Supplementary Information

Post-mitotic G1 phase survivin drives mitogen-independent cell divisions of B lymphocytes

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Material and Methods:

Mice

All experimental mice used were 8–12 weeks old C57BL/6J mice purchased from The Jackson Laboratory and were maintained in house facility at NIA, Baltimore. FUCCI mice (1) were provided by the RIKEN BRC through the National BioResource Project of the MEXT, Japan to Dr. Isabel Beerman (NIA, Baltimore) and were kindly gifted by her lab for the experiments. Mice were treated humanely in accordance with federal government guidelines. The protocol was approved by the Animal Care and Use Committee of the NIA Intramural Research Program.

B cell isolation and activation culture

Primary B cells were isolated using EasySep B cell kit (StemCell Technologies) according to manufacturer's protocol and were ≥95% pure based on CD19 staining. Purified B cells ($1.5x10^6$/ml) were cultured in c-RPMI (RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS (Gemini), 55μM β-mercaptoethanol (Sigma), 2mM L-glutamine and 100-IU penicillin and 100 μg/ml streptomycin (Gibco)) at humidified 37°C incubator. Phase-I cells were cultured in 100mm tissue culture dishes (Corning) while Phase-II cells were cultured in 24 well tissue culture plates (Corning).

For proliferation assays, B cells were labeled with cell trace dyes either Carboxyfluorescein succinimidyl ester (CFSE) or Cell Trace Violet (CTV) (Invitrogen) according to manufacturer protocol. These labeled or non-labeled cells were pulsed or continuous activated with goat anti-mouse IgM F(ab´)2 (Jackson Immunoresearch) as described earlier (2). After 6h hamster anti-mouse CD40 monoclonal antibody (BD Pharmingen) was added, and proliferation was measured by dilution of cell trace dye at different time points by flow cytometry. For other mitogenic stimulations, CD40 alone, 5µg/ml anti-mouse CD40 monoclonal antibody (BD Pharmingen) while LPS 5µg/ml or CpG (ODN) 50ng/ml (Invivogen) was added after CFSE or CTV labelling for indicated times. For washing of mitogen, 2 washes were performed by addition of 10X of complete RPMI (37°C).

Flow cytometry

For non-fixed cells live cells were identified using either TOPO3 (Invitrogen) or DAPI (Invitrogen) negative staining. Fixed live cells were identified by either forward and side scatter gates or by using fixable viability dye (FVD780) (BD Biosciences). Cells were counted using TC20 (BioRad) or Precision Counting Beads (Biolegend) according to manufacturer's protocol. Divided and non-divided B cells were sorted based on cell trace dyes levels using FACS Aria II or FACS Aria Fusion (BD Biosciences). Sort sensitivity was set to fine-tune, and post sort purity was consistently ≥95%. For Phase-II, cells were washed with warm complete RPMI after sorting, counted and plated ($1.5x10^6$/ml) in c-RPMI at humidified 37°C incubator for 1h to recover and then indicated treatments were performed.

Antibodies and reagents

Complete list of antibodies related to activation, flow cytometry, western blotting and CyTOF are listed in Supplementary Table 1. Inhibitors and other reagents are listed in Supplementary Table 2.

Cell cycle and intracellular staining analysis

Proliferating cells were pulse labelled for 1h or indicated times with EdU (Invitrogen) and Click-iT reaction was performed according to manufacturer's protocol to label incorporated EdU with Alexa Flour dyes. For flow cytometry analysis of intracellular proteins cells were fixed using 1.6% methanol free paraformaldehyde (Electron Microscopy Science, EMS) for 15min at room temperature and washed twice with PBS and resuspended in ice cold chilled 500µl perm-buffer III (BD Biosciences) and incubated for at least 30 minutes at 4°C or stored in -20°C till next step. After two washes cells were resuspended in 100µl saponin buffer (Invitrogen) and primary antibody was added with optimal concentration and incubated at 4°C for 1h. For non-conjugated primary antibodies, fluorochrome labeled secondary antibody were used according to manufacturer's protocol. For cell cycle analysis using DNA content, fixed cells were incubated for 30 min at 37°C in staining buffer prepared of saponin buffer (Invitrogen) containing DAPI (1µg/ml) (Sigma) and RNAse-A (10µg/ml) (Invitrogen).

For CyTOF staining, proliferating B cell cultures were pulsed for 1h labelled with IdU (10µM) (Sigma) for S phase cell identification at indicated time points. After harvesting at indicated time, for dead cell staining, cultured B cells were resuspended in 5ml of Cisplatin (50µM) (Sigma) in PBS for precisely 60s and quenched immediately with 2X volume of complete RPMI. Cells were further washed 2x with stain buffer (BD Bioscience) and fixed with 1.6% final concentration of methanol free paraformaldehyde for 15 minutes at room temperature. After washing cell pellets

were stored in 2% BSA in PBS at -20°C till staining with surface and intracellular antibodies was performed according to previously described method (3).

All measurements for flow cytometry were carried out using FACS CantoII or FACS Aria Fusion (BD Biosciences) and CyTOF acquisition was performed on CyTOF^{TM} mass cytometer (DVS Sciences, Toronto, Canada). Data was analyzed using Cytobank (tSNE and SPADE) (https://cytobank.org/) or FlowJo software (Tree Star).

Western blotting

Whole cell extracts (WCE) were made using RIPA buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific). Proteins were separated by electrophoresis through 4-12% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane using iBLOT2 (Thermo Scientific). After blocking with 5% BSA in Tris HCl-0.05% Tween (TBST) for 1h at room temperature, membranes were incubated with primary antibodies overnight at 4°C, washed in TBST, and incubated for 1h with horseradish peroxidase (HRP)–coupled secondary antibodies (Cell Signaling). Blots were developed using the enhanced chemiluminescence (ECL) systems (Pierce) and captured by GeneSys Syngene or LI-COR Odyssey XF Imaging System.

RNA-seq and gene expression analysis

Two independent biological replicates each consisting pools of 4-5 mice were used for Phase-I RNA-studies (Fig S4A). For Phase-II, divided (div12) B cells were further cultured in presence of 0.12 µg/ml of anti-CD40 for next 48h and further FACS sorted in divided (div12(48)d) and non-

divided (div12(48)u) cells. Total RNA from $1x10^6$ (Phase-I) and $5x10^5$ (Phase-II) cells was isolated using Qiazol (Qiagen) kit according to manufacturer's protocol. RNA-sequencing of total RNA was performed at the Johns Hopkins Deep Sequencing and Microarray Core facility using standard protocol for NextSeq 500 sequencer. Briefly, ribosomal RNA was depleted from 100ng of total RNA and barcoded library was made and 50bp single end reads were generated from each sample. Reads were analyzed using online server Galaxy RNA-seq pipeline (usegalaxy.org). Adapter trimmed sequences were aligned to mouse genome (mm10) using HISAT2 (4). Estimation of transcripts was done using Salmon (5) algorithm (TPM) and featureCounts (6) and htseq-count (7) were used for differential gene expression analysis using DESeq2 (8). Gene ontologies and PID analysis were performed using ToppFun (9) suite online server at ToppGene (https://toppgene.cchmc.org/). For PID weight score calculation, following equation was used:

$$Weight = \frac{\text{the number of genes enriched in Phase (I or II)from a PID}}{total \text{ number of genes in PID}} x100$$

Inhibitor experiments

Two independent biological replicates each consisting pools of 2-3 mice were used for CDK inhibitor experiments. After sorting cells from Phase-I into divided (div12) and non-divided (div0) B cells were cultured in presence of various CDK inhibitors or DMSO for Phase-II (next 48h). During Phase-II divided div12 B cells cultures were having only medium while div0 cells were supplemented with anti-IgM (10µg/ml) for re-stimulation. Inhibitors and their doses are listed in Supplementary Table 2. Proliferation was measured by CFSE dilution and cell cycle was analyzed using DNA content analysis by DAPI or TOPRO-3 staining.

For survivin inhibition experiments two independent biological replicates each consisting pools of 3 mice were used. Survivin inhibitor, LLP3 (Sigma) and S12 (Selleckchem) dissolved in DMSO was added at indicated dosages and times.

FUCCI-BJAB cell line preparation and sorting experiments

BJAB cells were transduced with pBOB-EF1-FastFUCCI-Puro (10) and after puromycin selection for 4 days, single cell clones were isolated using FACS and propagated for further experiments. We termed this cell line as FUCCI-BJAB cells. Cell cycle stages were identified as preciously described (10) and freshly divided cells (no color) and G2M cells (high green fluorescence) were sorted using FACS and re-cultured in pre-warm complete RPMI. Survivin inhibitors were added to the cultures at indicated dosages and were analysed using flow cytometer for cell cycle progression.

Supplementary Table S1: List of reagents, inhibitors, mitogens and other chemicals.

