBS	Life Technologies Europe
Zeocin	Life Technologies Europe
blasticidin	Sigma-Aldrich

Accutase	Sigma-Aldrich
CellTiter-Glo 3D®	Promega
Caspase-Glo 3/7 Assay (10ml) ®	Promega
EGF	PeproTech
FGF-2	PeproTech
PDGF-AA	PeproTech
PDGF-BB	PeproTech
IGF-1	R&D Systems

Supp. **Table 3: Cell culture products origin.**

Table S4.

Products Western blot	**Obtained**
phosphatase inhibitor mixture	Roche Molecular Biochemicals
Pierce™ BCA Protein Assay Kit	Life Technologies Europe
β-mercaptoethanol	Sigma-Aldrich
SDS-PAGE gels	Pre-cast, Bio-Rad
10x Tris/glycine/SDS buffer	Biorad
nitrocellulose	Biorad
Immun-Blot PVDF Membrane, 26 cm x 3.3 m, 1 roll	Biorad
Tris/Glycine buffer	Biorad
methanol	Fisher Scientific
BSA	Sigma-Aldrich

Supp. Table 4: Western blots products origin.

Table S5.

Antibodies	**ID**	**Dilution**	**Obtained**
rabbit monoclonal antibody to RRM2 [EPR11820] (ab172476) 100µg	ab172476	1/2000	Abcam (Netherlands) B.V.
Phospho-Chk1 (Ser345) Antibody	MA5-15145	1/1000	Bioké BV
total CHK1 antibody	sc-8408	1/1000	Cell Signaling Technology
Anti-RPA32/RPA2 (phospho S33) antibody	ab211877	1/500	Abcam (Netherlands) B.V.
anti-RPA32 (total protein)	ab2175	1/1000	Abcam (Netherlands) B.V.

rabbit polyclonal antibody to gammma H2A.X	ab2893	1/2000	Abcam (Netherlands) B.V.
Monoclonal Anti-Vinculin antibody produced in mouse	V9131	1/10000	Sigma-Aldrich
ATR	2790S	1/1000	Cell Signaling Technology
phospho-ATR	2853S	1/750	Cell Signaling Technology
DNA-PK	ab168854	1/5000	Abcam (Netherlands) B.V.
phospho-DNA-PK	ab18192	1/5000	Abcam (Netherlands) B.V.
mouse anti-BrdU, clone B44	347580	1:100	BD Benelux NV
Rat monoclonal Anti-BrdU antibody [BU1/75 (ICR1)]	ab6326	1:50	Abcam (Netherlands) B.V.
MYCN	santa-cruz, sc-53993	1/2000	Bio-Connect
TH	P40101-150	1/5000	Gentaur Molecular Products
GFP	#2956	1/400	Cell Signalling, Bioké BV
Phospho-Chk1 (Ser296) Antibody	#2349p	1/1000	Cell Signaling Technology
p-4E-BP1(Thr37/46)	9459	1/50	Bioké BV (NETHERLANDS)
4E-BP1 (53H11)	9644S	1/50	Bioké BV (NETHERLANDS)

Supp. Table 5: Western blots products origin.

Table S6.

Primers	**Forward**	**Reverse**
RRM2	AGGACATTCAGCACTGGGAA	CCATAGAAACAGCGGGCTTC
CDKN1A	CCTCATCCCGTGTTCTCCTTT	GTACCACCCAGCGGACAAGT

RRM2B	CCTTGCGATGGATAGCAGAT	TCAGGCAAGCAAAGTCACAG
BAX	GATGCGTCCACCAAGAAGCT	CGGCCCCAGTTGAAGTTG
PUMA	GCAGGCACCTAATTGGGCT	ATCATGGGACTCCTGCCCTTA
NOXA	GCGCAAGAACGCTCAA	GTTCAGTTTGTCTCCAAATCTC
HEXIM1	TGACTCCGAGGCCAGTAAGT	GGCTCTGTTTCTCGTCGAAC
Hprt_F_mm	TGCTCGAGATGTCATGAAGG	TATGTCCCCCGTTGACTGAT
Ubc_F_mm	GCAGATCTTTGTGAAGACCC	GAAGGTACGTCTGTCTTCCT
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC
YWHAZ	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT
Hatn10	ACTAATGAAGACAGCAGAAGTCA	CAGTAAACATGTCAGGCTAAATAAT
Tdr7	GCAGCATAATTGAGTACACCC	TTGCCTATATTCACTGAGAAATGGA
Loopern4	TGAGCTGAAACTTTACAGACACAT	AGACTTTGGTGTCTCCAGAATG
zRrm2	TGTTTTACCAAACACTCCAAGC	CGACTGCCCCTCTGATTCAT
hMYCN	AGGACACCCTGAGCGATTC	AGGCATCGTTTGAGGATCAG
zMycn	AACAAGAGGGAGAATGCCAGC	CCTCGTCCGGGTAGAAACAC
eGFP	GACCACATGAAGCAGCAC	TTGTCGGCCATGATATAGAC
mCherry	CTGAAGGGCGAGATCAAGCA	TAGTCCTCGTTGTGGGAGGT
TH	GAACATGGCGGGAGGTCTAC	GGCTGTAGCCGCAATGTTTC
DBH	GCAGCTCTTTCCTGGTGACT	ACATGGGTATGGGGCTCTCT
RRM2 -PCR	GGACGCGTATGGGAAGGGTCGGAGGCATGGC	TACCTTGGATGCTTCTAACATATGGA

Supp. Table 6: Primers design.

Table S7.

Cell lines	3AP		
	IC50	IC30	IC15
IMR-32	689.5 nM	295.5 nM	122 nM
CLB-GA	955.1 nM	409.3 nM	169 nM
NIH3T3	928.5 nM	397.9 nM	163.9 nM
SK-N-BE(2)-C	3200 nM	1372 nM	564 nM

Supp. Table 7: IC50, 30 and 15 of 3AP for IMR-32, CLBGA, NIH3T3 and SK-N-BE-(2)C at 48 hours.

Table S8.

Zebrafish maintenance & generation of stable line	**Obtained**
d*b*h:RRM2	Invitrogen
RRM2-pDONR221, n°GC-Z9335	Genecopoiea

Supp. Table 8: Origin of products used for zebrafish maintenance.

Table S9.

Generation of RRM2 Overexpression inducible cell line	**Obtained**
Human RRM2, n° GC-Z9335-GS	Genecopoeia
pLVX-TRE3G-Zsgreen1 cat no: 631353	Takara
Lenti-X 293T Cells, Cat no: 632180	Takara
pLVX-pEF1a-Tet3G cat no: 631353	
Lenti-X Packaging Single Shots (VSV-G) cat No. 631275	
TetR monoclonal antibody (Clone 9G9) cat no. 631131	

Supp. Table 9: Origin of products used for plasmid construction.