Supplemental figures and legends

Figure S1

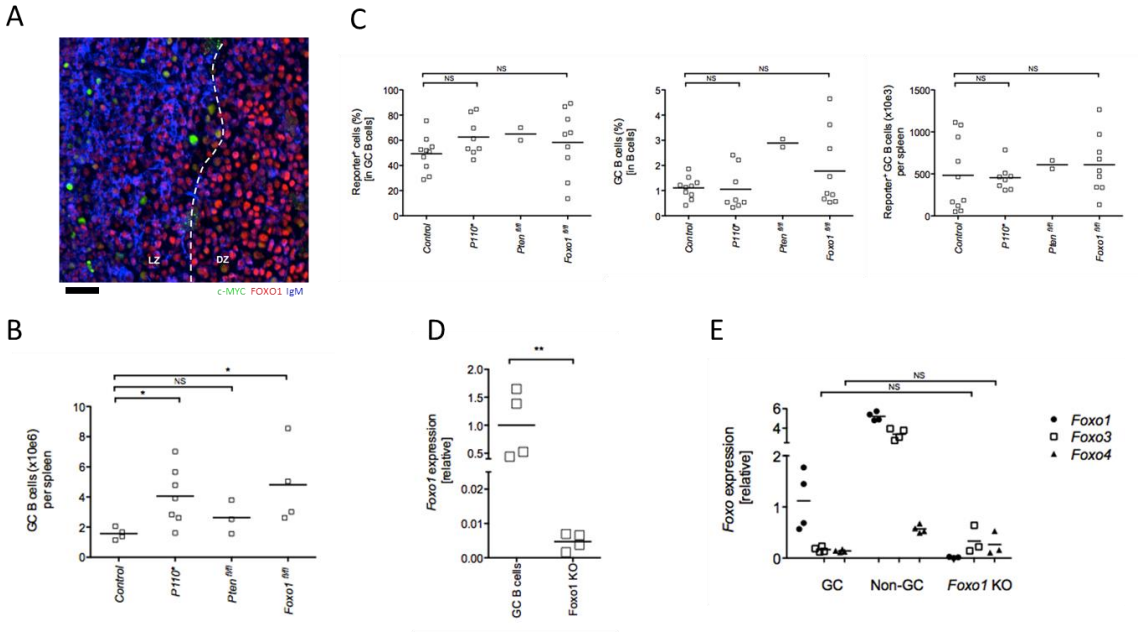

Figure S1, related to Figure 1. PI3K activation and FOXO1 deficiency in GC B cells do not interfere with GC B cell generation. **(A)** Immunofluorescence analysis of spleen sections from immunized control animals (day 10 after SRBC administration). Antibodies detecting FOXO1, IgM and c-MYC were used. Dashed line delineates DZ and LZ. These areas were defined by FDC network staining in consecutive tissue sections. Two individual animals were analyzed. Scale bar: 25 µm. **(B)** Summary and quantification of absolute splenic GC B cells in control and mutant animals at day 10 after SRBC injection. Each symbol represents an individual animal; horizontal lines indicate the mean. **(C)** Summary and quantification of splenic GC B cells in control and mutant animals at day 20 after SRBC injection (presented as in (B)). Left: reporter$^+$ cells (%); center: GC B cells (%); right: reporter$^+$ GC B cells (x10e3). **(D)** Quantitative RT-PCR analysis of *Foxo1* expression in GC B cells of control animals and *Foxo1* knockout (*Foxo1* KO) GC B cells isolated 10 days after SRBC immunization. Results are normalized to those of GC B cells and are presented relative to expression of the control gene *Hprt*. Each symbol represents an individual animal; horizontal lines indicate the mean. **(E)** Quantitative RT-PCR analysis of *Foxo1*, *Foxo3* and *Foxo4* expression in GC and non-GC B cells of control animals as well as *Foxo1* knockout (*Foxo1* KO) GC B cells isolated 10 days after SRBC immunization (presented as in (D)). Results are normalized to the expression of *Foxo1* in GC B cells and are presented relative to expression of the control gene *Hprt*. NS, not significant ($P > 0.05$); *$P \leq 0.05$ and **$P \leq 0.001$ (Wilcoxon-Mann-Whitney test).