Reagents	Cat#	Vendor
Cisplatin	P4394	Millipore-Sigma
Paraformaldehyede	15710	Electron Microscopy Science
Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit	C10419	Thermo Fisher Scientific
Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit	C10425	Thermo Fisher Scientific
IdU	I7125	Millipore-Sigma
DAPI	D1306	Thermo Fisher Scientific
Phosphoflow Perm Buffer III	558050	BD Biosciences
Perm/wash Buffer	554723	BD Biosciences
4-(3,5-Bis(benzyloxy)phenyl)-6-(5-chloro-2-hydroxyphenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile, LLP3	SML0991	Millipore-Sigma
Palbociclib (PD0332991) Isethionate, CDK 4/6 inhibitor	S1579	Selleck Chemicals
Cdk1/2 Inhibitor III - CAS 443798-55-8 CDK 2/1 inhibitor	217714	Millipore-Sigma
Lipopolysaccharide from Escherichia coli 0111:B4	tlrl-3pelps	Invivogen
ODN 2088 (CpG oligonucleotide)	tlrl-2088-1	Invivogen
RPMI 1640 Medium	11875093	Thermo Fisher Scientific
Fetal Bovine Serum (FBS) heat inactivated	100-106	Gemini Bio-products
Penicillin-Streptomycin-Glutamine (100X)	10378016	Thermo Fisher Scientific
QIAzol Lysis Reagent	79306	Qiagen
miRNeasy Mini Kit	217004	Qiagen
RNase-Free DNase Set	79254	Qiagen
Trypan Blue solution	T8154	Milipore Sigma
Precision Count Beads	424902	Biolegend

Supplementary Table 2:

List of functional antibodies used in experiments, flow cytometry antibodies, primary and secondary antibodies used for western blotting and the CyTOF antibodies and their conjugates.

Target Protein	Clone	Vendor	Concentration (ug/ml)	Element	Isotope
AffiniPure F(ab') Fragment Goat Anti-Mouse IgM, µ chain specific	Polyclonal	Jackson Immunoresearch	varies (5-10ug/ml)		
anti-CD40	HM-40	BD	varies (125ng/ml-5ug/ml)		
anti-CD40	FGK	Novus Biologicals	varies (125ng/ml-5ug/ml)		
CD19	6D5	Biolegend	1:200		
pRb (S807/811)	D20B12	CST	1:500		
cMyc	D84C12	CST	1:200		
pCDK2 (Thr160)	Thr160	CST	1:50		
PLK1 Antibody	OTI1D4	Novus Biologicals	1:100		
Ki-67	D3B5	CST	1:50		
Survivin	71G4B7	CST	1:500		
Rabbit IgG (isotype)	DA1E	CST	1:500		
anti-rabbit IgG Fab2 PE		CST	1:1000		
anti-rabbit IgG Fab2 AF-647		CST	1:500		
p27	SX53G8.5	CST	1:1000		
CDC20	D6C2Q	CST	1:1000		
Aurora B/AIM1	Polyclonal	CST	1:1000		
CENP-A	C5H3	CST	1:1000		
b-actin	AC-74	Sigma	1:5000		
anti-rabbit IgG-HRP		SANTA CRUZ BIOTECHNOLOGY	1:2000		
anti-mouse IgG-HRP		SANTA CRUZ BIOTECHNOLOGY	1:2000		
CD45	30-F11	Biolegend	3	In	115
pSTAT3	4	BD	4	La	139
IgD	11-26c.2a	BD	2	Ce	140
B220	RA3-6B2	BD	0.5	Pr	141
pHH3	HTA28	Biolegend	4	Nd	142
pMAPKAPK2	27B7	CST	2	Nd	144
pCREB	87G3	CST	3	Nd	145
pPLCg2	K86-689.37	BD	0.25	Nd	146
pSTAT1	4a	BD	4	Nd	150
pErk	D13.14.4E	CST	6	Eu	151
Cyclin B1	GNS-1	Fluidigm	1ul	Eu	153
CD27	LG.3A10	Biolegend	0.5	Sm	154
CD3	17A2	BD	2	Gd	157
pAKT	J1-223.371	BD	0.375	Gd	158
pSTAT5	47	BD	4	Tb	159
p4EBP1	236B4	CST	2	Dy	162
IkB	L35A5	CST	3	Dy	163
pRb	J112-906	BD	1.5	Dy	164
Ki67	SolA15	eBioscience	3	Ho	165
pSrc	K98-37	BD	3	Er	167
p-p38	36/p38	BD	3	Er	168
pNFkB	K10-895.12.50	BD	4	Tm	169
pSyk/ZAP70	17a	BD	3	Er	170
cleaved PARP	F21-852	BD	4	Yb	171
pS6	N7-548	Fluidigm	1ul	Yb	172
CD19	6D5	Biolegend	1.5	Yb	173
IgM	RMM-1	Biolegend	2.25	Yb	174
CD23	B3B4	BD	1.5	Lu	175
MHC II	M5/114.15.2	Biolegend	0.1875	Yb	176

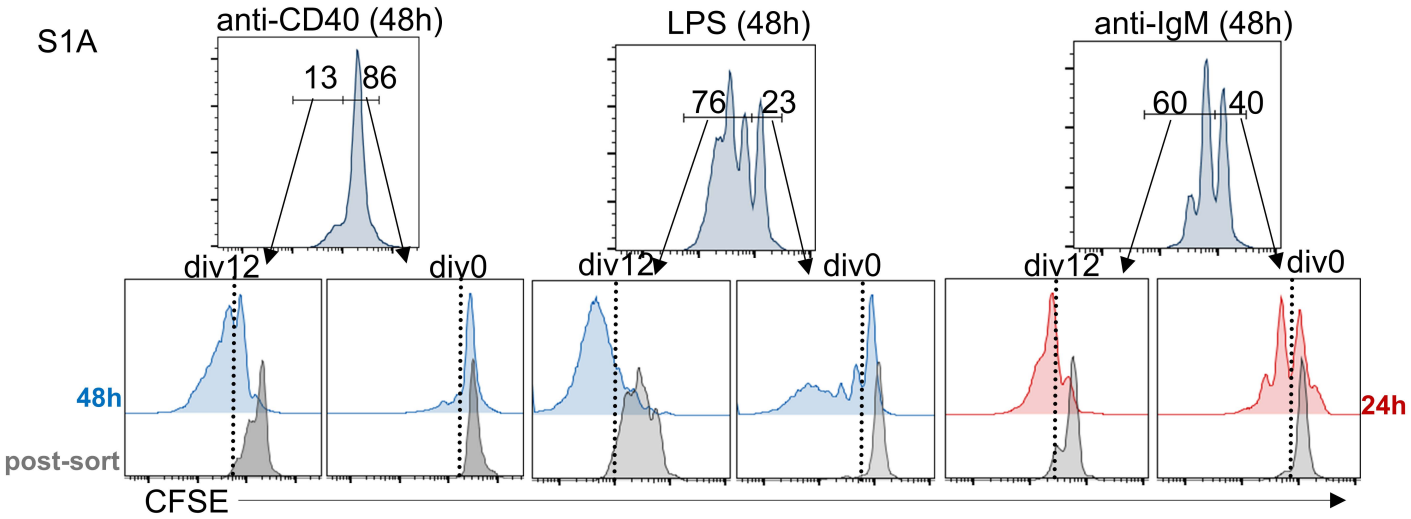
Supplementary Material Methods References:

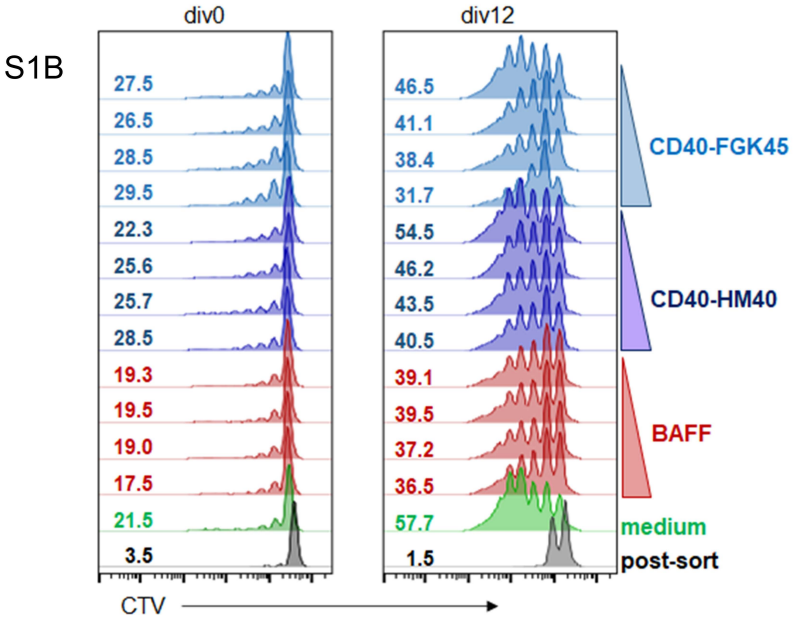
1. A. Sakaue-Sawano *et al.*, Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* **132**, 487-498 (2008).
2. B. Damdinsuren *et al.*, Single round of antigen receptor signaling programs naive B cells to receive T cell help. *Immunity* **32**, 355-366 (2010).
3. G. K. Behbehani, S. C. Bendall, M. R. Clutter, W. J. Fantl, G. P. Nolan, Single-cell mass cytometry adapted to measurements of the cell cycle. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* **81**, 552-566 (2012).
4. D. Kim, B. Langmead, S. L. Salzberg, HISAT: a fast spliced aligner with low memory requirements. *Nature methods* **12**, 357-360 (2015).
5. R. Patro, G. Duggal, M. I. Love, R. A. Irizarry, C. Kingsford, Salmon provides fast and bias-aware quantification of transcript expression. *Nature methods* **14**, 417-419 (2017).
6. Y. Liao, G. K. Smyth, W. Shi, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics (Oxford, England)* **30**, 923-930 (2014).
7. S. Anders, P. T. Pyl, W. Huber, HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)* **31**, 166-169 (2015).
8. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).
9. J. Chen, E. E. Bardes, B. J. Aronow, A. G. Jegga, ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic acids research* **37**, W305-311 (2009).
10. S. B. Koh *et al.*, A quantitative FastFUCCI assay defines cell cycle dynamics at a single-cell level. *Journal of cell science* **130**, 512-520 (2017).

