5. Materials and Methods

Nucleic acids

Extraction of nucleic acids

mRNA was extracted from cultured cells with Oligotex Direct mRNA Mini Kit (Qiagen); total RNA was extracted using either Trizol (Invitrogen) or RNeasy (Qiagen); genomic DNA with DNeasy Tissue kit (Qiagen); and plasmid DNA with QIAprep-Spin Miniprep, Midi or Maxi kit (all Qiagen) according to the manufacturer's specifications.

cDNA synthesis

0.5-1.0 µg RNA, 1 µl oligo dT_{40} (10 µM), H_2O added to 10 µl were incubated for 5 min at 70°C. The sample was placed on ice, and 4 µl 5 x RT buffer (Invitrogen), 1 µl 10 mM dNTP, 2 µl 0.1 mM DTT, 0.5 µl RNase inhibitor (Roche), 0.25 µl Superscript II (Invitrogen), and 2.25 µl H_2O were added and mixed. The sample was incubated for 1 hr at 37°C. For conventional PCR and Taqman, cDNA was diluted 1:5 with H_2O.

PCR

Table 1: Primer

#	Name	Primer sequence in 5'-3' orientation
1	5' AID old	CGC(CTCGAG)(GCCACC)ATGGACAGCCTTCTGATG
2	3' AID old	ATGGTT(CCAATTTAATGG)TCAAAATCCCAACATACGAAATGC
3	5' AIDNC	AGGGAGTCAAGAAAGTCACGCTG
4	3' AIDNC	AGAGCATCATTCACGACCCAAAGTC
5	5' AID exon 2	AGATAGTGCCACCTCCTGCTCAC
6	5' AID exon 3	TTACCGCGTCACCTGGTTCACC
7	5' AID exon 4	TCAAAGCCTGGGAAGGGCTACATG
8	5' Vgeneric	AGGT(CG)(AC)A(AG)CTGCAG(CG)AGTC(AT)GG
9	3' Cµ1	GGTTCTGATACCCTGGATGACTTCAG
10	5' V_HWEHI	CTTATGCCATACTATAGGAAAACAGGG
11	3' JH2	CCAGAGATTTATAGGGATCCTGGCCA
12	5' genCµ	GCAAGAAGACAGATTCTTACCCC
13	3' genCµ	GGGAGGGTTTGGTTCTTACCTGG
14	5' mUNG	AGGTCCGTGCTCAGCGCAGGC
15	3' mUNG	GGCGGCGGTAGCTGAAAGCACC
16	5' Ku70	ACCTTCTGCTGTCCCAGTGG
17	3' Ku 70	AAGTGGCTGGGCTTCTGAGC
18	5' Ku 80	TCAAATCACCTGAGGACCAGC

#	Name	Primer sequence in 5'-3' orientation
19	3' Ku 80	ACTCTTGGATTCCCCACACATC
20	5' 70-topfor	GACATCATCACCACCGCTGAG
21	3' 70-toprev	CAGCGGTGGTGATGATGT
22	5' 70-btmfor	TCCTCTGGGTACACGAAC
23	3' 70-btmrev	TGTTCGTGTACCCAGAGGAG
24	5' 80-topfor	GCCCCAACTTGTCTATAAAG
25	3' 80-toprev	CAATGGTCAGTTGGCAGG
26	5' 80-btmfor	CTCCTGGAGATGTAAGGCT
27	3' 80-btmrev	AGCCTTACATCTCCAGGAGC
28	5' GAPDH	TGAAGGTCGGTGTGAACGGATTTGGC
29	3' GAPDH	CATGTAGGCCATGAGGTCCACCAC.
30	3'IgE-CH2	CTCCGAGTGTGGGCCAAATA
31	5' Iε	TGGGCATGAATTAATGGTTACTAG
32	5' Iγ2b	CTTCACGCGGCTCCACATGTGA
33	3' Iγ2b-CH1	TGGGTGAGCAACGCTGCAGGT
34	5' Iα	ACTCTCTACCATAGGGAAGATAGCC
35	3' IgGα-CH2	CAGGATTTCTCAGGCCATTCAGAG
36	5' YFP-XF	AATTCTCGAGTCGCCACCATGGTGAGCAAG
37	Y3' FP-XB	AATTCTCGAGTTACTTGTACAGCTCGTCCATGCC
38	5' IgA	AGTCTGCGAGAAATCCCACC
39	3' IgA	ATGTTGCACGGAACATTTCACG
40	5' IgG2-1/2	ACACATTCTCCTCTCTTGCAG
41	3' IgG2-1/2	AGTGGAGCTCTGGTAGTGAC
42	5' IgG2-3/4	ATTTCCATCTCTCCTCATCAGC
43	3' IgG2-3/4	TACCTTGGAAAGAACCAGGAC
44	5' CμE2-F	AGATCTGCATGTGCCCATTCCAGG
45	3' CμE2-R	CATGTTCGGGTGGCATTGGCCATA
46	5' CμE3-F	ATGCCTAGCCCTCCCAGATTAGG
47	3' CμE3-R	AAGAGGACCTGCCCTCCCTATG
48	5'-CμE4-F	TCCAATTGCAGGACCCTTCCCG
49	3' CμE4-R	AGAACAGGCCCGTTTGTAAGTGTCC
50	5' Cμ-M-F	AGACTTGGCTTGACCCTCCCTC
51	3' Cμ-M-R	CTGTCAACACCGCAGGAAAGGTT
52	5' CμRT	AGAGTCAGTCCTTCCCAAATGTCTTC
53	3' CμRT	TCCATGTGACATTTGTTTACAGCTCAGC
54	5' Vκgen	GGCTGCAG(CG)TTCAGTGGCAGTGG(AG)TC(AT)GG(AG)AC
55	3' Jκ5	CTCCTAACATGAAAACCTGTGTCTTACACA
56	5' κ promotor	TACTGCAACGCTTACTTGGA
57	3' κ intron	TACATGTTCCCTTACATCTGG
58	5' κ leader	ATTTGCCTGTTCATCTCTTGGTGCTTC
59	5' V-active	AGGAAACAAACTGGAGTGGATGGGC

