Supplemental information for

Cooperative transcriptional repression by BCL6 and BACH2 in germinal center B-cell differentiation

Supplementary Methods

Cell line culture and siRNA electroporation

DLBCL cell lines OCI-LY1 and OCI-LY7 were maintained in medium containing 90% Iscove and 10% fetal calf serum (FCS) and supplemented with antibodies. The DLBCL cell lines Toledo, OCI-LY3, SU-DHL4, SU-DHL6, Karpas422 and VAL were maintained in medium containing 90% RPMI and 10% FCS supplemented with antibodies, L-glutamine and HEPES. Control siRNA (siNT), BCL6 siRNA (siBCL6#1 and siBCL6#2) and BACH2 siRNA (siBACH2#1 and siBACH2#2) were purchased from Invitrogen. SiRNAs were transiently electroporated into lymphoma cell lines using cell line nucleofactor transfection kit (AMAXA, Cologne, Germany) according to the manufacturer's instruction. The sequence for each siRNA is listed in **Table S1**

Flow cytometry and Immunochemistry

Antibodies with the following specificities were used for flow cytometry. Anti-mouse B220-PE or FITC or APC (RA3-6B2), anti-mouse IgM APC (II/41), anti-mouse IgD PE (11-26c.2a) and anti-Fas PE-Cy7 (jo2) were from BD Bioscience. Anti-mouse CD21 FITC (8D9), anti-mouse CD4 APC (GK1.5), anti-mouse CD23-PE (B3B4), anti-mouse CD43 FITC (eBioR2/60), anti-mouse CD44-PE (IM7), anti-mouse CD62L FITC (MEL-14) and anti-mouse CD8a FITC (53-6.7) were from eBiosciences. All flow cytometry data were acquired on BD LSR and analyzed using Flow software Package (Tri-Star). Immunochesmistry was performed as previously descripted[1]. Tissue sections were viewed with a light microscope (AxioSkop2; Carl Zeiss Microimaging, Thornwood, NY) using Plan-Neofluar lens at a 4×/0.50 air objective or 20×/0.50 air objective. Images were taken using a color camera (AxioCam; Carl Zeiss Microimaging) and were analyzed using Axiovision software (Carl Zeiss Microimaging).

RT-QPCR

Total RNA was prepared with TriZol reagent (Invitrogen) and cDNA was synthesized using Superscript reverse transcriptase and random primers (Invitrogen). Quantitative PCR (qPCR) was performed using Power SYBR Green PCR master mix (Applied Biosystems) to examine the mRNA level. Primers are listed in **Table S2**.

Antibodies, Western Blot and Immunoprecipiation

BACH2 polyclonal antibodies were raised in rabbits immunized with GST-Bach2 fusion protein (amino acids 230-450) according to a standard protocol and purified by IgG/A beads (Bio-Rad). Whole cell lysates were prepared in RIPA and proteins were resolved by SDS-PAGE and immunoblotting using the following antibodies: anti-BCL6 (D8, Santa Cruz), anti-MAFK (C-16, Santa Cruz), anti-BACH2, and actin (C-11,Santa Cruz). Detection was performed using the ECL system (Pierce) and images

were quantified with ImageJ software. Otherwise, cells were lysed with buffer: 0.5% Triton NP40, 50Mm Tris, 150mM NaCL, 10% Glycerol, 0.5 mM EDTA and protease inhibitor cocktail. The cell lysates were incubated with anti-BCL6 (D8, Santa Cruz) or anti-BACH2 antibodies for immunoprecipitation (IP).

BCL6 and BACH2 expression vectors, lentivirus production and infection

cDNA fragments encoding BCL6 and BACH2 were sub-cloned into pcDH-EF-T2A-puromycin Lentivirus expression vector (System Biosciences). Viral production and concentration were followed according to the standard protocol. Lymphoma cells were infected with concentrated virus in the presence of 8 μg/ml polybrene (Sigma). Five days after infection, puromycin-resistant cells were selected by adding 2 μg/ml puromycin (Invitrogen).