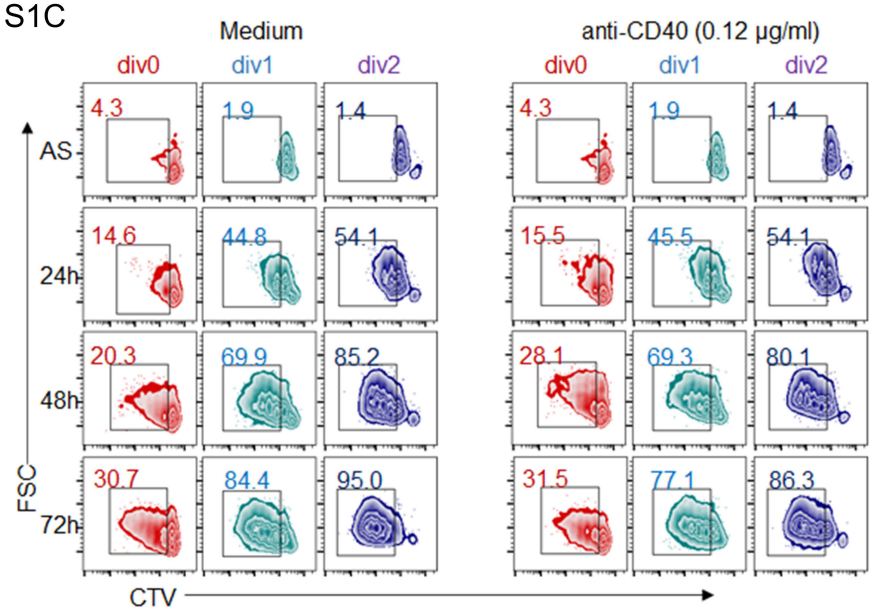
Supplementary figure S1:

Effect of different mitogenic stimulus leading to first mitotic division and subsequently leading to mitogen-independent B cell proliferation. CFSE labelled splenic B cells were activated for 48h with indicated mitogens (anti-CD40 (5µg/ml), LPS (5µg/ml) and anti-IgM Fab'2 (10µg/ml)). Divided (div12) and non-divided (div0) B cells were sorted using FACS and further cultured without any mitogen for next 48h. Proliferation was readout as CFSE dilution measured using flow cytometry.

A) Representative histograms of mitogen-dependent proliferation profile of indicated mitogen at 48h (top). Gates within represent the divided and non-divided B cells. CFSE profiles after post-sort and 48h later for anti-CD40 or LPS activated and post-sort and 24h for anti-IgM activated B cells (bottom). Dotted horizontal line represents distinction between divided and non-divided B cells.
B) Influence of survival signals from BAFF and anti-CD40 antibody on mitogen independent B cell proliferation. div0 and div12 B cells were sorted as Figure 1C, and further cultured for 48h without any stimulus (medium) or varying dose of BAFF (100-10ng) or two different clones (HM40 and FGK45) of anti-CD40 antibody (5-0.12µg/ml). Histograms representing CTV dilution at 48h post-sort of div0 (left) and div12 (right) with indicated stimulus on right (bold letters). Numbers represent the percentage of divided B cells during 48h after sorting.
C) Change in size (forward scatter, "FSC") during mitogen-independent B cell proliferation. Zebra-plot showing FSC and CTV dilution for no-stimulation (left, "medium") or anti-CD40 (right, "anti-CD40 0.12 µg/ml") after 72h post sort from B cells isolated as Figure 1C. Numbers represent the percentage of divided B cells.


S1A
anti-CD40 (48h)
13
86
LPS (48h)
76
23
anti-IgM (48h)
60
40
div12
div0
div12
div0
div12
div0
48h
post-sort
24h
CFSE


S1B
div0
div12
27.5
26.5
28.5
29.5
22.3
25.6
25.7
28.5
19.3
19.5
19.0
17.5
21.5
3.5
46.5
41.1
38.4
31.7
54.5
46.2
43.5
40.5
39.1
39.5
37.2
36.5
57.7
1.5
CD40-FGK45
CD40-HM40
BAFF
medium
post-sort
CTV


S1C
Medium
anti-CD40 (0.12 µg/ml)
div0
div1
div2
div0
div1
div2
AS
24h
48h
72h
FSC
4.3
1.9
1.4
14.6
44.8
54.1
20.3
69.9
85.2
30.7
84.4
95.0
4.3
1.9
1.4
15.5
45.5
54.1
28.1
69.3
80.1
31.5
77.1
86.3
CTV

Supplementary figure S2:

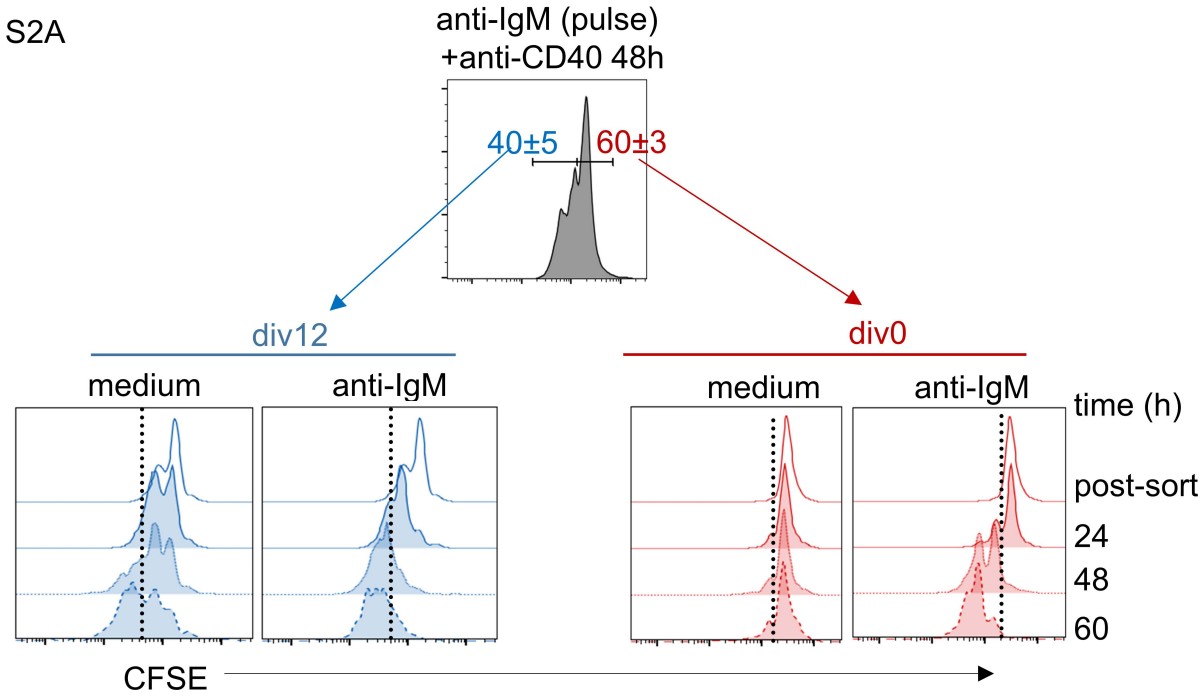
A) Non-divided B cells are not refractory to proliferation. Divided (div12) and non-divided (div0) B cells were sorted as Figure 1C and cultured further with (anti-IgM) or without stimulus (medium) for 60h. Representative histogram representing CFSE dilution with indicated time post-sort. Horizontal dotted line indicates distinction between divided and non-divided B cells.

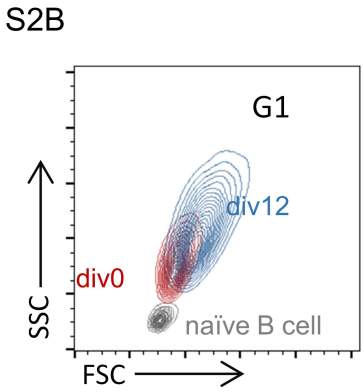
B) Activated yet not divided (div0) cells are bigger than their precursor naive B cells and divided B cells (div12) are even bigger than non-divided (div0). Contour plot representing forward scatter (size, FSC) and side scatter (granularity, SSC) from the G1 phase of naïve (resting), activated but non-divided div0 or divided div12.

