

SUPPLEMENTAL FIGURES

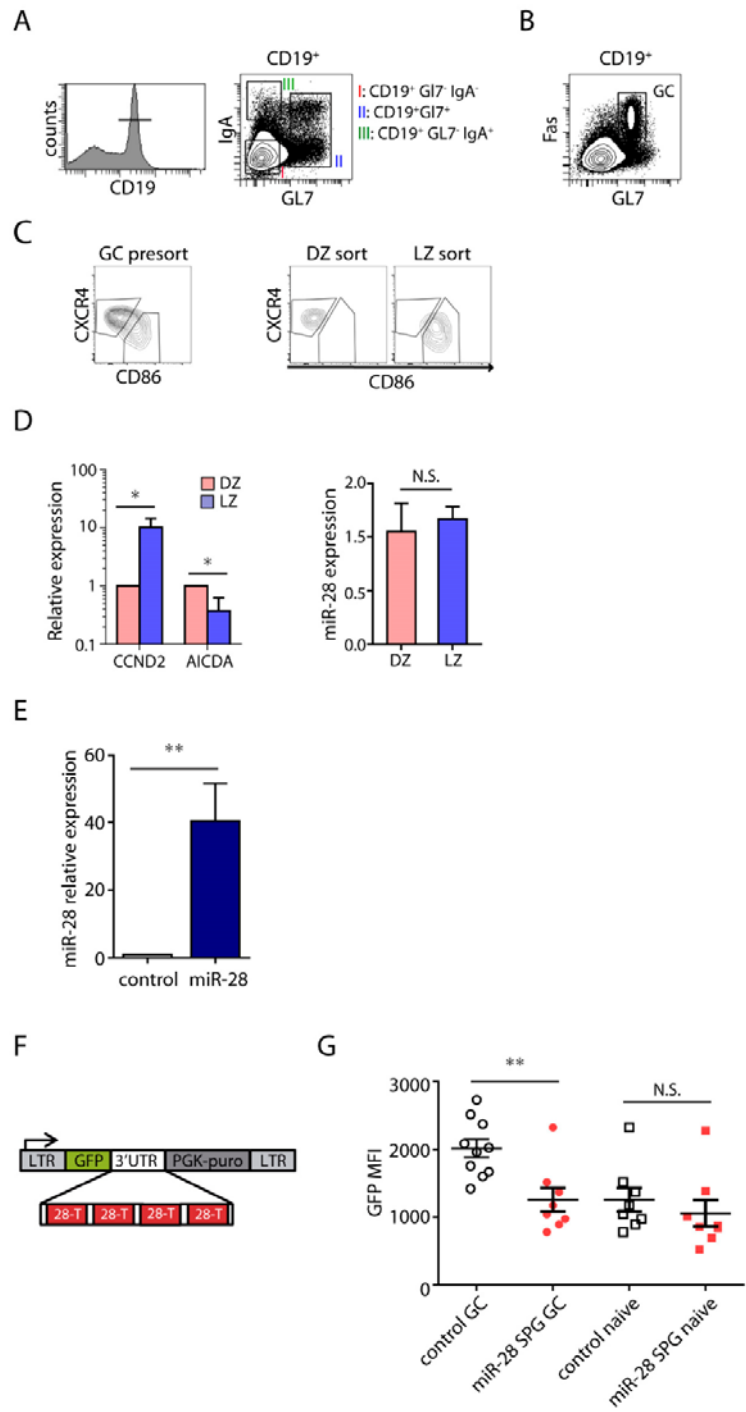


Figure S1

**Fig. S1. Experimental systems used to characterize miR-28 function in B cells. (A-B)** Gating strategy of sorting isolation of B cell subpopulations for quantification of miR-28 expression. **A)** Naïve B cells (CD19<sup>+</sup>Fas<sup>-</sup>GL7<sup>-</sup>); GC B cells (CD19<sup>+</sup>Fas<sup>+</sup>GL7<sup>+</sup>) and IgA-switched post-GC B cells (CD19<sup>+</sup>Fas<sup>-</sup>GL7<sup>-</sup>IgA<sup>+</sup>) were isolated from Peyer's patches. **B)** GC B cells were isolated from spleen cells from immunized mice (graph on the right) defined as CD19<sup>+</sup>Fas<sup>+</sup>GL7<sup>+</sup>. **(C-D)** miR-28 expression in Light Zone and Dark Zone GC subsets. **C)** Gating strategy for sorting isolation of Light Zone centrocytes (LZ) and Dark Zone centroblasts (DZ) GC subsets (left) and post-sort reanalysis (right). **D)** Gene expression in LZ and DZ GC B cells. Cyclin D2 (CCND2) and AID (AICDA) were quantified by q-RT-PCR as a validation of LZ/DZ cell isolation (left). miR-28 expression in DZ and LZ cells measured by q-RT-PCR is shown on the right. n=3, \*, p<0.05; N.S. not significant, unpaired