Figure S2

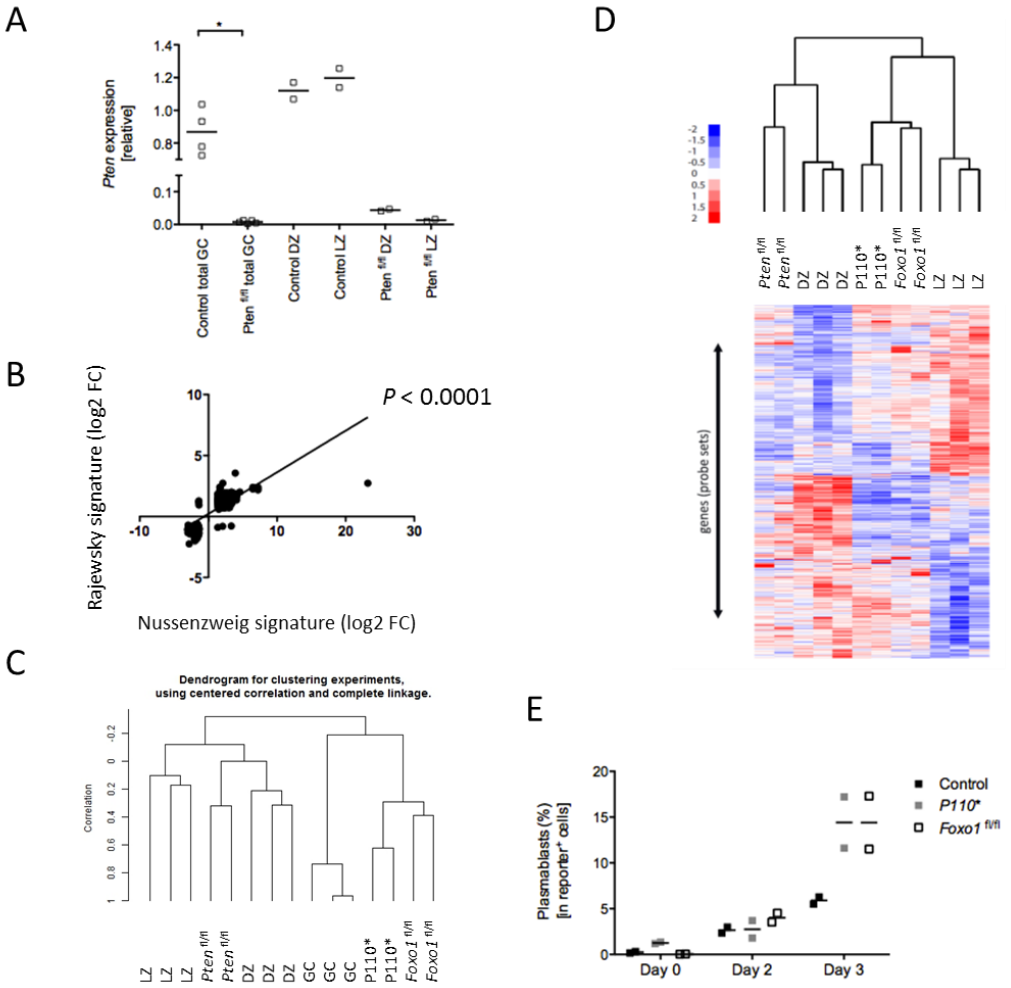

Figure S2, related to Figure 2. Mutant GC B cells activate LZ-like and other gene programs. **(A)** Quantitative RT-PCR analysis of *Pten* expression in total GC B cells as well as DZ and LZ GC B cells of control and *Pten* $^{fl/fl}$ animals isolated 10 days after SRBC immunization. Results are normalized to those of total control GC B cells and are presented relative to expression of the control gene *Hprt*. Each symbol represents an individual animal; horizontal lines indicate the mean. **(B)** Correlation of expression data for genes shared between the previously published DZ signature gene set (=Nussenzweig signature) and the signature set derived in control animals (=Rajewsky signature). **(C)** Unsupervised hierarchical clustering based on 11920 differentially expressed probe sets (GC = total germinal center B cells; microarray data was derived from Sander et al. 2012). **(D)** Hierarchical clustering as in Figure 2D after removal of 121 probe sets (106 genes) containing the gene ontology terms “proliferation”, “cell cycle”, and “growth function”. **(E)** Summary and quantification of reporter expressing plasmablasts (B220^{lo}, CD19$^{lo/-}$, CD138^{hi}) in LPS+IL4 treated splenic B cells at indicated time points after stimulation. Data of two independent experiments are summarized in the graph; horizontal lines indicate the mean. NS, not significant ($P > 0.05$); *$P \leq 0.05$ and **$P \leq 0.001$ (Wilcoxon-Mann-Whitney test).

