CDCA7 is a critical mediator of lymphomagenesis that selectively regulates anchorage-independent growth

Raúl Jiménez-P.,[1] Carla Martín-Cortázar,[1] Omar Kourani,[1] Yuri Chiodo,[1] Raul Cordoba,[2,†] María Purificación Domínguez-Franjo,[3,‡] Juan Miguel Redondo,[4,5] Teresa Iglesias[6,7] and Miguel R. Campanero[1,5]

[1]Department of Cancer Biology, Instituto de Investigaciones Biomédicas Alberto Sols, CSIC-UAM, Madrid; [2]Department of Hematology, University Hospital Infanta Sofía, San Sebastián de los Reyes, Madrid; [3]Department of Pathology, University Hospital Infanta Sofía, San Sebastián de los Reyes, Madrid; [4]Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares, (CNIC) Madrid; [5]CIBERCV, Spain; [6]Department of Endocrine and Nervous Systems Pathophysiology, Instituto de Investigaciones Biomédicas Alberto Sols, CSIC-UAM, Madrid and [7]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain

[†]Present address: Lymphoma Unit, Department of Hematology, Fundacion Jimenez Diaz University Hospital, Health Research Institute IIS-FJD, Madrid, Spain

[‡]Present address: Department of Pathology, Hospital Rey Juan Carlos, Mostoles, Madrid, Spain

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Correspondence: mcampanero@iib.uam.es

TITLE:

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AUTHORS:

[1]Raúl Jiménez-P., [1]Carla Martín-Cortázar, [1]Omar Kourani, [1]Yuri Chiodo, [2,†]Raul Cordoba, [3,‡]María Purificación Domínguez-Franjo, [4,5]Juan Miguel Redondo, [6,7]Teresa Iglesias, and [1,5]Miguel R. Campanero.

SUPPLEMENTARY DATA

Supplementary Materials

Patients and Cells.

Human BL (DG-75, Ramos, BL2, Mutu-I, and Akata), Diffuse Large B-Cell Lymphoma (DB, Toledo, OCI-LY-19, Karpas-422, and SUDHL.4), Follicular Lymphoma (RS11846 and WSU-NHL), Mantle Cell Lymphoma (Z138 and Granta 519), uncharacterized Non-Hodgkin Lymphoma (SUDHL.4 and RL), Myeloid leukemia (Hel, U937 and K562), Acute lymphoblastic B-cell (380) or T-cell leukemia (Jurkat and Molt-4), and LCLs derived from healthy donors (X50-7, JY, JC5, IB4, Alewife, and Boston) were cultured in RPMI-1640 (ThermoFischer Scientific, Waltham, MA USA). Human embryonic kidney HEK-293T, colorectal carcinoma HCT-116, cervical carcinoma HeLa, human osteosarcoma (U2OS and SAOS2) cell lines, and human primary diploid skin IMR-90 fibroblasts were cultured in DMEM (ThermoFischer Scientific). Cell lines were authenticated at the Genomic Facility of Instituto de Investigaciones Biomédicas Alberto Sols (Madrid, Spain) by genetic profiling using polymorphic short tandem repeat loci (D21S11, TH01, TPOX, vWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317 y D5S818). All media were supplemented with 10% fetal calf serum (ThermoFischer Scientific), 2 mM glutamine, 100U/ml penicillin, and 100µg/ml streptomycin. Cells were maintained at 37°C in a humidified incubator in the presence of 5% CO_2. All cells were *mycoplasma*-negative.

All cases consisted of existing frozen and paraffinated de-identified anonymous biopsy specimens obtained from Hospital Infanta Sofia and from the Spanish Tumor Bank Network in Centro Nacional de Investigaciones Oncológicas (Madrid, Spain). The study was approved by the CSIC Ethics Committee and by the Ethics Committee of Hospital Universitario La Paz (ref. HULP: PI-1658). Biopsy specimens from BL cases were reviewed by R. Córdoba and M.P. Domínguez-Franjo using the criteria of the World Health Organization (1). Twenty specimens corresponding to BL from patients, together with 3 reactive tonsils were finally selected for RNA analysis and 9 BL specimens were selected for immunohistochemistry. Cases associated with HIV or HCV infections or previous immunosuppressive treatments were excluded.