t test. **(E)** q-RT-PCR analysis of miR-28 expression in LPS+IL-4 stimulated primary spleen B cells 2 days after transduction with miR-28 or control vectors (n=2). **(F)** miR-28-sponge (miR-28 SPG) retroviral construct for miR-28 inhibition. Four miR-28 perfect complementary sites (28-T), separated by 4-nt spacers, were placed downstream of GFP in the MGP vector. **(G)** GFP mean fluorescence intensity (MFI) in GC and naïve spleen B cells from immunized control and miR-28 SPG chimera are shown. Each symbol represents an individual mouse. \*, p<0.05, \*\*, p<0.01, unpaired t test.

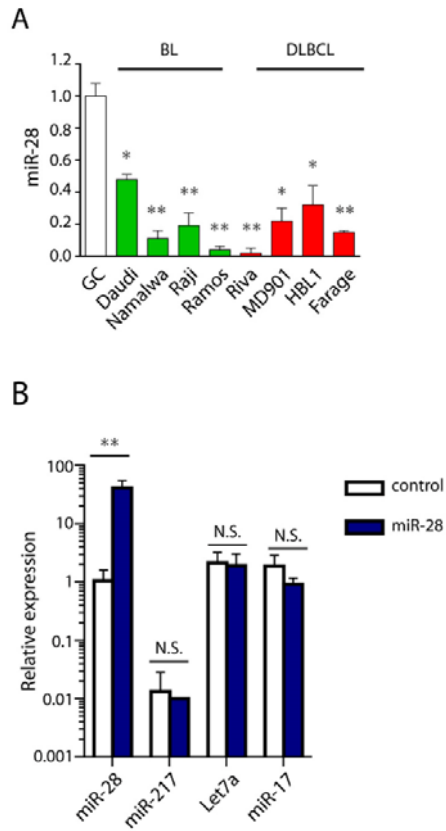


Figure S2

**Figure S2. miR-28 expression in human lymphoma cell lines** (A) miR-28 is downregulated in human and mouse GC-derived lymphomas. q-RT-PCR analysis of miR-28 expression in a collection of human BL (in green) and DLBCL (in red) cell lines is represented relative to GC B cell levels. (B) q-RT-PCR analysis of miR-28, miR-217, Let7a and miR-17 in RFP<sup>+</sup> Ramos BL cells transduced with scramble (control) or miR-28 vectors, as used for transcriptome and proteome analysis in the study (n=3). \*, p<0.05; \*\*p<0.01, unpaired t test.

