## Supplementary Information

# MicroRNA-17~92 Plays a Causative Role in Lymphomagenesis by Coordinating Multiple Oncogenic Pathways

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**Inventory of Supplementary Information**

**Supplementary data**



**Supplementary materials and methods**

**Supplementary references**

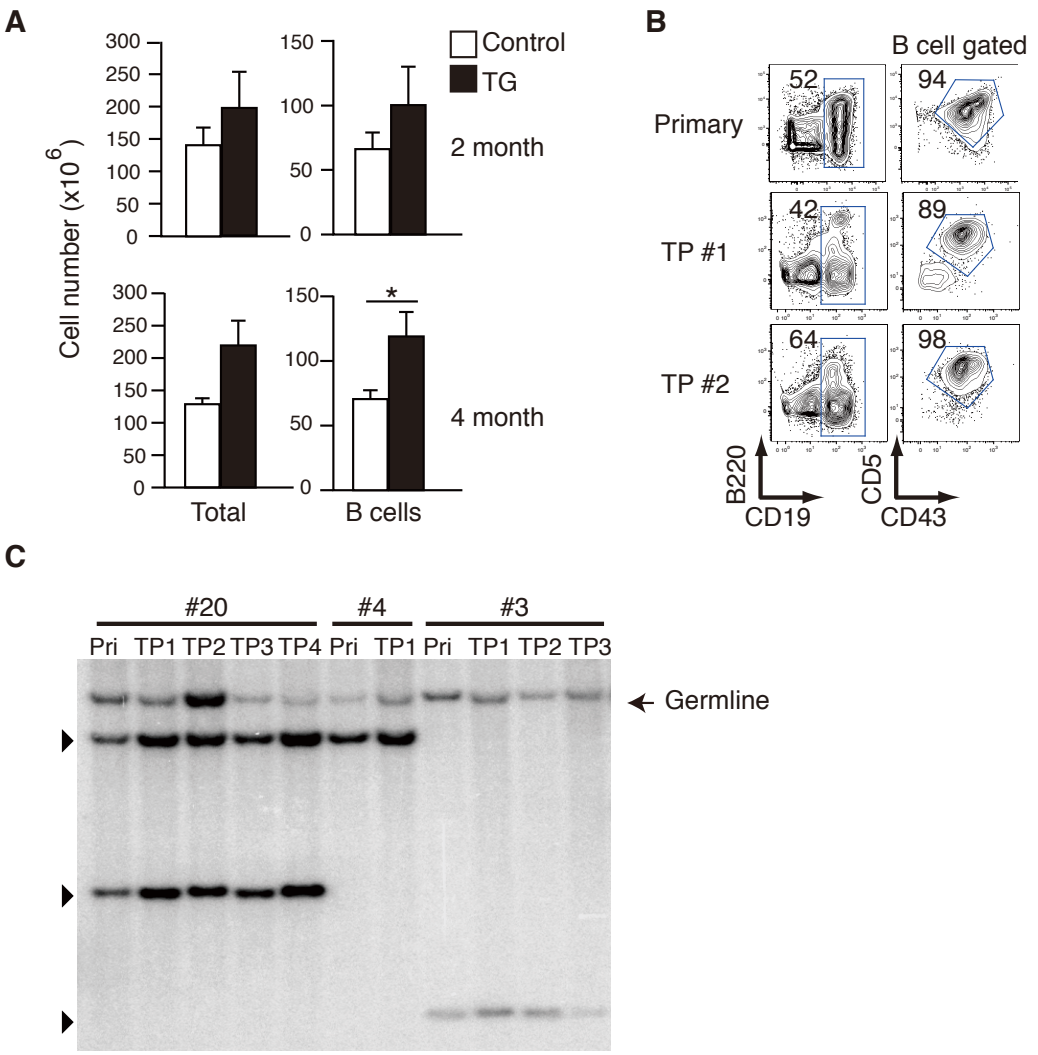


**Figure S1. Cell number, immunophenotype, and lymphoma clonality analysis in TG mice and transplant recipients.**

(**A**) Numbers of total splenocytes and B cells in mice of indicated ages.

(**B**) Representative immunophenotype of primary lymphoma and secondary lymphoma in transplant recipient mice (TP).

(**C**) Southern blot analysis of tumor clonality using the $J_H4$ probe of IgH locus. Arrowheads indicate clonal bands corresponding to VDJ or DJ rearrangements. Note that tumors developed in recipient mice show the same clonal bands as primary tumor (Pri). #20, #4, and #3 indicate IDs of lymphoma-bearing TG mice.