#	Name	Primer sequence in 5'-3' orientation
60	3' JH2	ACTGTGAGAGTGGTGCCTTGGC
61	5' V-silent	ATGGTGTACACTGGGTTCGCCAG
62	3' JH3	AGAGTCCCTTGGCCCCAGTAAG
63	5' Sμ	AGGACAGTGCTTAGATCCAAGGTGAGTG
64	3' Sμ	ACAGCTCAGTCTAGCACATCTGAGTCC
65	3' Cμ2exon	TCCACGAGCTTCCCATCCTTTAGC
66	dT40	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
67	3' Cκ	GTGGTGGCGTCTCAGGACCTTTGT
68	5' Neo direct	CGACGGCGAGGATCTCGTCGTGAC
69	5' G3 AID KO	GGGCCAGCTCATTCCTCCACTC
70	5' 811 AID WT	CTGAGATGGAACCCTAACCTCAGCC
71	3' G4 (AID E4)	CACGATTTTCTACAAATGTATTCCAGC

Table 2: PCR

PCR product	Template	5' primer	3' primer	Enzyme system	# Cycles	Denaturing step	Annealing step	Elongation step
AID coding	RNA	1	2	Superscript[1]	40	94°C 30 sec	50°C 30 sec	72°C 1 min
AID non-coding	cDNA	3	4	Pfu[2]	35	95°C 1 min	52.2°C 1 min	72°C 1 min
AID exon 2	cDNA	5	4	Taq[3]	35	94°C 1 min	55°C 1 min	72°C 1 min
AID exon 3	cDNA	6	4	Taq	35	94°C 1 min	55°C 1 min	72°C 1 min
AID exon 4	cDNA	7	4	Taq	35	94°C 1 min	55°C 1 min	72°C 1 min
VDJ-Cμ1	DNA	8	9	Superscript	40	94°C 30 sec	53.4°C 30 sec	72°C 1 min
WEHI V_P	DNA	10	11	Pfu Turbo[2]	40	95°C 45 sec	58°C 45 sec	72°C 1 min
Cμ1	DNA	12	13	Pfu Turbo	40	95°C 1 min	60°C 1 min	72°C 30 sec
UNG	RNA	14	15	Superscript	40	94°C 30 sec	62°C 30 sec	72°C 1 min
Ku70	cDNA	16	17	Pfu	30	95°C 45 sec	62.5°C 45 sec	72°C 2.5 min
Ku80	cDNA	18	19	Pfu	30	95°C 45 sec	62.5°C 45 sec	72°C 2.5 min
GAPDH	RNA	28	29	Superscript	22-30	94°C 30 sec	55°C 1 min	72°C 1 min
GAPDH	cDNA	28	29	Pfu/Taq	22-30	94°C 1 min	55°C 1 min	72°C 1 min
IgE H chain	cDNA	8	30	One step[4]	40	94°C 45 sec	51°C 45 sec	72°C 1 min
Iε	RNA	31	30	One step	40	94°C 45 sec	51°C 45 sec	72°C 1 min
Iγ2b	RNA	32	33	One step	40	94°C 45 sec	55°C 45 sec	72°C 1 min
Iα	RNA	34	35	One step	42	94°C 45 sec	52°C 45 sec	72°C 1 min
GFP	DNA	36	37	Native Pfu[2]	30	95°C 1 min	60.6°C 1 min	72°C 1 min
Ig α exons 1/2	DNA	38	39	Pfu	35	95°C 1 min	55.9°C 1 min	72°C 1 min
Ig γ2b exons 1/2	DNA	40	41	Pfu	35	95°C 1 min	55.9°C 1 min	72°C 1 min
Ig γ2b exons 3/4	DNA	42	43	Pfu	35	95°C 1 min	57.2°C 1 min	72°C 2 min
Cμ2	DNA	44	45	Native Pfu	35	95°C 45 sec	58°C 45 sec	72°C 1.5 min
Cμ3	DNA	46	47	Native Pfu	35	95°C 45 sec	58°C 45 sec	72°C 1.5 min
Cμ4	DNA	48	49	Pfu	35	95°C 45 sec	61.5°C 45 sec	72°C 1.5 min
Cμ membrane exons	DNA	50	51	Pfu	35	95°C 45 sec	62.6°C 45 sec	72°C 1.5 min

