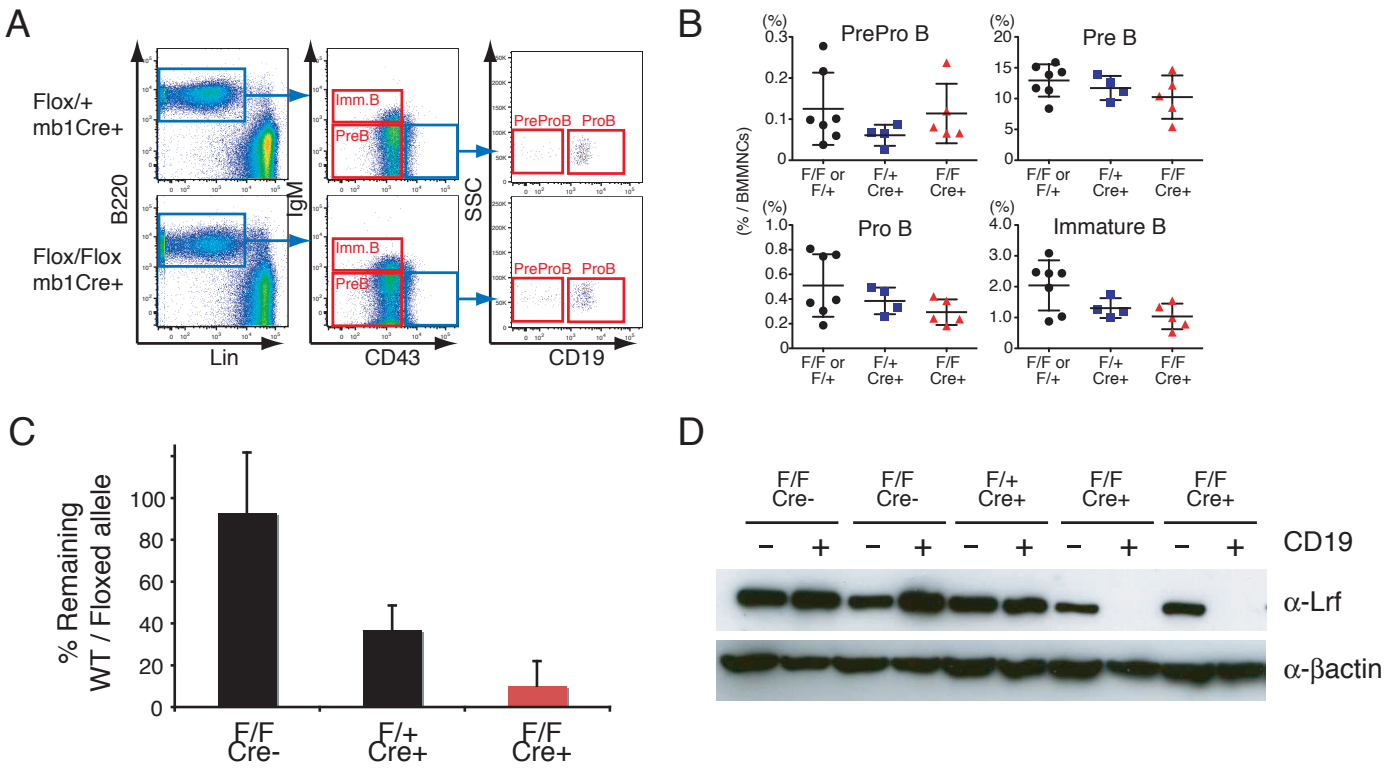
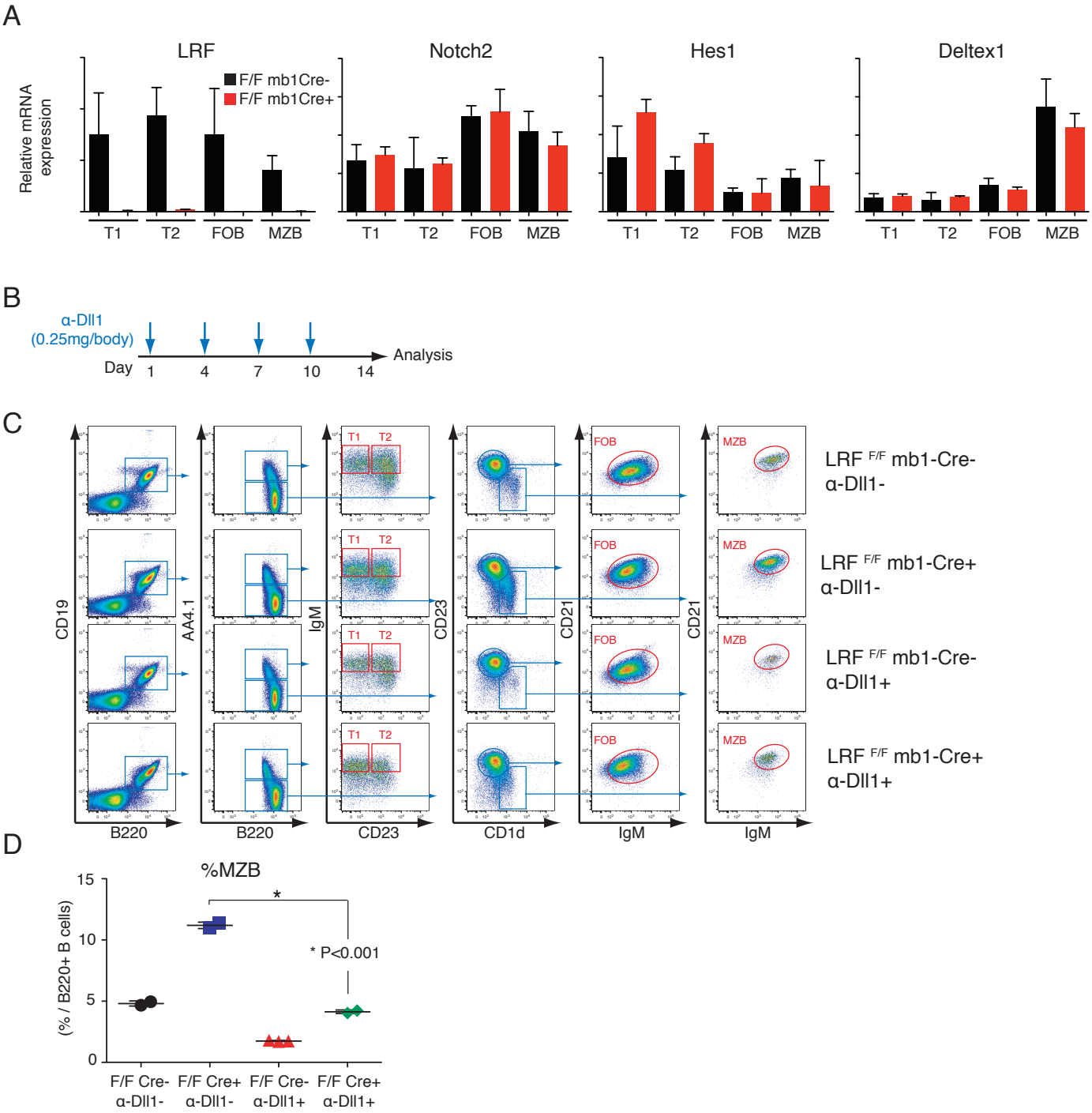