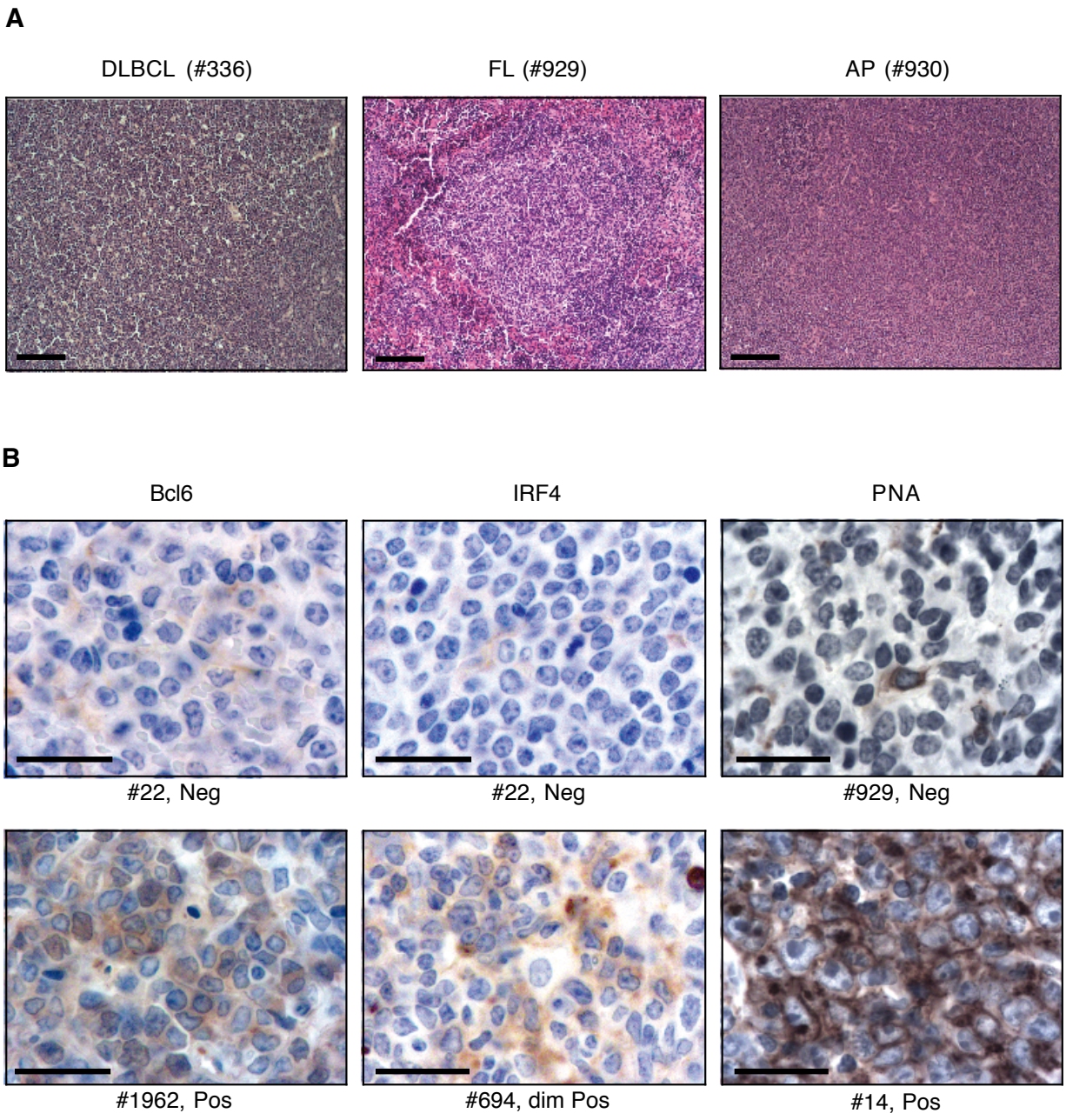


**Figure S2**. **Histological analysis of lymphoma in TG mice.**
(**A**) Haematoxylin and Eosin (H&E) staining of lymphoma tissues in TG mice. Scale bars, 100 μm. Note the nodular structure in follicular lymphoma (FL), and sheets of lymphoid cells in diffuse large B-cell lymphoma (DLBCL) and anaplastic plasmacytoma (AP).
(**B**) Representative immunohistochemical staining of Bcl6, IRF4, and PNA. Neg, negative; dim Pos, dim positive; Pos; positive. Scale bars, 20μm. Numbers indicate IDs of mice bearing primary lymphomas.

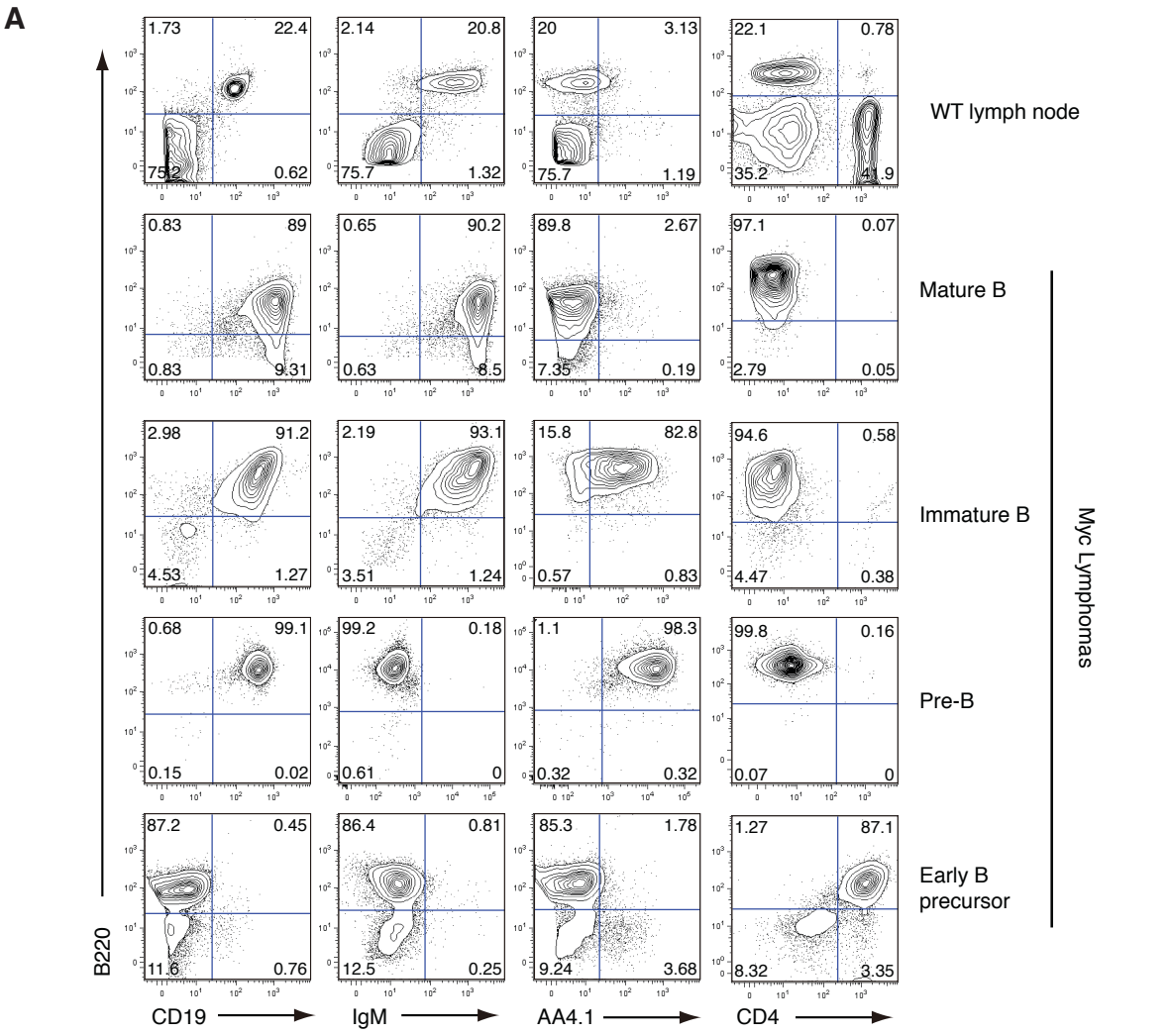
A
1.73
22.4
75.2
0.62
2.14
20.8
75.7
1.32
20
3.13
75.7
1.19
22.1
0.78
35.2
41.9
WT lymph node
0.83
89
0.83
9.31
0.65
90.2
0.63
8.5
89.8
2.67
7.35
0.19
97.1
0.07
2.79
0.05
Mature B
2.98
91.2
4.53
1.27
2.19
93.1
3.51
1.24
15.8
82.8
0.57
0.83
94.6
0.58
4.47
0.38
Immature B
0.68
99.1
0.15
0.02
99.2
0.18
0.61
0
1.1
98.3
0.32
0.32
99.8
0.16
0.07
0
Pre-B
87.2
0.45
11.6
0.76
86.4
0.81
12.5
0.25
85.3
1.78
9.24
3.68
1.27
87.1
8.32
3.35
Early B precursor
Myc Lymphomas
B220
CD19
IgM
AA4.1
CD4
B
Myc; miR fl/fl lymphomas
Myc; miR fl/fl; CD19Cre lymphomas
Imm
Pre-B
Imm
Imm
EP
EP
EP
EP
EP
EP
EP
EP
EP/Pre-B
Germline

