Cancer Cell, *19*

Supplemental Information

ATMIN Is Required for Maintenance of Genomic Stability and Suppression of B Cell Lymphoma

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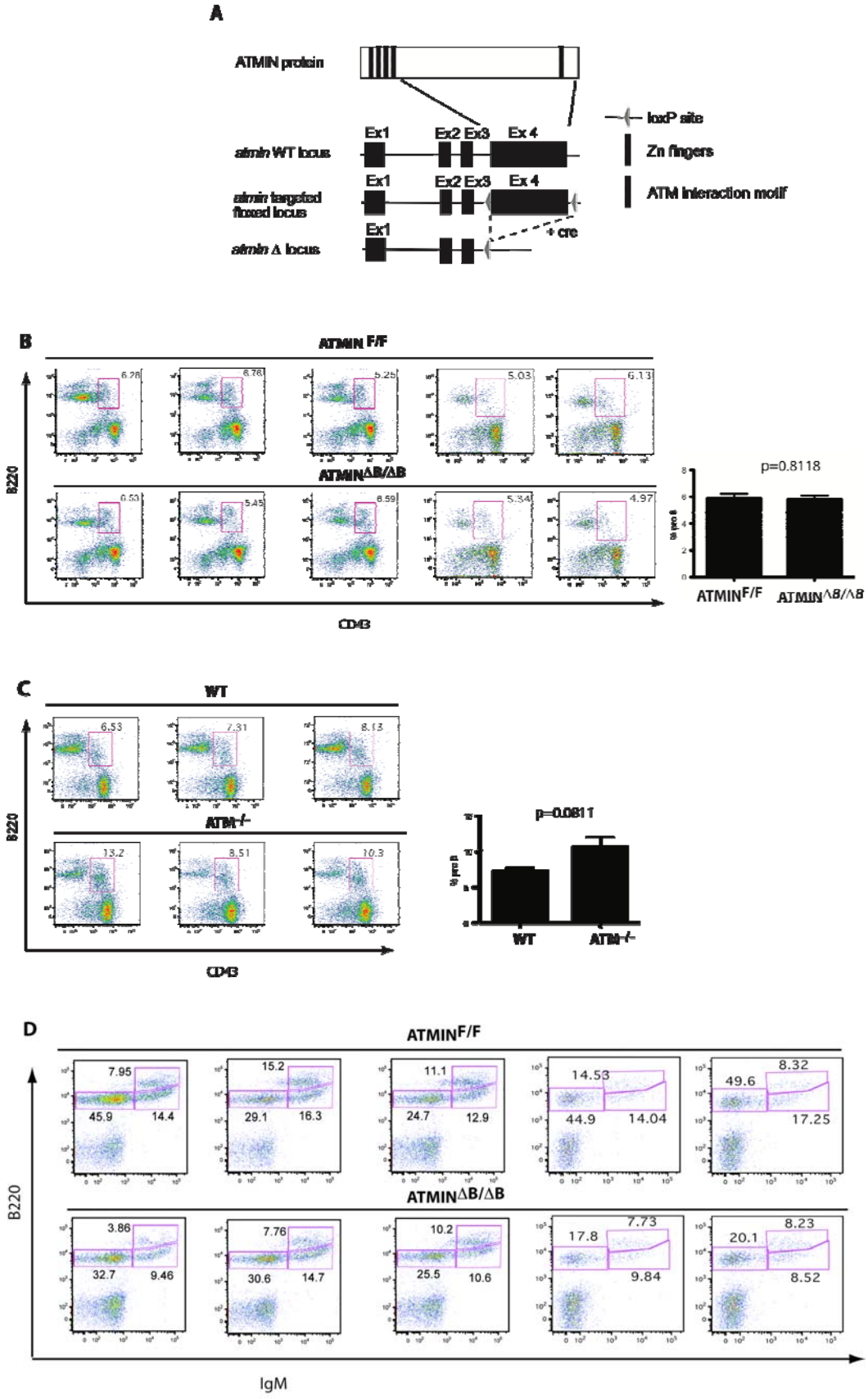
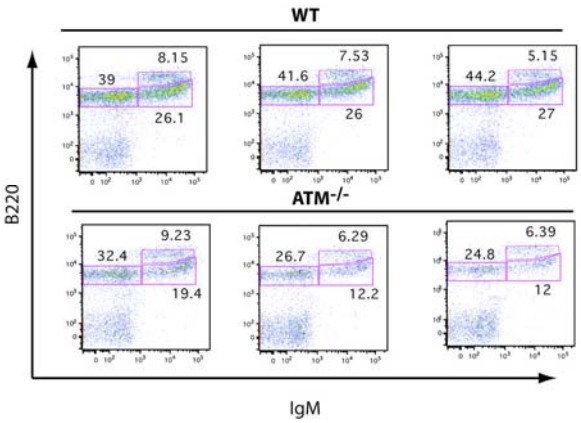

A
ATMIN protein
loxP site
Zn fingers
ATM interaction motif
Ex1
Ex2 Ex3
Ex 4
atmin WT locus
atmin targeted floxed locus
atmin Δ locus
+ cre
B
ATMIN F/F
ATMINΔB/ΔB
B220
CD3
6.28
6.76
5.25
5.03
6.13
6.53
5.45
6.59
5.34
4.97
p=0.8118
ATMINF/F
ATMINΔB/ΔB
C
WT
ATM-/-
6.53
7.31
8.13
13.2
8.51
10.3
p=0.0811
WT
ATM-/-
D
ATMINF/F
ATMINΔB/ΔB
B220
IgM
7.95
45.9
14.4
15.2
29.1
16.3
11.1
24.7
12.9
14.53
44.9
14.04
49.6
8.32
17.25
3.86
32.7
9.46
7.76
30.6
14.7
10.2
25.5
10.6
17.8
7.73
9.84
20.1
8.23
8.52

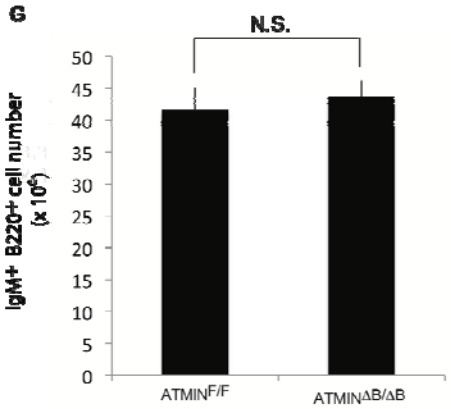
Figure S1 continued

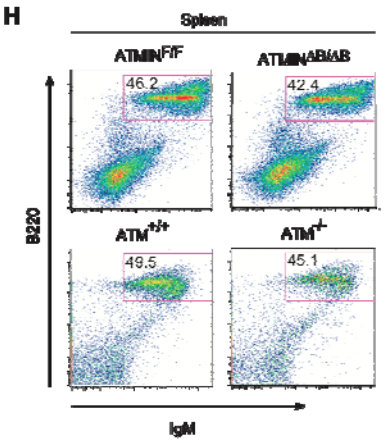
E



F

	Mouse ID	Total number ($x10^6$)	% IgM+; B220+	Number IgM+; B220+ ($x10^6$)	Mean
$ATMIN^{F/F}$	70	102.75	46.2	47.4705	41.66725
	78	86.25	41.1	35.44875	
	80	90.5	46.5	42.0825	
$ATMIN^{\Delta B/\Delta B}$	69	122.25	38.9	48.77775	43.7365833
	72	98	42.4	41.552	
	71	91.25	44.8	40.88	





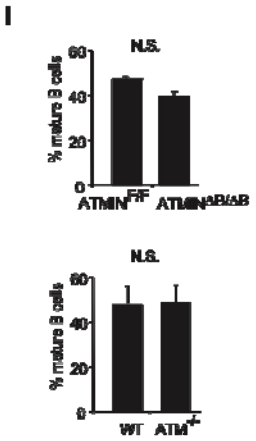


Figure S1, related to Figure 1.