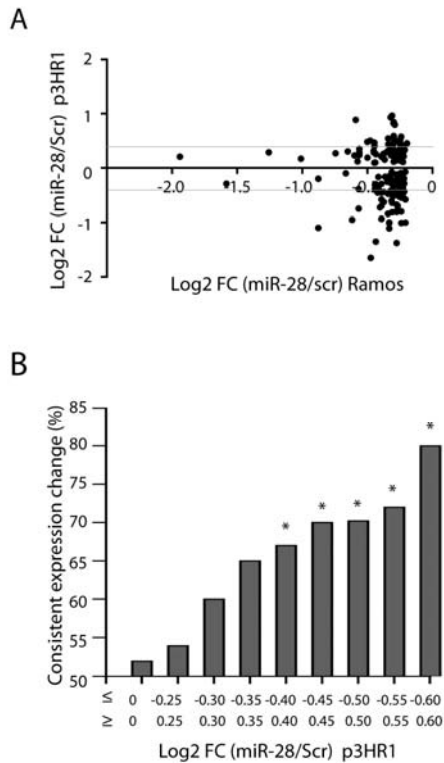


Figure S3

**Figure S3. Comparison of transcriptome analysis in miR-28 expressing Ramos and p3HR1 cells.** (A) X-axis, Log2 fold change values (miR-28/control) of mRNAs downregulated upon miR-28 ( $p < 0.05$ ) that were identified by RNAseq in Ramos B cells. Y-axis Log2 fold change values of all mRNAs whose expression is altered (either upregulated or downregulated,  $p < 0.05$ ) by miR-28 expression in p3HR1 cells, as reported in <sup>1</sup>. Grey lines show the Log2 fold change threshold from which the expression changes found in both studies are significantly consistent, as explained below. (B) mRNAs showing consistent changes of expression upon miR-28 expression in Ramos and p3HR1 cells. Bars show the proportion of mRNAs found downregulated in p3HR1 cells. Numbers underneath show the Log2 fold change thresholds used as cutoff in each case. \*  $p < 0.05$  fisher exact t test.

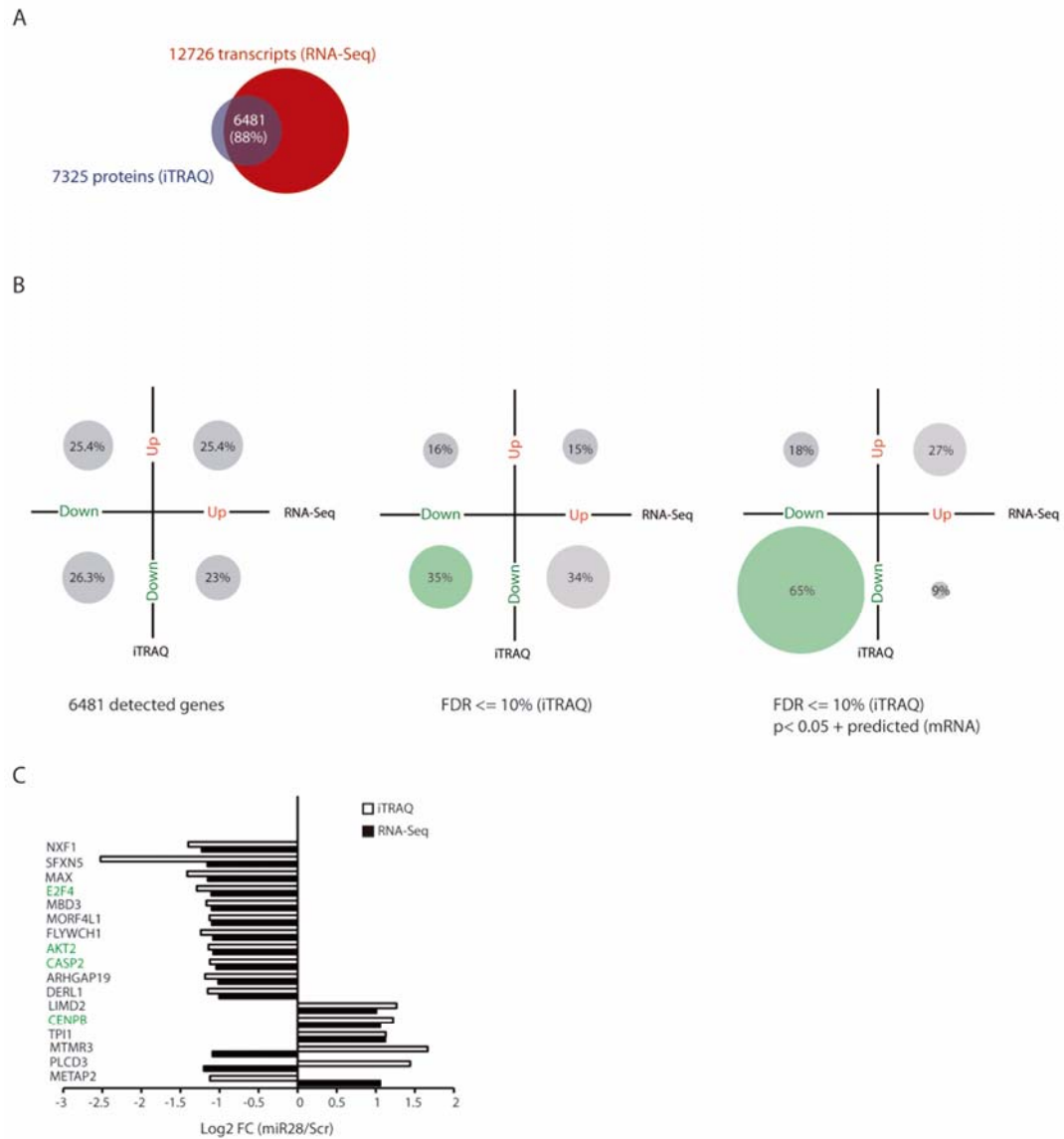
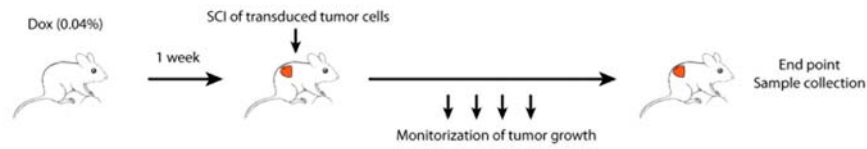