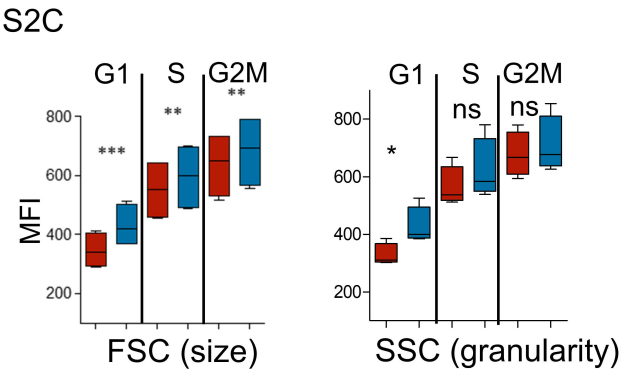
C) Quantification of part B for div0 and div12 from 5 independent experiments. Forward scatter (left) and side scatter (right) are shown by box and whisker plot demonstrating the mean fluoresce intensity (MFI) and error bars SEM with paired t-test. ***=$p<0.0001$, **=$p<0.001$, *=$p<0.05$ and ns=not significant.

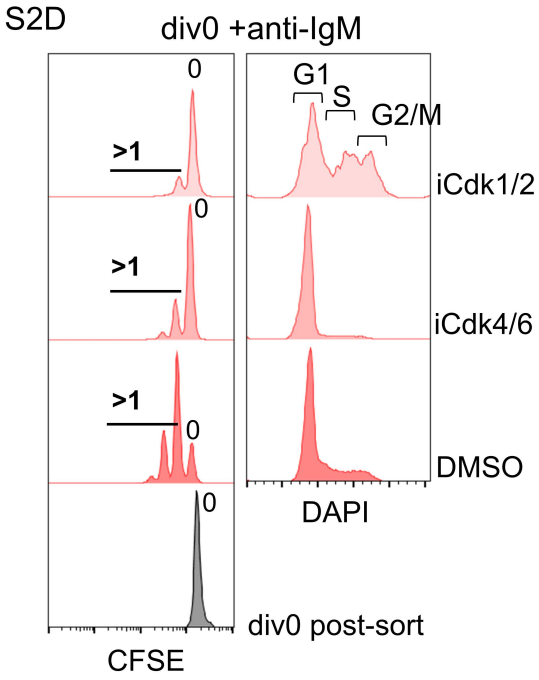
D) Representative histograms of cell division (left) and cell cycle stages (right) measured after 48h culture of purified div0 cells re-stimulated with anti-IgM in the presence of no inhibitor (labelled DMSO) or CDK inhibitors as indicated. Cell division numbers are indicated within CFSE profiles.

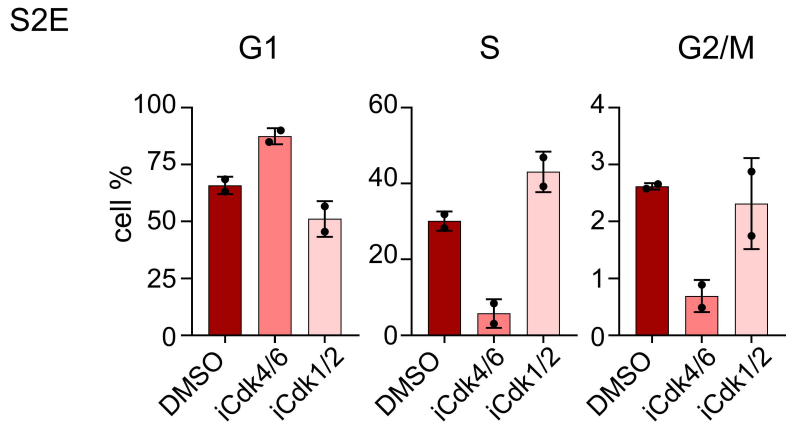
E) Two independent inhibitor experiments from part D were quantified to identify percentage of cells present in each stage of the cell cycle. Error bars represent SEM (unpaired t-test).


S2A
anti-IgM (pulse)
+anti-CD40 48h
40±5
60±3
div12
div0
medium
anti-IgM
medium
anti-IgM
time (h)
post-sort
24
48
60
CFSE


S2B
G1
div12
div0
naïve B cell
SSC
FSC


S2C
G1
S
G2M
MFI
FSC (size)
SSC (granularity)
ns
ns


S2D
div0 +anti-IgM
G1
S
G2/M
>1
iCdk1/2
iCdk4/6
DMSO
DAPI
div0 post-sort
CFSE


S2E
G1
S
G2/M
cell %
DMSO
iCdk4/6
iCdk1/2

Supplementary figure S3:

A) Parameters used during CyTOF analysis. Parameters in italics were used in tSNE and SPADE analysis.

B) Gating strategy on SPADE plots for identification of various stages of cell cycles. First row, mitotic cell (M-phase) nodes identified by high levels of pH3, second row, G2 phase cell nodes identified by high levels of Cyclin B1 and third row, shows S phase cell nodes as identified by high levels of IdU. Absence of all these marks is demonstrated in forth row as G0/G1 cells.

C) Nodes in SPADE plots representing S phase cells during mitogen-independent proliferation if Phase-II for div0 (left) and div12 (right).

D) SPADE plots for indicated markers during Phase-I (left 3 columns) and Phase-II (right 2 columns)

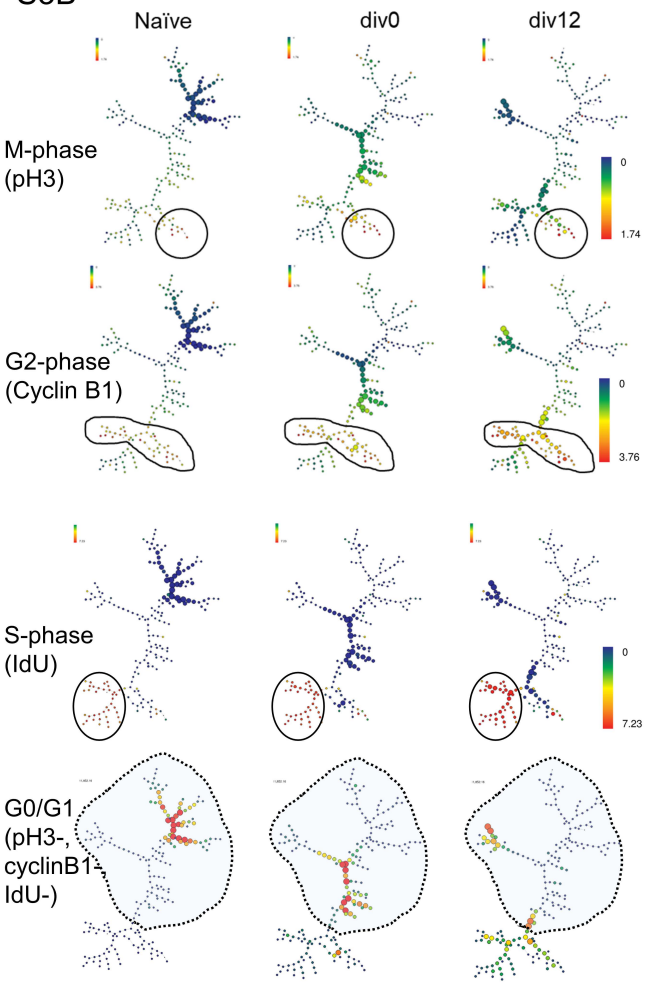
E) SPADE plots representing CFSE levels during Phase-II mitogen-independent proliferation.

F) Analysis of signaling proteins in SPADE clusters of Phase-II. Heatmap representation from nodes (n=4) of divided (div12(48), blue) and undivided (div0(48), red) B cells is shown. Hierarchical cluster analysis was carried out as in part Fig 4D.

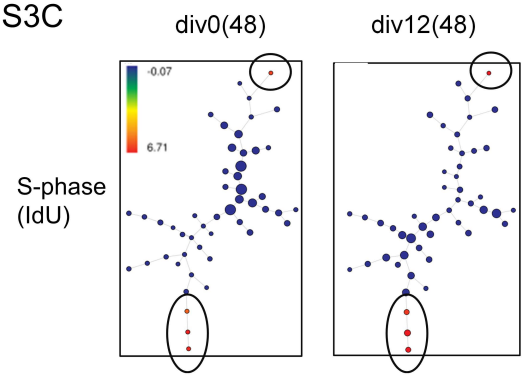
S3A

Gating (9)	Cell cycle (5)	Signaling (18)		Others (7)
CD19	pRb	p4EBP1	pCREB	IgM
B220	CyclinB1	IkBa	pPLCg2	IgD
CD3	IdU	pSrc	pSTAT5	MHCII
Cisplatin	Ki67	pp38	pSTAT1	CD23
DNA1	pHH3	pSyk	pNFkB	CD27
DNA2		pErk	cPARP	CD45
Event Length		pAkt	pS6	CFSE for phase II
Time		pSTAT3		
Bead Density		pMAPKAPK2		