**Figure S3. Immunophenotype and clonality analysis of lymphomas developed in Myc;miR$^{fl/fl}$ and Myc;miR$^{fl/fl}$;CD19Cre mice.**

(**A**) Immunophenotypes of representative lymphoma cases. Lymph node cells from WT mouse were used as control.

(**B**) Southern blot analysis of tumor clonality using the $J_H4$ probe of IgH locus. Arrowheads indicate clonal bands corresponding to VDJ or DJ rearrangements. Imm, immature B cells. EP, early B-lymphocyte precursors.

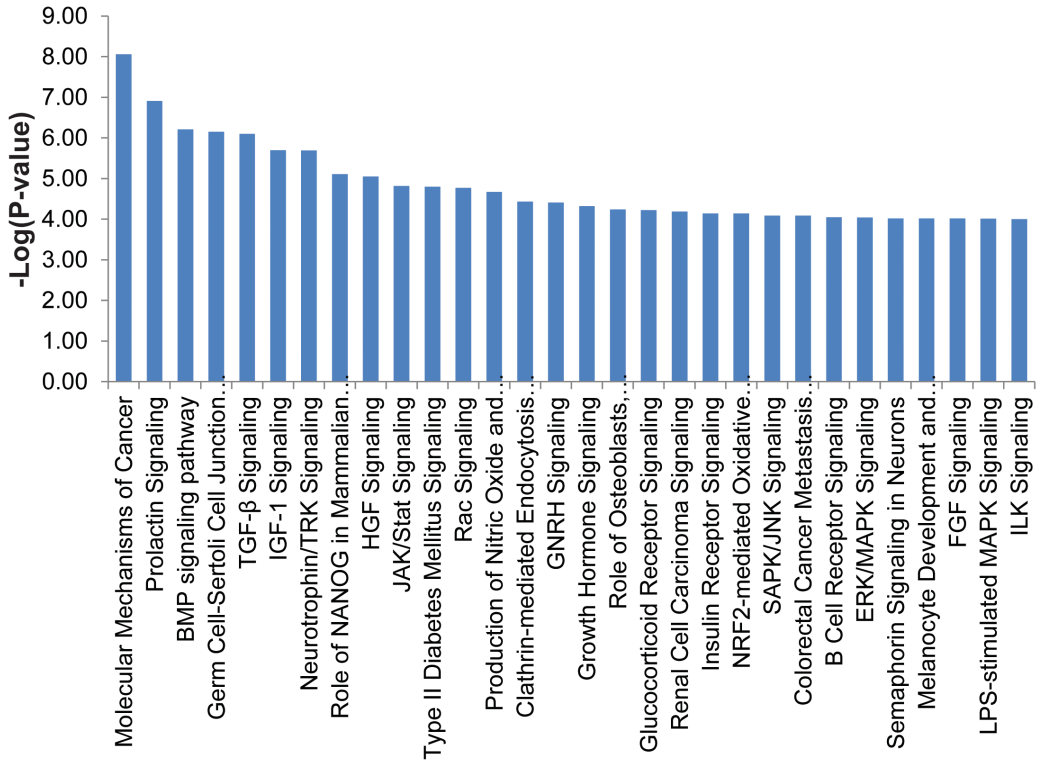


**Figure S4**. **Pathway analysis of miR-17~92 target genes.**
The 868 PAR-CLIP identified miR-17~92 target genes were analyzed using Ingenuity Pathway Analysis software. Pathways with the Fisher's Exact test significance values less than 0.0001 (P-value<0.0001) were plotted.