Figure S4

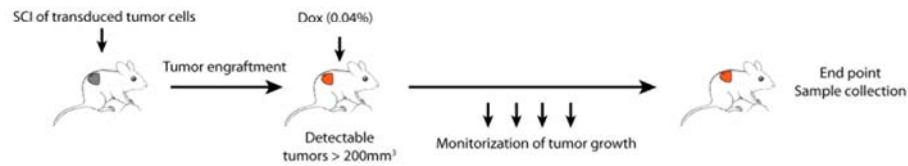
**Figure S4. Integrative analysis of miR-28 transcriptome and proteomic data.** (A) Venn diagram shows the overlap between the mRNA and protein species detected by RNAseq and iTRAQ. (B) Sign of expression changes detected at the mRNA level (X-axis) and the protein level (Y-axis) upon miR-28 re-expression in Ramos cells. Plot on the left represents all 6481 detected species without any statistical filtering. Plot in the middle shows only those species whose protein levels were significantly changed (FDR <= 10%). Plot on the right shows only those species whose proteins were significantly changed (FDR <= 10%) and whose mRNAs were significantly changed (p < 0.05) and predicted as miR-28 targets. (C) Bars show the Log2 FC values (miR28/scramble) of the individual species shown in the right plot above (S4B). Gene

names involved in BCR signaling pathway (Ingenuity Pathway Analysis shown in Figure 2G) are highlighted in green.