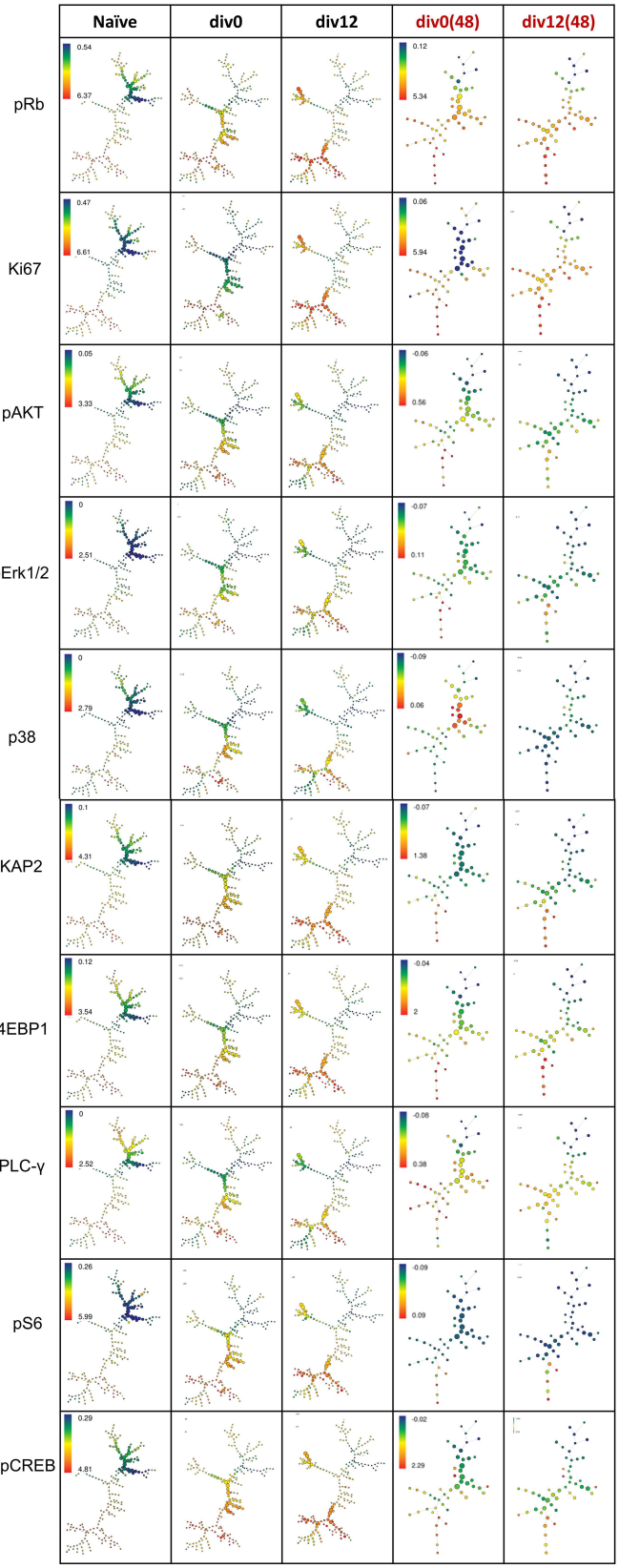
S3B



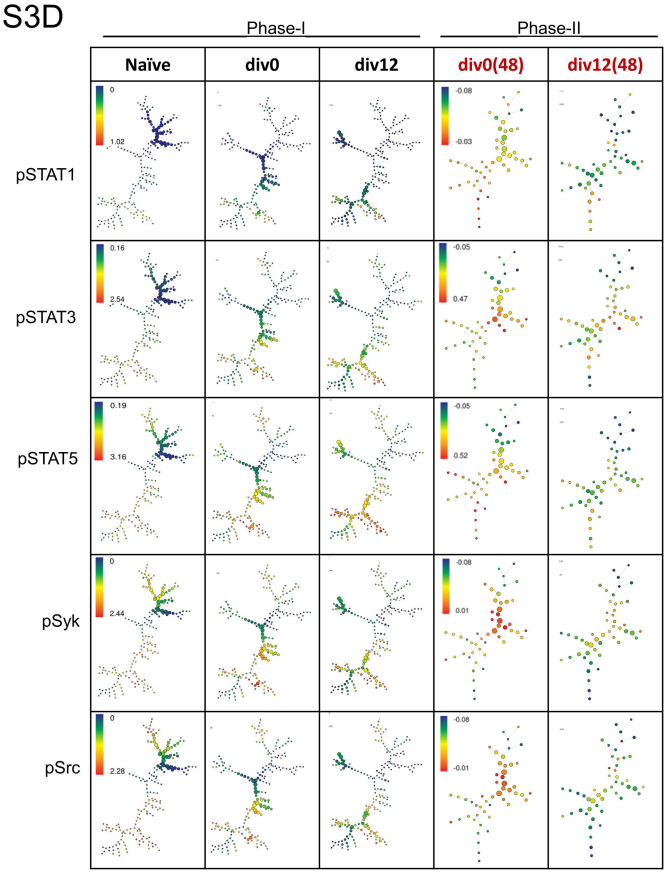
S3C



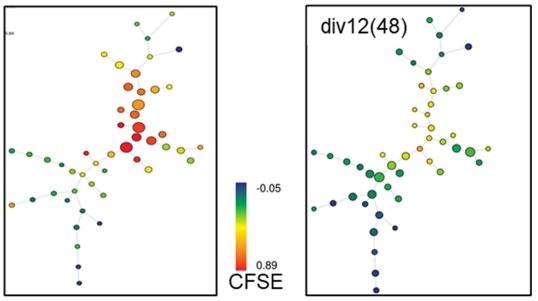
S3D



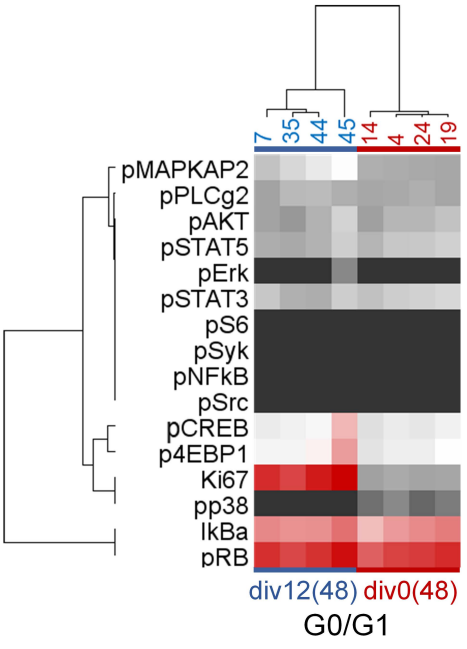
S3D


Phase-I
Phase-II
Naïve
div0
div12
div0(48)
div12(48)
pSTAT1
pSTAT3
pSTAT5
pSyk
pSrc

S3E


div12(48)
-0.05
0.89
CFSE

S3F


7
35
44
45
14
4
24
19
pMAPKAP2
pPLCg2
pAKT
pSTAT5
pErk
pSTAT3
pS6
pSyk
pNFkB
pSrc
pCREB
p4EBP1
Ki67
pp38
IkBa
pRB
div12(48)
div0(48)
G0/G1

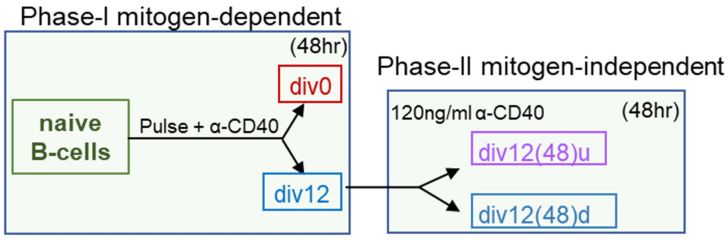
Supplementary figure S4:

A) Schema of RNA seq experiment, 3 populations form Phase-I (naïve, div0 and div12) and 2 populations from Phase-II (div12(48)u and div12(48)d) were analyzed for their transcriptome analysis using RNA-seq.

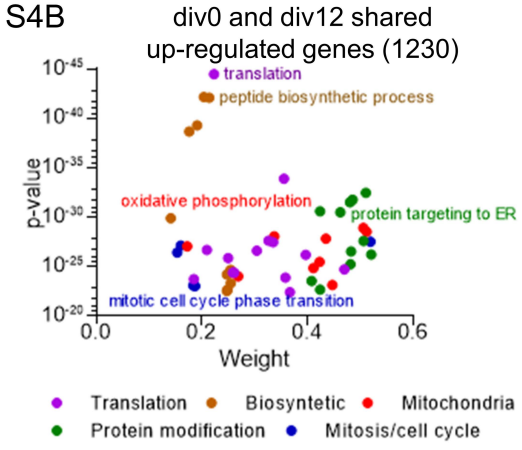
B-G) Scatter plots representing enriched Biological Process identified by GO analysis using TOPP gene for indicated comparisons. Plots demonstrated p-value and weight of corresponding biological process. Similar biological process is represented by same color and representative process is shown on the graphs.

H-J) Pathway Interaction Database (PID) networks for indicated network. Phase-I nodes are circled red while Phase-II by blue color.