Microarray Experiment Data Analysis

Total RNA from BL and LCL cell lines was extracted using RNeasy (Qiagen; Venlo, Netherlands) following manufacturer's instructions. The RNA from X50-7, IB-4, and Dana LCL cell lines was pooled in equimolar amount, labeled in Cy3 and used as a universal control for the whole experiment. The RNA from DG-75, Ramos, BL2, Mutu-I, and Akata BL cell lines was labeled in Cy5. Labeling of total RNA was done with the Amino Allyl Labeling Kit for Array analysis (ThermoFisher Scientific). Each of the 5 BL samples was mixed with an equal amount of universal control pool and hybridized overnight at 42ºC on 5 independent *Homo sapiens* 22K-oligo microarrays (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL9955; CapitalBio Corporation; Beijing, China). After standard washing, the arrays were scanned using a LuxScan 10K Scanner (CapitalBio). Data were extracted from raw tiff images using LuxScan3 software (CapitalBio). Data analysis was performed at the Bioinformatics Unit in Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain) on R statistical framework (http://www.r-project.org/) using the Marray package (http://www.bioconductor.org/packages/2.10/bioc/html/marray.html) for preprocessing and quality control of the data. To eliminate putative background irregularities and to make the 5 microarrays comparable, the following transformations were applied: (i) background

correction by subtraction; (ii) internal array normalization by within-print-tip-group scale normalization; and (iii) normalization between arrays by MAD scale transformation genes (2, 3). Lineal models were implemented by the Limma package (http://www.bioconductor.org/packages/release/bioc/html/limma.html) (2, 3) to find genes similarly expressed in BL cell lines but differentially expressed relative to the pool of LCL cell lines. Genes with similar expression within BL cell lines were ranked according to their p-value for differential expression (adj. P value <0.05). Gene expression data are available at http://www.ncbi.nlm.nih.gov/projects/geo/ under accession number GSE41865.

Northern blotting and quantitative PCR analysis

Total RNA was extracted from $5x10^6$ lymphocytes using RNeasy (74104; Qiagen, Venlo, Netherlands). Northern blotting was performed as previously described (4) using ^{32}P-labeled 5' *CDCA7-2* and *ACTG* DNA fragments. The Genomic Facility at Instituto de Investigaciones Biomedicas synthesized complementary DNA using M-MLV retro transcriptase (ThermoFischer Scientific) and analyzed gene expression by real-time quantitative RT–PCR (qPCR) using TaqMan Gene Expression Assays (ThermoFischer Scientific) specific for both isoforms of human *CDCA7* (Hs00230589_m1), CDCA7-1 (Hs00912235_m1), CDCA7-2 (Hs00914361_m1), or *TBP* (Hs00427621_m1). *TBP* was chosen as a control gene on the basis of its homogeneous expression in used cell lines and tissue specimens. Calculations were made from measurements of 3 replicates of each sample.

Construction of CDCA7 expression vectors.

Full-length Human *CDCA7-1* and *CDCA7-2* coding sequences were obtained by PCR amplification from cDNA obtained from DG-75 cells employing primers JPO1-F1 (5'-ctaagcatcgatatggacgctcgccgcgtgc-3') and JPO1-R-STOP (5'-tgcatgtctagattatgcttgcatttcaaattcctgtttcaggc-3') and cloned immediately downstream from the HA-tag into pCDNA3 (ThermoFischer Scientific) to create pcDNA3-HA-CDCA7-1 and pcDNA3-HA-CDCA7-2. All plasmids were sequence verified.

Cell Transfection, lentivirus production and cell transduction.