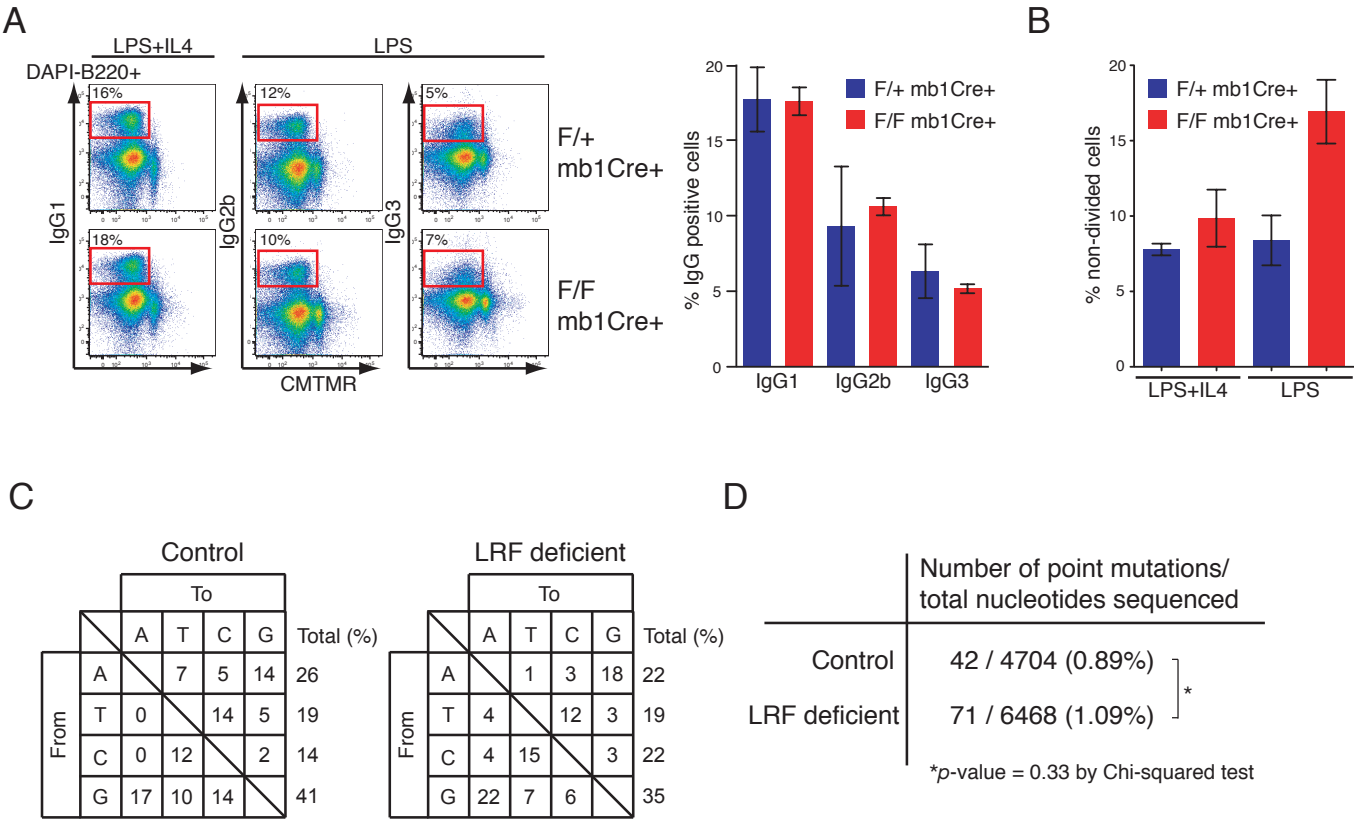