Figure S3

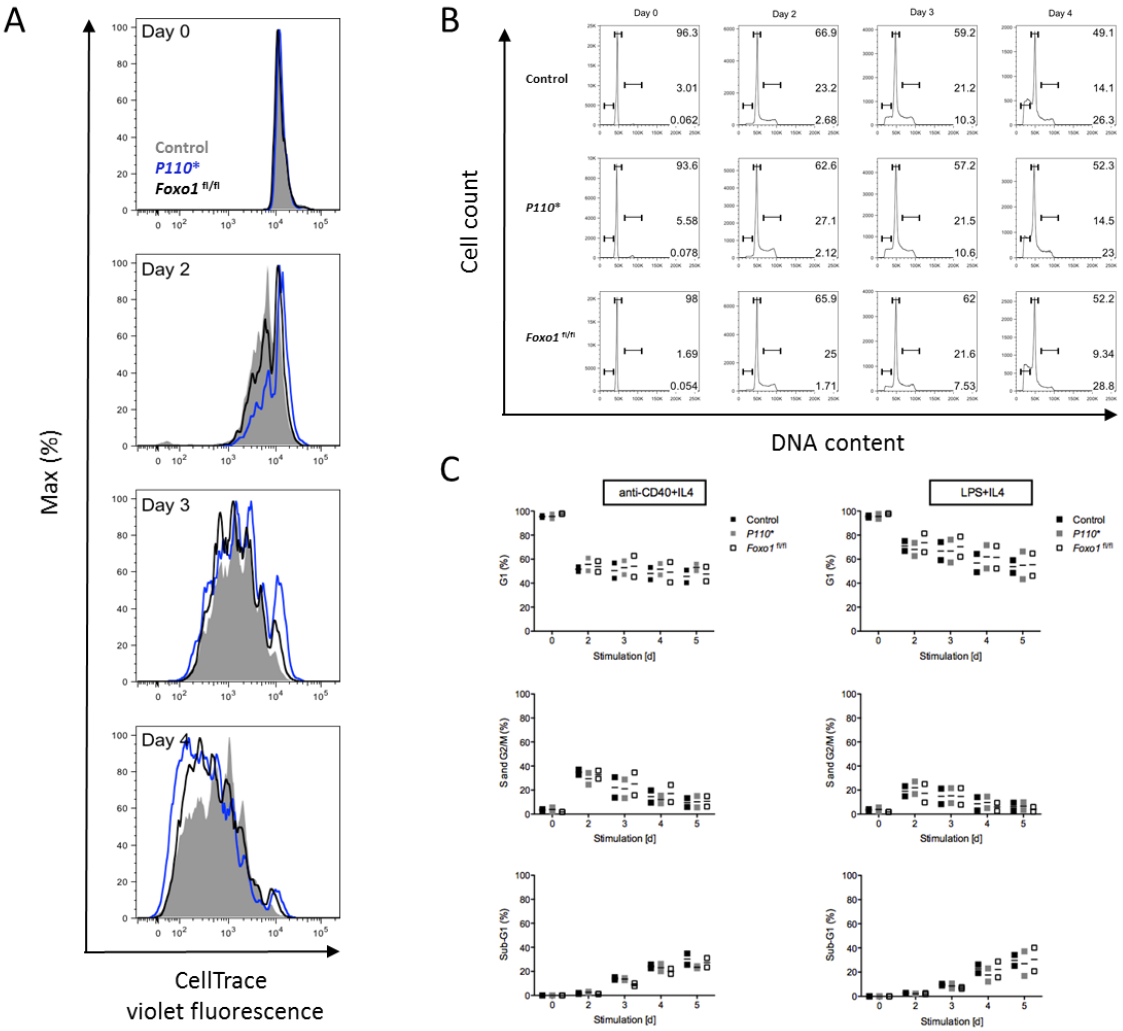

Figure S3, related to Figure 3. PI3K pathway activated or *Foxo1* deleted B cells proliferate like control cells after stimulation. **(A)** CellTrace analysis in LPS+IL4 stimulated B cells of various genotypes (Control= solid grey line; *P110**= blue line; *Foxo1* $^{fl/fl}$= black line). Day 0 corresponds to the labeling efficiency of the B cells before stimulation. At days 2, 3 and 4 after stimulation only reporter positive cells were analyzed. Data are representative of two independent experiments. **(B)** Representative cell cycle analysis in LPS+IL4 stimulated B cells of control and mutant animals. **(C)** Summary and quantification of flow cytometry data as shown in (B) corresponding to anti-CD40+IL4 (left) or LPS+IL4 (right) treated B cells at indicated time points after stimulation. Data of two independent experiments per stimulation condition are summarized in the graph; horizontal lines indicate the mean.