HEK-293T cells were seeded in 10-cm dishes and transfected at 70-80% confluence using the calcium phosphate method (5) with 30µg of pCDNA3-HA (empty), pCDNA3-HA-CDCA7-1 or pCDNA3-HA-CDCA7-2. Transfected cells were washed 16h later and harvested for protein extraction the following day. Lentiviral particles were produced as previously described (6). MISSION pLKO.1-puro-based vectors encoded either a non-targeting shRNA (SHC002) or the following *CDCA7* targeting shRNAs: sh-25 (TRCN0000140725), sh-40 (TRCN0000139240), sh-56 (TRCN0000139556) sh-83 (TRCN0000145183). Conditioned medium was harvested and filtered through 0.45-µm filters.

DG-75, Ramos, BL2, Toledo and Molt-4 cell lines were transduced using 2 rounds of infection with a time-gap of 12h between them. The cells were incubated with viral particles in culture medium with 8µg/ml protamine sulfate during 24h. S1F cells were incubated with viral particles for 16h in culture medium supplemented with 8µg/ml polybrene. Cells were washed to remove viral particles and transduced cells were selected in the presence of 1µg/ml puromycin for at least 96h.

Antibodies, immunoblotting and immunohistochemistry.

The anti-CDCA7 S99 polyclonal rabbit serum was raised against the following KLH-conjugated peptide CRGRHPLPGSDSQSRRPR (Immunostep, Salamanca, Spain) as described (7). Anti-Ki67 rabbit monoclonal antibody was from Abcam (ab16667), while anti-alpha-Tubulin (T9026) and anti-Beta-actin (A5441) mouse monoclonal antibodies were from Sigma-Aldrich.

Cells were harvested and suspended in lysis buffer (125 mM Tris-HCl pH 6.8, 4% SDS, and 20% glycerol). Cell lysates were boiled for 15 minutes and protein concentration was determined by the Lowry method using DcProtein Assay kit (Bio-Rad laboratories; Hercules, CA, USA). After quantification, β-mercaptoethanol (1:100 v/v) and bromophenol blue powder were added. Protein samples were resolved in SDS-PAGE and transferred to nitrocellulose membranes as described (6). Protein loading equivalence was verified by Ponceau red (Sigma-Aldrich) staining. Membranes were probed with anti-CDCA7 S99 and

mouse anti-ß-actin or anti-Tubulin monoclonal antibodies, followed by anti-mouse or anti-rabbit antibodies respectively conjugated to IR-680Dye (926-32213) or IR-800Dye (926-32220) (both from LI-COR Biosciences, Lincoln, NE, USA), and scanned using an Odyssey® infrared imaging system (LI-COR Biosciences).

Paraffin-embedded tissue samples were stained with anti-CDCA7 S99 and counterstained with hematoxylin, as previously described (8). The specimens were analyzed by microscopy, using an Olympus BX60 (Olympus Optical). Images were taken with an Olympus DP50 camera and analyzed with ImageJ software (http://imagej.nih.gov/ij). CDCA7 staining was quantified by thresholding the brown signal using the hue-saturation-intensity color model and determining the percentage of stained area.

Cell growth, cell proliferation, and cell cycle analysis.

To determine cell growth, cells (1 x 10^5/mL) were cultured in 24-well plates by triplicate. Growth was measured by cell count at different intervals. Cell viability was assessed by trypan blue exclusion. Cell proliferation was assessed by EdU incorporation during 16h of culture. Cells were harvested and the incorporated EdU was detected using the Click-iT EdU Alexa fluor 488 Flow Cytometry Assay Kit (ThermoFisher Scientific) following manufacturer´s instructions. Cell cycle analysis was performed as previously described (4).

Transformation assays *in vitro* and *in vivo*.

Cells (2 x10^4) were suspended in 0.35% noble agar (Difco) and laid over 6-well culture plates previously coated with 0.5% noble agar. Agar was prepared in RPMI medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100U/ml penicillin and 100 µg/ml streptomycin. The plates were kept at 37°C in a humidified incubator in the presence of 5% CO_2. The number of colonies formed after 3 weeks was counted in triplicate plates.