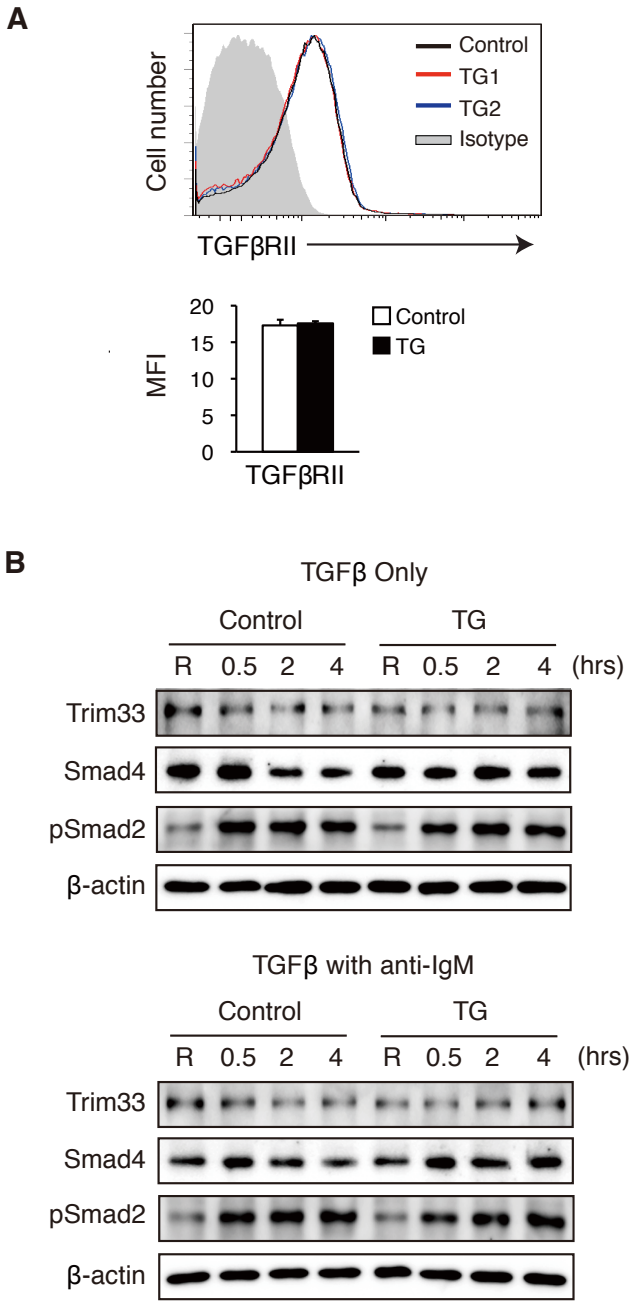


**Figure S5. TGFβ signaling in TG B cells.**
(**A**) Cell surface TGFβRII expression levels on control and TG B2 cells were analyzed by flow cytometry. Bar graphs represent TGFβRII mean fluorescence intensity (MFI) (n=4 for each group).
(**B**) Western blot analysis of Trim33 and phospho-Smad2 in B2 cells activated *in vitro* with indicated stimulants (anti-IgM, 2 μg/ml; TGFβ, 5ng/ml). R, rested in B cell medium for 3 hrs without stimulation.

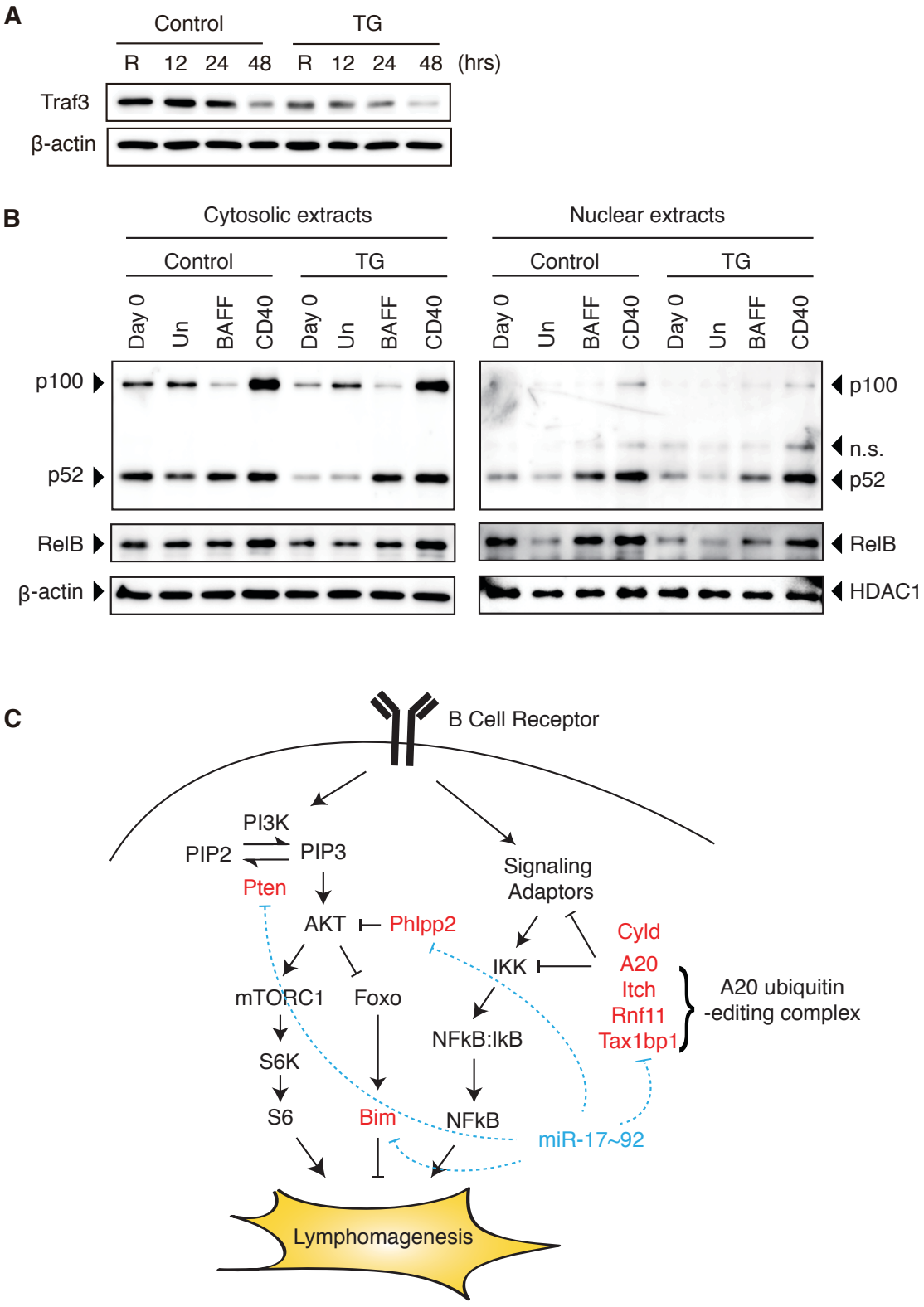
A
Control
TG
R 12 24 48 R 12 24 48 (hrs)
Traf3
β-actin
B
Cytosolic extracts
Nuclear extracts
Control
TG
Day 0
Un
BAFF
CD40
p100
p52
RelB
β-actin
n.s.
HDAC1
C
B Cell Receptor
PI3K
PIP2
PIP3
Pten
AKT
Phlpp2
Signaling Adaptors
Cyld
A20
Itch
Rnf11
Tax1bp1
IKK
A20 ubiquitin -editing complex
mTORC1
Foxo
NFkB:IkB
S6K
S6
Bim
NFkB
miR-17~92
Lymphomagenesis