PCR product	Template	5' primer	3' primer	Enzyme system	# Cycles	Denaturing step	Annealing step	Elongation step
Cμ RT-PCR	cDNA	52	53	Taq	35	94°C 1 min	58.8°C 1 min	72°C 1 min
Generic κ L chain	cDNA	54	55	Herculase[5]	40	94°C 1 min	64.8°C 1 min	72°C 2 min
κ L chain promotor	cDNA	56	57	Pfu	35	95°C 45 sec	58.8°C 45 sec	72°C 1 min
WEHI κ L chain	cDNA	58	67	Taq	35	94°C 1 min	58°C 1 min	72°C 1 min
VP-JH2	cDNA	59	60	Taq	35	94°C 1 min	66°C 1 min	72°C 1 min
VNP-JH3	cDNA	61	62	Taq	35	94°C 1 min	66°C 1 min	72°C 1 min
S region	DNA	63	64	IProof[6]	35	98°C 10 sec	65°C 20 sec	72°C 1.5 min
V_P-Cμ2	RNA	59	65	Superscript	35	94°C 30 sec	58.5°C 30 sec	72°C 1 min
V_{NP}-Cμ2	RNA	61	65	Superscript	35	94°C 30 sec	58.5°C 30 sec	72°C 1 min
C_H1-C_H2	DNA	12	65	Pfu	35	95°C 45 sec	58°C 1 min	72°C 1 min
Genotype AID KO 1	DNA	69	71	IProof	35	98°C 10 sec	60°C 30 sec	72°C 30 sec
Genotype AID KO 2	DNA	68	4	IProof	35	98°C 10 sec	57°C 20 sec	72°C 1.5 min
Genotype AID WT 1	DNA	70	71	IProof	35	98°C 10 sec	60°C 30 sec	72°C 30 sec
Genotype AID WT 2	DNA	6	4	IProof	35	98°C 10 sec	65°C 20 sec	72°C 1.5 min
AID-Neo	cDNA	68	2	Taq	35	94°C 1 min	60°C 1 min	72°C 1 min
Primers 20-27 were used for primer walking to sequence Ku70 and Ku80.								

[1]SuperScript one-step RT-PCR with Platinum Taq (Invitrogen)

[2]Pfu (= cloned Pfu), native Pfu or turbo Pfu (Stratagene)

[3]Taq DNA polymerase, recombinant (Invitrogen)

[4]One step RT-PCR kit (Qiagen)

[5]Herculase (Stratagene)

[6]iProof High-Fidelity DNA Polymerase (Biorad)

Detection of nucleic acids

Nucleic acids were mixed with loading buffer and run on a 1 % agarose/ethidium bromide/1xTAE gel. 0.7 µg Ready load 1 kb DNA ladder (Invitrogen) was run as a standard.

Loading buffer: 20 % Ficoll 400, 0.1 M Na_2EDTA, pH 8, 1.0% sodium dodecyl sulfate, 0.25 % bromphenol blue, 0.25 % xylene cyanol.

50 x TAE electorophoressis buffer (1l): 242 gTris base, 57.1 ml glacial acetic acid, 37.2 g $Na_2EDTA•2H_2O$, adjust to pH 8.5.

1000 x ethidium bromide solution (0.5 mg/ml): 50 mg ethidium bromide, 100 ml H_2O.

Subcloning in bacteria and sequencing

For cloning into bacteria, 3' A overhangs were added to the amplification product by incubation for 10 min at 72°C with Taq polymerase. TOPO TA Cloning (Invitrogen) was done according to the manufacturer's specifications using the pCR2.1-TOPO vector. Bacteria were transformed with the TOPO vector containing the amplification product; single colonies were picked; plasmid DNA was extracted (miniprep, Qiagen). Minipreps were analyzed by restriction digest with EcoRI, which cuts out the insert. Restriction digest: 2 µl DNA + 1 µl EcoRI buffer (NEB) + 0.5 µl EcoRI (NEB) + 6.5 µl H_2O. Digest was incubated for 1 hr at 37°C. Sequencing was done by Davis Sequencing Inc.

Quantitative PCR/Taqman

Quantitative PCR was carried out using the ABI 7700 of Applied Biosystems, according to the manufacturer's instructions. Probes for real-time PCR were purchased from Applied Biosystems; at the 5' end, they were conjugated to the fluorochrome FAM; and at the 3' end to the quencher TAMRA. First strand cDNA was synthesized using random hexamer primers. The cycle conditions for real-time PCR were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Following primers and probes were used:

<u>Alpha and epsilon germline transcripts taqman</u>

"I" primers and "I probe" denote (part of) the germline transcript that includes the I exon and the C region exons.

5' Iγ2b: 5'-GAAGAGTTCAGAGTTCTCACACACAGA;

3' Iγ2b: 5'-AGTTGTATCTCCACACCCAGGG;

Iγ2b probe: 5'-6-FAM-ACCACCAAAACAACACCCCCATCAGTCTAT-TAMRA.

5'Iε: 5'-CCACTCACTTATCAGAGGACCTCA;

3'Iε: 5'-AGCAGTGCCTTTACAGGGCTT,

Iε-Probe: 5'-6-FAM-CCTCTATCAGGAACCCTCAGCTCTACCCC-TAMRA.

5' HPRT: 5'-TGGAAAGAATGTCTTGATTGTTGAA;

3' HPRT: 5'-AGCTTGCAACCTTAACCATTTTG;

HPRT probe: 5'-6-FAM-CAAACTTTGCTTTCCCTGGTTAAGCAGTACAGC-TAMRA.