Supplementary Figure 1. (A) FACS profiles of BM B cells of control ($LRF^{Flox/+}$ mb-1 Cre+) and knockout mice ($LRF^{Flox/Flox}$ mb-1 Cre+). **(B)** Dot graphs show proportions of early B cell compartments in the BM in Cre-negative control (Flox/Flox or Flox/+; n= 7), LRF heterozygous (Flox/+ Cre+; n=4) and B cell-specific LRF KO mice (Flox/Flox Cre+; n=5). Horizontal black bars: average value; error bars: standard deviation. **(C)** Quantitative genotyping of BM $CD19^+$ B cells. The percentage remaining WT or Floxed allele of the LRF gene was calculated as described (26). **(D)** Western blot analysis for LRF in purified $CD19^+$ or $CD19^-$ BM cells, confirming specific LRF inactivation in B cells.

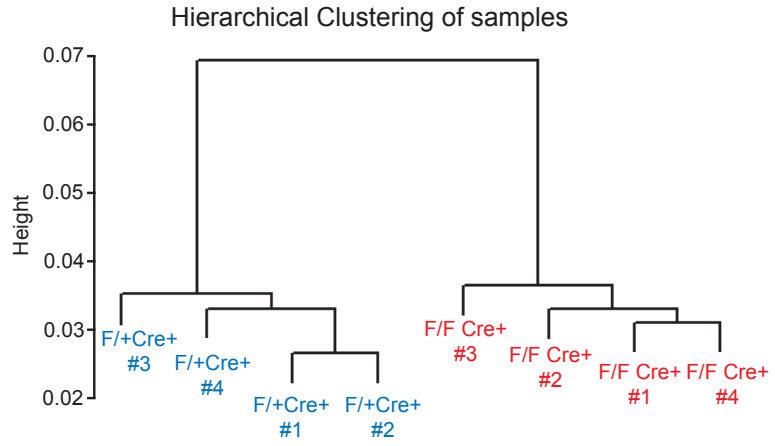


Supplementary Figure 2. **(A)** The Notch target Hes1, but not Notch2 itself, is upregulated in LRF-deficient LT-HSCs. RNA samples were obtained from FACS-sorted mature B cells (three mice per genotype) and q-RT-PCR performed. Relative expression levels for LRF, Notch2 Hes1 and Deltex1 are shown. Data shown are mean with SD. **(B)** Anti-Dll1 antibody was administered intraperitoneally four times at 3-day intervals and splenic B cell development analyzed 14 d after the first injection. **(C)** Representative FACS profiles of mature B cell compartments in non-treated WT ($LRF^{Flox/Flox}$ mb-1 Cre-, α-Dll1-), non-treated LRF knockout ($LRF^{Flox/Flox}$ mb-1 Cre+, α-Dll1-), treated WT ($LRF^{Flox/Flox}$ mb-1 Cre-, α-Dll1+) and treated LRF knockout ($LRF^{Flox/Flox}$ mb-1 Cre+, α-Dll1+) mice. **(D)** Dot graphs demonstrate proportions of splenic MZB cells in each group (n=3). Horizontal black bars: average value.



Supplementary Figure 3. **(A)** CD43 negative (CD43$^-$) naive splenic B cells were collected from control (LRF$^{Flox/+}$ mb-1 Cre+) and LRF-deficient mice (LRF$^{Flox/Flox}$ mb-1 Cre+) and cultured in the presence of cytokines to induce CSR in vitro. Cells were stimulated with LPS or LPS plus IL-4 to induce CSR to the IgG1 and IgG2b/IgG3 isotypes, respectively. Cell division was monitored by labeling with the cell tracker dye CMTMR (Orange-fluorescent tetramethylrhodamine). Prior to the cytokine stimuli, CD43$^-$ B cells were labelled by incubation with CMTMR. Cells were harvested 4 d later, and expression levels of the class-switched immunoglobulins on the cell surface determined by FACS. Data represent mean with SD. **(B)** Bar graph showing proportions of non-divided cells (CMTMR-retaining cells) 4 d after stimulation. Data represent mean with SD. **(C)** Analysis of mutations in the V186.2 gene region of WT and LRF-deficient splenic GCB cells. Tables demonstrate the proportions of nucleotide substitution. **(D)** Mutation frequencies in the V186.2 gene region of WT and LRF-deficient splenic GCB cells. The total number of point mutations was divided by the total number of nucleotides sequenced in the V186.2 gene region.