**Figure S6. The alternative NFκB pathway in TG B cells.**

(**A**) Western blot analysis of Traf3 in B2 cells stimulated with 2 μg/ml anti-IgM for indicated time. R, rested in B cell medium for 3 hrs without stimulation.

(**B**) Western blot analysis of p52 and RelB in B2 cells stimulated with BAFF (100 ng/ml) or anti-CD40 (2 μg/ml) for 24 hrs in vitro. Day 0, freshly purified B2 cells without resting; Un, rested in B cell medium for 24 hrs without stimulation. n.s., non-specific signal.

(**C**) Schematic depiction of oncogenic pathways regulated by miR-17~92. Red indicates miR-17~92 target genes validated in this study.

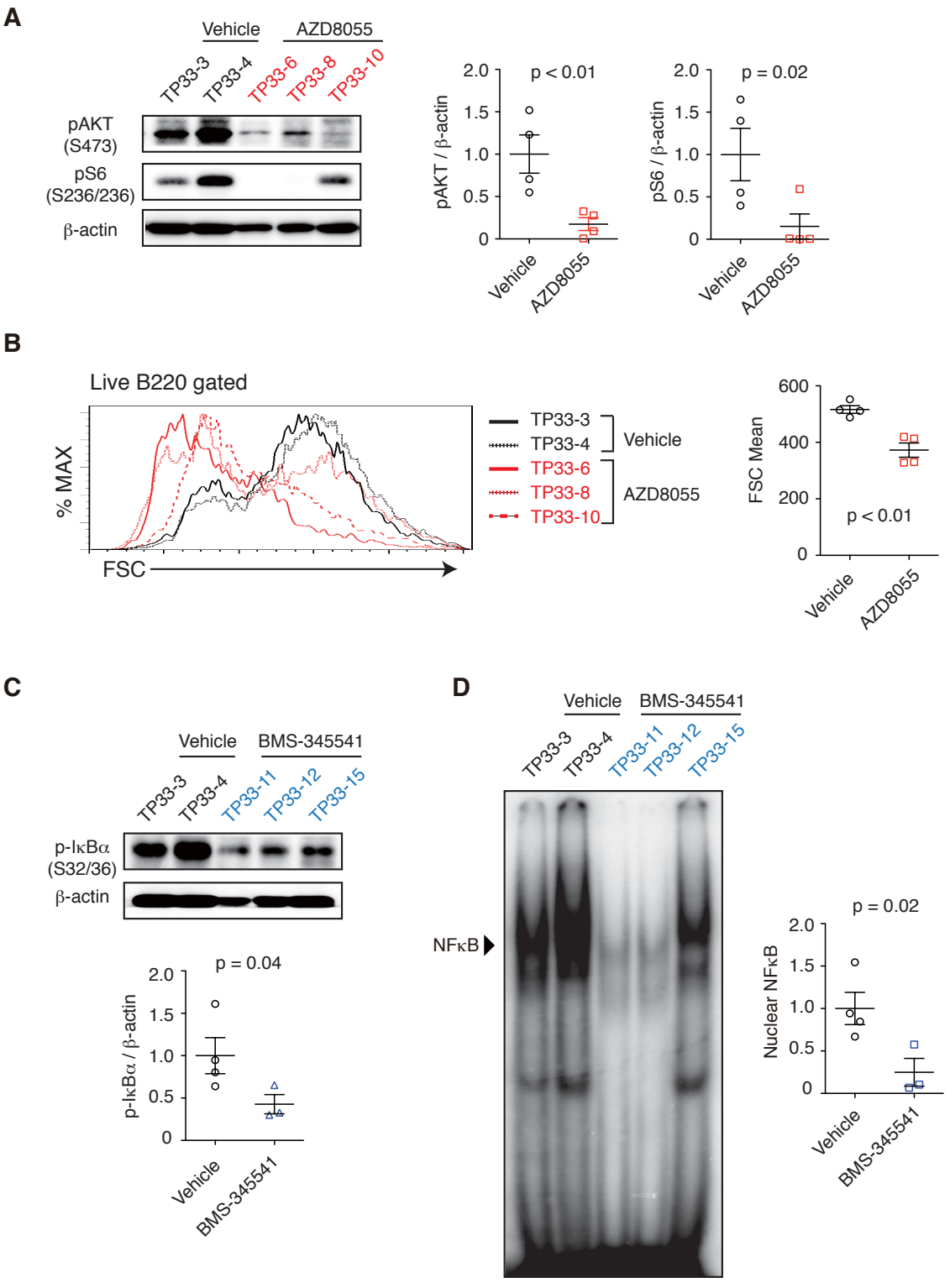
A
Vehicle
AZD8055
TP33-3
TP33-4
TP33-6
TP33-8
TP33-10
pAKT (S473)
pS6 (S236/236)
β-actin
pAKT / β-actin
p < 0.01
pS6 / β-actin
p = 0.02
Vehicle
AZD8055
B
Live B220 gated
% MAX
FSC
TP33-3
TP33-4
TP33-6
TP33-8
TP33-10
Vehicle
AZD8055
FSC Mean
p < 0.01
C
Vehicle
BMS-345541
TP33-3
TP33-4
TP33-11
TP33-12
TP33-15
p-IκBα (S32/36)
β-actin
p-IκBα / β-actin
p = 0.04
D
NFκB
Nuclear NFκB
p = 0.02

**Figure S7. Chemical inhibitors reduced NFκB and PI3K pathway activities in TG lymphoma cells *in vivo*.**

(**A**) Western blot analysis of phospho-AKT and phospho-S6 in transplanted TG lymphoma cells in AZD8055 treated mice. TP number indicates recipient mouse ID. The mean value of vehicle treated group was set as 1.