(A) Schematic representation of ATMIN protein, *atmin* WT locus, *atmin* targeted floxed locus, and the deleted *atmin* locus after CD19-cre-mediated recombination (*atmin* Δ locus). Exons are represented by black rectangles, intronic DNA is shown as a black line. Lox P sites are indicated by triangles. (B) Pro-B cells ($B220^{+}CD43^{+}$) are unchanged in $ATMIN^{\Delta B/\Delta B}$ (n=5) and (C) $ATM^{-/-}$ (n=3) bone marrow (BM), compared with their controls. (D) FACS analyses of pre-B, immature B and recirculating mature B cell populations in $ATMIN^{F/F}$ and $ATMIN^{\Delta B/\Delta B}$ mice and (E) WT and $ATM^{-/-}$ mice. (F) Total cell number, percentage of mature B cells ($IgM^{+}B220^{+}$) and total number of mature B cells are unchanged in $ATMIN^{\Delta B/\Delta B}$ mice compared to $ATMIN^{F/F}$ mice. (G) Bar chart of F. (H) FACS scan of F, including WT and $ATM^{-/-}$ mice (n=3). (I) Bar chart of H. (J) H&E staining of spleen of $ATMIN^{F/F}$ and $ATMIN^{\Delta B/\Delta B}$ mice. RP=red pulp; GC=germinal centre. Data are presented as mean and s.d. Statistical analyses were calculated by a Student's t-test with two-tailed distribution. p values are indicated. Scale bar = 100 μm.

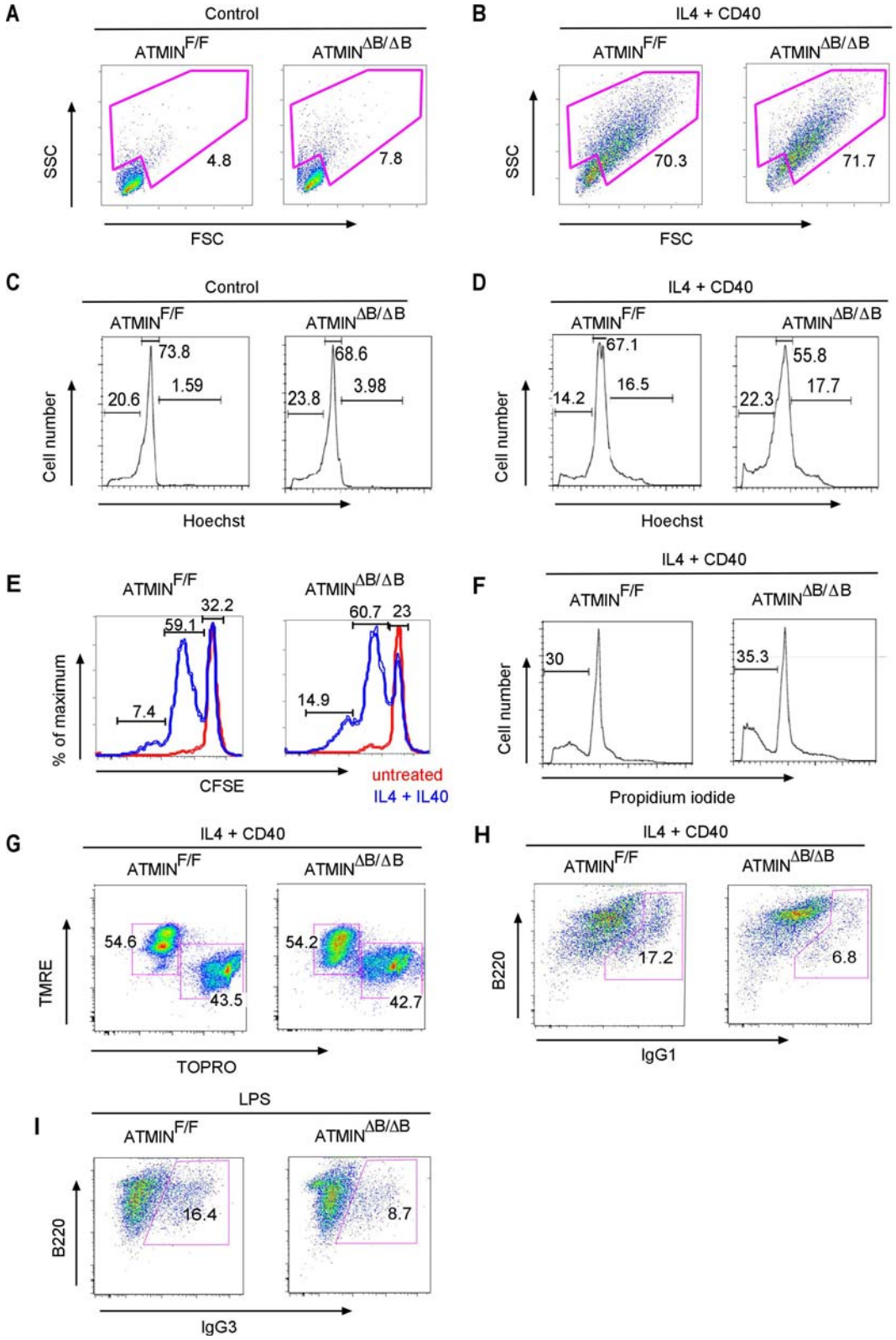

A
Control
ATMIN F/F
ATMIN ΔB/ΔB
SSC
FSC
4.8
7.8
B
IL4 + CD40
ATMIN F/F
ATMIN ΔB/ΔB
SSC
FSC
70.3
71.7
C
Control
ATMIN F/F
ATMIN ΔB/ΔB
Cell number
Hoechst
73.8
1.59
20.6
68.6
3.98
23.8
D
IL4 + CD40
ATMIN F/F
ATMIN ΔB/ΔB
Cell number
Hoechst
67.1
16.5
14.2
55.8
17.7
22.3
E
ATMIN F/F
ATMIN ΔB/ΔB
% of maximum
CFSE
32.2
59.1
7.4
60.7
23
14.9
untreated
IL4 + IL40
F
IL4 + CD40
ATMIN F/F
ATMIN ΔB/ΔB
Cell number
Propidium iodide
30
35.3
G
IL4 + CD40
ATMIN F/F
ATMIN ΔB/ΔB
TMRE
TOPRO
54.6
43.5
54.2
42.7
H
IL4 + CD40
ATMIN F/F
ATMIN ΔB/ΔB
B220
IgG1
17.2
6.8
I
LPS
ATMIN F/F
ATMIN ΔB/ΔB
B220
IgG3
16.4
8.7