A



B

GCTGGCTGTCACCGCGATGGGTGGCGAGCGAAGCGAGCGGGAT
CCGGAGCGTGCCCTGCGCGGGAGGCAGCGGGACCCCGGATGC
GGCAGGGCCCGCGCGCGCCTCCCCCTGGGCGCCTCTGGGAAG
CTTTCCCGCGCGACTGGGGACCGCGCGCCTTGGGACTCGGGGG
CGCGCCTGAGGGCGGAGATGGGCGTGGAGCAAAGATGGGCCG
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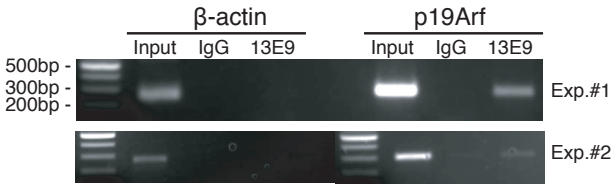
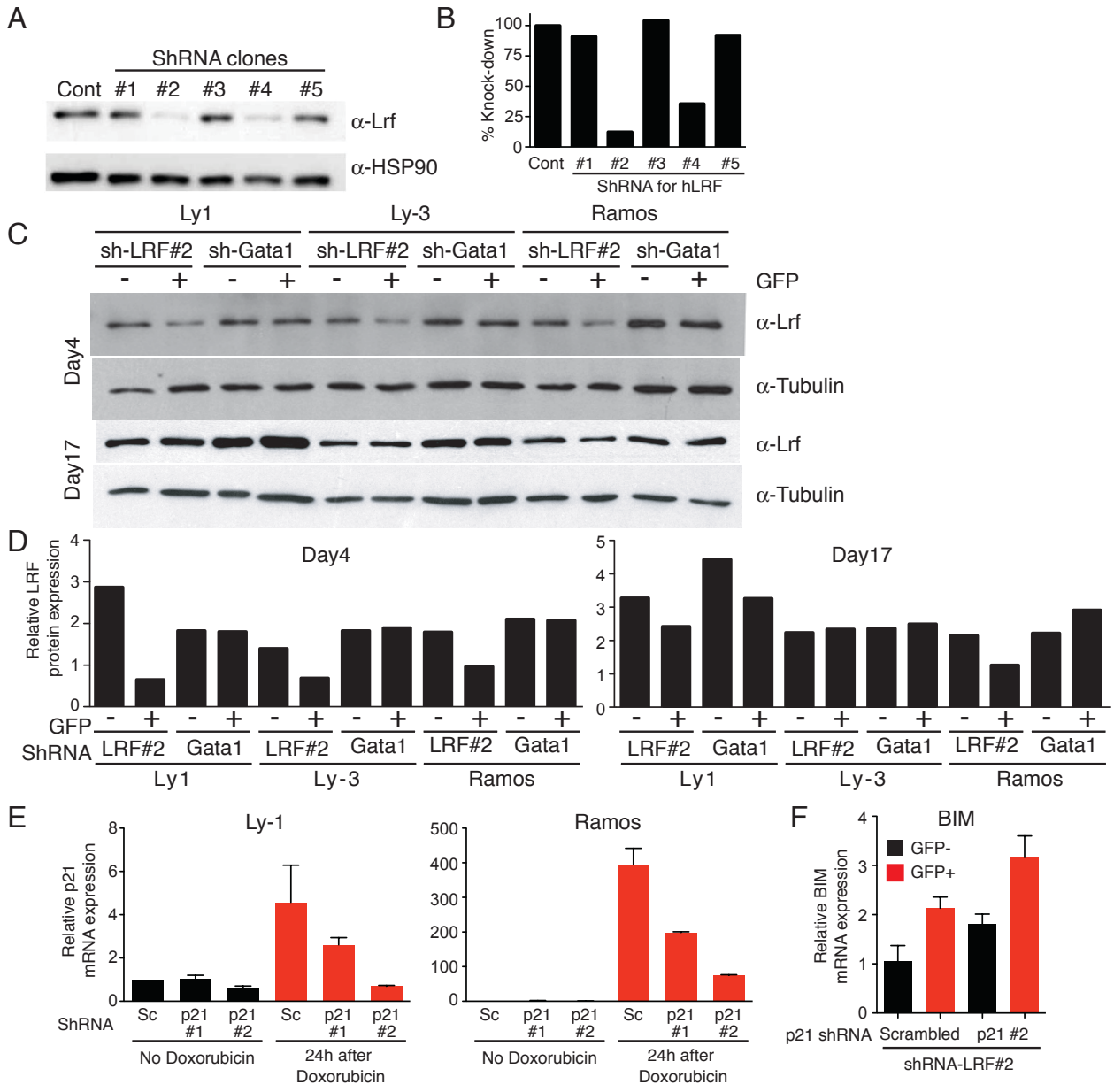
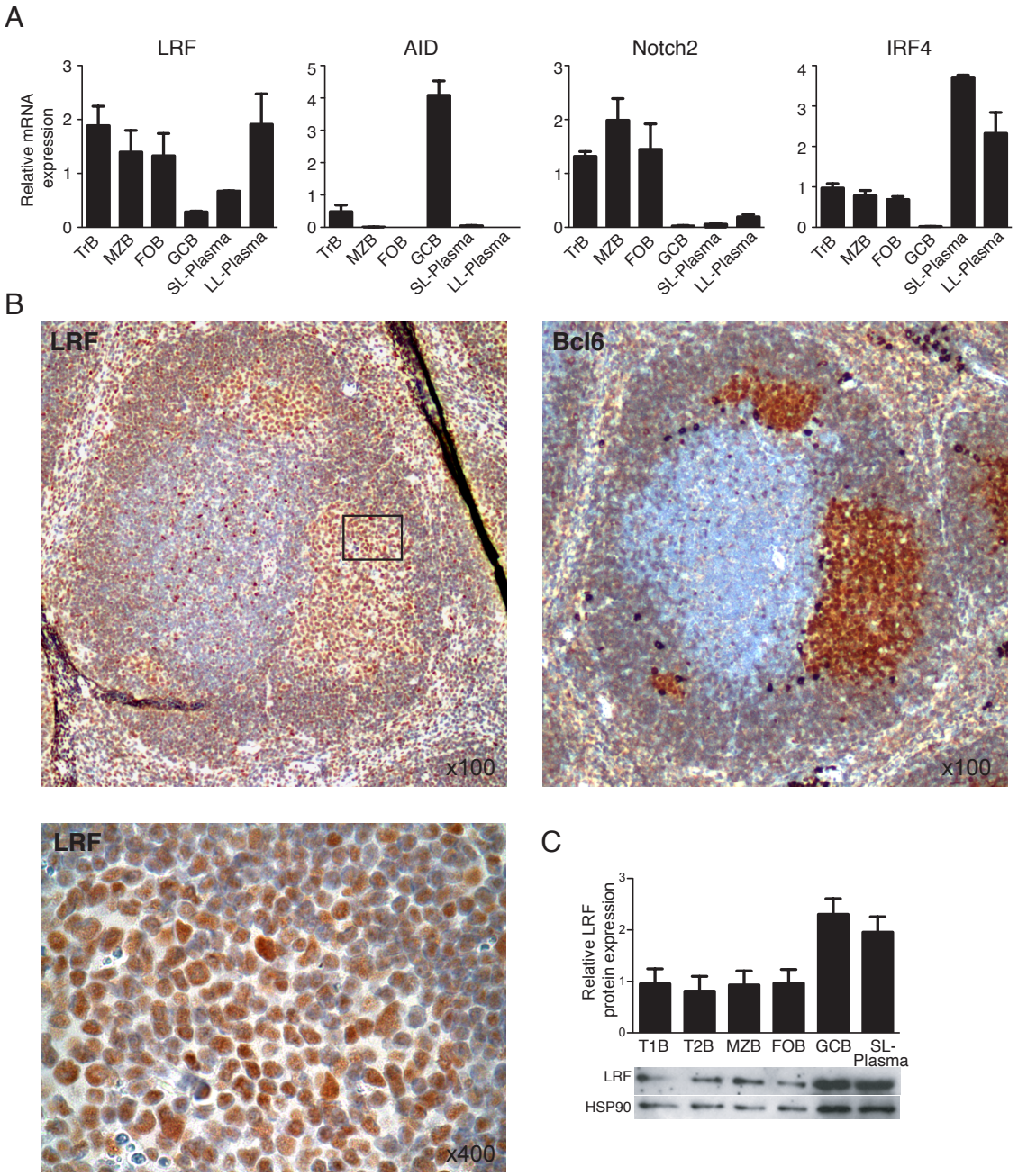


Supplementary Figure 4. **(A)** Hierarchical clustering analysis was generated by average linkage of the samples, using Pearson dissimilarity as the distance matrix. Four samples from the same genotype clustered together, suggesting that replicates were similar. **(B)** Mouse GCB cells were FACS-sorted and ChIP assay performed using anti-LRF antibody (13E9). Mouse p19Arf promoter sequences upstream of the transcription start site (purple); putative LRF binding site (red); and primer sequences used for ChIP assay (highlighted in yellow) are shown. Mouse beta-actin gene sequence was also amplified as an unbound control. Data from immunoprecipitations performed with normal hamster IgG are shown as controls. Results from two independent experiments are shown.