Cells (2 x 10^6) in 0.1 ml of PBS were injected subcutaneously in the dorsal flanks of 8- to 10-week-old female NOD.CB17-Prkdcscid/J mice (Charles River Laboratories, Wilmington, MA, USA). All mice were inoculated with control cells in one flank and CDCA7-silenced cells in the opposite flank. Tumor masses were removed after 3 weeks and weighted. All animal procedures were approved by the CSIC Ethics Committee and by the

Madrid Regional authorities (ref. PROEX 31/14), and conformed to EU 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. Overall mouse health was assessed by daily inspection for signs of discomfort, weight loss, or changes in behaviour, mobility, and feeding or drinking habits.

Statistical analysis

Graphpad Prism software 6.01 was used for the analysis. Differences were analyzed by one-way analysis of variance (ANOVA) with Bonferroni post-tests, or by paired samples t-test, as appropriate. Differences were considered significant at $P<0.05$.

The numbers of animals used are described in the corresponding figure legends. All experiments were carried out with at least three biological replicates. Experimental groups were balanced in terms of animal age and weight. Animals were caged together and treated in the same way. Appropriate tests were chosen according to the data distribution. Variance was comparable between groups throughout the manuscript. No randomization was used to allocate animals to experimental groups and investigators were not blinded to the group allocation during experiments or outcome assessments.