Figure S2 continued

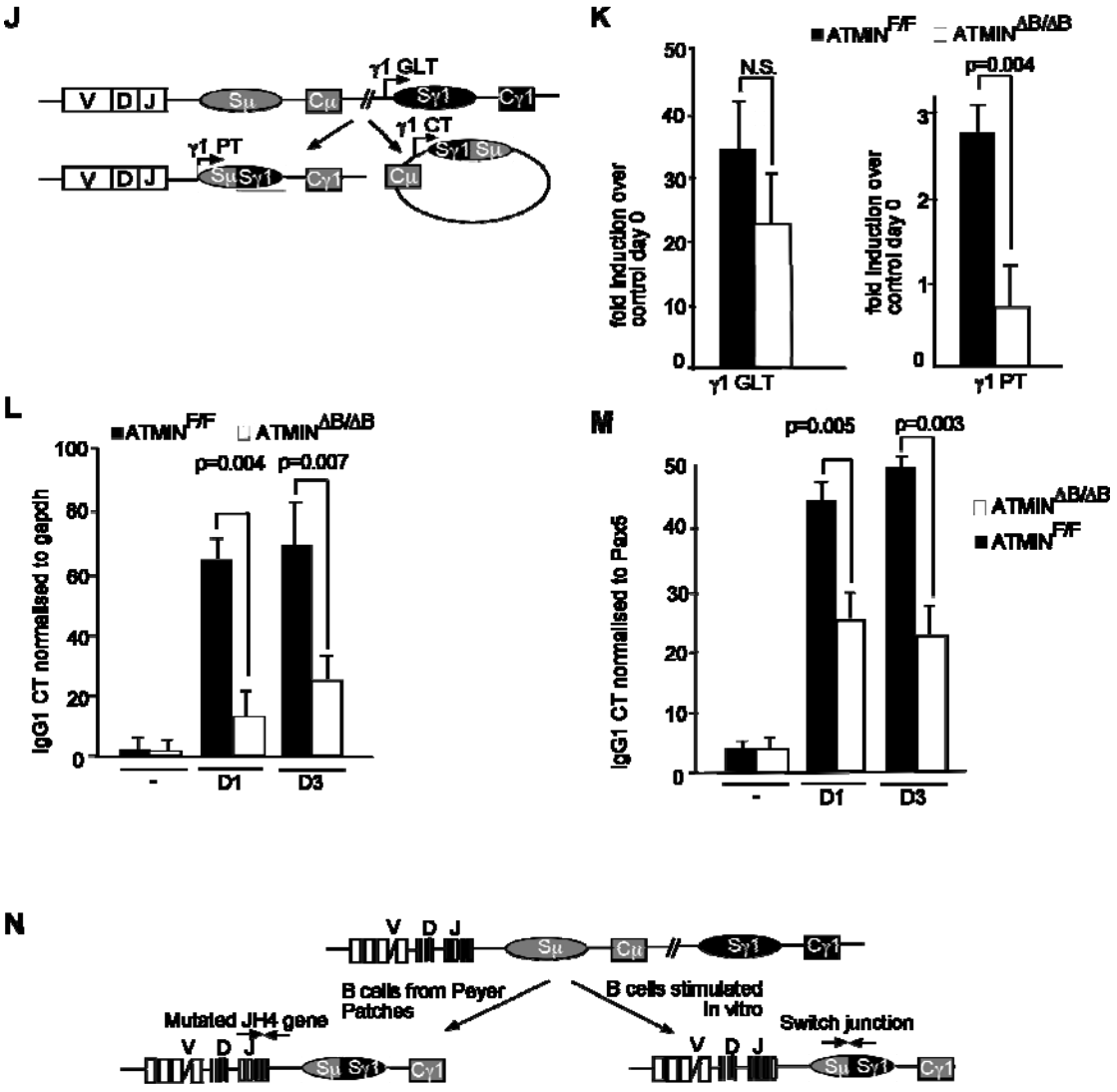


Figure S2 continued

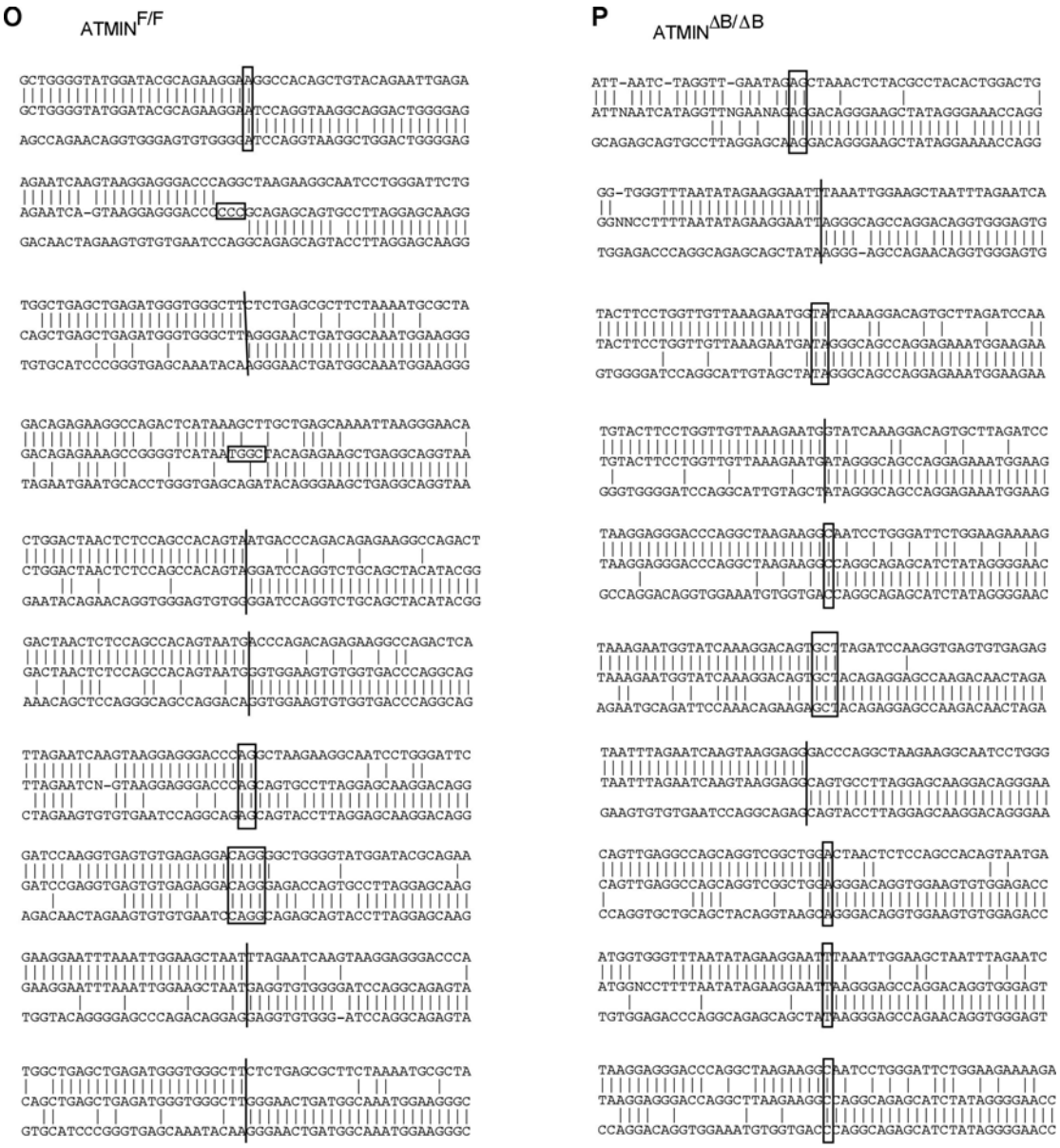


Figure S2, related to Figure 2.