GFP taqman

5' GFP: 5'- CCACATGAAGCAGCACGACT

3' GFP: 5'- TGCGCTCCTGGACGTAGC

GFP Probe: 5'-6-FAM-TTCAAGTCCGCCATGCCCGAA-TAMRA.

AID taqman

5' AID: 5'-GAAAATTCTGTCCGGCTAACCA

3' AID: 5'-TCGCAAGTCATCGACTTCGT;

AID probe: 5'-6-FAM-TCGGCGCATCCTTTTGCCCTT-TAMRA;

5' β-actin: 5'-AGGTCATCACTATTGGCAACGA;

3' β-actin: 5'-CACTTCATGATGGAATTGAATGTAGTT;

β-actin probe: 5'-6-FAM-TGCCACAGGATTCAATACCCAAGAAGG-TAMRA.

RNAi

Three different oligonucletides (designated as shRNA 1-3) encoding small hairpins were ordered 5' phosphorylated and PAGE purified. In the following, these oligonucleotides are listed; the underlined nucleotides form the stem; bold labeled nucleotides form the loop; grey background indicates the terminator, and italic the HpaI restriction site. A second reverse complementary primer was designed for each shRNA primer (indicated by Rev. Oligo).

ShRNA #1 at position 346 of the 3' UTR:

For. Oligo: 5'-TGGACACCGATGCTATTTATTTCAAGAGAATAAATAGCATCGGTGTCCTTTTTTC

Rev. Oligo.:

5'-*TCGA*GAAAAAAGGACACCGATGCTATTTATTCTCTTGAAATAAATAGCATCGGTGTCCA

ShRNA #2 at position 10 of the 3' UTR:

For. Oligo: 5'-TGGACTCTGGTTACCTCTGATTCAAGAGATCAGAGGTAACCAGAGTCCTTTTTTC

Rev. Oligo:

5'-*TCGA*GAAAAAAGGACTCTGGTTACCTCTGATCTCTTGAATCAGAGGTAACCAGAGTCCA

ShRNA #3 at position 312 of the 3' UTR:

For. Oligo: 5'-TGCAGCTCAATTGTCTATGTTTCAAGAGAACATAGACAATTGAGCTGCTTTTTTC

Rev. Oligo:

5'-*TCGA*GAAAAAAGCAGCTCAATTGTCTATGTTCTCTTGAAACATAGACAATTGAGCTGCA

Annealing step: 23 µl ddH_2O + 1 µl sense oligo (100 µM) + 1 µl antisense oligo (100 µM) + 25 µl 2 x annealing buffer (200 mM potassium acetate, 60 mM HEPES-KOH pH7.4, 4 mM Mg acetate) were mixed incubated for 5 min at 95°C. Sample was gradually cooled down to room temperature using a PCR machine. For the ligation step, 1 µl of 1:20 diluted annealed oligos, 50-100 ng HpaI-XhoI digested dephosphorylated (CIPed) vector, 2 µl 5 x ligation buffer (Invitrogen), 1 µl T4 ligase (Invitrogen), add to 10 µl with ddH_2O were mixed and incubated at 14°C overnight. 1 µl of ligation was transformed in top 10 cells (Invitrogen).

Constructs and cloning

pSico

Fig. 64 shows the Cre-lox regulated vector (pSico) for conditional activation of RNA interference (Ventura et al., 2004). In this vector the mouse U6 promoter has been modified by including a hybrid between LoxP site and TATA. Expression of Cre will lead to a recombination of the TATA-lox sites and conclude in the expression of the short hairpin RNA (shRNA). The pSico vector was obtained from M. McManus.

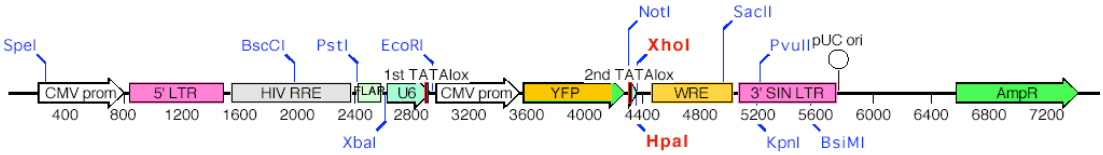


Fig. 64. pSico vector (Ventura et al., 2004)

MIG-Cre-GFP

The virus-encoding vector MSCV-IRES-GFP (MIG) was originally cloned by K. Humphries lab (Terry Fox Laboratory, Vancouver, BC, Canada) (Antonchuk et al., 2001; Antonchuk et al., 2002). Cre was cloned in the MIG vector with EcoRI (personal communication with M. Manus lab).

Fig. 65. MIG vector

pCMV-Myc-Supt6h or pCMV-HA-Supt6h

Supt6h was cut out of the vector KIAA0162 (Bluescript II) with SalI and NotI. This vector was obtained from the Kazusa DNA Research Institute, Japan (clone name: ha03982). Supt6h was ligated into the pCMV-HA and pCMV-Myc vector (Invitrogen).

