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 succinimidy 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™ 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:

\[
\text{Weight} = \frac{\text{the number of genes enriched in Phase (I or II) from a PID}}{\text{total number of genes in PID}} \times 100
\]

**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 previously 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               |
| Paraformaldehyde                                          | 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)                                    | S1579 | Selleck Chemicals             |
| Isethionate, CDK 4/6 inhibitor                             |       |                               |
| Cdk1/2 Inhibitor III - CAS 443798-55-8 CDK 2/1 inhibitor   | 217714| Millipore-Sigma               |
| Lipopolysaccharide from Escherichia coli 0111:B4           | tlr-3pelps | Invivogen                |
| ODN 2088 (CpG oligonucleotide)                             | tlr-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                        |
| miRNasy 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 (µg/ml) | Element | Isotope |
|------------------------|---------|-------------------------|-----------------------|---------|---------|
| AffiniPure F(ab')      | Polyclonal | Jackson Immunoresearch | varies (5-10µg/ml)    |         |         |
| Fragment Goat Anti-Mouse IgM, µ chain specific |         |                         |                       |         |         |
| anti-CD40               | HM-40   | BD                      | varies (125ng/ml-5µg/ml) |         |         |
| anti-CD40               | FGK     | Novus Biologicals       | varies (125ng/ml-5µg/ml) |         |         |
| CD19                   | 6D5     | Biolegend               | 1:200                 |         |         |
| pRb (S807/S811)        | D20B12  | CST                     | 1:500                 |         |         |
| cMyc                   | D84C12  | CST                     | 1:200                 |         |         |
| pCDK2 (Thr160)         | Thr160  | CST                     | 1:50                  |         |         |
| PLK1 Antibody          | OT11D4  | Novus Biologicals       | 1:100                 |         |         |
| Ki-67                  | D38B    | CST                     | 1:50                  |         |         |
| Survivin               | 71G4B7  | CST                     | 1:500                 |         |         |
| Rabbit IgG (isotype)   | DA1E    | CST                     | 1:500                 |         |         |
| anti-rabbit IgG Fab2 PE |         |                         |                       |         |         |
| p27                    | SX53G8.5| CST                     | 1:1000                |         |         |
| pCD20                  | D6C2Q   | CST                     | 1:1000                |         |         |
| Aurora B/AIM1          | Polyclonal |                        | 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                  | 155     |         |
| pSTAT3                 | 4 BD    | BD                      | 2 La                  | 139     |         |
| pSTAT5                 | 47 BD   | BD                      | 4 Tb                  | 159     |         |
| p4EBP1                 | 236B4   | CST                     | 2 Dy                  | 162     |         |
| pAKT                   | J1-223.371 BD | 0.375 Gd              | 0.375 Gd              | 158     |         |
| pS6                    | N7-548  | Fluidigm                | 1ul                   | 153     |         |
| MHC II                 | M5/114.15.2 | Biolegend             | 0.1875 Yb             | 176     |         |
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**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.
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).
**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.
|                | Phase-I                  | Phase-II                 |
|----------------|--------------------------|--------------------------|
|                | Naïve | div0 | div12 | div0(48) | div12(48) |
| pSTAT1         | ![Graph](image)          | ![Graph](image)          | ![Graph](image) | ![Graph](image) | ![Graph](image) |
| pSTAT3         | ![Graph](image)          | ![Graph](image)          | ![Graph](image) | ![Graph](image) | ![Graph](image) |
| pSTAT5         | ![Graph](image)          | ![Graph](image)          | ![Graph](image) | ![Graph](image) | ![Graph](image) |
| pSyk           | ![Graph](image)          | ![Graph](image)          | ![Graph](image) | ![Graph](image) | ![Graph](image) |
| pSrc           | ![Graph](image)          | ![Graph](image)          | ![Graph](image) | ![Graph](image) | ![Graph](image) |

**S3E**

- `div12(48)`

**S3F**

- Heatmap: `pMAPKAP2`, `pPLCg2`, `pAKT`, `pSTAT5`, `pErk`, `pSTAT3`, `pS6`, `pSyk`, `pNFkB`, `pSrc`, `pCREB`, `p4EBP1`, `Ki67`, `pp38`, `IkkBa`, `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.
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.
**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.
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.