Figure S4

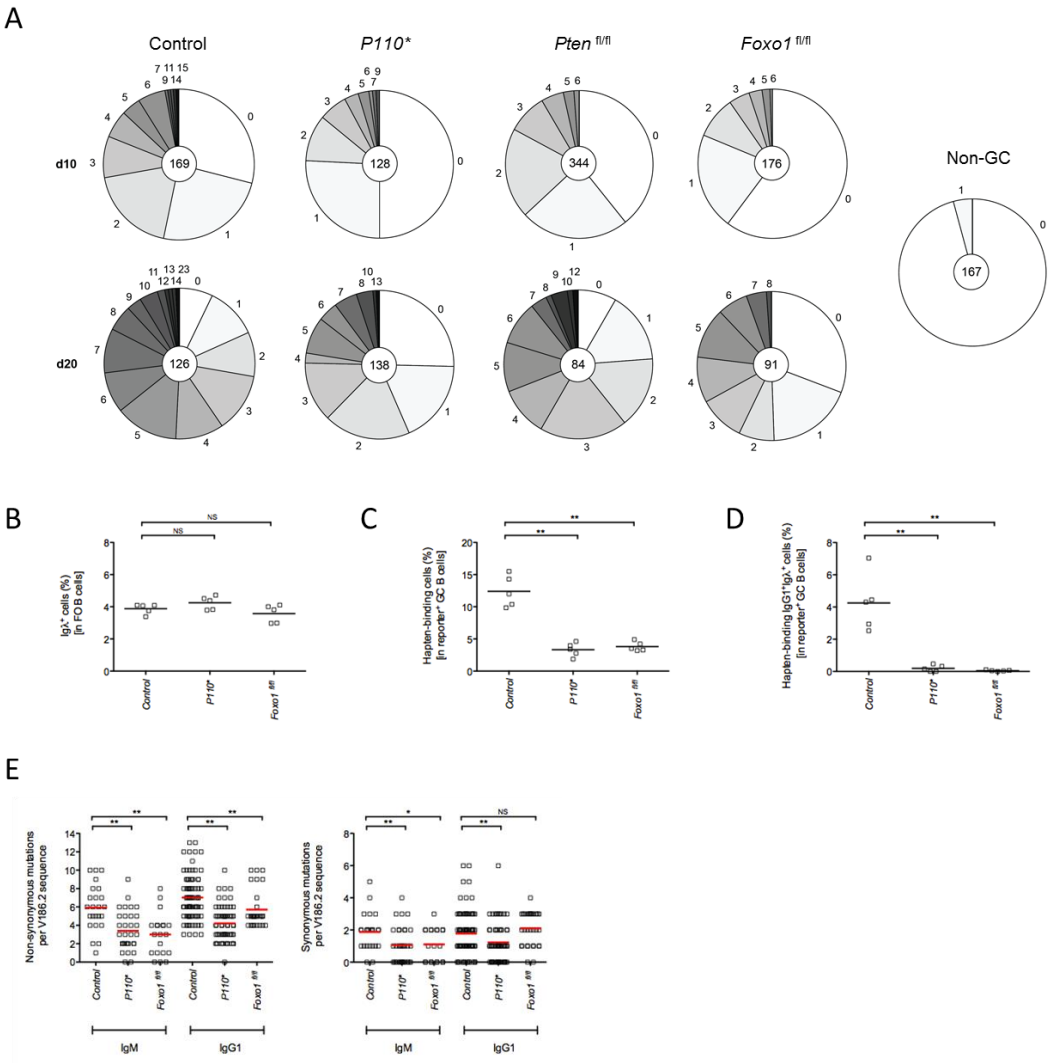

Figure S4, related to Figure 4. Mutant GC B cells are characterized by impaired acquisition of somatic mutations and abnormal selection. **(A)** Mutation analysis in the intron downstream of J_H4 in splenic GC B cells of SRBC injected animals at day 10 (d10) and day 20 (d20) after immunization. Sorted non-GC B cells of control animals were included in the analysis. Each pie chart shows the distribution of clones carrying the indicated number of mutations in the region assayed, with the number of reads analyzed shown at the center of each pie chart. Data of at least two animals per genotype are summarized in the diagrams. **(B)** Summary and quantification of flow cytometry analyses performed on splenic follicular B cells. Each symbol represents an individual animal; horizontal lines indicate the mean. **(C)** and **(D)** Summary and quantification of flow cytometry analyses performed on splenic GC B cells of immunized control and mutant animals (day 20 after NP-CGG immunization) (presented as in (B)). **(E)** $V_H186.2$ mutation analysis in splenic GC B cells of NP-CGG immunized animals (day 20 after immunization). Two individual animals per genotype were analyzed. Each symbol represents an individual sequence; red horizontal lines indicate the mean. NS, not significant ($P > 0.05$); *$P \leq 0.05$ and **$P \leq 0.001$ (Wilcoxon-Mann-Whitney test).