ChIP-QPCR and ChIP-seq

Lymphoma cell lines were cross-linked with 1% formaldehyde for 10 minutes at room temperature, lysed, and DNA was sonicated to a fragment size of 150-350bp. After sonication, immunoprecipitations were performed with the following antibodies: anti-BCL6 (N3, Santa Cruz), anti-BACH2, anti-MAFK (C-18,Santa Cruz) or rabbit control IgG (ChIP-grade, Abcam). DNA fragments enriched by ChIP were identified by quantitatively PCR (ChIP-QPCR) using the Fast SYBR green kit. The GAPDH gene, which is not a BCL6/BACH2 target, was used as negative control. The enrichment for each antibody at examined loci is expressed as percentage of input. Primers are listed in **Table S3**. ChIP-seq libraries were prepared using Illumina ChIP-seq Library preparation Kit according to the manufacturer's instructions with 10ng of purified ChIP DNA and sequenced for 36 cycles using Illumina GAIIx sequencer. Peak calling and downstream analysis was performed using the ChIPseeqer package[2]. The ChIP-seq data have been uploaded to GEO under accession number GSE47785. Peaks in ±2 kb vicinity of gene bodies were assigned to genes to identify the bound target genes. Two peaks with 10 bp overlap are considered to be coincident.

Pathway analysis

We compared the lymphoid-specific gene expression signatures (http://lymphochip.nih.gov/signaturedb/)[3] with BACH2-MAFK target genes to identify the enriched pathway regulated by BACH2 and MAFK. Fisher exact test was used to calculate enrichment *P* values, and the Benjamini Hochberg method was used for the multitest adjustment and false discovery rate control.

Supplementary References

1. Huang C, Hatzi K, Melnick A. Lineage-specific functions of Bcl-6 in immunity and inflammation are mediated by distinct biochemical mechanisms. *Nature immunology* 2013, **14**(4)**:** 380-388.

2. Giannopoulou EG, Elemento O. An integrated ChIP-seq analysis platform with customizable workflows. *BMC Bioinformatics* 2011, **12:** 277.

3. Shaffer AL, Wright G, Yang L, Powell J, Ngo V, Lamy L*, et al.* A library of gene expression signatures to illuminate normal and pathological lymphoid biology. *Immunological reviews* 2006, **210:** 67-85.