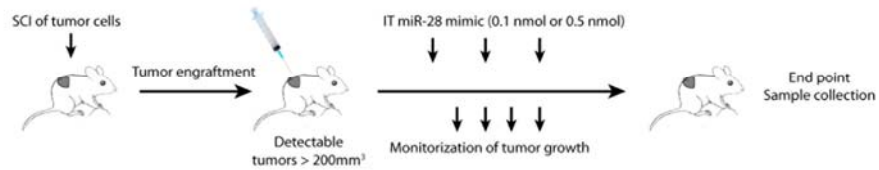
#### A Xenograft tumor establishment



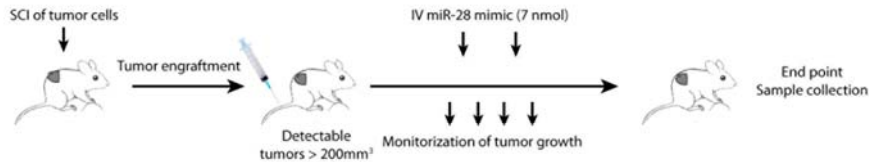
#### B Xenograft tumor regression



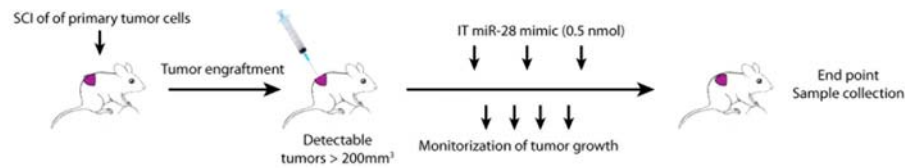
#### C Xenograft tumor treatment with intratumor miR28 mimic



#### D Xenograft tumor treatment with intravenous miR28 mimic



#### E Primary lymphoma treatment with intratumor miR28 mimic



#### F Primary lymphoma treatment with intravenous miR28 mimic

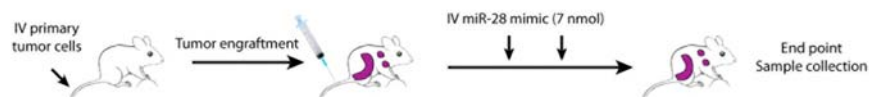


Figure S5

**Fig. S5. Schematics of the therapeutic protocols used in this study.** (A) Xenograft tumor establishment; used for the experiments shown in Figure 4 A to D. (B) Xenograft tumor regression used; for the experiments shown in Figures 5A and B. (C) Xenograft tumor treatment with intratumoral miR-28 mimic; used for the experiments shown in Figures 5C and D. (D) Xenograft tumor treatment with intravenous miR-28 mimic administration; used for the experiments shown in Figure 5E. (E) Primary mouse  $\lambda$ -MYC transgenic local BL model treatment with intratumor miR-28 mimic used for the experiments shown in Figure 6 A and B. (F) Primary mouse  $\lambda$ -MYC transgenic systemic BL model treatment with intravenous miR-28 mimic administration; used for the experiments shown in Figure 6 G, H and I



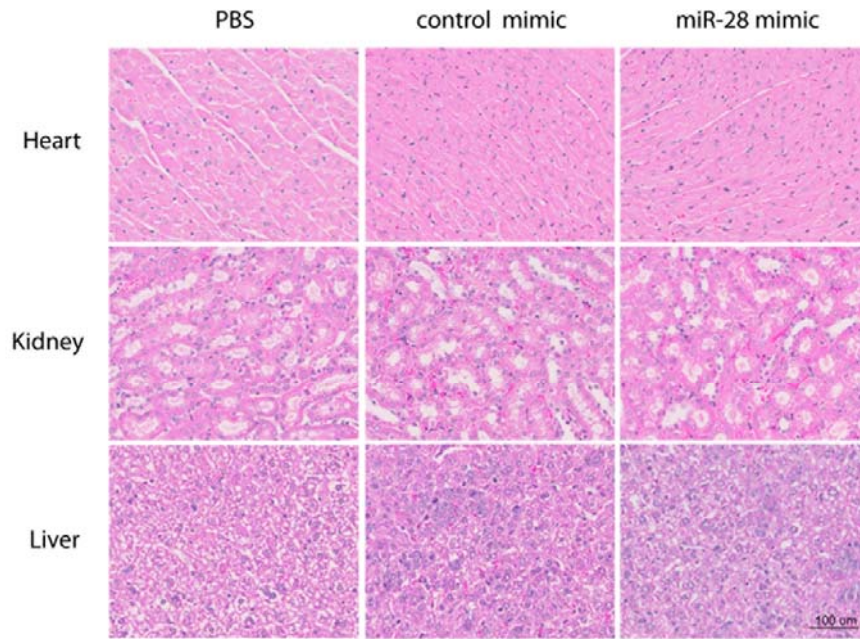


Figure S6

**Fig. S6. Intravenous miR-28 mimic administration shows no evidence of heart, kidney and liver toxicity.** Lymphoma xenograft of Ramos BL cells established NOD scid gamma mice were treated with PBS, 7 nmol of control or miR-28 mimic as described in Figure 5D. Mice were sacrificed 20 days after tumor establishment and heart, kidney and liver were formalin/paraffin fixed and stained with hematoxylin and eosin.