**(B)** FACS analysis of transplanted TG lymphoma cell size in AZD8055-treated mice. FSC (forward side scatter) indicates cell size.

**(C)** Western blot analysis of phosphor-IκBα and **(D)** EMSA analysis of nuclear NFκB activity in transplanted TG lymphoma cells in BMS-345541 treated mice. Mean values of vehicle treated group were set as 1 in dot graphs.

For all the experiments, representative Western blots, histogram, and EMSA result are shown together with dot graphs summarizing quantification results from multiple independent experiments. Each dot indicates one experimental mouse.

## Supplementary Table 2. Analysis of somatic mutations in TG lymphomas

| Mouse ID | Diagnosis | Ki-67 | Bcl6 | IRF4 | CD138 | PNA | Surface IgM | IgH | Productive Rearrangment | No. Colonies | No. Nucleotides | No. Mutations (Frequency, %) | IgL | Productive Rearrangment | No. Colonies | No. Nucleotides | No. Mutations (Frequency, %) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| #1962 | DLBCL | 98% | ++ | int | - | - | int | Ighv6-3 ; Ighj2 | Yes | 12 | 332 | 20 (6.0%) | Igkv14-111 ; igkj2 | Yes | 8 | 422 | 18 (4.3%) |
| #22 | DLBCL | 95% | + | - | - | - | + | Ighv11-2 ; Ighj1 | Yes | 7 | 357 | None | Not detected | | | | |
| | | | | | | | | Ighv1-15 ; Ighj2 | Yes | 6 | 341 | None | | | | |
| | | | | | | | | Ighv5-4 ; Ighj2 | No | 4 | 321 | None | | | | |
| #16 | DLBCL | 90% | + | - | - | - | + | Ighv3-8 ; Ighj4 | Yes | 12 | 453 | None | Igkv1-110 ; igkj4 | Yes | 1 | 437 | None |
| #694 | DLBCL | 80% | + | + | - | - | + | Ighv11-2 ; Ighj1 | Yes | 8 | 466 | None | Igkv14-126 ; igkj2 | Yes | 2 | 424 | None |
| | | | | | | | | Ighv1-76 ; Ighj4 | Yes | 6 | 360 | 1 (0.3%) | Igkv6-20 ; igkj1 | Yes | 6 | 427 | None |
| | | | | | | | | Ighv1-75 ; Ighj3 | Yes | 4 | 332 | None | | | | |
| #696 | DLBCL | 95% | + | + | - | - | + | Ighv1-31 ; Ighj1 | Yes | 15 | 356 | None | Not detected | | | | |
| #930 | AP | 90% | + | + | ++ | - | + | Ighv11-2 ; Ighj1 | Yes | 12 | 470 | None | Igkv14-126 ; igkj2 | Yes | 3 | 424 | None |

Abbreviations:
DLBCL, Diffuse Large B cell Lymphoma; AP, Anaplastic Plasmacytoma; int, intermediate levels of expression; ++, strong positive; +, posive; -, negative; No. Colonies, numbers of colonies examined; No. Nucleotieds, numbers of nucleotides of V and J regions analyzed; No. Mutations, numbers of mutations found in V and J regions.

## Supplementary materials and methods

### Flow Cytometry

Cell surface staining and flow cytometric analysis were performed following established protocols. Intracellular staining of Ki-67 (BD Biosciences) was performed following fixation and permeabilization according to manufacturer's instructions. Stained cells were analyzed on FACSCalibur or LSR II (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Ashland, OR). Antibodies and reagents with the following specificities were used for staining: Annexin V, anti-CD4 (RM4.5), anti-CD5 (53-7.3), anti-CD23 (B3B4), all from BioLegend, San Diego, CA; anti-CD21 (eBio4E3), anti-IgD (11-26) and anti-CD93 (AA4.1) from eBioscience, San Diego, CA; anti-CD19 (1D3), anti-CD43 (S7), anti-B220 (RA3-6B2), BrdU (3D4), Ki-67 (B56), all from BD Bioscience; anti-Tgfbr2 (FAB532A, R&D Systems) and anti-IgM (115-097-020, Jackson ImmunoResarch, West Grove, PA).

### PAR-CLIP

(*P*hoto*a*ctivatable-*R*ibonucleoside-Enhanced *C*ross*l*inking and *I*mmuno*p*recipitation)

Lymphoblastoid cell lines were established by infecting primary human B cells with EBV B95.8 (EF3D-AGO2, LCL35) or EBV B95.8 Bacmid (LCL-BAC-WT) and were maintained in RPMI 1640 containing 15% (v/v) FBS. EF3D-AGO2 stably expresses a FLAG-tagged Ago2. PAR-CLIP libraries were generated as previously described[4,5]. Briefly, ~1 x $10^9$ cells were cultured in the presence of 100 μM 4-thiouridine (4SU) (Sigma-Aldrich, St. Louis, MO) for 16-18 hrs and then irradiated at UV 365 nm. Cells were lysed on ice in NP40-lysis buffer and cross-linked Ago2:RNA complexes were immunoprecipitated using either anti-FLAG (Sigma-Aldrich) for EF3D-AGO2 or monoclonal antibodies to endogenous Ago2 (clone 9E8.2, Millipore, Billerica, MA) for LCL35 and LCL-BAC-WT. Ago2-bound RNAs were radiolabelled, gel-purified, and sequentially ligated to 3' and 5' Illumina adapters for deep sequencing. Ligated RNAs were reverse transcribed using SsIII (Invitrogen) and cDNAs were PCR amplified (22-28 cycles) following a pilot PCR to determine conditions. Sequencing was performed at the Duke University IGSP Sequencing Core Facility using an Illumina GA2 Sequence Analyzer.