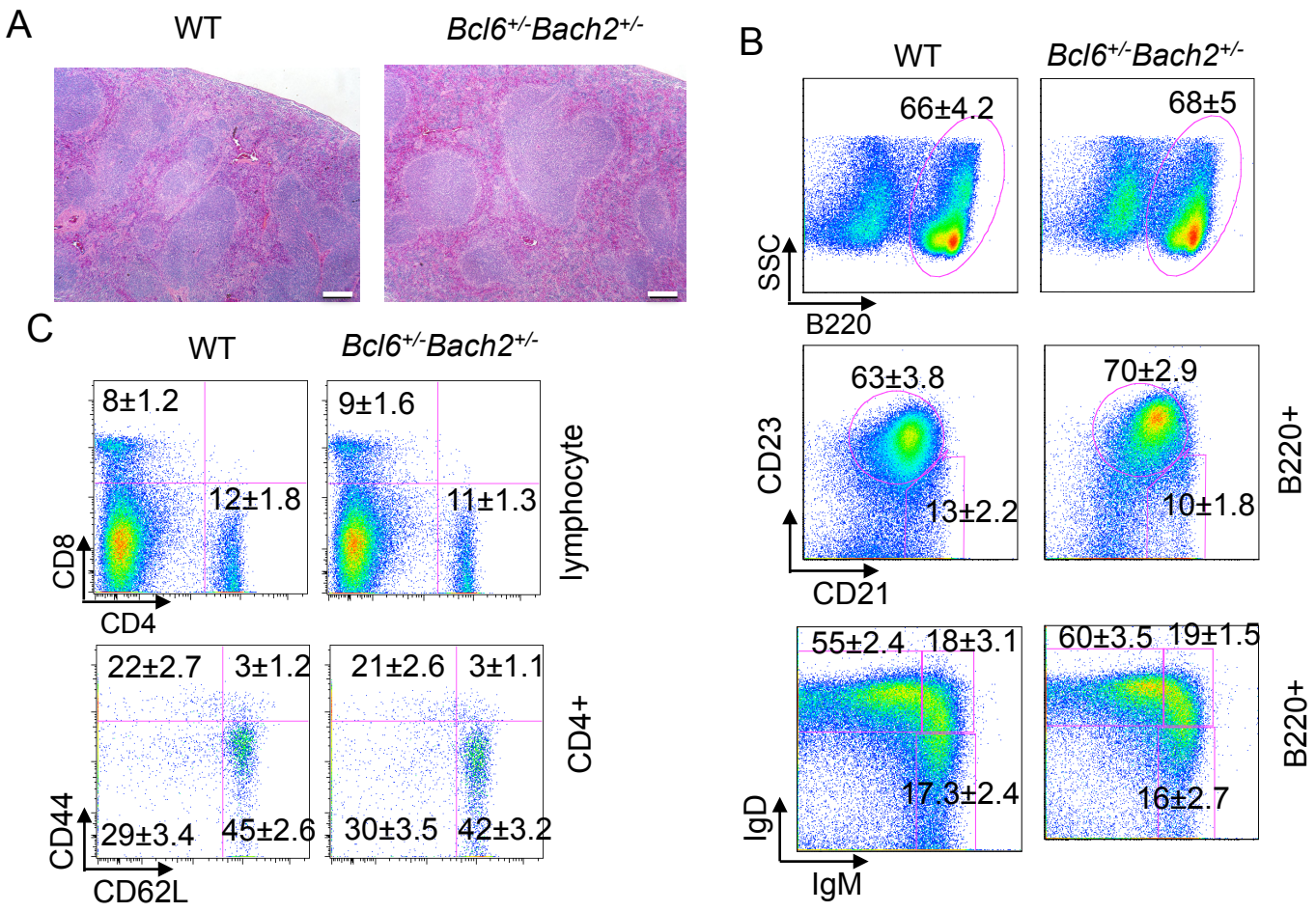

Figure S1. *Bcl6*$^{+/-}$*Bach2*$^{+/-}$ mice display normal splenic lymphoid follicles and development of B cells.

A. Hematoxylin and eosin (HE) staining of spleens from WT and *Bcl6*$^{+/-}$*Bach2*$^{+/-}$ mice. Scale Bars: 200μm.

B. Phenotypic flow cytometer analysis of FSC, B220, CD23, CD21, IgM and IgD expression on splenic B cells from WT and *Bcl6*$^{+/-}$*Bach2*$^{+/-}$ mice.

C. Phenotypic flow cytometer analysis of CD4, CD8, CD44 and CD62L expression on splenic cells from WT and *Bcl6*$^{+/-}$*Bach2*$^{+/-}$ mice. In **B** and **C**, data is from three mice and present as means±SEM.