(A) Splenic B cells were purified from ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ mice and cell size was measured by forward and sideward scatter. (B) Splenic B cells were purified from ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ mice and stimulated for 3 days with IL4/CD40 following which blast formation was measured by forward and

sideward scatter. (C) ATMIN$^{\Delta B/\Delta B}$ and ATMIN$^{F/F}$ B lymphocytes enter the cell cycle following stimulation comparably. Cells were treated as in (A) and then stained with Hoechst to ascertain their cell cycle profile without stimulation (C) or with IL4/CD40 stimulation (D) (n=4 per genotype). (E) Cell proliferation is not perturbed in ATMIN$^{\Delta B/\Delta B}$ B lymphocytes. ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ B lymphocytes were incubated with CFSE to track cell divisions and then stimulated to proliferate as in (A and B). (F) ATMIN$^{\Delta B/\Delta B}$ B lymphocytes stimulated *in vitro* have a similar survival rate as ATMIN$^{F/F}$ B lymphocytes. Cells were stimulated as in (B) following which apoptosis was assessed by measuring the sub-G1 population. (G) Splenic B cells were purified from ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ mice and were stimulated *in vitro* with IL4/CD40 for 3 days and then stained with TOPRO and TMRE. Living cells are defined as TMRE^{+}TOPRO^{-}. ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ purified splenic B lymphocytes were stimulated with either (H) IL4/CD40 for 5 days and then stained with anti B220 and IgG1 antibodies (n=4 per genotype) or (I) LPS for 5 days and then stained with anti B220 and IgG3 antibodies (n=4 per genotype). (J) Schematic representation for the production of γ1 germline transcript (GLT) in unstimulated B cells and γ1 productive transcript (PT) and γ1 circle transcript (CT) following stimulation with LPS/IL4. (K) Real time PCR analysis of GLT and PT IgG1 transcripts in ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ B lymphocytes. Total RNA was extracted from B cells stimulated *in vitro* with LPS/IL4 for 3 or 6 days to measure GLT or PT respectively. The data were normalized to gapdh. Real time PCR analysis of CT of IgG1 in ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ B lymphocytes. Total RNA was extracted from unstimulated B cells (-), B cells stimulated *in vitro* with LPS/IL4 for 1 day (D1) or 3 days (D3) and the data

were normalized to (L) GAPDH as well as (M) Pax5. Data for K-M presented as mean and s.d. Statistical analyses were calculated by a Student's t-test with two-tailed distribution. p values are indicated. Accuracy of CSR is unaffected in ATMIN$^{\Delta B/\Delta B}$ B lymphocytes. (N) Schematic representation for the PCR strategy to analyze either Sμ-Sγ1 (GenBank/EMBL/DDBJ sequences J00440 corresponds to Sμ and D78344 corresponds to Sγ1). CSJ following LPS/IL4 stimulation of ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ B cells or SHM within the J_H4-C_H intron in 10-month old ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ mice. Analysis of microhomology at the Sμ-Sγ1 junctions derived from *in vitro* stimulated B lymphocytes (4 days) reveals no difference in microhomolgy between (O) ATMIN$^{F/F}$ and (P) ATMIN$^{\Delta B/\Delta B}$ (n=4) B lymphocytes. Overlap was determined by identifying the longest region at the switch junction of perfect uninterrupted donor/acceptor identity. The Sμ and Sγ1 germline sequences are shown above and below each junction sequence respectively.

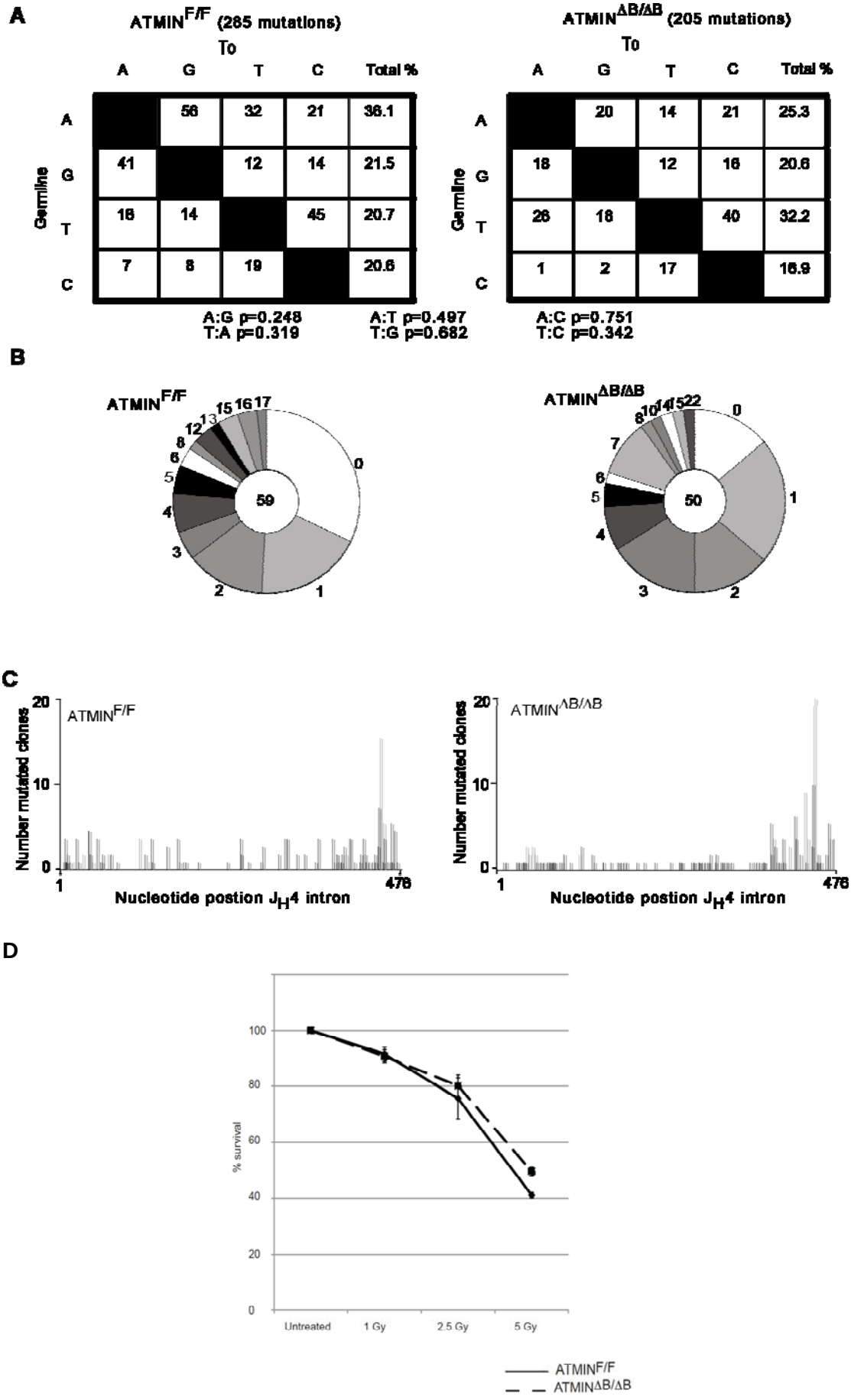

A
ATMIN F/F (265 mutations)
To
A G T C Total %
A 56 32 21 36.1
G 41 12 14 21.5
T 18 14 45 20.7
C 7 8 19 20.6
Germline
A:G p=0.248
T:A p=0.319
A:T p=0.497
T:G p=0.682
ATMIN ΔB/ΔB (205 mutations)
To
A G T C Total %
A 20 14 21 25.3
G 18 12 16 20.6
T 26 18 40 32.2
C 1 2 17 16.9
Germline
A:C p=0.751
T:C p=0.342
B
ATMIN F/F
59
ATMIN ΔB/ΔB
50
C
ATMIN F/F
ATMIN ΔB/ΔB
Number mutated clones
Nucleotide postion JH4 intron
1
476
D
% survival
Untreated
1 Gy
2.5 Gy
5 Gy
ATMIN F/F
ATMIN ΔB/ΔB

Figure S3, related to Figure 3.