## SUPPLEMENTAL MATERIALS AND METHODS

### *Cell culture*

Mouse primary B cells were purified from spleens of C57/BL6 mice by anti-CD43 immunomagnetic depletion (Miltenyi Biotech) and cultured in RPMI medium containing 10% fetal calf serum (FCS), 25 µg/ml LPS (Sigma) or 10 µg/ml anti-mouse IgM (Jackson ImmunoResearch), 10ng/ml IL-4 (Peprotech), 10mM Hepes (Gibco), and 50µM β-mercaptoethanol (Gibco). The human B cell lymphoma cell lines (Ramos, Raji and MD901) were cultured in RPMI medium containing 10% FCS and 10mM Hepes (Gibco).

### *Bone marrow reconstitution and immunizations*

Total bone marrow cells ( $5 \times 10^6$ ) from CD45.1 B6.SJL or R26-Tomato<sup>ki/+</sup> AID<sup>Creki/+</sup> mice (generated by crossing the R26-Tomato<sup>ki/+</sup> B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato) Hze/J from Jax Mice with the AID<sup>Creki/+</sup> 2) donor mice were injected intravenously into 8- to 12-week-old CD45.2 C57BL/6J mice that had been lethally irradiated (2 x 550 cGy) 1 day before. Neomycin (2 mg/ml) was added in the drinking water for four weeks. For immunization, groups of 7-9 littermate mice were injected in the footpads with 50 µg of NP-CGG (Biosearch Technologies) in complete Freund's adjuvant. Mice were sacrificed for analysis 14 days after immunization. Alternatively, mice were immunized by tail vein injection with  $100 \times 10^6$  sheep red blood cells (SRBC).

### *FACS isolation and analysis of B cell subsets*

Naïve B cells (CD19<sup>+</sup>Fas<sup>-</sup>GL7<sup>-</sup>) and GC B cells (CD19<sup>+</sup>Fas<sup>+</sup>GL7<sup>+</sup>) were purified by flow-activated cell sorting from Peyer's patches or after immunization from spleen. Live lymphoid populations (7AAD or DAPI negative) from bone marrow, spleen and Peyer's patches were analyzed in cell suspensions after blocking with anti-mouse CD16/CD32 antibodies (BD Pharmingen) and staining with combinations of the following antibodies: anti-CD19-APC, anti-CD19-biotin, anti-CD3-APC, anti-CD45.2-PerCpCy5, anti-CD23-biotin, anti-CD138-PE, anti-IgD-biotin, anti-IgG1-biotin, anti-IgM-biotin, anti-IgA-biotin, PE-Cy7-streptavidin, and anti-CD21-PE (all from BD Pharmingen); anti-B220-vioblue (Miltenyi Biotech); anti-GL7-FITC, anti-GL7-Alexa647, and anti-CD93-APC (BD eBioscience); and anti-IgM-PE (Invitrogen). Labeled cells were analyzed on FACSCanto or Fortessa flow cytometers (BD Biosciences). Apoptosis was measured with the Annexin V eFluor450 kit (eBioscience) or by staining for anti-active caspase-3 (BD Pharmingen). Proliferation was assessed with Cell Trace Violet (Invitrogen), and cell cycle was analyzed using propidium iodide staining and the FITC BrdU Flow Kit (BD Pharmingen).