Supplementary Figure 5. **(A)** LRF knockdown efficiencies were determined by Western blot. Each shRNA-LRF clone (pLKO.1-puro) was transduced into Ramos cells and protein samples prepared 48 h after puromycin treatment. **(B)** Bar graph showing LRF protein levels relative to that of parental Ramos cells. **(C)** Cells were transduced with lentivirus encoding Dox-inducible shRNA and cultured for 17 d in the presence of Dox. GFP-positive and-negative fractions were FACS sorted on days 4 and 17. Western blot shows significant reduction of LRF protein expression in shRNA-LRF transduced cells on day 4. **(D)** Bar graphs represent LRF protein levels normalized to the corresponding tubulin protein levels. **(E)** Cells were treated with either vehicle or Doxorubicin (1 μM for 24 h). p21 response to Doxorubicin treatment and efficiencies of p21 knockdown by shRNA-p21 clones were validated by q-RT-PCR. p21 mRNA expression levels were normalized to corresponding PBGD mRNA level. Bar graph: p21 expression levels relative to untreated control cells (scrambled ShRNA). SC: scrambled shRNA control. **(F)** shRNA against p21 or scrambled shRNA was stably expressed in Ly-1 cells, which were previously transduced with the lentivirus encoding Dox-inducible shRNA-LRF#2. Four days after Dox treatment, GFP-positive and-negative fractions were FACS sorted and q-RT-PCR for BIM was performed. Bar graph: mRNA expression level of BIM normalized to corresponding PBGD mRNA level.



Supplementary Figure 6. **(A)** Quantitative RT-PCR analysis of the LRF gene in FACS-sorted mature B cells (Transitional [TrB], Marginal Zone [MZB], Follicular [FOB], Germinal Center [GCB], short-lived [SB] and long-lived [LL] Plasma cells). Relative mRNA expression levels of both the LRF gene and the corresponding β-actin gene were measured in each sample. mRNA expression levels were normalized to β-actin mRNA (bar graphs); error bars: standard deviation. SL-Plasma: short-lived plasma cell in spleen. LL-Plasma: long-lived plasma cell in the BM. **(B)** Immunohistochemical analysis for LRF in the spleen of an immunized WT mouse (left). Bcl6 staining of the consecutive section (right). High magnification of LRF stain within the GC (inset) is also shown (bottom). **(C)** Western blot analysis for LRF and Hsp90 protein in mature B cells. Bar graph: protein expression level of LRF normalized to corresponding Hsp90 protein level. Data represent mean with SD.

Supplementary Methods

PCR primers for mouse genotyping

Mutant mice were genotyped by PCR of tail-DNA with the following primers. For LRF flox mice, PCFW1: 5′- TCTGAGGCCCGGTGCAT-3′, PCFW2: 5′- AGGGTGGTGCTCCCTCTAGAC-3′, PCFrt: 5′- ACCGCGGTCTAGGGATCC-3′ and PCRV: 5′- GCTTGGGCTCCCCATGTAG-3′. For the mb1-Cre transgene, mb1hCreFW: 5′-CCCTGTGGATGCCACCTC-3′ and mb1hCreRV: 5′-GTCCTGGCATCTGTCAGAG-3′. For Notch2 flox mice, Notch2FW1: 5′-ACTTGGAGAGAGGAAAGAGAAGGG-3′, Notch2RV: 5′-GCCTGACTAGAGCAATGCTTCCTG-3′ and Notch2FW2: 5′-CGGGGATCAATTCGAGCTCGC-3′. For Cγ1-Cre mice, Cγ1-CreFW1: 5′-TGTTGGGACAAACGAGCAATC-3′, Cγ1-CreRV: 5′- GTCATGGCAATGCCAAGGTCGCTAG-3′ and Cγ1-CreFW2: 5′- GGTGGCTGGACCAATGTAAATA-3′. Quantitative genotyping for the LRF flox allele was performed as previously described (1).

Flow cytometric analysis (FACS) and cell sorting

FACS data were collected using a LSRII FACS analyzer (BD), and data analysis performed with FlowJo software (Tree Star). Single cell suspensions were prepared from spleen and BM by passing cells through 70 μm cell strainers (BD). Cells were then re-suspended in PBS containing 2% FBS and incubated (15 min, on ice) with fluorochrome-conjugated (or with biotin-conjugated) antibodies. Lineage antibody cocktail was prepared as described previously (1). DAPI (100 ng/ml) was added for live/dead discrimination before analysis. GC B cells and other B cell compartments were FACS-sorted using a MoFlo (DAKO) at the COH Analytical Cytometry Core facility. Each B cell compartment was defined based on expression of the following surface markers: PreProB ($Lin^-B220^+IgM^-CD43^+CD19^-$); ProB ($Lin^-B220^+IgM^-$

$CD43^+CD19^+$); PreB ($Lin^-B220^+IgM^-CD43^-CD19^+$); Immature B ($Lin^-B220^+IgM^+CD43^-CD19^+$); Transitional B1 ($B220^+CD19^+$ $AA4.1^+$ IgM^+CD23^-); Transitional B2 ($B220^+CD19^+$ $AA4.1^+$ IgM^+CD23^+); FOB ($B220^+CD19^+AA4.1^-CD1d^-CD23^+IgM^{low/-}CD21^{low}$); MZB ($B220^+CD19^+AA4.1^-CD1d^+CD23^-IgM^{high}CD21^{high}$); GCB ($B220^+CD19^+FAS^+CD38^{dim}PNA^+$); IgG_1+ Memory B cells ($B220^+CD19^+FAS^-CD38^+IgG_1^+$) and splenic short- and BM long-lived plasma cells ($B220^-CD138^+$). For 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, mice were intraperitoneally injected with EdU (160 μg/g) 5 h before analysis, and splenic GC B cells analyzed for the degree of EdU incorporation utilizing a Click-iT™ EdU Alexa Fluor® 647 (Invitrogen) in combination with surface marker staining (APC-Alexa750-B220, FITC-Fas and biotin-CD38/ streptavidin PerCP-Cy5.5) plus DAPI DNA staining (1 μg/ml) on day 7 after immunization.