(A-C) The percentage, number and position of nucleotide substitutions at the J_H4-C_H intron are unchanged in ATMIN$^{\Delta B/\Delta B}$ B lymphocytes. Germinal centre B cells (B220$^+$PNA$^+$) sorted from Peyer's Patches of ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ mice (n=3 per genotype) were analyzed for mutations at the J_H4 3' flanking sequence of endogenous rearrangements of the VhJ558 family members. A 476-bp fragment was analyzed as in Martomo et al., 2006 and the data in the ''%'' column corrected for base composition. (A) Although we noted a few subtle differences in the hotspot targeting preferences in both control ATMIN$^{F/F}$ and in ATMIN$^{\Delta B/\Delta B}$ mice compared with other studies we cannot exclude that the differences might reflect mouse strain differences. Statistical analyses were calculated by a Student's t-test with two-tailed distribution. (B) The mutation load in the J_H4-C_H intron is unaffected in ATMIN$^{\Delta B/\Delta B}$ B lymphocytes. The number of sequences with the same number of mutations accumulated in the 476-bp fragment are represented as pie sectors. 59 ATMIN$^{F/F}$ and 50 ATMIN$^{\Delta B/\Delta B}$ non-clonal sequences were analyzed. (C) Distribution of mutations in ATMIN$^{\Delta B/\Delta B}$ B lymphocytes compared with control ATMIN$^{F/F}$. The position and number of mutations at the J_H4-C_H intron is unchanged in ATMIN$^{\Delta B/\Delta B}$ B lymphocytes compared with controls (n=3 per genotype). ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ non-clonal sequences were analyzed on the basis of their unique CDR3 sequence. (D) ATMIN$^{\Delta B/\Delta B}$ B cells do not display sensitivity to ionising radiation. B cells were cultured with LPS overnight following which cells were either left untreated or treated with 1, 2.5 or 5 Gy ionising radiation and then cultured for a further 48 hours (n=3).

Percentage survival was assessed by trypan blue exclusion. Error bars denote s.d.

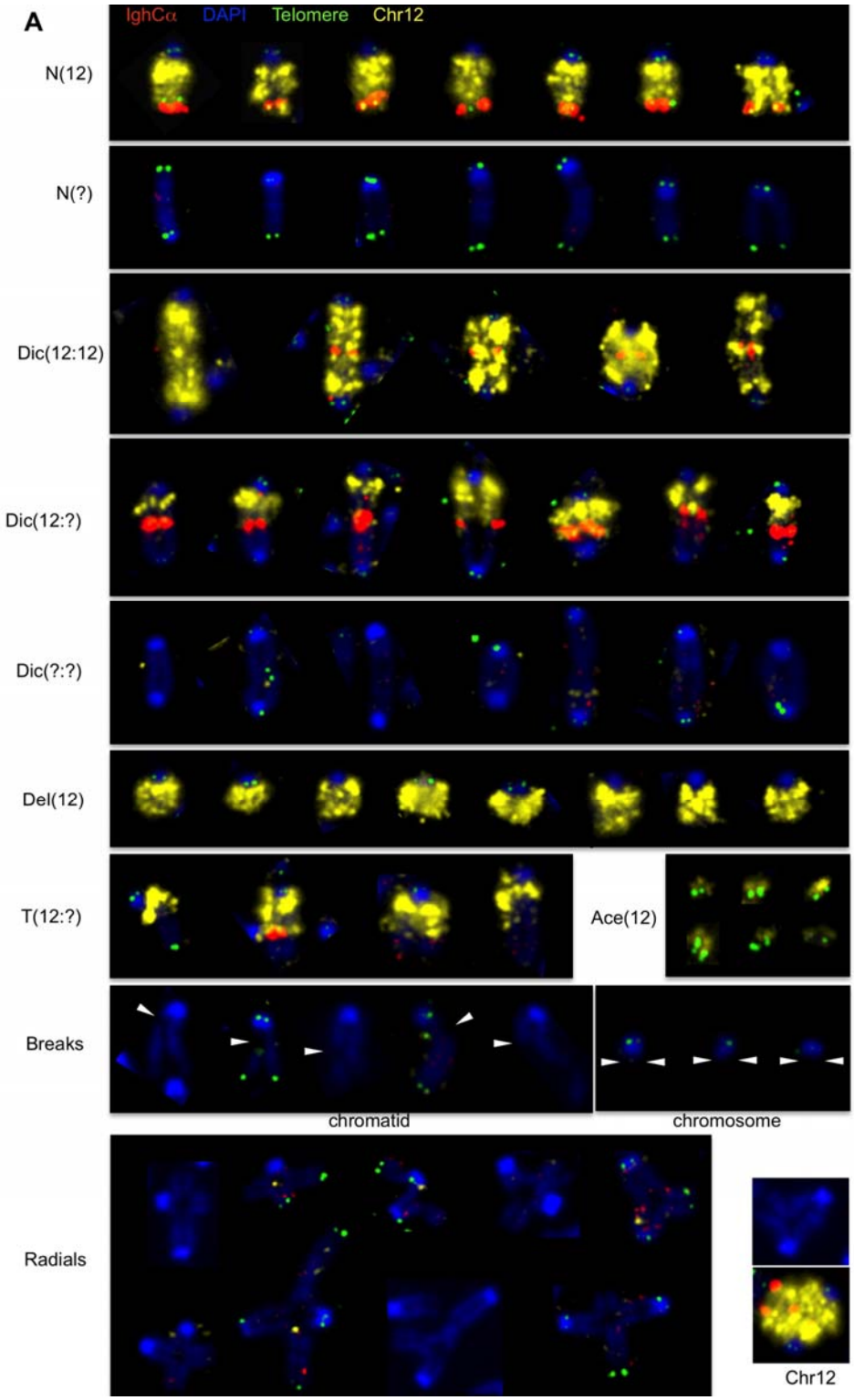

A
IghCα
DAPI
Telomere
Chr12
N(12)
N(?)
Dic(12:12)
Dic(12:?)
Dic(?:?)
Del(12)
T(12:?)
Ace(12)
Breaks
chromatid
chromosome
Radials
Chr12