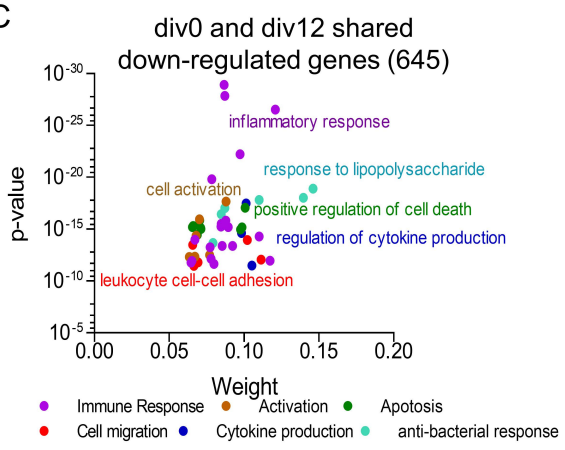
S4A



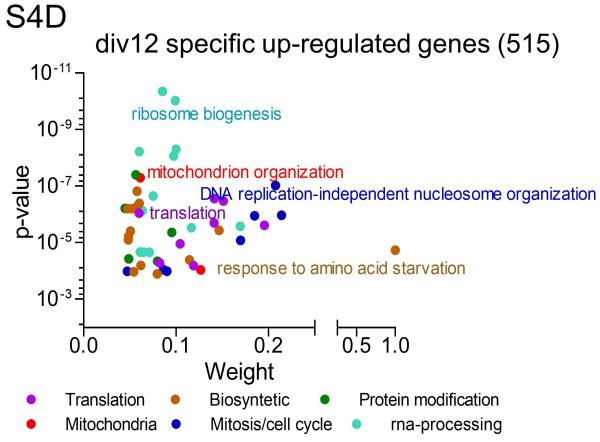
S4B



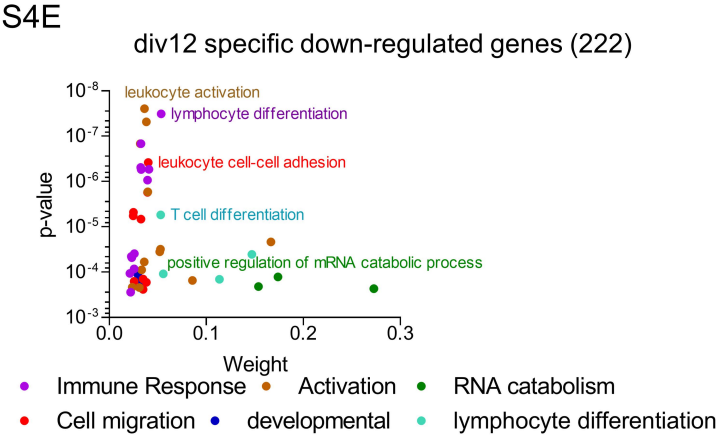
S4C



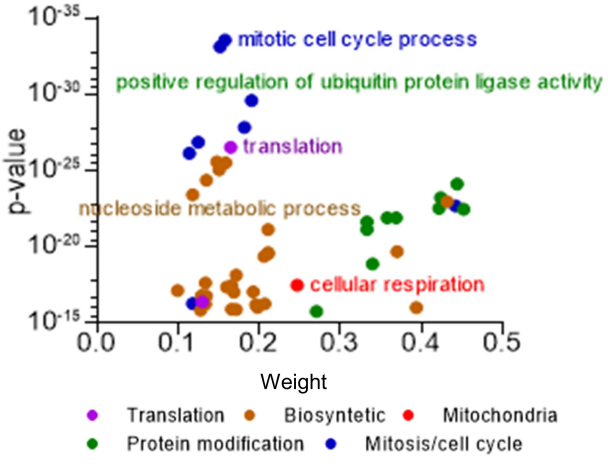
S4D



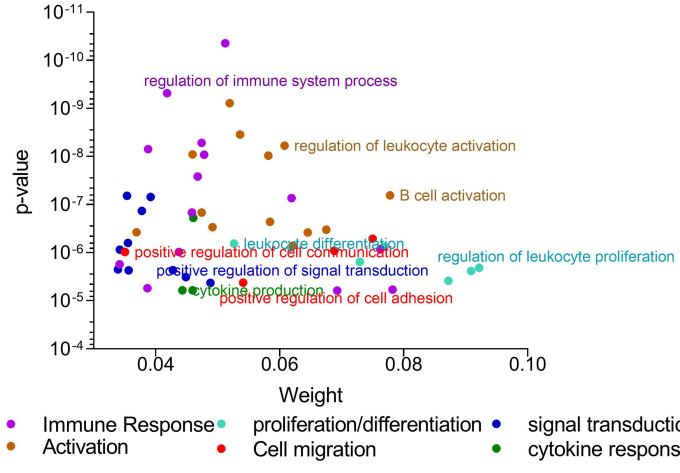
S4E



S4F



S4G

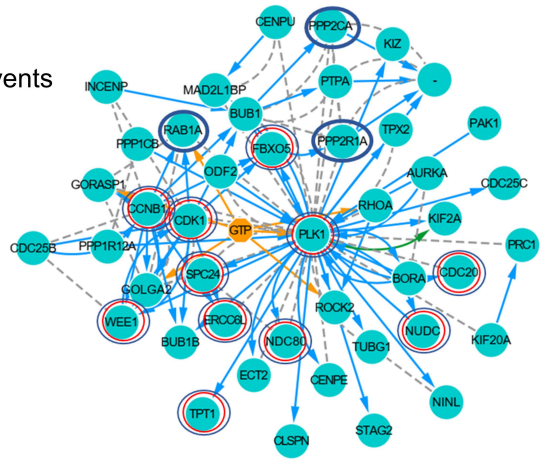


S4H

PLK1 signaling events

Phase-I

Phase-II

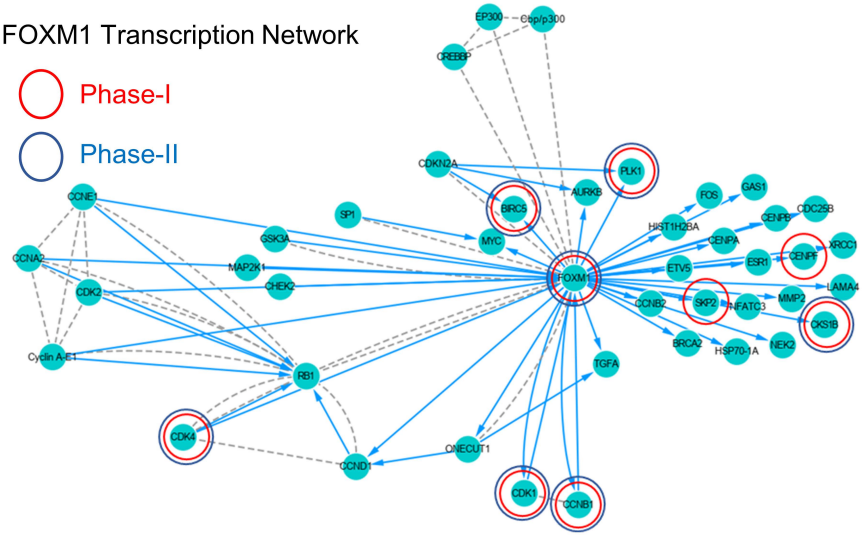


S4I

FOXM1 Transcription Network

Phase-I

Phase-II

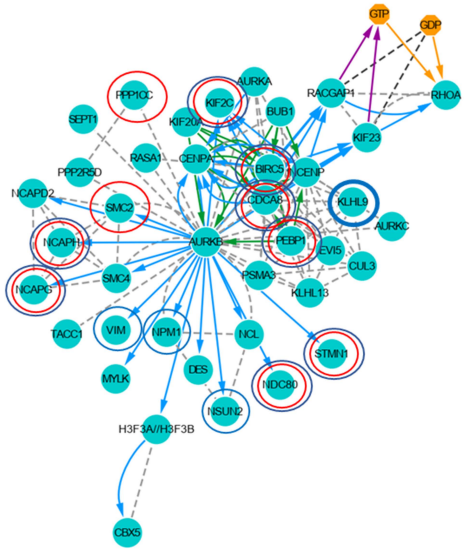


S4J

Aurora B signaling

Phase-I

Phase-II



Supplementary figure S5:

A) Highly expressed proteins in Phase-I G2/M cluster (n=4 nodes) identified by CyTOF (Fig S3D) and their relative expression in G1 phase of div12 and div0 clusters (n=4 nodes). MHCII expression is taken as control.