Figure S5

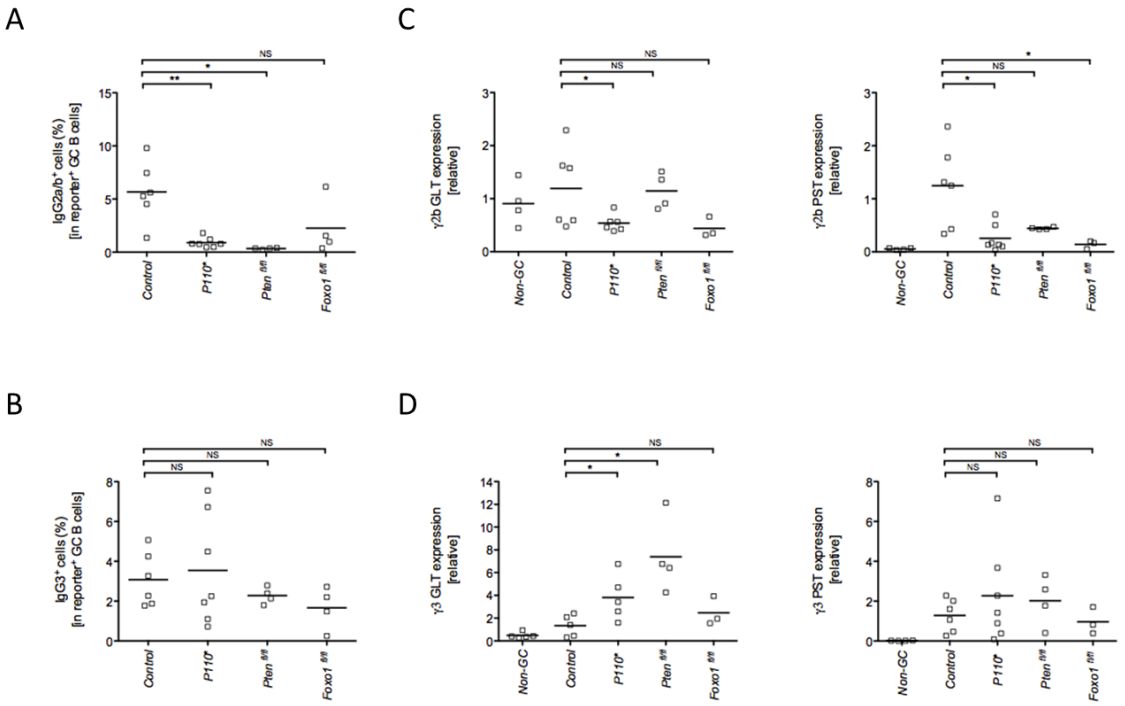

Figure S5, related to Figure 6. CSR is largely blocked in PI3K pathway activated or *Foxo1* deleted GC B cells. **(A)** and **(B)** Summary and quantification of IgG2 (A) and IgG3 (B) expressing splenic GC B cells (as determined by flow cytometry) at day 10 after SRBC immunization. Each symbol represents an individual animal; horizontal lines indicate the mean. **(C)** Quantitative RT-PCR analysis of γ2b GLT (left) and PST (right graph) expression in splenic GC B cells of control and mutant animals (day 10 after SRBC immunization). Non-GC B cells of control animals were included in the analysis. Results are normalized to those of control cells and are presented relative to expression of the control gene *Hprt*. Each symbol represents an individual animal; horizontal lines indicate the