Antibodies for FACS

Antibodies used for this study were (purchased from eBioscience, unless otherwise indicated): FITC-CD1d (1B1), PE-IgM (II/41), PerCP-Cy5.5-IgM (II/41), PE-Cy7-CD23 (B3B4), APC-AA4.1, APC-Alexa750-B220 (RA3-6B2), PerCP-Cy5.5- B220 (RA3-6B2), PacificBlue-B220 (RA3-6B2), Alexa700-CD19 (eBio1D3), PacificBlue-CD21 (eBio4E3), FITC-CD38 (90), Alexa647-CD38 (90), PerCP-Cy5.5-CD38 (90), PE-CXCR4 (2B11), FITC-Fas (Jo2, BD), PE-Cy7-Fas (Jo2, BD), APC-CD138 (281-2, BD), FITC-CD43 (eBioR2/60), biotin-IgG_1 (RMG1-1, Biolegend), biotin-IgG_{2b} (RMG2b-1, Biolegend), biotin-IgG_3 (RMG3-1, Biolegend), biotin-CD38 (90), biotin-CD3 (145-2C11), biotin-CD4 (GK1.5), biotin-CD8 (eBioH35-17.2), biotin-Gr1 (RB6-8C5), biotin-CD11b (M1/70), biotin-TER119 and biotin-NK1.1 (PK136). Streptavidin-PerCP-Cy5.5 and Streptavidin-APC-Alexa750 were purchased from eBioscience and Streptavidin-PE-TexasRed from BD. FITC-conjugated PNA (L7381) was obtained from Sigma.

For apoptosis assays, PE Active Caspase-3 Apoptosis Kit and Alexa Fluor® 647 anti-Cleaved PARP (both from BD) were used.

Immunizations and anti-Dll1 treatment

Mice (8-10-wk-old) were immunized by intraperitoneally injecting NP_{26}-CGG (100 μg, Biosearch Technologies) in Imject alum (Pierce) or NP-Ficoll (30 μg, Biosearch Technologies) in PBS. For microarray analysis, mice were immunized intraperitoneally with 100 μl SRBCs (Colorado Serum Co. CS1112) and GCB cells collected *via* FACS sorting 7 d after immunization. Anti-Dll1 antibody (2) (0.25 mg/body) was administered intraperitoneally four times at 3-d intervals and splenic B cells analyzed by FACS 14 d after the first injection.

Detection of CSR in vitro

Single cell suspensions were prepared from spleens of 8-10-wk-old mice, and red blood cells (RBCs) eliminated by incubation (5 min, on ice) with RBC lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM EDTA at pH7.4). Splenocytes were then incubated with magnetic bead-conjugated anti-CD43 antibody (Miltenyi Biotec), and the $CD43^-$ fraction harvested using MACS separation LS columns (Miltenyi Biotec). Resultant $CD43^-$ naive B cells (2 x 10^6 cells/4 ml per well in 6-well plate) were cultured with B cell culture medium (RPMI supplemented with 1% glutamine, 1% sodium pyruvate, 53 μM 2β-mercaptoethanol, 10 mM HEPES and 10% FBS) in the presence of either LPS (20 μg/ml, L2630 from Sigma) or LPS (20 μg/ml) plus IL4 (25 ng/ml, I1020 from Sigma). CellTracker™ Orange (5 μM, Invitrogen) was added to the medium to monitor cell proliferation. After four days, cells were harvested and their surface immunoglobulin expression levels analyzed by FACS.

Detection of SHM

The frequency of SHM in the IgH locus was measured as described (3). Briefly, DNA was extracted from FACS-sorted GCB cells, and the V186.2 variable gene segment, which is a member of the J558 gene family and frequently rearranged and expressed in B cells responding to NP, and amplified *via* nested-PCR. The first PCR reaction was performed using the V186.2 FW primer (5′-TCTTTACAGTTACTGAGCACACAGGAC-3′) and JH2 RV primer (5′-GGGTCTAGAGGTGTCCCTAGTCCTTCATGACC-3′), and the second PCR reaction was performed using the same V186.2 FW primer and the V186.2 RV primer (5′-CAGTAGCAGGCTTGAGGTCTGGAC-3′) (3). The resultant 500 bp PCR amplicon was subcloned into the PCR2.1 TOPO vector using the TA cloning kit (Invitrogen). DNA sequencing was performed at the DNA Sequence Core facility at COH.

Enzyme-linked immunosorbent assay (ELISA)

Immune sera were collected from 8-10-wk=old mice by retro-orbital bleed. To determine baseline immunoglobulin titers, the IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA titers were measured using a Mouse Immunoglobulin Isotyping ELISA Kit (BD). For measurement of the NP-specific Ig titers, ELISA plates (Thermo Scientific) were coated with NP-BSA (Biosearch Technologies), and serially diluted serum samples and control anti-NP IgG1 antibody (kind gift from Dr. Michel Nussenzweig) were applied and incubated (1 h, room temperature [RT]). Plates were then incubated (1 h, RT) with HRP-conjugated rat anti-IgG_1 (or anti-IgM for TID response) or biotin-conjugated IgG_{1b} followed by streptavidin HRP (BD), and washed 6 times with 0.05% PBS-T to entirely remove unbound antibodies. Bound antibody titer was measured using a 1-Step TM Slow TMB-ELISA kit (Thermo Scientific).

Immunohistochemical (IHC) and immunofluorescence (IF) analysis

For IHC, all tissues were fixed in phosphate-buffered 10% formalin solution (Fisher Diagnostic) and embedded in paraffin. LRF staining was performed as previously described using anti-LRF antibody (clone 13E9) (4), and Bcl6 staining with anti-BCL6 antibody (Cell Marque) run on Ventana (Tucson, AZ) according to the manufacturer's instructions.

For IF, 200,000 GCB cells were FACS-sorted directly onto an L-Lysine coated glass slide, fixed with 4% paraformaldehyde and blocked with 10% goat serum. Samples were then incubated (overnight, 4^{o}C) with primary antibodies (anti-p19Arf, clone 5-C3, Abcam), washed and incubated (1 h, RT) with DAPI and fluorochrome-conjugated secondary antibodies (Alexa568 conjugated goat-anti Rat secondary antibody). Image data was acquired with a Zeiss Upright LSM510 2-Photon Microscope at the COH Light Microscopy Digital Imaging Core.