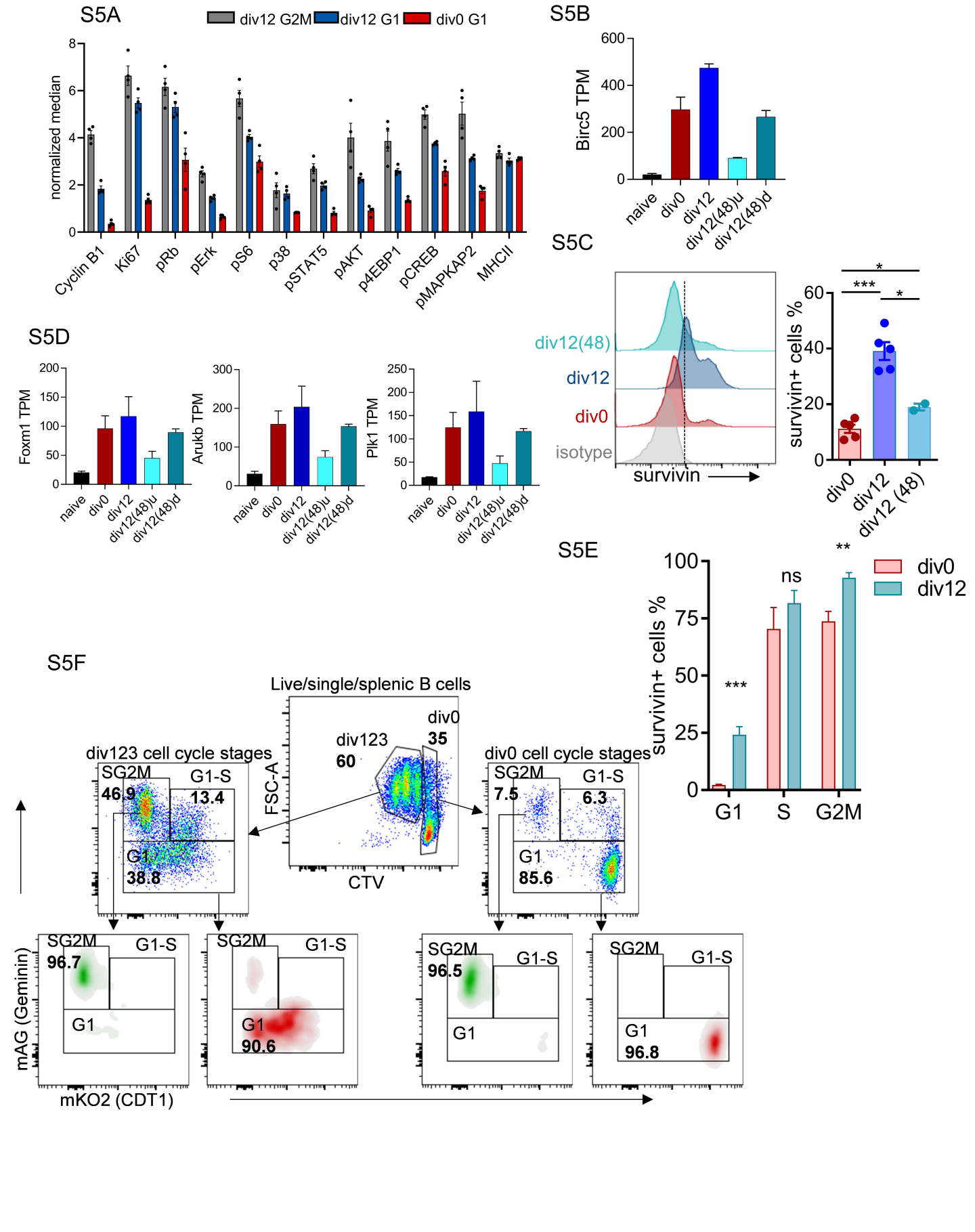
B) Bar graphs representing TPM values of *Birc5* from two biological replicates calculated using Salmon algorithm.

C) Survivin protein levels were assayed by flow cytometry. A representative histogram pattern is shown on the left. Div0 and div12 cells were obtained after 48h mitogenic activation as in A; div12(48) represents total cell population present after culture of div12 cells for 48h in the absence of mitogen. Right panel show the proportion of cells with survivin expression in different cell populations obtained from multiple experiments. Bars represent mean±SEM (n=5 for div0 and div12 (paired t-test) and n=2 for div12(48) (unpaired t-test))

D) *Foxm1, Aurkb* and *Plk1* mRNA tracks from two biological replicates from indicated cell populations (top). Bar graphs representing TPM values for *Foxm1, Aurkb* and *Plk1* from two biological replicates calculated using Salmon algorithm.

E) Quantification of cell cycle phase specific survivin expression from Fig 6B (n=3).

F) Gating strategy and post sort purity of G1 and SG2M phase B cells. CTV labelled splenic B cells were stimulated with LPS for 48h and divided and non-divided cells were sorted according to div123 and div0 gates. Cell cycle stages were identified using red (G1) and green (SG2M) fluorescence and gated accordingly and FACS sorted. Density plots shows post sort purity and these cells were further used for making WCE.


S5A
div12 G2M
div12 G1
div0 G1
normalized median
Cyclin B1
Ki67
pRb
pErk
pS6
p38
pSTAT5
pAKT
p4EBP1
pCREB
pMAPKAP2
MHCII
S5B
Birc5 TPM
naive
div0
div12
div12(48)u
div12(48)d
S5C
div12(48)
div12
div0
isotype
survivin
survivin+ cells %
S5D
Foxm1 TPM
Arukb TPM
Plk1 TPM
S5E
survivin+ cells %
ns
G1
S
G2M
S5F
Live/single/splenic B cells
div123 cell cycle stages
div0 cell cycle stages
FSC-A
CTV
SG2M
G1-S
G1
mAG (Geminin)
mKO2 (CDT1)

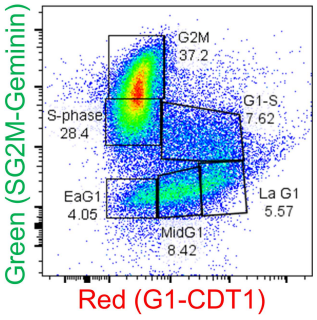
Supplementary figure S6:

A) Cell cycle stages identified by green and red fluorescence in FUCCI-BJAB cell line. Various gates identify different stages of cell cycle EaG1- Early G1 phase (no-color), MidG1 - Middle of G1 phase (dim red fluorescence), LaG1- Late G1 (bright red fluorescence), G1-S transitional cells identified by yellow fluorescence (loss of red and gain of green), S-phase (dim green) and G2/M (bright green fluorescence).

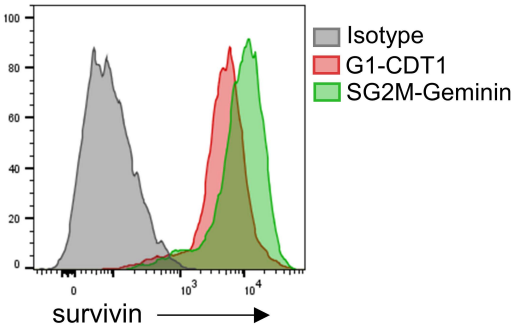
B) Cell cycle stage specific survivin expression in FUCCI-BJAB cell line, identified by intracellular staining of survivin.

C) G2/M and G1 specific inhibition of survivin with S12 and LLP3. FUCCI-BJAB cells were sorted in two different population based on the bright green fluorescence (G2/M stage) and no-color freshly divided cells, early G1 cells (EaG1). Cells were further cultured for 18 hours in presence of indicated dosages of survivin inhibitors, S12 or LLP3 and vehicle (DMSO). Cell cycle progression was observed using flow cytometry for both G2/M and EaG1 sorted FUCCI-BJAB cells. Representative FACS plots from two independent experiments are shown, upper panel (G2/M) and lower panel (G1). Major gates identify cell cycle stages as identified in Fig S6A, for G2/M populations mitotic block (M-Block) population is identified as double positive for green and red. Boxed dot plots show the optimum inhibitor dose. LLP3 treated cells have high autofluorescence and gates were adjusted accordingly.