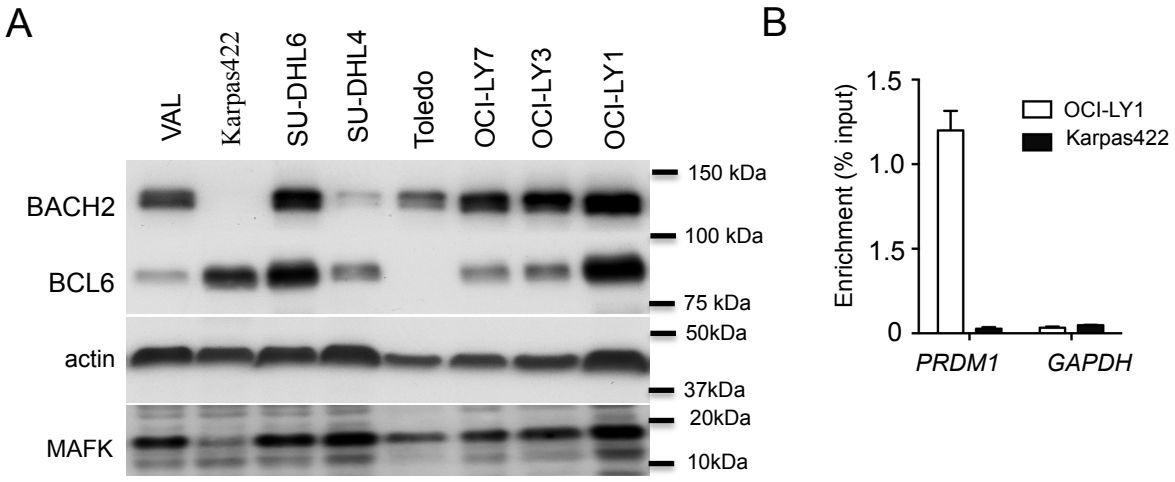

Figure S2. Characterization of anti-BACH2 antibodies

A. Immunoblot analysis of BACH2, BCL6 and MAFK in a panel of human lymphoma cell lines.

B. The enrichment of BACH2 at the promoter of *PRDM1*, but not negative *GAPDH* genomic locus, revealed by ChIP-QPCR. Data is from two independent experiments and present as means±SEM.

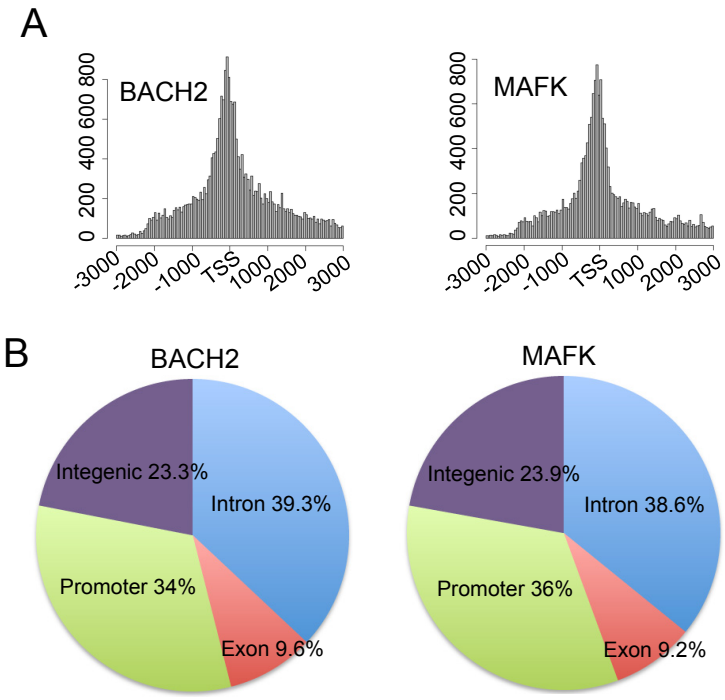

Figure S3. Genome-wide occupation of BACH2 and MAFK.
A. The density of BACH2 and MAFK peaks around the transcription stat site (TSS) and their distance to the TSS.
B. Genomic distribution of BACH2 and MAFK binding regions. The genomic features (exons, introns, and intergenic regions) were defined based on RefSeq gene (hg19) annotations. Promoter was defined as -2 kb to +2 kb relative to TSS. Integenic region was defined away from gene.