### *Expression constructs and transductions*

For retroviral overexpression of miR-28, a DNA fragment containing the precursor sequence of miR-28 and its flanking 50-bp-long genomic context were cloned into the XhoI-EcoRI sites of the pre-miRNA GFP vector, as previously described<sup>3</sup>. An empty p-miRNA vector was used as control. For retroviral inhibition of the endogenous miRNA, miR-28 sponges were generated as previously described<sup>4</sup>. For lentiviral

constructs, the miR-28 precursor sequence was cloned into the pTRIPZ vector (Thermo scientific). miR-28 sponges were generated by cloning 4 sequences complementary to mature miR-28 in tandem within the 3'UTR of GFP into the MGP vector by annealing and subsequent extension with Klenow DNA polymerase (New England Biolabs) of two partially complementary oligonucleotides: (forward) 5'-

tcgactagctagaacgcggccgctcaatagactgtgagctcctcgatctcaatagactgtgagctcctaccggtctcaat-3'; (reverse) 5'-

gaagtcagtcgcgattctcgagaaggagctcacagtctattgaggtgaaaggagctcacagtctattgagaccggtgaaggagctc-3'. Retroviral supernatants were obtained from 293T cells transfected with pCL-ECO (Imgenex) and with p-miR-28 and/or miR-28 sponge retroviral vectors and transduced into mouse splenic B cells and total bone marrow in the presence of 8 µg/ml polybrene (Sigma-Aldrich). Lentiviral supernatants were obtained from 293T cells transfected with VSVG, Δ9.8, and pTRIPZ plasmids. Transduced human lymphoma cell lines were selected by culture in the presence of 0.4µg/ml puromycin for 3 days. Expression of miR-28 or a scramble control sequence was induced with 0.5 µg/ml doxycycline (Dox).

### *Mice*

λ-MYC transgenic mice <sup>5</sup>, NOD/SCID/IL-2rnull (NSG) mice, C57BL/6J (CD45.2) mice, and B6.SJL (CD45.1) mice were bred in-house under pathogen-free conditions. All animal procedures conformed to European Union Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

### *miR-28 detection by qRT-PCR*

Total RNA was extracted with Trizol (Invitrogen) and miR-28-5p was measured by qRT-PCR using miR-28 miRCURY LNA primers (Exiqon). U6 amplification was used as a normalization control. Reactions were performed in a 7900HT fast real-time PCR thermocycler (Applied Biosystems).

### *RNAseq and iTRAQ analysis*

Ramos cells transduced with miR-28 or scrambled pTRIPZ vectors were selected with puromycin (0.4µg/ml) and induced for 2 days with Dox (0.5µg/ml); RFP<sup>+</sup> cells were isolated with a FACS Aria cell sorter and subjected to quantitative transcriptome and proteomic analysis. For RNA-seq, total RNA was extracted from 6 samples (3 pairs of miR-28 and scrambled samples) with RNeasy columns (Qiagen) and sequenced on an iLlumina HS2500 platform. Differential expression was analyzed using the edgeR package from Bioconductor. Proteomic analysis was performed in 4 replicates of miR-28 and scrambled samples. Cell pellets were homogenized in lysis buffer (50 mM Tris-HCl pH 8.0 – 4% SDS – 50 mM DTT) and boiled for 10 min; 160 µg of total protein for each sample were digested using the FASP protocol <sup>6</sup> and the resulting peptides subjected to isobaric labeling using the 8-plex iTRAQ Reagents Multiplex Kit (Applied Biosystems, Foster City, CA, USA). Labeled peptides were fractionated and analyzed by LC-MS/MS as described <sup>6</sup>. Peptides were identified with Proteome Discoverer using

SEQUEST-HT (Thermo Fisher Scientific) using the probability ratio method <sup>7</sup> and false discovery rates were calculated using the refined method <sup>8</sup>. Proteins were quantified from reporter ion intensities and data analyzed using QuiXoT, based on the WSPP statistical model <sup>8</sup>. In this model, protein log2-ratios are expressed in the form of standardized variables (Zqa values). From the 4 replicates of miR-28 transduced and scrambled transduced Ramos samples, 7324 proteins were identified with an FDR of 1%. The abundance of 277 proteins were found to be significantly altered ( $p < 0.0038$ , 10% FDR). The Systems Biology Triangle algorithm was used to detect functional category alterations produced by the coordinated actions of proteins <sup>9</sup>. Functional categories of proteins were constructed using the Ingenuity Knowledge Database (IPA) <sup>10</sup>, and CORUM <sup>11</sup> and DAVID <sup>12</sup> repositories. All proteomics data derived from this study are deposited in PeptideAtlas, and are accessible through the accession number PASS00874.