Figure S4 continued

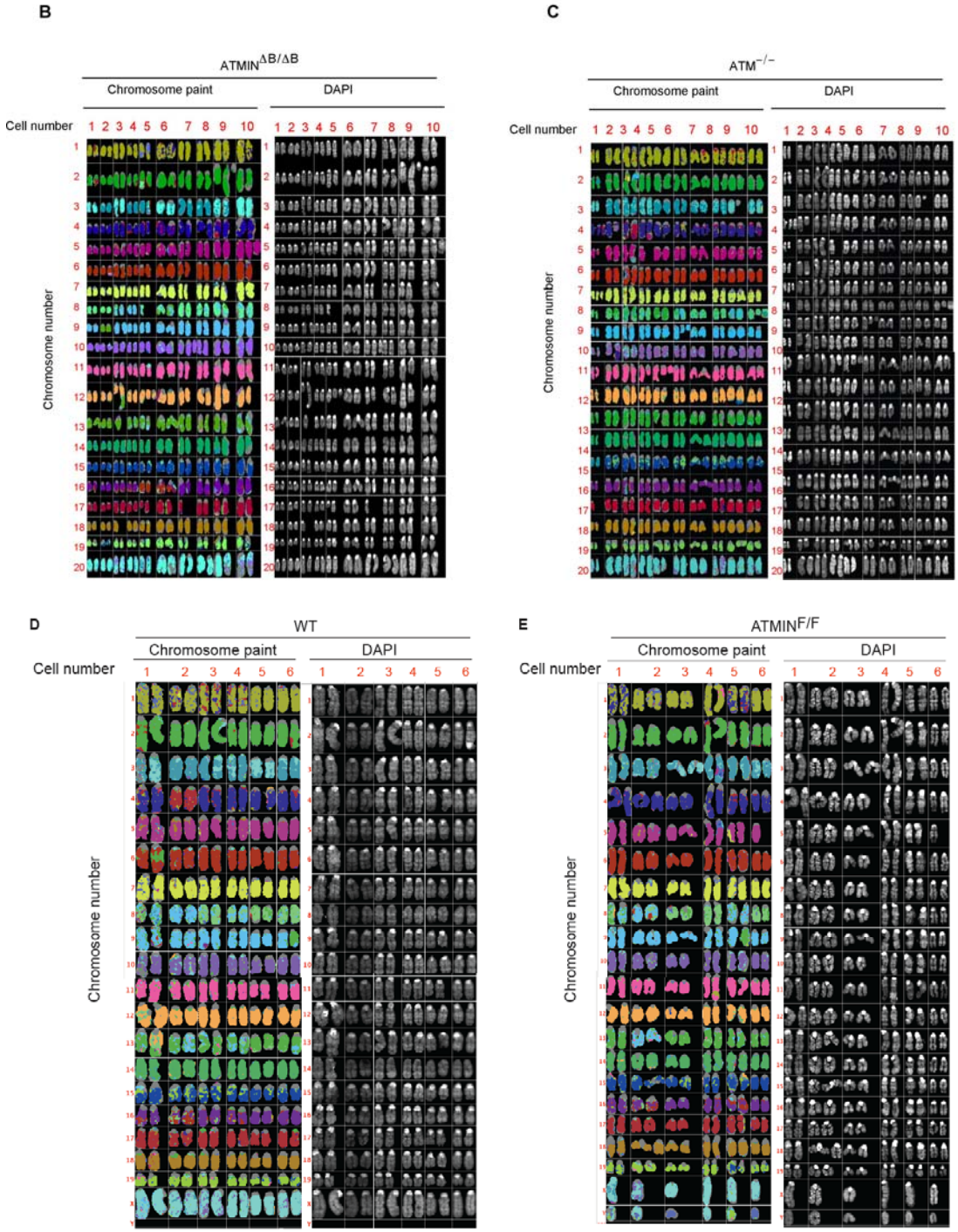


Figure S4, related to Figure 4.

(A) Examples of normal and aberrant chromosomal structures identified by metaphase FISH observed in ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ B cells. N = normal, Dic = dicentric, Del = deletion, T = translocation, Ace = acentric (fragment). Red = *Igh* C region probe, yellow = chromosome 12 paint, green = telomere probe. Counterstain (blue) = DAPI. (B) ATMIN$^{\Delta B/\Delta B}$ and (C) ATM$^{-/-}$ B lymphocytes show genomic instability. Splenic B cells were stimulated for 3 days with LPS/IL4 following which cells were arrested in metaphase and M-FISH was performed using chromosome painting as well as DAPI staining (n=4). (D) WT and (E) ATMIN$^{F/F}$ B lymphocytes were processed as in B-C

A

ATMIN$^{F/F}$

Resting	Number	%	Stimulated	Number	%
Colocalised	115	4.3%	Colocalised	130	5.6%
Separate	2567	95.5%	Separate	2167	93.9%
Break apart	6	0.2%	Break apart	10	0.4%
Total	2688			2307	

ATMIN$^{\Delta B/\Delta B}$

Resting	Number	%	Stimulated	Number	%
Colocalised	127	8.0%	Colocalised	105	6.3%
Separate	1452	91.6%	Separate	1553	92.6%
Break apart	6	0.4%	Break apart	19	1.1%
Total	1585			1677	

WT

Resting	Number	%	Stimulated	Number	%
Colocalised	118	6.3%	Colocalised	184	6.8%
Separate	1745	93.6%	Separate	2495	92.8%
Break apart	2	0.1%	Break apart	11	0.4%
Total	1865			2690	

ATM$^{-/-}$

Resting	Number	%	Stimulated	Number	%
Colocalised	109	7.8%	Colocalised	119	6.7%
Separate	1277	91.9%	Separate	1632	91.6%
Break apart	4	0.3%	Break apart	30	1.7%
Total	1390			1781	

B

Resting			Stimulated		
Observed	Break apart	Colocalised	Observed	Break apart	Colocalised
ATMIN$^{F/F}$	6	2682	ATMIN$^{F/F}$	10	2297
ATMIN$^{\Delta B/\Delta B}$	6	1579	ATMIN$^{\Delta B/\Delta B}$	19	1658
Expected	Break apart	Colocalised	Expected	Break apart	Colocalised
ATMIN$^{F/F}$	7.5	2680.5	ATMIN$^{F/F}$	16.8	2290.2
ATMIN$^{\Delta B/\Delta B}$	4.5	1580.6	ATMIN$^{\Delta B/\Delta B}$	12.2	1664.8
	p=0.354			p=0.010	

Resting			Stimulated		
Observed	Break apart	Colocalised	Observed	Break apart	Colocalised
WT	2	1863	WT	11	2679
ATM$^{-/-}$	4	1387	ATM$^{-/-}$	30	1751
Expected	Break apart	Colocalised	Expected	Break apart	Colocalised
WT	3.4	1861.6	WT	24.7	2665.3
ATM$^{-/-}$	2.6	1387.4	ATM$^{-/-}$	16.3	1764.7
	p=0.235			p=1.19x10^{-5}	

C

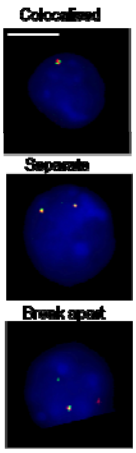


Figure S5, related to Figure 5.

ATMIN is required to maintain the stability of the *Igl* locus through peripheral V(D)J recombination. (A) Quantification of *Igl* allele colocalisation (5' and/or 3' probes from the two *Igl* loci within 0.5μm of each other), separation (both *Igl*

alleles intact but separated by >0.5μm) and breaks (cells that had at least one *Igl* break where the 5' and 3' probes are visibly separated) in $ATMIN^{F/F}$, $ATMIN^{\Delta B/\Delta B}$, WT and $ATM^{-/-}$ B cells either before stimulation (resting) or after 3 days of LPS stimulation (stimulated). (B) Chi-squared test analysis of the data in A. (C) examples of *Igl* colocalisation, separation and breaks. Scale bar = 5 μm. Statistical analyses were calculated by a Student's t-test with two-tailed distribution.

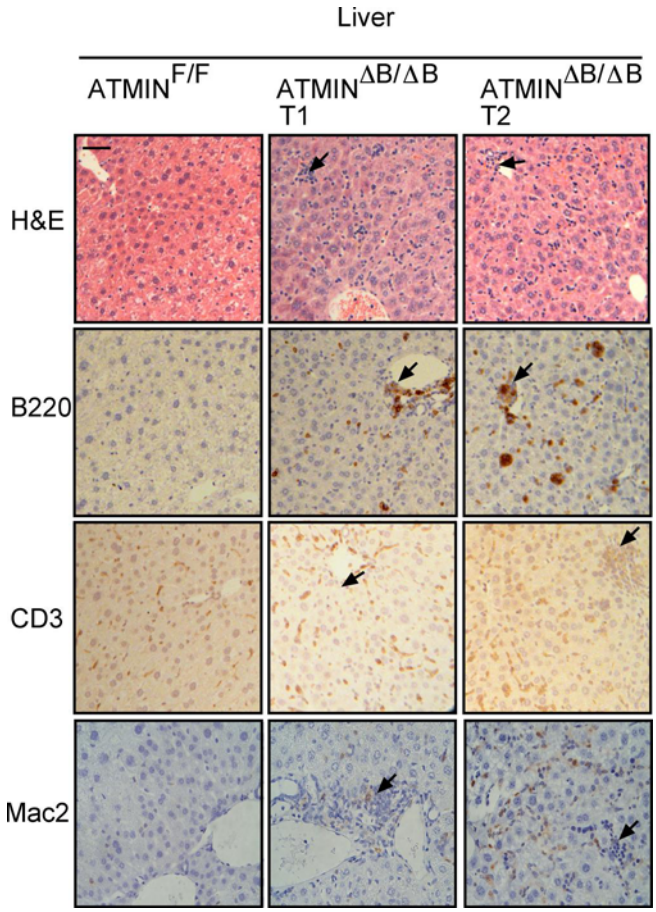


Figure S6, related to Figure 6.

Confirmation of B cell lymphomas in ATMIN$^{\Delta B/\Delta B}$ mice by IHC. H&E, B220, CD3, and Mac2 staining of liver tissue from two ATMIN$^{\Delta B/\Delta B}$ mice with B cell lymphoma (ATMIN$^{\Delta B/\Delta B}$ T1 and T2) demonstrates that the tumourigenic infiltrating cells (indicated by an arrow) are Mac2 negative, CD3 negative and B220 positive. Scale bar = 100μm.