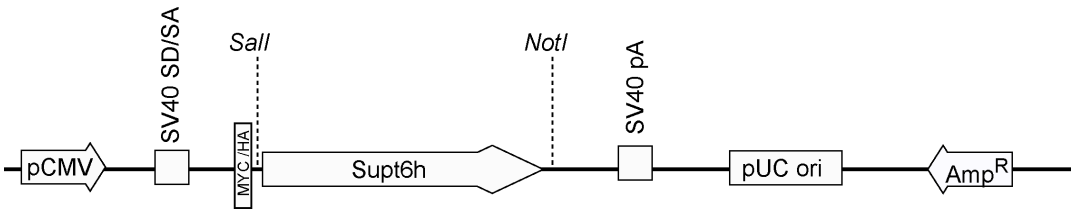


Fig. 66. Supt6h-Myc/Supt6-HA vector

AID Flag/HA

The original vector was obtained from Bernardo Reina (Nussenzweig lab). AID with the double tag FLAG and HA (2T-AID) on its N-terminus was cloned into the PMX-PIE (pMX-puromycin-IRES-EGFP) vector with BamHI/NotI. Double tag AID was cloned into a pcDNA3 vector (Invitrogen) using the same restriction sites.

Fig. 67. AID double tag vector

Point mutation reporter plasmid

The reporter plasmid is based on the retroviral CRU5-IRES-eGFP vector, which is derived from the p96-dsG plasmid (Lorens et al., 2000). Further features and derivates of this vector are described in (Klasen et al., 2005).

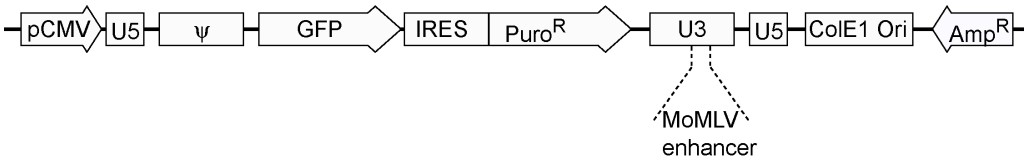


Fig. 68. Indicator plasmid Cru5

Switch construct SCI(μ,α)

Fig. 69 shows the structure of the CSR substrate SCI(μ,α) (Okazaki et al., 2002). pEF-1α, elongation factor 1α promotor; pTET, tetracycline-responsive promotor; Neo, neomycin resistance gene with the TK promotor; HyTK, gene for the fusion protein hygromycin phosphotransferase-thymidine kinase, with the CMV promotor; EC and TM indicate extracellular and transmembrane domains of CD8α, respectively. Before electroporation, SCI(μ,α) was linearized with ScaI.

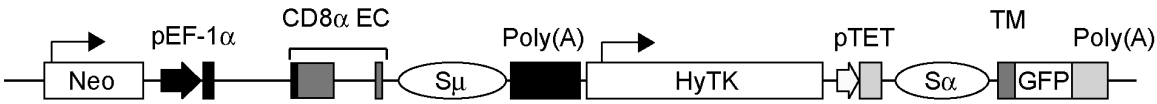
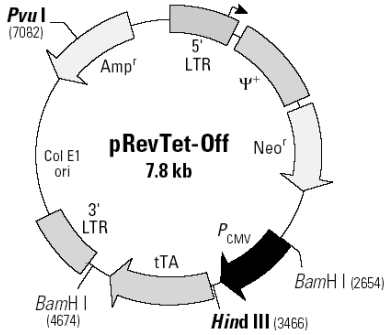


Fig. 69 . Switch construct SCI(μ,α)

pRevTet-Off™ vector (Clontech)



pRevTet-Off is a retroviral vector expressing the tetracycline-controlled transactivator (tTA) from the CMV promoter. The tTA binds to the Tet-response element (TRE) on a second vector, in our case SCI(μ,α), thus activation transcription in absence of Tetracyline. As tetracycline is added, transcription from the inducible promoter is turned off.

Fig.70. pRevTet-Off

Protein

Western blot

Cells were washed twice with cold PBS, counted, resuspended and lysed in an appropriate volume of lysis buffer (Tab. 1) for 20 min on ice. As a ratio cell/lysis buffer: approximately 2-3 x 10^7 cell per 1 ml lysis buffer was taken. Samples were spun down for 20 min at 14 000 g, 4°C. The supernatant was transferred to a fresh tube and the protein concentration was determined by the Bradford assay. Samples were mixed with 2 x SDS sample buffer (Tab. 1) and incubated at 95°C of 10 min. 30 µg whole cell lysate of each sample was loaded on a 10-15 % SDS gel, depending on the size of the protein of interest. As a standard, 10 µl of Precision Plus Protein all blue standards (Biorad) was loaded. Running and blotting the gel was done as described in Maniatis current protocols in molecular biology using buffers shown in Tab. 1. The gel was blotted onto nitrocellulose membrane (N+ bond, Amersham) and blocked with 5% milk/TBS-T (Tab. 1) either at 4°C over night or 1-2 hrs at room temperature. The primary antibody was applied over night at 4°C rotating (see below). Blots were washed twice for 10 min with TBS-T (Tab. 1) and blots were incubated with the secondary antibody for 1 hr at room temperature rotating. Blots were washed twice for 10 min with TBS-T. ECL (Amersham) was used according to the manufacture's instructions. The blot was exposed to an x-ray film and the film was developed. If necessary, the blot was stripped with stripping buffer (Tab. 3) for 1 hr and probed again.

Primary antibodies used in Western blots:

All primary antibodies were used in a 1:1000 dilution in 5% milk/TBS-T (Tab. 1) unless indicated otherwise. Primary antibody was incubated overnight at 4°C rotating.