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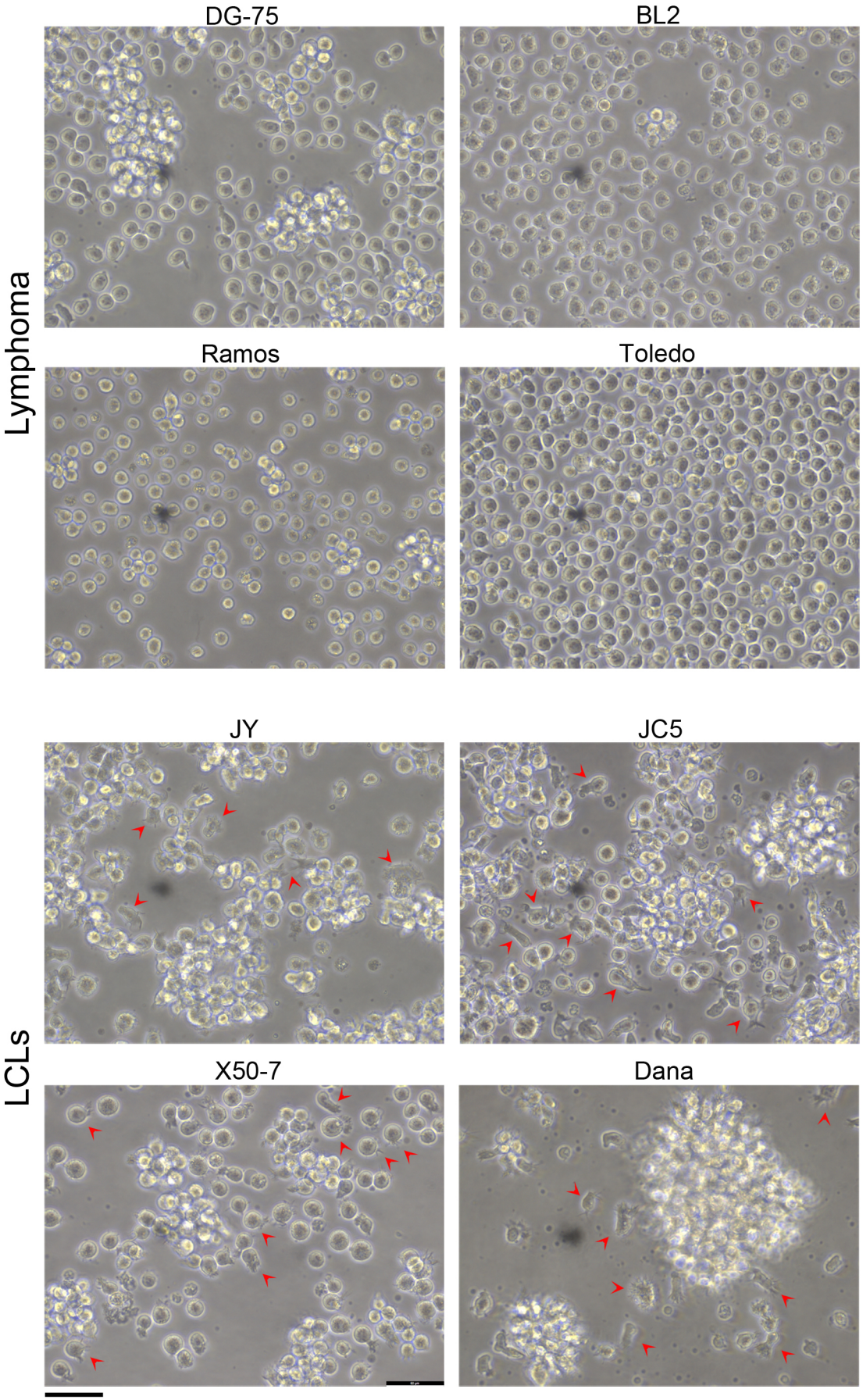
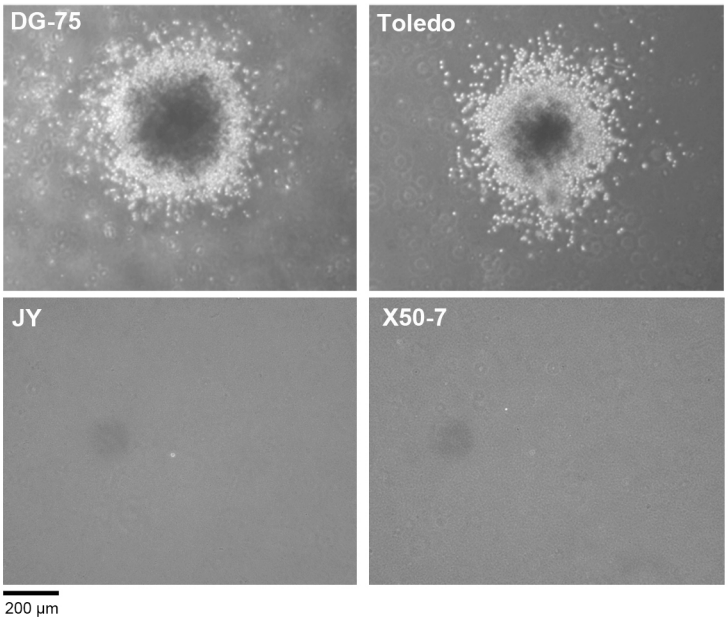
Supplementary Figures