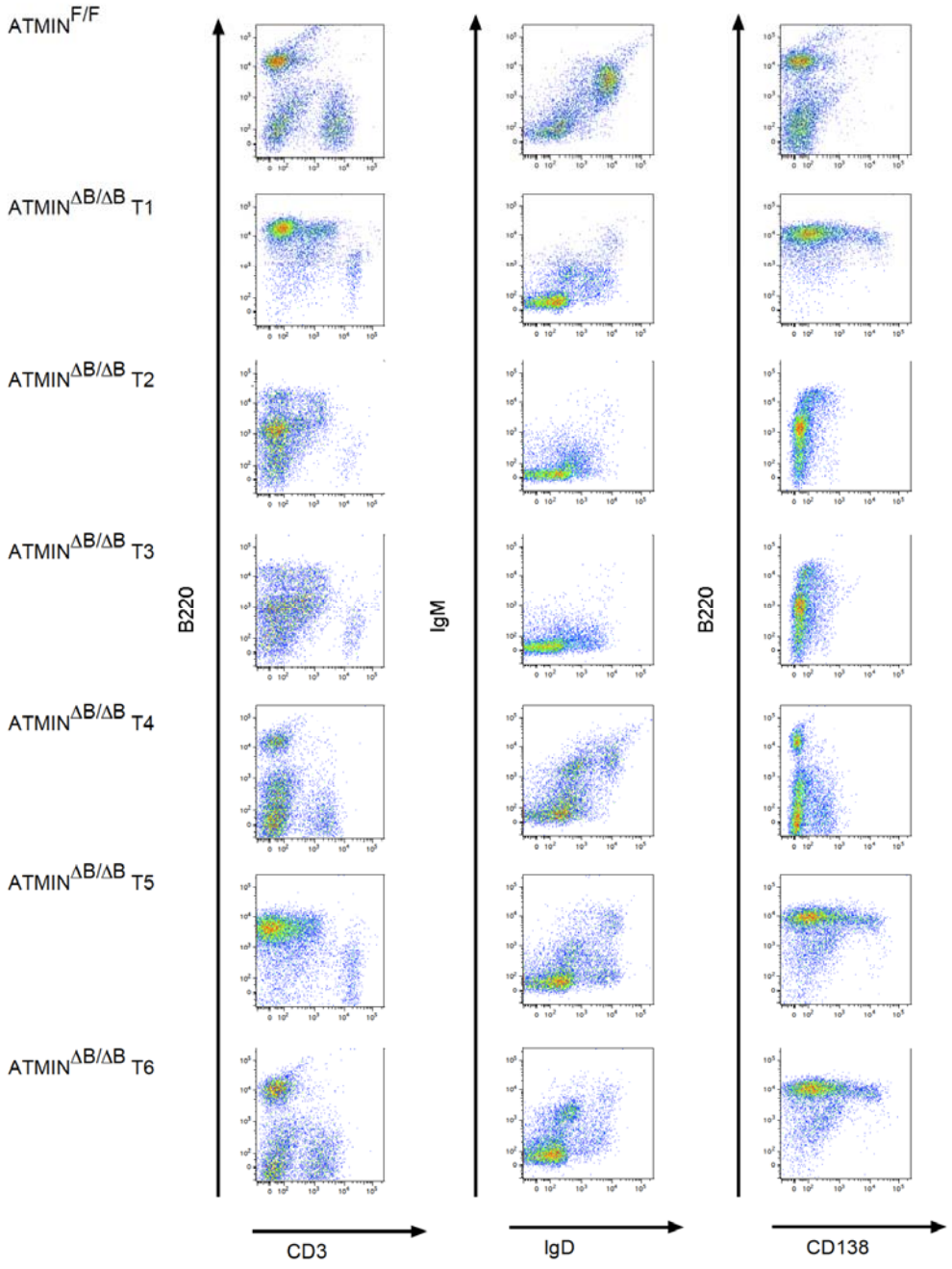


Figure S7, related to Figure 7.

FACS analysis of ATMIN$^{\Delta B/\Delta B}$ tumours. Cells from the spleens of control ATMIN$^{F/F}$ and ATMIN$^{\Delta B/\Delta B}$ T1-T6 were stained with anti-B220, CD3, IgM, IgD and CD138 antibodies and analysed by FACS.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Western blotting

Cells were extracted in RIPA lysis buffer (NEB) supplemented with protease inhibitors (Sigma). Western blots were performed using standard procedures. Protein samples were separated by SDS–PAGE, and subsequently transferred onto nitrocellulose membranes. All primary antibodies were used at 1:1000 dilution and secondary antibodies at 1:2000. The following antibodies were used: ATM 2C1 (Santa Cruz), P-S824 KAP1 (Bethyl Laboratories, Inc); KAP1 (Bethyl Laboratories, Inc); ASCIZ, (Millipore), RNA Pol II (Santa Cruz); β-actin (Sigma); HRP-conjugated goat anti-mouse/rabbit IgG (Sigma).

Southern blotting

To determine clonality of the B cell lymphomas by Southern blotting, DNA from spleens of control ATMIN$^{F/F}$ mice or ATMIN$^{\Delta B/\Delta B}$ mice with B cell lymphomas was digested overnight with *Bgl II*, resolved on 1% agarose gel, transferred to nitrocellulose and probed with a ^{32}P labeled-JH probe (1.6 KB HindIII–EcoRI fragment of J_H4 region as in Wang et al., 2008).

Immunohistochemistry and in situ hybridisation

Tissue was fixed overnight in 10% neutral buffered formalin, briefly washed with PBS and transferred into 70% ethanol, processed and embedded into paraffin. Sections were cut at 4 μm for H&E staining and

immunohistochemistry using an antibody against B220 (BD Pharmingen), CD3 (Dako), Mac2 (Cedarlane) and Ig2b (BD Pharmingen). Samples analysed for *atmin* RNA by *in situ* hybridisation were processed as described above by the Experimental HistoPathology Unit at the LRI.

Immunofluorescence

Purified B cells were adhered onto slides that had been pre-coated with poly-lysine and stained as described previously (Loizou et al., 2004). The antibody used was ATM P-S1981-FITC (10H11.E12) from LSBio and γH2AX (ser139) clone JBW301 biotin conjugate (from Millipore) followed by staining with an anti-streptavidin-PE (BD Pharmingen). Fixed cells were counterstained with DAPI.

Metaphase FISH

Purified B cells were stimulated as described above with LPS/IL4 for 3 days and then arrested with Colcemid (Invitrogen) at a final concentration of 0.12ug/ml for 3 hours. For FISH, metaphase spreads were prepared using standard procedures and then probed with BAC RP24-258E20 (containing Cδ-Cα of the *Igh* locus) directly labelled with alexa-555 (Invitrogen), a FITC labelled telomere-repeat specific peptide nucleic acid (PNA) probe (Panagene), and a chromosome 12 paint probe labelled with biotin (Cambio Starfish) and detected with streptavidin alexa-647. Metaphases were imaged using a Metasystems metafer automated capture system and counted manually using Volocity software (Improvision).

3D interphase FISH

3D FISH for *Igl* was essentially as described in Wang et al 2009 and references therein with minor modifications. BACs RP23-382P9 (5' *Igl*) and RP23-374P12 (3' *Igl*) were directly-labelled by incorporation of amino-allyl-dUTP (Ambion) by nick-translation followed by chemical coupling of succinimidyl-ester derivates of alexa-488 (382) and alexa-555 (374) (Invitrogen). Cells were imaged in 3D (0.5μm Z step) on a metacyte automated imaging system (metasystems) and FISH signals counted/distances between signals measured using a custom metacyte classifier. Cell image libraries were generated manually to correct for errors in classification.