#### *miR-28 transcriptomic analysis comparison*

To compare the transcriptome profile induced by miR-28 expression in human Ramos and with the effect described in p3HR1 B cells <sup>1</sup> raw data from GEO GSE56268 was downloaded and preprocessed using the ReadAffy package from R. Control (24h after induction: GSM1358019, GSM1358020, GSM1358021, GSM1358022, GSM1358023, GSM1358024) and miR28 (24h after induction: GSM1358025, GSM1358026, GSM1358027, GSM1358028, GSM1358029, GSM1358030) samples were used for the analysis. Data were normalized using gcrma method. A mixed model was fit using the lmFit function from the R limma package using the condition and the batch as fixed terms and the mice as random effect. The comparison between miR28 overexpressed samples and control samples was performed using the make Contrast function.

#### *Phosphorylation detection*

For immunoblotting, pellets from Ramos cells transduced with miR-28 or scrambled were lysed in RIPA buffer, sized-fractioned by SDS-PAGE, and transferred to PVDF membranes. Membranes were probed with anti-human ERK1/2 (Cell Signaling), anti-human p-ERK1/2 (Thr202/Tyr204; Cell signaling), anti-human p-AKT (S473; Cell signaling), and anti-human tubulin (Sigma-Aldrich). Band intensities were quantified with Image-J. For FACS analysis, transduced Ramos cells were activated for 5 minutes at 37°C with 10 µg/ml soluble anti-human-IgM (Rockland), treated with IC fixation and permeabilization buffer (eBioscience), and labeled with anti-phospho-AKT (S473) (eBioscience). Stained cells were analyzed in a FACS Canto flow cytometer (BD Biosciences).

#### *Lymphoma models*

For subcutaneous xenografts, 2-10x10<sup>6</sup> Ramos cells were resuspended in 100 µL PBS and mixed with 100 µL Matrigel (BD Biosciences) and injected into NOD Scid Gamma

(NSG) mice (8-14 weeks old). When transduced cells were used, Dox (Sigma-Aldrich) was administered at 0.04% in the drinking water. To establish subcutaneous  $\lambda$ -MYC primary tumors, enlarged lymph nodes were extracted from 4-to-8-month-old sick  $\lambda$ -MYC animals, and  $10^7$  cells were subcutaneously injected into the flanks of NSG mice. Subcutaneous tumor volume was measured 3 times per week with a digital caliper using the following formula: volume = (width)<sup>2</sup> x length/2. For the systemic model of BL,  $2 \times 10^6$  primary lymphoma cells from  $\lambda$ -MYC mice were intravenously injected into the tail vein of receptor NSG mice.

#### *B cell lymphoma treatment with miR-28 mimic*

miRNA mimics for miR-28 or control were purchased from Ambion. For intratumor administration, established tumors were injected with 0.1 or 0.5 nmol miRNA mimics together with InvivoFectamin (Ambion) 3 times, separated by 3-4 days. For intravenous administration, mice with established tumors were injected twice with 7 nmol miRNA mimics, with injections separated by 4 days.

#### *Histopathology and toxicity studies*

Organs were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (Sigma), anti-ki-67 (Abcam), anti-Bcl-2 (Dako), or anti caspase-3 (RyD systems). Signals were quantified using Image-J. Toxicity was evaluated by immunohistochemical analysis of tissue damage and clinical biochemistry of plasma. Tissue damage was assessed by H&E staining of kidney, liver, heart, and spleen. Clinical biochemistry included blood counts in an Abacus Junior hematology analyzer and biochemical profiling of indicators of liver, kidney and heart function, including AST/GOT, ALT/GPT, GGT, total cholesterol, alkaline phosphatase, triglycerides, glucose, and albumin.

## SUPPLEMENTAL MATERIAL BIBLIOGRAHY

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