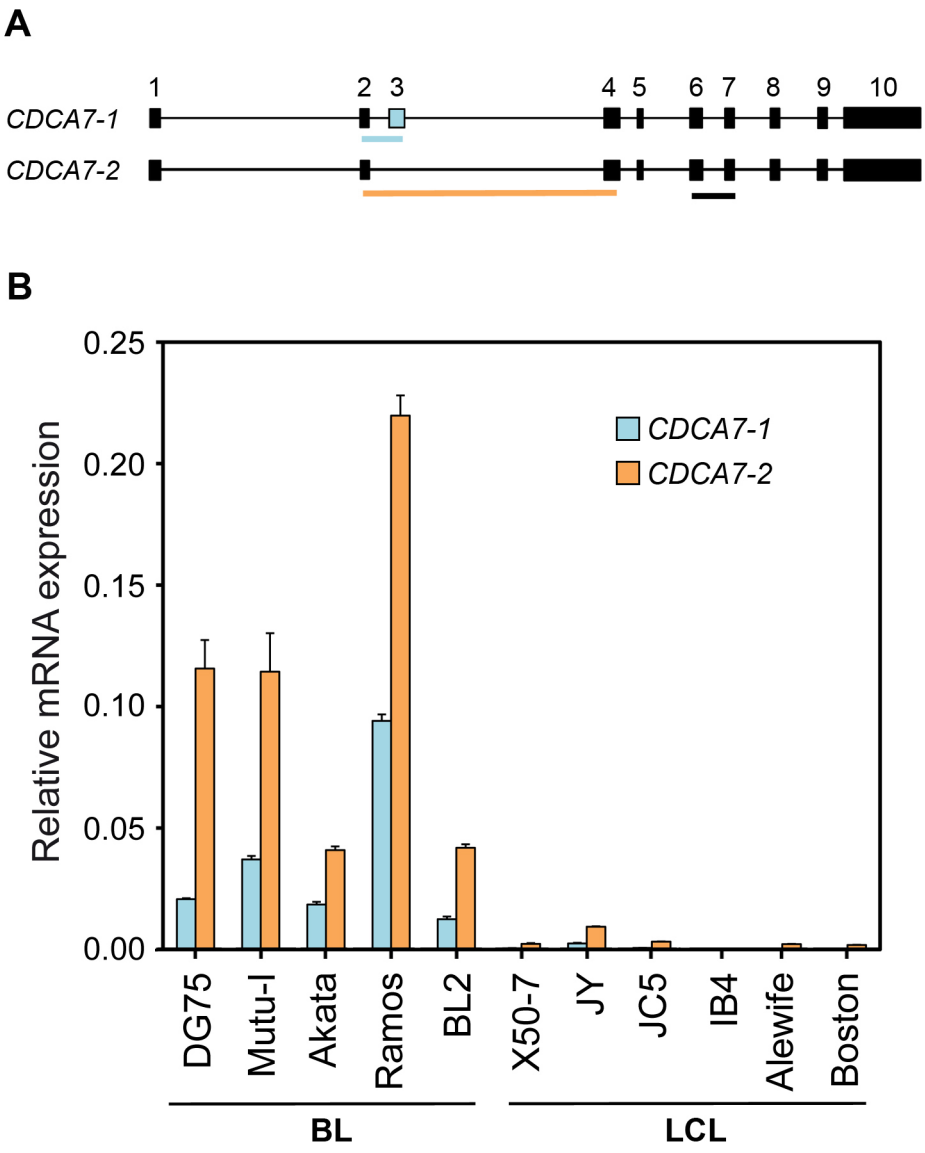


Supplementary Figure S1

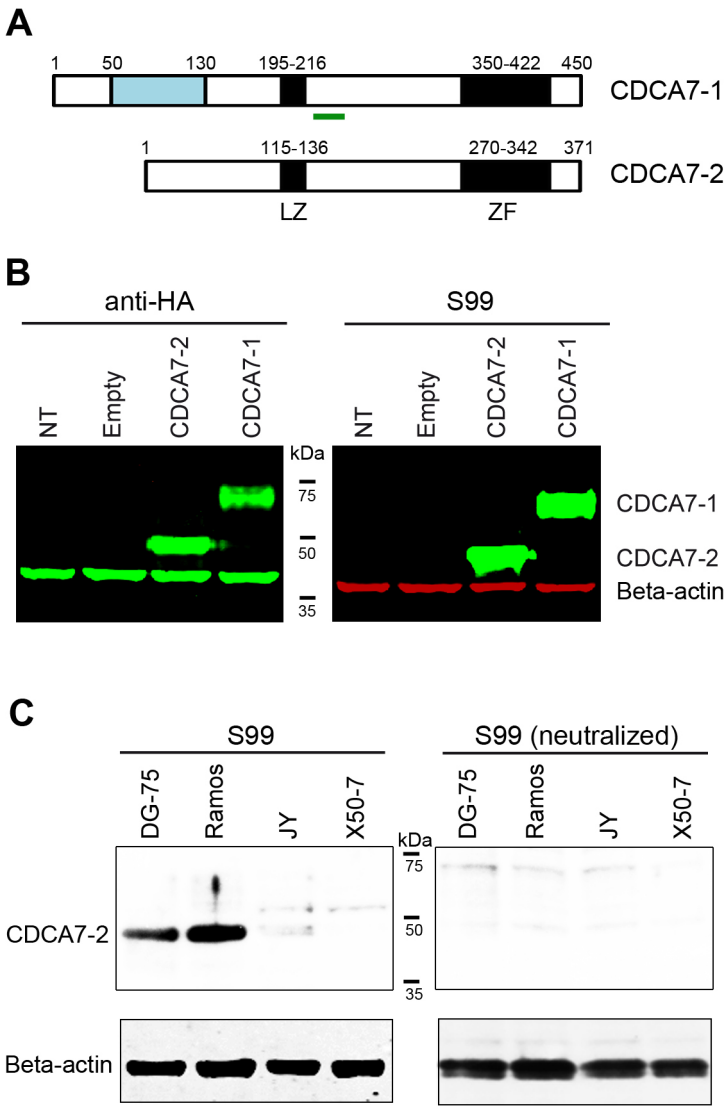
Supplementary Figure S1. LCLs grow anchored to other cells or to the culture vessel surface. Representative images of lymphoma (DG-75, BL2, Ramos, and Toledo) and LCL (JY, JC5, X50-7, and Dana) cell lines cultured in liquid medium on plastic. Scale bar, 