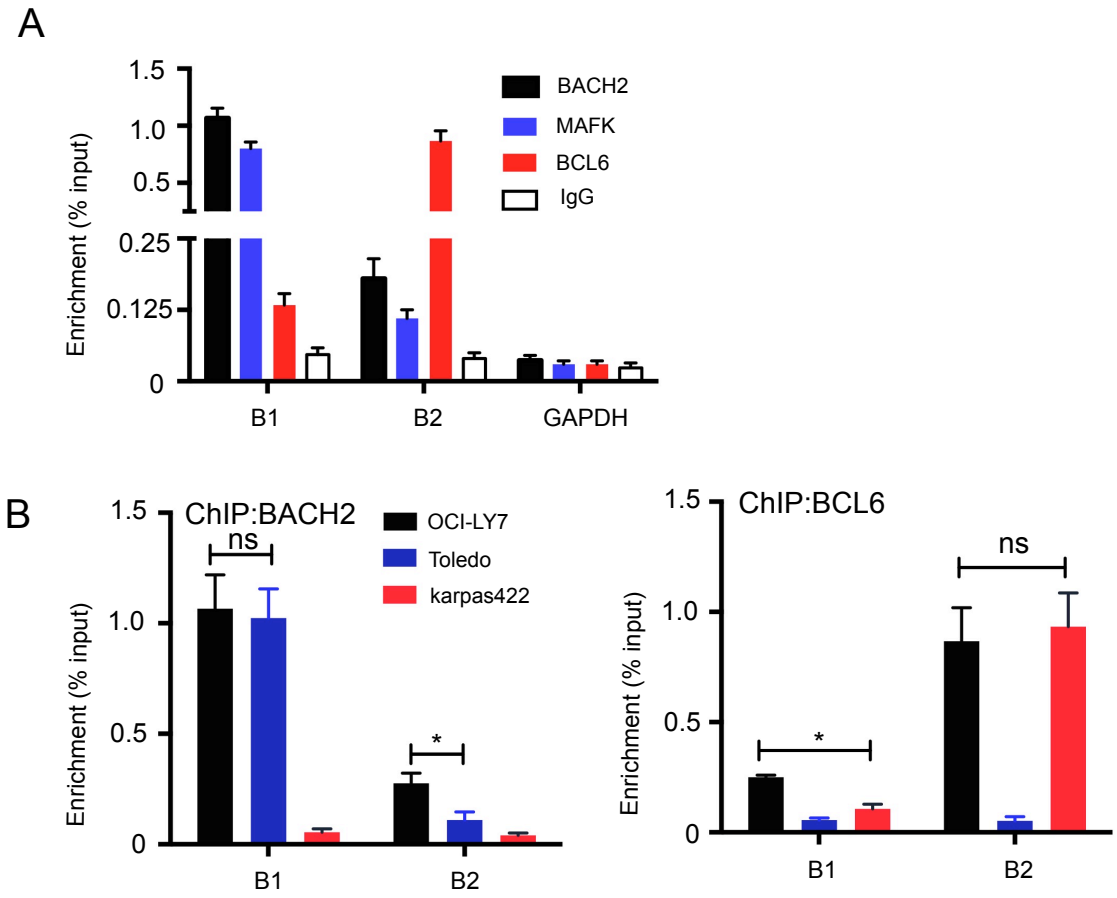

Figure S4. The binding of BACH2, BCL6 and MAFK at PRDM1 loci in a panel of DLBCL cell lines and tonsillar GC B cells.

A. ChIP-QPCR analysis of the recruitment of the indicated protein at the B1 and B2 loci of the *PRDM1* gene in normal GC B cells isolated from human tonsil.

B. ChIP-QPCR analysis of enrichment of BCL6 and BACH2 at B1 and B2 sites in OCI-LY7 ($BCL6^{+}BACH2^{+}$), Toledo ($BCL6^{-}BACH2^{+}$) and Karpas422 ($BCL6^{+}BACH2^{-}$) DLBCL cell lines. . Data are present as means±SEM from two independent experiments. ns, not significance; * $p<0.05$ (two-tailed *t* test)