Anti-AID: rabbit affinity purified anti-AID antibody against the C-terminus of AID (kindly provided by F. Alt) (Chaudhuri et al., 2003), dilution 1:250.

Anti-kappa: Goat anti-mouse kappa unknown concentration (Fisher Scientific lot 2392-7, OB1137-21).

Anti-GFP: antibody was produced in rabbit (kindly provided by M. Fukata, D. Bredt lab).

Anti-IgM: Affinity purified rabbit anti-mouse IgM, µ chain specific, 2.4 mg/ml (Jackson Immuno Research Laboratories)

Anti-TID: mouse monoclonal TID-1 Ab-2 Clone RS13 (Neomarkers).

Anti-Supt6h: rabbit anti-human Supt6h serum published in (Winkler et al., 2000).

Anti-Myc: mouse anti Myc antibody clone 9E10 (BD biosciences).
Anti-FLAG: ANTI-FLAG M2 monoclonal antibody (SIGMA product # F3165), working solution 10 µg/ml.
Anti-Actin: mouse monoclonal anti-actin (Ab-1) antibody (Oncogene).

Secondary antibodies

All secondary antibodies were obtained from Southern Biotechnology Associates, Inc, and were used in a 1: 5000 dilution in 5% milk/TBS-T (Tab. 1). The secondary antibody was incubated with the blot for 1 hr at room temperature.

Goat anti-rabbit Ig (H+L)-HRP, human absorbed, Lot G138-U318, Cat. # 4010-05.
Rabbit F(ab')2 anti-Goat IgG (H+L)-HRP, Lot C831-R591D, Cat. # 6020-05.
Goat anti-mouse Ig (H+L)-HRP, human absorbed, Lot D3403-QI13C, Cat # 1010-05.

Tab. 3. Buffers used for Western blotting:

2xSDS Sample buffer to 40 ml H_2O add: 1.52 g Tris base 20 ml glycerol 2.0 g SDS 2.0 ml β-mercaptoethanol 1 mg Bromphenol Blue Adjust to pH 6.8 with 1N HCl Add H_2O to 100 ml	10 ml Lysis buffer 0.5 ml 10% Triton X 0.5 ml 1M TRIS (pH 7.4) 0.5 ml 100 mM EDTA 1.5 ml 1M NaCl 7.0 ml H_2O → add protease inhibitors: 20 µl PMSF stock: 100 mM 2 µl Leupeptin stock: 1 mg/ml 1 µl Pepstatin stock: 2.5 mg/ml 5 µl Aprotinin stock: 2 mg/ml	100 ml Stripping buffer 140 µl β-mercaptoethanol 6.25 ml 1M Tris pH 6.8 10 ml 10% SDS 83 ml H_2O → Incubate for 1 hr @ 60°C
4 l 10 x Proteinbuffer 122 g Tris 576 g Glycine	1 l Running buffer 100 ml 10 x protein buffer 10 ml 10 % SDS add 1000 ml H_2O	100 ml 5% milk (blocking solution) 5 g dry milk fill up to 100 ml with 1xTBS-T
1 l Blotting buffer 100 ml 10 x protein buffer 10 ml 10 % SDS 200 ml methanol add 1000 ml H_2O	4 l 10 x TBS 96.8 g Tris 350.64 g NaCl → pH 7.6	5 l TBS-T 500 ml 10 x TBS 5 ml Tween 20 4.5 l H_2O

ELISA

96–well ELISA plates were coated with anti-IgM goat anti-mouse IgM FITC (cat. # 1020-02 Southern Biotechnology) or anti-kappa antibodies goat anti-mouse kappa-FITC (cat. # 1055-02 Southern Biotechnology) with 200 ng/well at 4°C overnight. On the next day plates were washed with PBS/0.1% Tween 20 and blocked with 2% milk/PBS for 2 hrs at room temperature. Equal numbers of FS and HM cells were cultured for 18 hrs. Supernatants of these cultures were applied to the ELISA plates in 3 step dilutions and incubated for 3 hrs at room temperature. Plates were washed and incubated with an HRP coupled secondary goat anti-mouse Ig antibody (cat. # 1010-05 Southern Biotechnology) for one hr at room temperature. A mouse IgM antibody (cat. # 101-01 Lot A088-M698 Southern Biotechnology) was used as a standard.

Co-immunoprecipitation

Transfected HEK cells were washed twice with cold PBS. Cells were lysed with 1.8 ml IAP buffer (20 mM Tris/Cl pH 7.5, 2 mM EDTA, 150 mM NaCl, 1 % Triton X, 50 µg/ml PMSF) for 10 min on ice. Cells were transferred into a 1.5 ml microfuge tube and spun for 30 min, 14 000 rpm at 4°C. The supernatant was transferred into a fresh tube and a 50 µl aliquot as a lysate control. During centrifugation the FLAG coupled agarose (anti-FLAG M2 Affinity Gel SIGMA product # A2220) was prepared. 60 µl slurry agarose per sample was washed 4 times with 1 ml IAP (10 000 rpm for 1 min at room temperature).