mean. **(D)** Quantitative RT-PCR analysis of γ3 GLT (left) and PST (right graph) expression in splenic GC B cells of control and mutant animals (day 10 after SRBC immunization) (presented as in (C)). NS, not significant ($P > 0.05$); *$P \leq 0.05$ and **$P \leq 0.001$ (Wilcoxon-Mann-Whitney test).

Figure S6

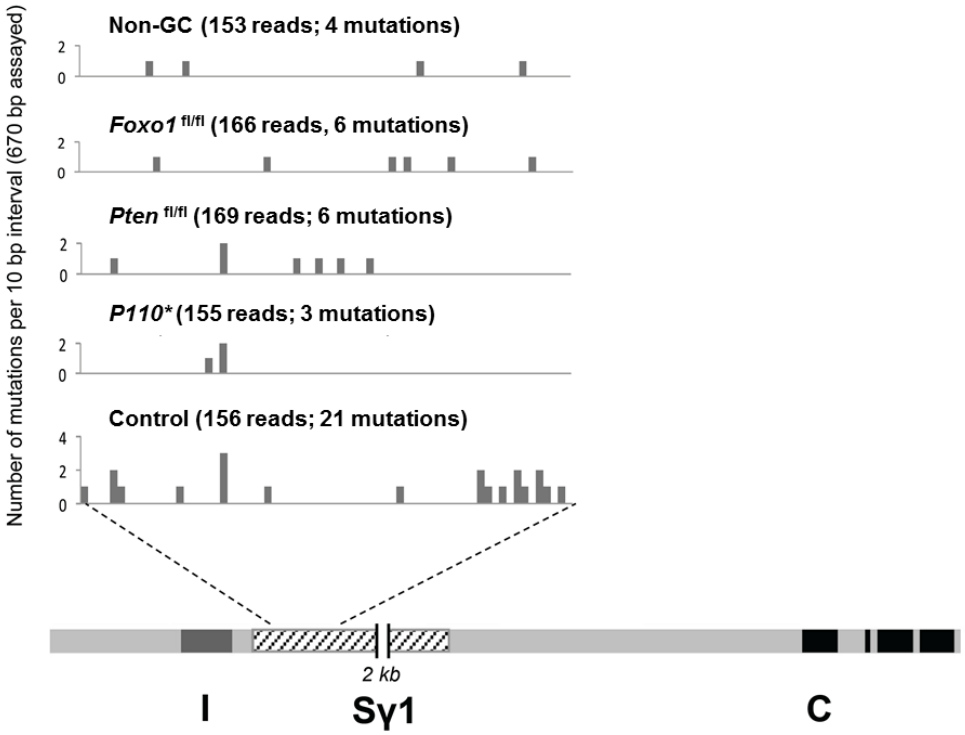

Figure S6, related to Figure 7. Reduced mutation load in the γ1 switch region (Sγ1) of PI3K activated or FOXO1 deficient GC B cells. The distribution of somatic mutations in the assay region (670 bp at the immediate 5' end of Sγ1) is shown for each genotype studied. The assay was performed by PCR and sequencing using the same genomic DNA samples described in Figure 7. Sorted non-GC B cells were included in the analysis as a negative control. Data of two animals per genotype are summarized.