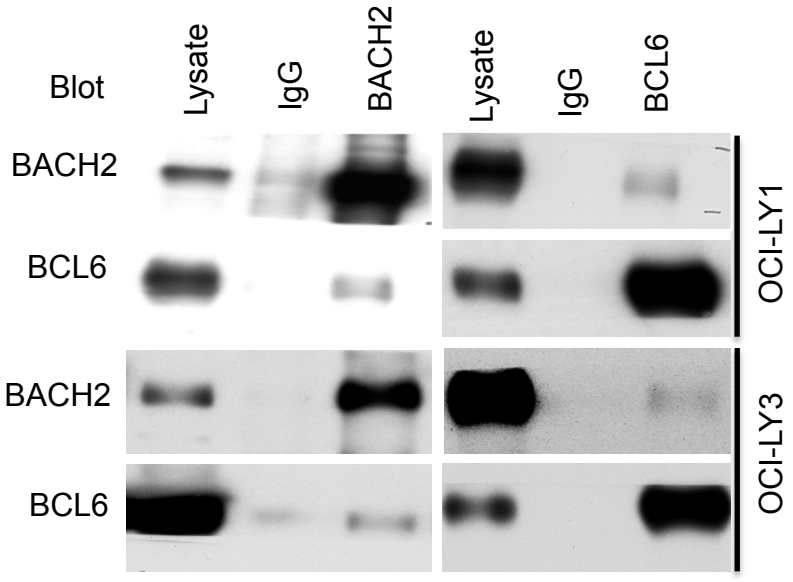

Figure S5. BCL6 forms a protein complex with BACH2 in OCI-LY1 and OCI-LY3 cells. Immunoprecipitation and immunoblot analysis of the interaction between BCL6 and BACH2 in OCI-LY1 and OCI-LY3 cells.

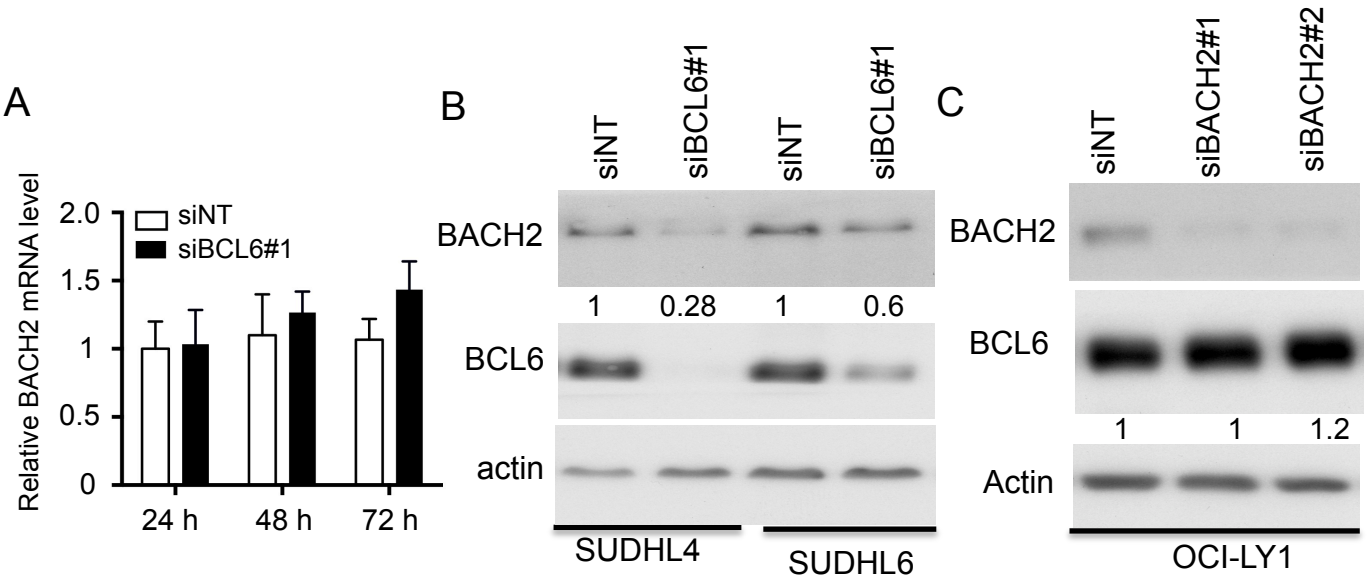

Figure S6. BCL6 maintains BACH2 protein stability.
A. RT-QPCR analysis of BACH2 mRNA level in OCI-LY1 transfected with either siNT or siBCL6 for indicated time.
B. Immunoblot analysis of BACH2 and BCL6 in SUDHL4 and SUDHL6 cells transfected with siNT or siBCL6 for 72 hours.
C. Immunoblot analysis of BACH2 and BCL6 in OCI-LY1 cells transfected with siNT or siBACH2 (#1 and #2) for 72 hours. In **B** and **C**, Relative expression levels were determined by densitometry.