Mulitplex-Fluorescence In Situ Hybridization (M-FISH)

Metaphase spreads were prepared as for metaphase FISH. Mouse 21 colour M-FISH paint was made essentially following the "pooling" strategy described in Jentsch et al., 2001. Briefly, individual mouse chromosome-specific DOP-PCR products were grouped into five re-amplifiable pools based on the fluorescence label and subsequently labeled with biotin-16-dUTP (Roche), Texas red-12-dUTP, Cy3-dUTP and Cy5-dUTP, Cy3-, Cy5-dUTP (GE Healthcare), SpectrumGreen-dUTP (Abbott Molecular) or Chromatide® Texas Red-12-dUTP (Invitrogen) via PCR. The labeled DNA was mixed and ethanol precipitated together with mouse Cot-1 DNA. The pellet was resuspended in hybridization buffer containing 50% formamide, 10% dextran sulfate and 2×SSC in 1× Denhardt solution (Sigma). Following hybridization and post-hybridization washes, biotin-labeled probe was detected with one layer of

Cy5.5-conjugated mouse anti-biotin (5 µg/ml, Rockland Immunochemicals). After post-hybridisation washes, slides were mounted with SlowFade Gold® mounting solution containing DAPI (Invitrogen). Metaphases were examined with a Zeiss Axioplan Imaging microscope equipped with narrow bandpass filters for Cy5.5, Cy5, Texas red, Cy3, fluorescein and DAPI fluorescence. Images were captured via a cooled CCD camera (ORCA-ER, Hamamatsu) and processed with the SmartCapture and SmartType software (Digital Scientific).

Flow cytometry

Cells were stained for 1 hour at 4°C with the indicated antibodies in PBS supplemented with 1% FCS. For analysis of cell surface markers, flow cytometry was performed on living cells on the basis of forward and side scatter with the exclusion of $DAPI^+$ cells. The following fluorochrome-conjugated antibodies in various combinations (BD Pharmingen), as described in the relevant sections, were used: IgG1-PE, IgG3-FITC, IgM-PE, B220-FITC, B220-APC, CD43-FITC, CD3-PE, IgD-biotin (followed by anti-streptavidin-APC), CD138-PE. For analysis of mitochondrial integrity as a measure of apoptosis cells were stained with TMRE and TOPRO using standard methods. For cell cycle profiling and identification of subG1 populations, cells were fixed in 70% ethanol, stained with propidium iodide (PI) or Hoechst and analyzed. For CFSE staining cells were analysed as described by the manufacturer (Invitrogen). Cells were cultured under the conditions described to induce class switching, harvested at various time points after stimulation and analyzed using a Becton Dickinson FACScan.

Antibody detection by ELISA

To measure antigen-specific immunoglobulins, 96-well plates (Nunc) were coated with 2.5μg/well TNP-OVAL (Biosearch Technologies, Inc.), blocked with 2% BSA/PBS and incubated with serum diluted in 2% BSA/PBS. Plates were then incubated with purified biotin-conjugated goat anti-mouse IgE, IgG2b, IgG3, IgG1 or IgM (Southern Biotechnology Inc.) and detected with HRP-conjugated goat anti-mouse avidin (Southern Biotechnology Inc). For detection of total IgG1, 96-well plates (Nunc) were coated with purified IgG1, blocked with 2% BSA/PBS and incubated with serum diluted in 2% BSA/PBS. Plates were then incubated with purified biotin-conjugated goat anti-mouse (Southern Biotechnology Inc.) and detected with HRP-conjugated goat anti-mouse avidin (Southern Biotechnology Inc). Wells were developed with pNPP tablets (Sigma) dissolved in Tris buffer and the optical density (OD) was measured at 405 nm. Titers were determined by interpolation of the dilution of serum that gave a 50% OD of the maximum absorbance achieved.

Quantitative Real Time PCR

Total RNA was extracted using RNAEasy (Qiagen), reverse transcribed with random hexamers and superscript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was accomplished with SYBR Green incorporation (Platinum® Quantitative PCR SuperMix-UDG w/ROX, Invitrogen) using an ABI7900HT (Applied Bioscience), and the data were analyzed using the SDS 2.3 software. For the analysis of γ1 GLT and PT results were normalized to those obtained for GAPDH and results are presented as fold induction over

control day 0. For the analysis of γ1 CT the results were normalized to those obtained for GAPDH. The following primers were used, as in Lumsden et al., 2004; Reina-San-Martin et al., 2003; Schotta et al., 2008:

GAPDH-F 5'-TGAAGCAGGCATCTGAGGG-3'

GAPDH-R 5'-CGAAGGTGGAAGAGTGGGAG-3'

γ1 GT-F 5'-TCGAGAAGCCTGAGGAATGT-3'

γ1 GT-R 5'-ATAGACAGATGGGGGTGTCG-3'

γ1 PT-F 5'-TCGAGAAGCCTGAGGAATGT-3'

γ1 PT-R 5'-ATAGACAGATGGGGGTGTCG-3'

γ1 CT-F 5'-TCGAGAAGCCTGA-GGAATGTG-3'

γ1 CT-R 5'-GAAGACATTTGGGAAGGACTGACT-3'

ATM-F 5'-AATTTTCTACTTGACTGATCACCACCTA-3'

ATM-R 5'-CAGCACGTATCCTACGAATGGA-3'

Pax5-F 5'-AACTTGCCCATCAAGGTGTC-3'

Pax5-R 5'-CTGATCTCCCAGGCAAACAT-3'

For the analysis of ATMIN RNA levels in B cells of $ATMIN^{F/F}$ and $ATMIN^{\Delta B/\Delta B}$ mice, B-cells were sorted as $B220^{+}$ and then RNA was extracted. Primers used were:

Actin-F: 5'-GGATGCAGAAGGAGATCACTG-3'

Actin-R: 5'-CGATCCACACGGAGTACTTG-3'

ATMIN-F: 5'-CAAGCACTCGGTGTCAATGG-3'

ATMIN-R: 5'-CACAGTGCGCAGGCATCT-3'

Switch junction analysis

Splenic B cells from four mice of each genotype were stimulated for 4 days with LPS and IL4 following which DNA was extracted and Sγ1-Sμ junctions were amplified essentially as described (Martomo et al., 2004; Martomo et al., 2006). The following primers were used: for the μ switch region the first forward primer is 5'-AGATAAAATGGATACCTCAG-3' and second forward primer is 5'-ACTCTAGATGGTTTTTAATGGTGGGTTT-3'. For the γ1 switch region the first reverse primer is 5'- CAATTAGCTCCTGCTCTTCTGTGG -3' and second reverse primer is 5'-ACGAATTCAGCTCCTGCTCTTCTGTGG-3'. Nested PCR was done using Expand Long Template PCR System (Roche). The first amplification was 30 cycles in which the first 10 cycles were done at an annealing temperature of 55°C for 30 sec, and extension at 68°C for 1 min. This was followed by another 20 cycles using the same conditions except for an extension time of 2.5 min. The second PCR was done as the first except the annealing temperature was changed to 58°C. PCR products of 1000-500bp were then cloned into pGEM-T Easy (Promega) and sequenced.

Hypermutation analysis

Germinal centre CD45R(B220)$^+$PNA$^+$ (PE and FITC-conjugated antibodies from BD Pharmingen and Sigma, respectively) B cells were sorted from Peyer's Patches of approximately 10-month old mice. The J_H4 3' flanking sequence of endogenous rearrangements of the VhJ558 family members were amplified by PCR using Turbo Pfu (Stratagene) and primers FR105 5'-GGGAATTCGCCTGACATCTGAGGACTCTGC-3' and FR106 5'-GACTAGTCCTCTCCAGTTTCGGCTGAATCC-3' as in Jolly et al., 1997. The resulting products were cloned into TOPOBlunt (Invitrogen) and sequenced.

Sequences were aligned and analyzed by using Seqman software. A 476-bp segment was analyzed for mutations.

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