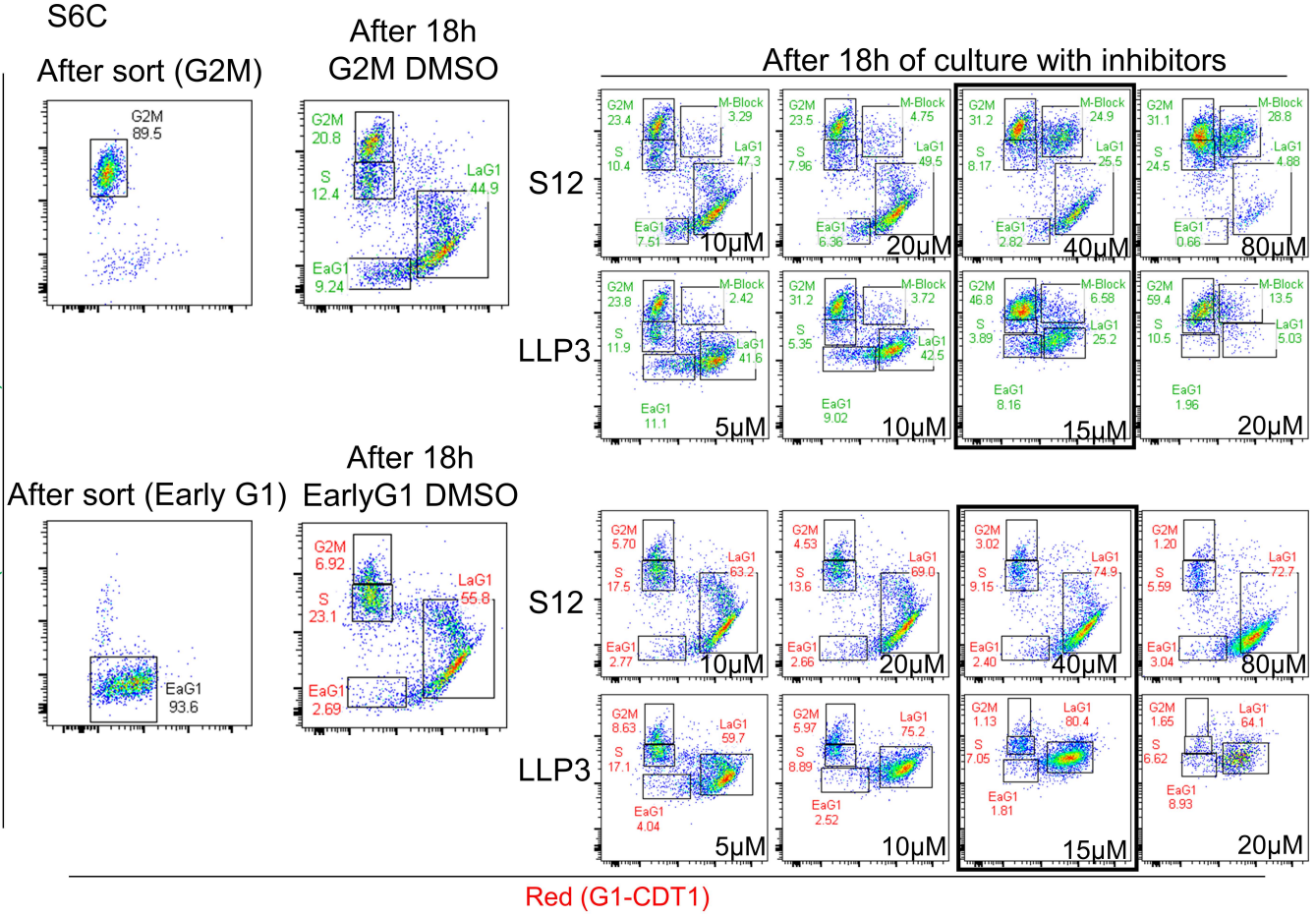
S6A


G2M
37.2
S-phase
28.4
G1-S
7.62
EaG1
4.05
La G1
5.57
MidG1
8.42
Green (SG2M-Geminin)
Red (G1-CDT1)

S6B


Isotype
G1-CDT1
SG2M-Geminin
survivin

S6C


After sort (G2M)
After 18h
G2M DMSO
After 18h of culture with inhibitors
S12
LLP3
After sort (Early G1)
After 18h
EarlyG1 DMSO
5μM
10μM
15μM
20μM
40μM
80μM
Green (SG2M-Geminin)
Red (G1-CDT1)

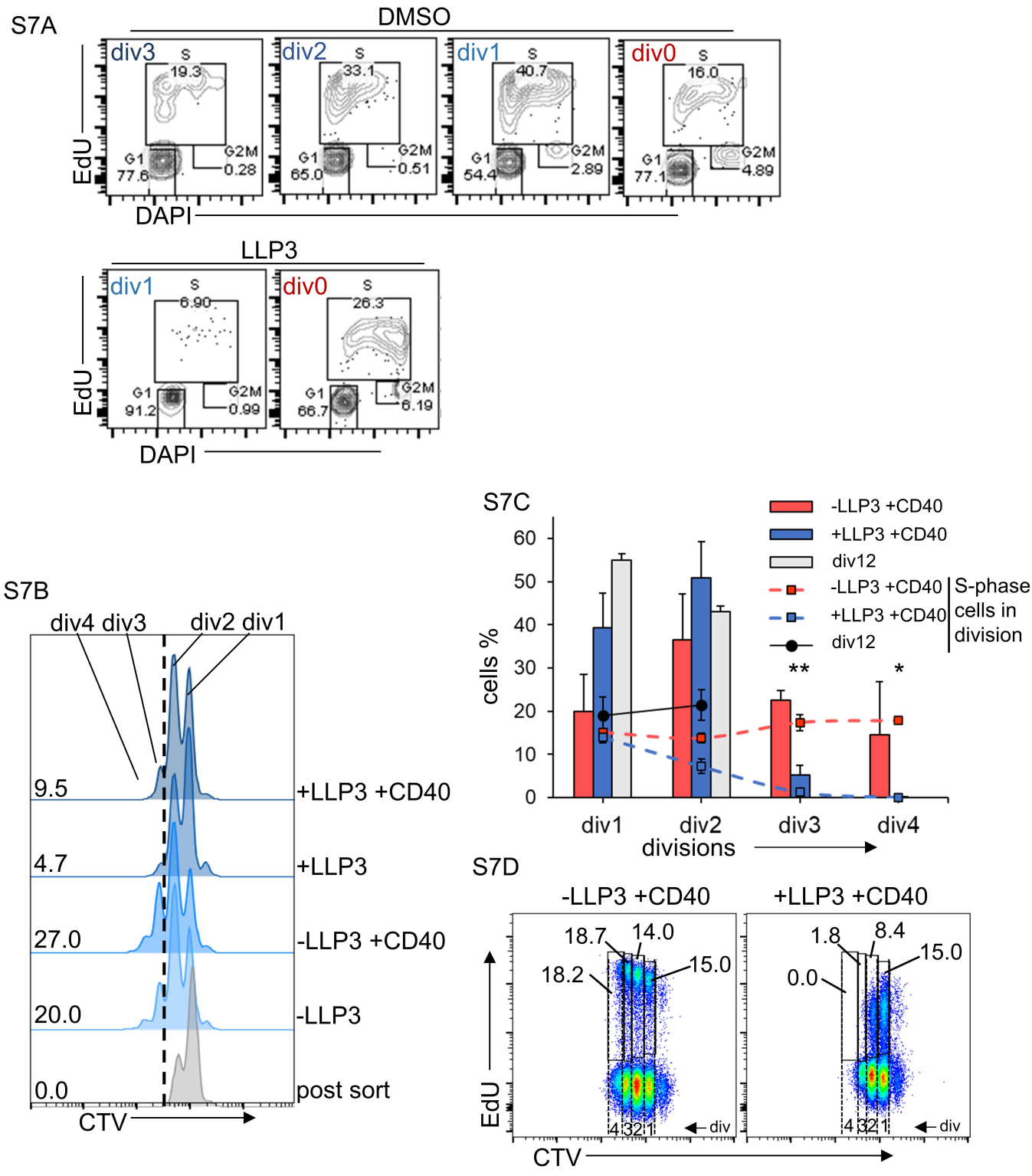
Supplementary figure S7:

A) Cell cycle analysis demonstrating G1, S and G2/M phase from each division of DMSO (top panel) and LLP3 (lower panel) treated cells as in Fig 7A.

B) After Phase I (Figure 4A), sorted div12 cells (that had undergone 1or 2 divisions) were cultured (Phase II) with survivin inhibitors. Cell division was determined by CFSE or CTV dilution and cell-cycle stages were determined by pulse labeling cells with EdU prior to flow cytometric analysis. Div12 cells were further cultured in medium alone or with LLP3 (15μM), half the cultures contained non-mitogenic anti-CD40 antibody (0.12μg/ml). Proliferation was assessed after 36h culture by CTV dilution. Representative histograms showing CTV dilution for indicated treatments (right); numbers within plots represent the percentage of newly divided div3 or div4 (dotted line) cells in each population.

C) Quantification of two independent experiments of Fig. S7B; bars represent the percent of cells in each division. Starting post-sort population is shown in grey and population of cells after 36h culture without and with LLP3 are shown as red and blue bars, respectively. Dashed lines represent percentage of S phase cells in each division quantified from two experiments as described in part Fig. S7D.

D) Effect of LLP3 on G1 to S progression in mitogen-independent B cells proliferation. After 36h culture, div12 B cells were pulse-labelled with EdU for 1h and stained using Click-iT reaction before flow cytometry. Representative dot-plot showing EdU-positive S phase cells and CTV dilution of cells in the absence or presence of LLP3 supplemented with non-mitogenic dose of anti-CD40 antibody.


S7A
DMSO
div3
S
19.3
G1
77.6
G2M
0.28
div2
S
33.1
G1
65.0
G2M
0.51
div1
S
40.7
G1
54.4
G2M
2.89
div0
S
16.0
G1
77.1
G2M
4.89
EdU
DAPI
LLP3
div1
S
6.90
G1
91.2
G2M
0.99
div0
S
26.3
G1
66.7
G2M
6.19
EdU
DAPI
S7B
div4 div3
div2 div1
9.5
+LLP3 +CD40
4.7
+LLP3
27.0
-LLP3 +CD40
20.0
-LLP3
0.0
post sort
CTV
S7C
-LLP3 +CD40
+LLP3 +CD40
div12
-LLP3 +CD40
+LLP3 +CD40
div12
S-phase cells in division
cells %
60
50
40
30
20
10
0
**
*
div1
div2
div3
div4
divisions
S7D
-LLP3 +CD40
+LLP3 +CD40
18.7
14.0
18.2
15.0
1.8
8.4
0.0
15.0
4 3 2 1
div
4 3 2 1
div
EdU
CTV