50 μm. Red arrowheads point to examples of cells markedly spread on the culture vessel surface.



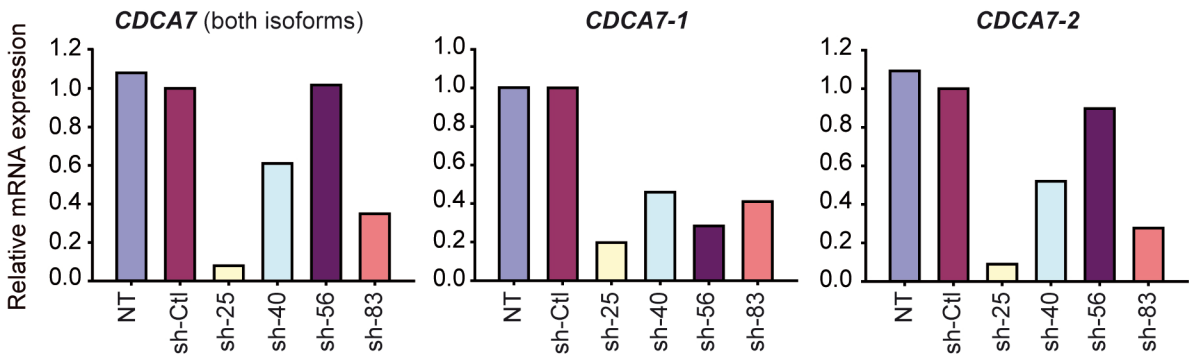
Supplementary Figure S2. Lymphoma cells, but not LCLs, form colonies in soft agar. Images of representative wells containing lymphoma cells (DG-75 and Toledo) or LCLs (JY and X50-7) seeded in soft agar. Scale bar, 200 μm.