Table S1: Sequence for siRNA

siBCL6#1: 5- CCAUUGUGAGAAGUGUAACCUGCAU-3
siBCL6#2: 5-GCCAGCCGGCUCAAUAACAUCGUU-3
siBACH2#1: 5- GAGCACCUCGGUGCAUUCUUAUUCU-3
siBACH2#2: 5- UGAACAGAGACCUUAAGCAGGAGGG-3

Table S2: Primers for RT-QPCR

mPrdm1F: 5- AACCAGGAACTTCTTGTGTGGT-3
mPrdm1R: 5- ACTGTATTGCTTTGGGTTGCTT-3
hPrdm1βF: 5-GCCCATTTGCCATTCACT-3
hPrdm1βR:5-TCATGTTCGGGAAAATAACAGA -3
hPrdm1αF: 5-CAGCACTGTGAGGTTTCAGG -3
hPrdm1αR: 5-GCATCCTCCATGTCCATTTT-3
mXbp1F: 5- TGACACTGTTGCCTCTTCAGAT-3
mXbp1R: 5-GGAGTTCCTCCAGACTAGCAGA-3
mCD138F: 5-TCATTGTGGGGAGGTCTACTTT-3
mCD138R: 5-ACAGACTTGCCTTACCCCAGTA-3
mHprtF: 5-TTAAGCAGTACAGCCCCAAAAT-3
mHprtR: 5-CCAACAACAAACTTGTCTGGAA-3
hHPRT5'-AAAGGAACCCCACGAAGTGTT-3'
hHPRT5'-TCAAGGGCATATCCTACAACAA-3'

Table S3: Primers or ChIP-QPCR

BACH1F: 5- AAAGTTGGGTTGCTGAGTCATT-3
BACH1R: 5- GTGGAGGCATGTGATGAGAGTA-3
BACH2F: 5- CCTACCTGGCAAAAACAAAAAC-3
BACH2R: 5-TCTTTTTGAGCAGTGGCATAGA-3
CD69F: 5-CTCCAGTGGACCAGACAATGTA -3
CD69R: 5-CACTTTCCTCCTGCTACACCTT-3
GADD45AF: 5-GGAAGAGATCCCTGTGAGTCA-3
GADD45AR: 5-TCTGCCCTGCTAAAGGAATTAG-3
GADD45BF: 5-TCAAATGATGACTCAGCTCCAT-3
GADD45BR: 5-CTGCAAAGATGAACAAAACGAG-3
Prdm1intro3(B2)F: 5-CGAAGAGTACAAGAGCGATGG-3
Prdm1intro3(B2)R: 5-ACAAGGCTGGGTGAATTCTG-3
Prdm1prof(B1)F: 5-GGCCAGCCTTCAGTATGACTAC-3
Prdm1prof(B1)R: 5-GCCTGCTCAAGAGGGTTTATTA-3
GAPDHF: 5-ACGTAGCTCAGGCCTCAAGA-3
GAPDHR: 5-GCTGCGGGCTCAATTTATAG-3
CDKN1BF: 5-AAGAATGGTGGAGTTGAGTGCT-3
CDKN1BR: 5-CCAAATGTTTCTGCGAAGGT-3
MDM2F: 5-GTTCCGAAACTGCAGTAAAAGG-3
MDM2R: 5-ACCTGGATCAGCAGAGAAAAAG-3

Table S4: Lymphoid gene sets enriched in BACH2-MAFK target genes. **A.** Detailed explanation for each lymphoid gene set. **B.** Full list of enriched lymphoid gene sets. *P* values are calculated by Fisher's Exact test and multi-testing adjusted by Benjamini and Hochberg (BH) method.