Real time PCR assay

RNA was extracted from FACS-sorted cells using Trizol (15596-018, Invitrogen), according to the manufacturer’s specifications, and subsequently treated with DNase I (Invitrogen) to eliminate remaining genomic DNA. cDNA was synthesized using the SuperScript™ III First-Strand Synthesis Super Mix System (Invitrogen), and real-time PCR performed using LightCycler 480 SYBR Green I Master and a LightCycler 480 (Roche). For Notch target gene detection in mature B cells, the following Taqman probes (Applied Biosystems) were used: LRF/Zbtb7a: Mm00657132_m1, Notch2: Mm00803077_m1, Gapdh: Mm99999915_g1, Hes1: Mm00468601_m1, Dtx1: Mm00492297_m1 and Hes5: Mm00468601_m1. Relative mRNA expression levels of the target gene and corresponding control Hprt gene in each sample were measured and the target mRNA expression level normalized as described previously (1). All primer sequences for SYBR green, except for LRF (mouse and human), p19Arf, Hprt and

human PBGD were obtained from the PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html).

Primer sequences and their PrimerBank IDs were as follows:

For mouse genes: LRF FW: 5′-GAGAAGAAAATCCGGGCCAAG-3′, LRF RV: 5′-GCAGCTATCGCACTGGTATGG-3′; HPRT FW: 5′-CACAGGACTAGAACACCTGC-3′, HPRT RV: 5′-GCTGGTGAAAAGGACCTCT-3′; p19Arf FW: 5′-CGGAATCCTGGACCAGGTG-3′, p19Arf RV: 5′-ACCAGCGTGTCCAGGAAGC-3′; IRF4 FW: 5′-TGACTTTGAGGAATTGGTCGAG-3′, IRF4 RV: 5′-GTGCTGTCATGGGGTAGGG-3′ (7305519a3); p53 FW: 5′-CTCTCCCCCGCAAAAGAAAAA-3′, p53 RV: 5′-CGGAACATCTCGAAGCGTTTA-3′(200203a1); p21 FW: 5′-CCTGGTGATGTCCGACCTG-3′, p21 RV: 5′-CCATGAGCGCATCGCAATC-3′; Notch2 FW: 5′-ATGTGGACGAGTGTCTGTTGC-3′, Notch2 RV: 5′-GGAAGCATAGGCACAGTCATC-3′ (33859592a1) and Bcl6 FW: 5′-CCTAAGAGCGCACAAGGCA-3′, Bcl6 RV: 5′-CCGGCAGAGAGAGTACATCCA-3′ (6671616a2).

For human genes: PBGD FW: 5′-GGCAATGCGGCTGCAA -3′, PBGD RV 5′-GGGTACCCACGCGAATCAC-3′; human p21 FW: 5′-ACCTGGAGACTCTCAGGGTC-3′, human p21 RV: 5′-GCGTTTGGAGTGGTAGAAATCT-3′ (11386203a2); BIM FW: 5′-GACAGAACCGCAAGGTAATCC-3′, BIM RV: 5′-ACTTGTCACAACTCATGGGTG-3′ (2895500a1) and LRF FW: 5′-GAAGCCCTACGAGTGCAACATC-3′, LRF RV: 5′-GTGGTTCTTCAGGTCGTAGTTGTG-3′ (2895500a1).

Plasmids

Xpress- and Flag-Tagged human LRF expression vectors were generated by subcloning the human LRF cDNA into the pcDNA 3.1 His (Invitrogen) and pCMV-Tag2 Vectors (Stratagene),

respectively. pcDNA3.1hGLuc empty vectors, Zip-hGLuc1 and Zip-hGLuc2 vectors were kind gifts from Dr. Stephen Michnick. LRF-POZ-hGluc1 and LRF-POZ-hGluc2 were generated by introducing a PCR-amplified human LRF-POZ domain sequence. The MSCV-PIG (Puro-IRES-GFP) retrovirus vector was described elsewhere (4). For the CSEμmarCD19-IRES-GFP vector, an EμmarCD19 sequence was obtained from the EL19-GFP vector (5). Mutated LRF expression vectors were constructed by using a QuikChange Mutagenesis Kit (Stratagene).

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay for the mouse p19Arf promoter was performed as described (4). Primer sequences were as follows: Arf-ChIP-FW1: 5′-GCTGGCTGTCACCGCGATG-3′, Arf-ChIP-RV1: 5′-CGGCCTCACTGTGACAAGCG-3′ (amplicon=296bp), β-actin-ChIP-FW: 5′-TCGATATCCACGTGACATCCA-3′ and β-actin-ChIP-RV: 5′-GCAGCATTTTTTTACCCCCTC-3′ (amplicon=269bp).

Cell toxicity assay

Lentiviral shRNA vectors (pLKO.1-puro) were purchased from Sigma. For human LRF knockdown, 5 different shRNA clones were validated: TRCN0000136852 (Clone#1), TRCN0000137891 (Clone#2), TRCN0000138084 (Clone#3), TRCN0000136851 (Clone#4) and TRCN0000138142 (Clone#5). For p21 knockdown, two pLKO-shRNA-p21 vectors were used: TRCN0000040123 (Clone#1) and TRCN0000040124 (Clone#2). Efficacies of shRNA-mediated p21 knockdown were validated by q-RT-PCR upon Doxorubicin treatment (1μM for 24h). An inducible GATA1 shRNA, pLVUTHshGATA1-tTR-KRAB (6), was purchased from Addgene (plasmid 11650). Inducible LRF shRNA vectors (pLVUTHshLRF-tTR-KRAB) were generated by introducing a U6-shRNA-LRF cassette from a pLKO-shRNA-LRF(Clone#2 or #4) into the pLVUTH-tTR-KRAB empty vector, which we generated from vectors

pLVUTHshGATA1-tTR-KRAB and pLVTHM (Addgene: plasmid 12247). Virus production and transduction of lymphoma cell lines were performed as described (7) with minor modifications. Two days after transduction, medium was replaced with medium containing Dox (day 0); the fraction of GFP positive cells was measured over time by FACS. For p21 knockdown, shRNAs (p21 or scrambled) were lentivirally expressed in the lymphoma cell lines that were transduced with inducible-shRNA vectors (empty vector or shRNA-LRFclone#2). Cells that stably expressed the second shRNA (shRNA-p21 or scrambled) were established upon puromycin selection and cell toxicity assays performed as above.

Co-Immunoprecipitation and Western blot analysis

Co-IP and Western blot was performed with standard protocols. The following antibodies were used: anti-LRF (clone 13E9) (4), anti-p19Arf (clone 5-C3, Abcam), anti-Flag (M2, Sigma), anti-Xpress (Invitrogen), anti-HSP90 (610418, BD), anti-Tubulin (T5168, Sigma) and anti-β actin (A5316, Sigma). Protein was visualized by using the Fast Western kit or SuperSignal western blot kit (Pierce). Signal intensity was measured by ImageJ 1.34S software (http://rsb.info.nih.gov/ij/).