Supplemental tables

Table S1, related to experimental procedures. Primer sequences

	Primer sequence (5'-3')	Reference
qRT-PCR		
IgG1_GLT_Fw	CAGCCTGGTGTCAACTAG	Park et al. Eur J Immunol 2005
IgG1_GLT_Rv	CTGTACATATGCAAGGCT	Park et al. Eur J Immunol 2005
IgG2b_GLT_Fw	GGGAGAGCACTGGGCCTT	Park et al. Eur J Immunol 2005
IgG2b_GLT_Rv	AGTCACTGACTCAGGGAA	Park et al. Eur J Immunol 2005
IgG3_GLT_Fw	CAAGTGGATCTGAACACA	Park et al. Eur J Immunol 2005
IgG3_GLT_Rv	GGCTCCATAGTTCCATT	Park et al. Eur J Immunol 2005
Im_Fw	TTCCAATACCCGAAGCATTTAC	Perlot et al. PNAS 2008
Im_Rv	GTCCATGAGCAGCCAGGTG	Perlot et al. PNAS 2008
Imu_sense_Fw	ACCTGGGAATGTATGGTTGTGGCTT	Park et al. Eur J Immunol 2005
IgG1_PST_Rv	CTGTACATATGCAAGGCT	Park et al. Eur J Immunol 2005
IgG2b_PST_Rv	AGTCACTGACTCAGGGAA	Park et al. Eur J Immunol 2005
IgG3_PST_Rv	GGCTCCATAGTTCCATT	Park et al. Eur J Immunol 2005
Foxo1_Fw	TGAAGAGCGTGCCCTACTTC	*This study*
Foxo1_Rv	TGAGCATCCACCAAGAACTCT	*This study*
Foxo3_Rv	TCATTCTGAACGCGCATGAAG	PrimerBank-MGH-PGA; ID:9789951a1
Foxo3_Fw	CTGGGGGAACCTGTCCTATG	PrimerBank-MGH-PGA; ID:9789951a1
Foxo4_Rv	ACAGGATCGGTTCGGAGTGT	PrimerBank-MGH-PGA; ID:9055158a1
Foxo4_Fw	CTTCCTCGACCAGACCTCG	PrimerBank-MGH-PGA; ID:9055158a1

Pten_Fw	CTGCAGAAAGACTTGAAGGTGT	*This study*
Pten_Rv	AGTTCTAGCTGTGGTGGGTT	*This study*
Aicda_Fw	CGGCTAACCAGACAACTTCG	Sayegh et al. Nat Immunol 2003
Aicda_Rv	GCATCTCGCAAGTCATCGA	Sayegh et al. Nat Immunol 2003
Hprt_Fw	TGGCTGAGTTTCTGAGATGGAACCCTAACCTCAGCCTGAG	*This study*
Hprt_Rv	TCAAAATCCCAACATACGAAATGCATCTCGCAAGTCATCG	*This study*
SHM analysis		
J_H4_intron_Rv	CTCCACCAGACCTCTCTAGACAGC	Jolly et al. Nucleic Acids Res 1997
VHA_Fw	ARGCCTGGGRCTTCAGTGAAG	Jolly et al. Nucleic Acids Res 1997
VHE_Fw	GTGGAGTCTGGGGGAGGCTTA	Jolly et al. Nucleic Acids Res 1997
m.2_Fw	GCGAATTCAGTCAGTGACGTGAAGGGCTTCTAAG	Xue et al. J Exp Med 2006
m.2_Rv	CGGGATCCGCTACTCCAGAGTATCTCATTTCAGATC	Xue et al. J Exp Med 2006
g1.2_Fw	GCGAATTCTTCCTACCTTCTCCCCTGAGTCTCAA	Xue et al. J Exp Med 2006
g1.2_Rv	CAACAAGCTTCACCTGGATCAGTTTCTCTGTGACTGC	Xue et al. J Exp Med 2006
V186.2-Fw	TTCTTGGCAGCAACAGCTACA	Kaji et al. J Exp Med 2012
IgG1-Rv-external	GGATCCAGAGTTCCAGGTCACT	Kaji et al. J Exp Med 2012
IgM-Rv-external	AAATGGTGCTGGGCAGGAAG	Kaji et al. J Exp Med 2012
IgG1-Rv-internal	GGAGTTAGTTTGGGCAGCAG	Kaji et al. J Exp Med 2012
IgM-Rv-internal	AGCCCATGGCCACCAGATT	Kaji et al. J Exp Med 2012

Table S2, related to Figure 2. Probe sets differentially expressed in LZ and DZ samples

List of probe sets differentially expressed among classes “LZ” and “DZ”; probe sets displayed are significant at 0.05 level of the univariate test (Two-sample T-test with random variance model).