After the last wash 70 µl IAP was added to agarose, mixed with the protein lysate and incubated overnight at 4°C rotating. On the next day sample was spun for 1 min 10 000 rpm and supernatant was discarded. The agarose was then washed 7 times with 2 ml IAP. The supernatant was completely aspirated after the last wash. Flagged protein was competitively eluted with 150 µl Flag peptide (SIGMA product # F3290) (0.25 mg/ml) at 4°C, overnight, rotating. The sample was spun down, and the supernatant was transferred into a fresh tube. The sample was mixed with 2 x SDS sample buffer (Tab. 1) and incubated for 10 min at 95°C.

For precipitation of Myc tagged Supt6h, anti-c-Myc agarose conjugate (SIGMA product # A7470) was used. Protein was eluted by incubation at 95°C for 5 min with 2 x SDS sample buffer (Tab. 1).

Deglycosylation of IgM

35 µl denatured protein lysate (= 43.75 µg) were incubated with 3.5 µl G7 buffer (NEB product # B0701); 2.1 µl PNGase F (NEB product # P0704) and 3.5 µl 10% NP40. Samples were incubated for 75 min at 37°C. Cells were then loaded on an 8% SDS gel and blotted as described above.

Cell Culture

Cells were kept under conventional cell culture conditions at 37°C, 5% CO_2.

Media

18-81 media (for 18-81, WEHI-231, 70Z, Hybridomas)
500 ml RPMI 1640 (CCF, UCSF)
50 ml Fetal calf serum (Invitrogen, Biomeda)
5 ml Pen/Strep (10 000 mcg/ml Streptomycin SO_4, 10,000 units/ml Penicillin"G") (CCF, UCSF)
5 ml sodium pyruvate (11 mg/ml, 100 x) (CCF, UCSF)
25 µl β-mercaptoethanol

DMEM media (for NIH3T3, 293 HEK cells)
500 ml DMEM (CCF, UCSF)
50 ml newborn calf serum (Invitrogen)
5 ml Pen/Strep (10 000 mcg/ml Streptomycin SO_4, 10,000 units/ml Penicillin"G") (CCF, UCSF)

Immunofluorescence

$7x10^4$ cells were spun onto slides with a cytocentrifuge. Cells were fixed for 5 min in absolute alcohol and rehydrated with PBS-F (PBS / 1% BSA / 1% NaN_3) overnight. Fixed cells were incubated with 1:30 diluted primary antibodies goat anti-mouse kappa unlabeled (cat. # OB1137-21 Fisher Biotech) and goat anti-mouse IgM (cat. # 1020-04 Southern Biotechnology) for 10 min covered by a cover slip. The cover slip was washed of and slide was incubated in PBS-F for 10 min. 100 µl of 1:100 diluted secondary antibody rabbit F(ab)2 anti-goat IgG FITC (cat. # OB132921 Fisher Biotech) was applied and incubated for 10 min. Slides were washed with PBS-F. Mounting media (biomeda) was applied and coverslip was sealed with nail polish.

Antibodies and control cells used for Ig class identification

For flow cytometry, using fluorescence coupled antibodies, cells were stained in the following ratio: $2x10^6$ cells were stained in 50 µl PBS / 2% FCS with 0.2-0.5 µg antibody, for 30 min on ice, and then cells washed twice with PBS / 2% FCS.
For the proof of absent CSR, the following FITC-coupled antibodies were used: anti IgM^b (AF6-78; Pharmingen); IgM^a (DS-1; Pharmingen); goat anti-IgG_{2a} (Fisher Scientific); anti-IgG_1 (Pharmingen), anti-IgG_{2b} (R12-3; Pharmingen); anti-mouse IgG_3

(R40-82; Pharmingen); anti-IgE, (R35-72; Pharmingen); anti-IgA, (R5-140 Pharmingen); and polyclonal anti-IgD was PE coupled (Southern Biotechnology). As positive control served cells from GCL 28-22 (IgD), PC1-56.0 (IgG3), OKT3.5 (IgG2a), GK14-1 (IgG2b), IGELb4-2 177), ALFA2.16 (IgA); and spleen cells were taken from a C57BL/6 mouse and were cultured with 50 µg/ml LPS and 100ng/ml IL-4 for 4 days.

For the CSR indicator experiments, cells were stained with R-PE anti-mouse CD8α (Ly2; Pharmingen). For isolation of B cells from total spleen, cells were stained with PE anti-mouse CD45R/B220 (RA3-6B2; Pharmingen) before FACS. To examine IgD expression in WEHI-231 anti-IgD^a-FITC AMS 9.1 (cat. # 553507; Pharmingen) and anti-IgD^b-PE (cat. # 05075B; Pharmingen) were used.

Spleen cell preparation

The spleen was extracted from the mouse, placed in a 10 cm dish with 20 ml cold 18-81 media, and minced with the plunger of a 5 cc syringe (BD) until most of the spleen cells are in suspension. The cell suspension is filtered through a 70 µm nylon cell strainer (Falcon) and spun down and supernatant was decanted. Erythrocytes were lysed with 1 ml sterile ddH_2O for 3 sec, and adding 30 ml of 18-81 media stopped the lysis. Cells were filtered and washed twice afterwards.