Sucrose gradient analysis

Sucrose gradient assay was performed as described (8). Nuclear extracts prepared from Ly-1 cells were fractionated using 10–40% sucrose gradients along with molecular weight markers (Bio-Rad) containing a mixture of thyroglobulin (670 kD), γ-globulin (158 kD) and ovalbumin (44 kD). Each fraction corresponding to the 10–20% range of the gradient was then loaded onto a 10% SDS-PAGE for analysis.

Protein-fragment complementation assay (PCA) and Luciferase reporter assay

PCA was performed as described (9) with minor modifications. Plasmids harbouring PCA fusions were co-transfected into HEK293 cells, and luciferase assays were performed 48 h later using a Gaussia Luciferase Assay Kit (NEB). For reporter assays, 5000 immortalized LRF-deficient MEFs ($LRF^{-/-}p19Arf^{-/-}$) (4) per well were plated into 48-well plates in triplicate 16 h before transfection, and cells were transfected with Lipofectamine 2000. At 24 h after transfection, cells were assayed with a dual luciferase assay kit (Promega).

Analytical ultracentrifugation

The Beckman Proteome XLI analytical ultracentrifuge was used to collect the sedimentation equilibrium data. Radial scans at 10, 20, 30 and 40 kRPM and at 20 ^{o}C were performed after 10 h and 12 h at speed and compared to ensure the sample achieved equilibrium. Data were analyzed using conventional equilibrium equations incorporated into IgorPro (Wavemetrics) (10). The partial specific volume for each construct was calculated using SEDNTERP (11). The extinction coefficient at 280 nm was calculated from the primary sequence. For the association constants, molecular weight, vBAR, rho (density), and epsilon (extinction coefficient) were kept constant, the protein concentration was allowed to float and the association constant was fit.

Retrovirus infection and generation of growth curve

Retrovirus-mediated gene expression in MEFs was performed as described (4). $LRF^{+/+}$ or $LRF^{-/-}$ MEFs were prepared from embryos at 13.5 days post coitus. MEFs at passage one or two were used for all experiments. Retovirally-transduced MEFs were cultured in the presence of puromycin (2 μg/ml) for 48 h, and growth curves generated by seeding 2.5 x 10^4 cells (per well) in 12-well plates in triplicate. At 2-day intervals, cells were fixed and stained with crystal violet. After extensive washing, crystal violet was resolubilized in 10% acetic acid and quantified at 595 nm as a relative measure of cell numbers.

Lentivirus infection and stem cell transfer

Virus production was performed as previously described (http://www.brc.riken.jp/lab/cfm/Subteam_for_Manipulation_of_Cell_Fate/Protocols.html). BM HSCs were obtained from femurs and tibias from five CD45.2+ $LRF^{Flox/Flox}$ mb-1 Cre+ mice 5 days after 5-FU injection (250 mg/kg). Cells were cultured overnight with RPMI medium supplemented with 20% FBS, SCF (100 ng/ml), IL-3 (6 ng/ml), and IL-6 (10 ng/ml) (Peprotech). Cells were then mixed with viral supernatant containing polybrene (at a final concentration of 10 μg/ml) and subsequently spin-infected for 90 min at 2500 rpm. After 24 h, 1 x 10^6 cells were transferred to a lethally irradiated CD45.1+ recipient mouse (B6.SJL-$Ptprc^aPepc^b$/BoyJ from JAX).

References

1. Maeda, T., Merghoub, T., Hobbs, R.M., Dong, L., Maeda, M., Zakrzewski, J., van den Brink, M.R., Zelent, A., Shigematsu, H., Akashi, K., Teruya-Feldstein, J., Cattoretti, G., and Pandolfi, P.P. 2007. Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF. *Science* **316**:860-866.

2. Moriyama, Y., Sekine, C., Koyanagi, A., Koyama, N., Ogata, H., Chiba, S., Hirose, S., Okumura, K., and Yagita, H. 2008. Delta-like 1 is essential for the maintenance of marginal zone B cells in normal mice but not in autoimmune mice. *Int Immunol* **20**:763-773.

3. Esposito, G., Texido, G., Betz, U.A., Gu, H., Müller, W., Klein, U., and Rajewsky, K. 2000. Mice reconstituted with DNA polymerase beta-deficient fetal liver cells are able to mount a T cell-dependent immune response and mutate their Ig genes normally. *Proc Natl Acad Sci U S A* **97**:1166-1171.

4. Maeda, T., Hobbs, R.M., Merghoub, T., Guernah, I., Zelent, A., Cordon-Cardo, C., Teruya-Feldstein, J., and Pandolfi, P.P. 2005. Role of the proto-oncogene Pokemon in cellular transformation and ARF repression. *Nature* **433**:278-285.

5. Moreau, T., Barlogis, V., Bardin, F., Nunes, J.A., Calmels, B., Chabannon, C., and Tonnelle, C. 2008. Development of an enhanced B-specific lentiviral vector expressing BTK: a tool for gene therapy of XLA. *Gene Ther* **15**:942-952.

6. Szulc, J., Wiznerowicz, M., Sauvain, M.O., Trono, D., and Aebischer, P. 2006. A versatile tool for conditional gene expression and knockdown. *Nat Methods* **3**:109-116.

7. Szulc, J., and Aebischer, P. 2008. Conditional gene expression and knockdown using lentivirus vectors encoding shRNA. *Methods Mol Biol* **434**:291-309.

8. Tanese, N. 1997. Small-scale density gradient sedimentation to separate and analyze multiprotein complexes. *Methods* **12**:224-234.

9. Remy, I., and Michnick, S.W. 2006. A highly sensitive protein-protein interaction assay based on Gaussia luciferase. *Nat Methods* **3**:977-979.

10. Arkin, M., and Lear, J.D. 2001. A new data analysis method to determine binding constants of small molecules to proteins using equilibrium analytical ultracentrifugation with absorption optics. *Anal Biochem* **299**:98-107.

11. Harding, S.E., Rowe, A.J., and Horton, J.C. 1992. *Analytical Ultracentrifugation in Biochemistry and Polymer Science.* Royal Society of Chemistry, Cambridge.