For data analysis, 5' barcode and 3' adapter sequences were trimmed from raw sequences using the FAST-X toolkit (http://hannonlab.cshl.edu/fastx_toolkit). Unique reads ≥13 nt in

length were aligned to the human genome (hg19) using Bowtie[6], allowing for up to three mismatches. Only genomic locations with reads falling into the best stratum (i.e. lowest number of mismatches) were kept for further analysis. Additional analysis of PAR-CLIP reads was performed using the PARalyzer toolkit[7]. Briefly, reads aligning to unique genomic locations and overlapping by at least one nucleotide were grouped, analyzed for T>C conversions, and nucleotide strings containing a greater likelihood of converted T>Cs than non-converted Ts were extracted as clusters. Clusters were then interrogated for miRNA seed-match sites (requiring a minimum of 7mer match, i.e. 7mer-A1 or 7mer-m8)[8] using miRNA sequences identified in a matched small RNA sequencing library. We pooled clusters from three experiments (LCL35, EF3D-AGO2 and LCL-BAC-WT) and this yielded 3744 unique miR-17~92 binding sites.

MiR-17~92 binding sites in human B cells identified by PAR-CLIP were manually examined in UCSC Genome Browser to evaluate their conservation in mouse (assembly NCBI37/mm9). The minimal requirement for conservation was a perfect match at the seed region (7mer-1A or 7mer-m8) in these two species.

**Pathway Analysis of miR-17~92 Target Genes**

MiR-17~92 target genes were loaded and analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems Inc. Redwood City, CA). The Gene Ontology terms of each gene were mapped to Ingenuity's internal knowledge base, and was exported to Excel and grouped. Ingenuity uses Fisher's Exact test to generate statistical significance values (P value) for each group of canonical pathways that can be associated from the list of targets genes, and pathways with a P value <0.0001 were listed in Figure S4.

**Northern Blot**

MiRNA Northern blotting was done as described previously[9]. Briefly, 10μg total RNA from MACS-purified B2 cells was isolated and separated on 10% denaturing polyacrylamide gels, subsequently electrotransferred to nylon membrane. DNA oligonucleotides antisense to mature microRNAs were purchased from Integrated DNA Technologies (Coralville, IA) and used as probes. U6 snRNA was used as internal control for normalization. Northern blot results were acquired on a STORM 860 phosphorimager and analyzed using the ImageQuant software.

**Southern Blot**

Southern blot analysis was performed according to standard methods. Briefly, genomic DNA isolated from tumor specimens was digested with EcoRI (New England Biolabs, Beverly, MA), separated on a 0.8 % agarose gel, transferred to nylon membranes, and hybridized with a $^{32}P$ labeled $J_H4$ probe spanning the $J_H4$ exon and a part of the downstream intronic sequence of the IgH locus. Southern blot results were acquired on a STORM 860 phosphorimager.

**Western Blot**

B2 cells were purified as described in Experimental Procedures, and stimulated as indicated in the figures. At each time point, live cells were purified using Ficoll method according to manufacturer's instruction (GE Healthcare, Ficoll Paque PLUS, 17-1440-02) achieving >90% live and >98% B cells ($B220^+$ $CD19^+$). The purified cells were lysed in buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 1 mM sodium orthovanadate, 1 mM DTT and proteinase inhibitors, subjected to 4 –20 % SDS-PAGE and Western blotting (10 μg per lane). Antibodies used for western blotting are anti-Bim (2933), anti-Bcl6 (4242), anti-IκBα (9242), anti-phospho-IκBα (9246), anti-IκBβ (9248), anti-IRF4 (4964), anti-Pten (9559), anti-phospho-S6 (4856), anti-S6 (2217), anti-Traf3 (4729), anti-Trim33 (8972), anti-phospho-Smad2 (9341), from Cell Signaling; anti-c-Myc (sc-40), anti-Ccnd2 (sc-593), anti-Cdk4 (sc-601) anti-Rb (sc-50), anti-E2f1 (sc-193), anti-E2f2 (sc-633), anti-E2f3 (sc-879), anti-Cyld (sc-137139), anti-A20 (sc-16692), anti-c-Rel (sc-71), from Santa Cruz Biotechnology; anti-Rnf11 (ab57180), anti-Tax1bp1 (ab22049), from Abcam; anti-casp11 (Novus Biologicals, NB120-10454), anti-Phlpp2 (Bethyl, A300-661A-1), anti-Itch (BD Transduction Laboratories, 611199), anti-phospho-Rb (BD Pharmingen, 554136), anti-Cre (Novagen, 69050-3) and anti-β-actin (Sigma-Aldrich).

**Preparation of Nuclear Extracts**

20 x $10^6$ B cells before or after stimulation were harvested and resuspended in 100μl 0.1% NP-40 lysis buffer (10mM Tris-Cl, pH 7.5, 10mM NaCl, 3mM $MgCl_2$, 0.1% NP-40) and allowed to swell at 4°C for 5 minutes. The homogenates were centrifuged at 800 g for 5 minutes and the supernatant was stored at -80°C as cytoplasmic extracts. The nuclear pellet was washed twice with washing buffer (10mM Tris-Cl, pH 7.5, 10mM NaCl, 3mM $MgCl_2$) and

resuspended in 20μl nucleic lysis buffer (10mM HEPES, pH 8.0, 1.5mM $MgCl_2$, 0.4mM EDTA, 20% Glycerol, freshly added 0.5mM DTT) and 5μl of 3M KCl for following EMSA, or with 1%NP-40 lysis buffer for western blot anaylsis. After incubation at 4°C for 30 minutes with occasional vigorous vortex mixing, the nuclear extracts were centrifuged at 16,000 g for 10 minutes at 4°C and the supernatant was frozen in aliquots at -80°C.