Mutation indicator plasmid experiment

$2x10^5$ Phoenix E cells were transfected, using FuGENE 6 (Roche Applied Sciences), with a plasmid containing sequences of the Moloney virus and either GFP, or GFP with an amber stop codon (Klasen et al., 2005; Wang et al., 2004a). Two days after transfection of Phoenix E cells, cells of the lines 18-81, FS and 70Z were transduced with the virus-containing Phoenix E supernatant. Following cell numbers were transduced per well of a six well plate: 18-81: $2x10^6$, All WEHI-231 lines: $3x10^6$, 70Z: $4x10^6$. The experiment was done in duplicates for each cell line. Phoenix E supernatant was filtered through a 0.45 µm syringe filter and 4 µg/ml polybrene was added to the culture. 24 h after infection cells were selected with puromycin (FS, 0.25-0.5 µg/ml; 18-81, 2.5 µg/ml; 70Z, 1.5 µg/ml). On day 3 after transduction, GFP positive cells (i.e. pre-existing mutants) were purged by FACS. Cells were grown and analyzed by flow cytometry at different time points.

CSR indicator plasmid experiment

$2x10^5$ Phoenix E cells were transfected, using FuGENE 6 (Roche Applied Sciences), with the pRevTet-Off (Clontech) plasmid. Two days after transfection of Phoenix E cells, cells of the lines 18-81, FS and 70Z were transduced with the virus-containing Phoenix E supernatant. Following cell numbers were transduced per well of a six well plate: 18-81: $2x10^6$, All WEHI-231 lines: $3x10^6$, 70Z: $4x10^6$. Phoenix E supernatant was filtered through a 0.45 µm syringe filter and 4 µg/ml polybrene was added to the culture. 24 h after infection cells were selected with Geneticin (Gibco) (18-81, 0.5 mg/ml; FS, 1 mg/ml; 70Z, 0.5 mg/ml). To buffer the media two volumes $NaHCO_3$ were added for one volume of Geneticin. After one month of selection, the cultures were treated with Doxycycline (1µg/ml). One week later, the cells were electroporated with the CSR indicator plasmid SCI(µ,α) with the following conditions: 5.5 $x10^6$ cells were electroporated (250 V, 960 µF) with 13.5 µg linearized SCI(µ,α) DNA in 1 ml media (4 cm cuvette). As an electroporator, Gene Pulser II (Biorad) was used. All cell lines were selected 24 h after electroporation with 0.4 mg/ml Hygromycin and the Geneticin selection was reduced to 0.4 mg/ml in order to decrease the stress. Cells were selected for 2.5 weeks, then Hygromycin selection was stopped, and for each cell line half of the cells were washed and incubated without Doxycycline. One week after Doxyclycline withdrawal, cells were analysed by flow cytometry.

Production of lentivirus

293T cells were 70-80% confluent on the day of transfection. Cells were transfected with four viral constructs with FuGENE (Roche Applied Sciences) according to manufactures manual. 100 µl serum–free DMEM media + 0.3 – 1 µg pMD.G + 0.3 – 1 µg pRSV + 0.3 – 1 µg pMDLgag + 0.3 – 1 µg lentilox vector are mixed in a tube 3-9 µl FuGENE 6 were added and transfection solution was applied to the cells in 2 ml per well of a six well plate.

After 36-42 hrs virus-containing supernatant was harvested and filtered through a 0.45 µm syringe filter into a SW41 ultraclear centrifuge tube. To concentrate the virus, the supernatant was spun down for 60-90 min at 25 000 rpm (SW41 rotor) at 4°C. Supernatant was placed tube in inverted position at 4°C for 20 min. Pelleted virus was resuspended with 100-300 µl of 1 x PBS at 4°C over night.

For Mig virus production, transfection was executed with a two plasmid system is used and the virus containing supernatant was concentrated using centricon filter.

18-81 infection with lentivirus

$2x10^5$ 18-81 cells were plated in 100 µl 18-81 media into 24 well plates. Either 1 ml of media only (non-infected) or 100 µl media + 1 µl concentrated lentivirus encoding for shRNA and YFP as a marker. 4 µg/ ml polybrene were added to the cells. Cells were expanded 24 hrs after transduction. 5 days after transduction cell were analysed by flow cytometry. Because only 5-8 % of the cells were transduced, we sorted for YFP positives five days after transduction.

18-81 infection with Cre constructs

For expression of Cre, cells were infected with a second virus. The virus-encoding vector MSCV-IRES-GFP (MIG) was originally cloned by Keith Humphries lab (Terry Fox Laboratory, Vancouver, BC, Canada). $5x10^5$ cells target cells (18-18 non-transduced, 18-18+Y14, 18-18+Y22, 18-18+Y31) were plated in 100 µl 18-81 media into 24 well plates. Either 1 ml of media (non-infected) or ~800 µl Mig-Cre-GFP supernatant and 4 µg/ml polybrene were added to the cells.
Analysis of cells infected with Mig-Cre-GFP showed a 34.56 % GFP positive cells 48 hrs after infection. Three days after infection cells were enriched for GFP positives and YFP negatives.
Numbers of sorted cells: Cells sorted: Y14 + Mig: $1.6x10^5$; Y22 + Mig: $2.7x10^5$; Y31 + Mig: $3.2x10^5$.

Transfection of 293 HEK cells

One day before transfection $4x10^6$ HEK cells were plated in 15 ml antibiotic free media (DMEM + 10 % NBS) in a 10 cm cell culture dish. Transfection of HEK cells was performed using Lipofectamine (Invitrogen) according to the manufactors manual. 24 µg DNA was mixed with Lipofectamine 2000 and serum-free, antibiotic-free media and incubated for 5 min at room temperature. Mixture was then added to cells. Cells were lysed 48 hrs after transfection.