⇨ See attached Excel file

Table S3, related to Figure 2. Probe sets differentially expressed in mutants and DZ samples

List of probe sets differentially expressed among classes “mutants” (*Foxo1* $^{fl/fl}$ and *P110**) and “DZ”; probe sets displayed are significant at 0.05 level of the univariate test (Two-sample T-test with random variance model).

⇨ See attached Excel file

Supplemental experimental procedures

Flow cytometry and cell sorting

Single-cell suspensions were stained with the following monoclonal or polyclonal antibodies from BD Biosciences, Biolegends, eBioscience or LifeTechnologies: anti-CD19(ID3), anti-B220(RA3-6B2), anti-FAS(Jo2), anti-CD38(90), anti-CXCR4(2B11), anti-CD86(GL-1), anti-IgG1(A85-1), anti-IgA(mA-6E1), anti-IgG2a/b(R2-40), anti-IgG3(RMG3-1), anti-Igλ1,2,3(R26-46), anti-CD138(281-2), anti-GFP. TO-PRO-3 (Invitrogen) or propidium iodide (Sigma) was used to exclude dead cells. Anti-phospho-AKT antibody (S473) (D9E, Cell Signaling), goat-anti rabbit IgG (Invitrogen) and anti-phospho-AKT (S473) blocking peptide (Cell Signaling) were used in intracellular staining experiments. Samples were acquired on FACSCantoII and FACSFortessa (BD Biosciences) analyzers, and data was analyzed using FlowJo software (Tree Star). Reporter gene expressing GC B cells or non-GC B cells of immunized animals were sorted on a FACSAriaII (BD Biosciences) and used for RNA and DNA preparation.

Mass spectrometry based protein quantification

Proteins extracted from $2x10^5$ mouse GC B cells of control and mutant animals (day 10 after SRBC immunization) were digested by Lysyl endopeptidase (LysC) and trypsin and digested peptides were desalted with C18 Stage Tips (Rappsilber et al., 2003) prior to online liquid chromatography-tandem mass

spectrometry (nanoLC-MS/MS) analysis. Peptides were separated on a monolithic column (100 μm i.d. x 2,000 mm, MonoCap C18 High Resolution 2000 [GL Sciences] kindly provided by Dr. Yasushi Ishihama [Kyoto University]) using 6 hour gradient of increasing acetonitrile concentration at a flow rate of 300 nL/min. The Q Exactive instrument (Thermo Fisher Scientific) was operated in the data dependent mode with a full scan in the Orbitrap followed by top 10 MS/MS scans using higher-energy collision dissociation (HCD). MaxQuant software (v1.5.1.2) (Cox and Mann, 2008) was used to identify and quantify proteins. False discovery rate was set to 1% at both peptide and protein amount. Peptides were quantified based on peak areas.

References

Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 2008;26(12):1367-72.

Jolly CJ, Klix N, Neuberger MS. Rapid methods for the analysis of immunoglobulin gene hypermutation: application to transgenic and gene targeted mice. Nucleic Acids Res. 1997;25(10):1913-9.

Kaji T, Ishige A, Hikida M, Taka J, Hijikata A, Kubo M, Nagashima T, Takahashi Y, Kurosaki T, Okada M, Ohara O, Rajewsky K, Takemori T. Distinct cellular pathways select germline-encoded and somatically mutated antibodies into immunological memory. J Exp Med. 2012 Oct 22;209(11):2079-97.

Park SR, Seo GY, Choi AJ, Stavnezer J, Kim PH. Analysis of transforming growth factor-beta1-induced Ig germ-line gamma2b transcription and its implication for IgA isotype switching. Eur J Immunol. 2005;35(3):946-56.

Perlot T, Li G, Alt FW. Antisense transcripts from immunoglobulin heavy-chain locus V(D)J and switch regions. Proc Natl Acad Sci U S A. 2008;105(10):3843-8.

Rappsilber J, Ishihama Y, Mann M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem. 2003;75(3):663-70.

Sayegh CE, Quong MW, Agata Y, Murre C. E-proteins directly regulate expression of activation-induced deaminase in mature B cells. Nat Immunol. 2003;4(6):586-93.

Xue K, Rada C, Neuberger MS. The in vivo pattern of AID targeting to immunoglobulin switch regions deduced from mutation spectra in msh2-/- ung-/- mice. J Exp Med. 2006;203(9):2085-94.