**Electrophoretic Mobility Shift Assay**

MACS-purified B2 cells were stimulated with anti-IgM (2 μg/ml) at 37°C for indicated amounts of time after 3 hours of resting in B cell media in a tissue culture incubator (37°C). Nuclear extracts were then prepared as described above. Equal amounts (2 μg) of nuclear extracts were incubated at room temperature for 45 minutes in 20 μl of gel shift binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 40 mM KCl) with freshly added 100 μg/ml poly(dI-dC) and 2.5 μl of purified $\gamma$-$^{32}$P end-labeled NFkB oligo (Promega, Madison, WI). Samples were fractionated by electrophoresis in a 4% native gel. After drying, gels were exposed to a storage-phosphor screen overnight at room temperature. The images were acquired by a STORM 860 phophoimager (Molecular Dynamics).

**Genotyping PCR**

Genomic DNA purified from lymphoma and control samples was analyzed by genotyping PCR. For detection of miR-17~92 flox and deleted alleles, following primers were used: AVF4 5'-TCGAGTATCTGACAATGTGG-3', AVR5 5'-TAGCCAGAAGTTCCAAATTGG-3' and AV145 5'-ATAGCCTGAAACCAACTGTGC-3'. For detection of CD19Cre and CD19WT alleles, following primers were used: Cre (CreF 5'- CGATGCAACGAGTGATGAGG-3' and CreR 5'-CGCATAACCAGTGAAACAGC-3'); and CD19WT (CD19.8 5'-AATGTTGTGCTGCCATGCCTC-3' and CD19.9 5'-GTCTGAAGCATTCCACCGGAA-3'). PCR was performed with T3000 thermocycler (Biometra).

**V gene mutation analysis**

$IgV_H$ and IgVκ gene somatic hypermutation in purified lymphoma cells was first investigated by a 5'-RACE PCR strategy (Ambion, AM1700). To produce cDNA from full-length mRNA, total RNA was treated with calf intestinal phosphatase (CIP) to remove 5'-$PO_4$ from degraded mRNA. RNA from the reaction was further purified using the phenol:chloroform method. The

extracted RNA was treated with tobacco acid pyrophosphatase (TAP) to remove cap from full-length mRNA and the 5'-RACE adaptor was ligated. The first cDNA strand was synthesized using a primer recognizing Cμ region (Cμ primer) of IgH or a primer recognizing Cκ region (Cκ primer) to amplify the κ-light chain. The first cDNA strand was further amplified using an outer primer recognizing the 5'-RACE adaptor as 5'-primer and Cμ primer or Cκ primer as 3'-primers.

To confirm results from 5'-RACE, a direct PCR strategy using genomic DNA as template was also employed. We designed 25 5'-primers that are able to recognize the most commonly used 90 $V_H$ genes in mice, and 4 3'-primers that recognize each of the 4 $J_H$ genes. To reduce the complexity of 5'-primer and 3'-primer combinations, we grouped the 25 5'-primers into five groups (5 primers per group) and performed PCR using genomic DNA as template, with one of the 5'-primer groups and all 4 of the 3'-primers.

The PCR product from 5'-RACE PCR and genomic PCR were gel purified and cloned into pCR2.1-TOPO vector following manufacturer's instructions. For each lymphoma case, 16~40 colonies from genomic PCR and 12~24 colonies from 5'RACE PCR were picked for Sanger sequencing. The V and J region sequences from each colony were compared to germline sequences in NCBI Blast, UCSC Genome Browser Blat and IMGT/V-QUEST database. A mutation was counted only when observed in more than one colony from the same lymphoma specimen. The following are sequences of primers and adaptor used in this study:

5'-RACE adaptor:
5'-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3';

Outer primer: 5'-GCTGATGGCGATGAATGAACACTG-3':
Cμ primer: 5'-GCCACCAGATTCTTATCA GACA-3';
Cκ primer: 5'-TGTTCAAGAAGCACACGACTGA-3',

25 primers recognizing genomic VH regions:
5'-CAGTCTGGACCTGAGCTGAAGA-3',
5'-GGAGTCTGGAGGAGGCTTGGT-3',
5'-GTCTGGCCCTGGGATATTGCAG-3',

5’-TCTGGAGGAGGCTTGGTACAG-3’,
5’-GCAGCTTCAGGAGTCAGGACCT-3’,
5’-GAGGTGCAGCTTGTTGAGTCT-3’,
5’-AGGTCCAGCTGCAGCAGTCTG-3’,
5’-GAGGTCCAGCTGCAACAGTCTG-3’,
5’-GAGGTCCAGCTGCAACAATCTGGA-3’,
5’-GAGGTTCAGCTGCAGCAGTCTG-3’,
5’-GAGGTTCAGCTCCAGCAGTCTG-3’,
5’-CAGGTTCAGCTGCAACAGTCTG-3’,
5’-AGGTCCAGCTWCAGCAGTCTGGA-3’,
5’-CAGGTCCAGCTGAAGCAGTCTG-3’,
5’-AAGTGCAGCTGTTGGAGACTGGA-3’,
5’-AGGTGAAGCTTCTCCAGTCTGGA-3’,
5’-GGAGGCTTAGTGMAGCCTGGA-3’,
5’-CAGGTGCAGCTGAAGSAGTCAG-3’,
5’-CAGGTCCAACTGCAGCAGCC-3’,
5’-CAGGTTCAACTGCAGCAGTCTG-3’,
5’-TGCAGCAGTCTGGAGCTGA-3’,
5’-TGCAGCAGTCTGGACCTGA-3’,
5’-TGCAACAGTCAGGAGCTGAG-3’,
5’-CAGGTGCAGCTTGTAGAGA-3’,
5’-CACCTACAACAGTCTGGTTCT-3’;

4 primers recognizing $J_H$ regions:
5’-CAGCTTACCTGAGGAGACGGT-3’,
5’-ACTCACCTGAGGAGACTGTGA-3’,
5’-ACTCACCTGCAGAGACAGTGA-3’,
5’-CATTCTTACCTGAGGAGACGGT-3’.

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