A

Signature names	Signature identifiers (Shaffer et al, Immunol Rev. 2006)	Detailed explaination (Shaffer et al, Immunol Rev. 2006)	Reference for the signatures	Selection criteria (Shaffer et al, Immunol Rev. 2006)
Proliferation genes	Proliferation_DLBL	http://lymphochip.nih.gov/cgi-bin/signaturedb/signatureDB_DisplayGenes.cgi?signatureID=10	Rosenwald A et. al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002 Jun 20;346(25): 1937-47.	Supplementary appendix 1: proliferation genes in DLBCL
BCL6 target genes	BCL6_targets_CHIPCHIP	http://lymphochip.nih.gov/cgi-bin/signaturedb/signatureDB_DisplayGenes.cgi?signatureID=237	Polo JM et. al. Transcriptional signature with differential expression of BCL6 target genes accurately identifies BCL6-dependent diffuse large B cell lymphomas. Proc Natl Acad Sci U S A. 2007 Feb 27;104(9):3207-12.	BCL-6 binding in DLBCL by CHIP-CHIP using Nimblegen promoter arrays
PRDM1 repressed genes	Blimp_Bcell_repressed	http://lymphochip.nih.gov/cgi-bin/signaturedb/signatureDB_DisplayGenes.cgi?signatureID=89	Shaffer AL et. al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity. 2002 Jul;17(1): 51-62.	Blimp-1 repressed genes in human mature B cell (65 genes)
cell cycle genes	Cell_cycle_Whitfield	http://lymphochip.nih.gov/cgi-bin/signaturedb/signatureDB_DisplayGenes.cgi?signatureID=147	Whitfield ML et. al. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell. 2002 Jun;13(6): 1977-2000.	Genes periodically expressed in the human cell cycle and their expression in tumors
MYC target genes	Myc_ChIP_PET_Expr_Down	http://lymphochip.nih.gov/cgi-bin/signaturedb/signatureDB_DisplayGenes.cgi?signatureID=246	Zeller KI et. al. Global mapping of c-Myc binding sites and target gene networks in human B cells. Proc Natl Acad Sci U S A. 2006 Nov 21;103(47):17834-9.	Table ST5: 668 Myc direct responsive genes in P493 cell
PRDM1-repressed proliferation genes	Blimp_proliferation_repressed	http://lymphochip.nih.gov/cgi-bin/signaturedb/signatureDB_DisplayGenes.cgi?signatureID=88	Shaffer AL et. al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity. 2002 Jul;17(1): 51-62.	Blimp-1 repressed genes in human mature B cell (90 genes)

B

Signature Name	-log10(BH) BACH2	-log10 (BH) MAFK
Proliferation_genes	13.76	15.74
BCL6_target genes	9.256	12.56
PRDM1 target genes	8.271	5.638
Cell cycle genes	8.128	5.361
Myc_ChIP_PET_Expr_Down	8.089	2.482
Blimp_proliferation_repressed	8.039	3.264
Hematopoietic_Node1658	7.956	3.983
GC_B_cell_U133Plus	6.061	2.099
HIF1alpha_1.5x_down	5.801	5.845
Myc_ChIP_PET_Expr_Up	5.55	5.423
GC_T_helper_up2x_Chtanova	5.55	2.642
Germinal_center_Bcell_DLBCL	5.415	2.844
Glutamine_starve_up	4.882	2.44
Glutamine_starve_down	4.608	6.427
Glutamine_Glucose_starve_both_down	4.538	4.433
Cell_cycle_Liu	3.999	2.391
Tcell_PIrep4x_Feske_Fig6	3.873	2.29
Proliferation_Node1618	3.756	3.629
CD40_downregulated_Burkitt_lymphoma	3.756	2.714
Quiescence_heme_all	3.373	2.245
Leucine_starve_up	3.323	2.663
STAT3high_ABC_DLBCL_subgroup	3.168	2.403
ABC_gt_GCB_LC	2.87	2.177
XBP1_target_all	2.61	2.902
Leucine_starve_down	2.024	4.638