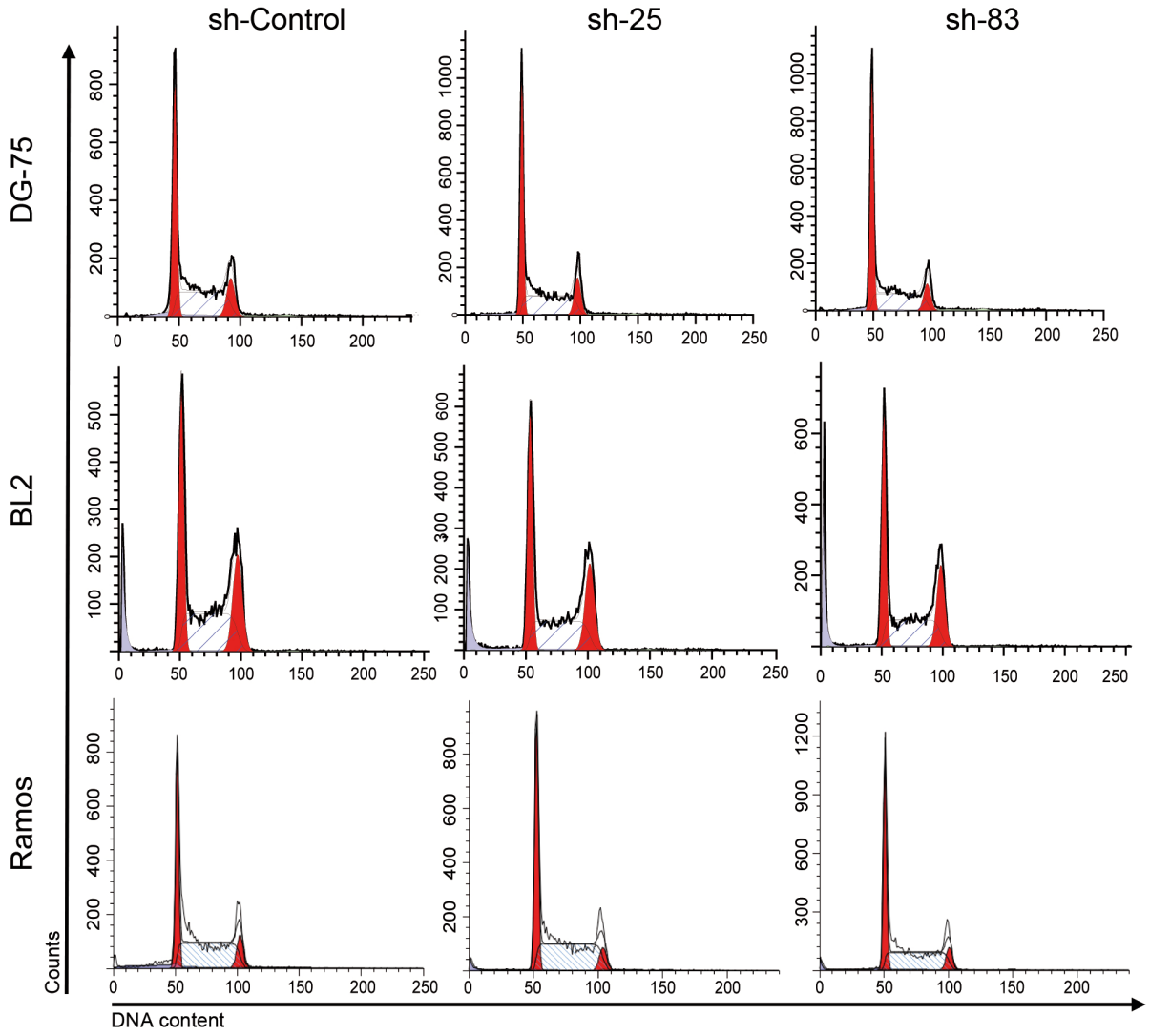
Supplementary Figure S3. *CDCA7-1* and *CDCA7-2* levels are higher in BL cell lines than in non-malignant cells. (**A**) Scheme depicting exon organization of *CDCA7-1* and *CDCA7-2* isoforms. Blue, orange, and black lines under exons indicate the regions analyzed by TaqMan assays for *CDCA7-1*, *CDCA7-2*, or both isoforms, respectively. (**B**) qPCR analysis of *CDCA7-1* and *CDCA7-2* mRNA levels in the indicated cell lines is shown normalized with *ACTB* as mean+s.e.m. (n=3).



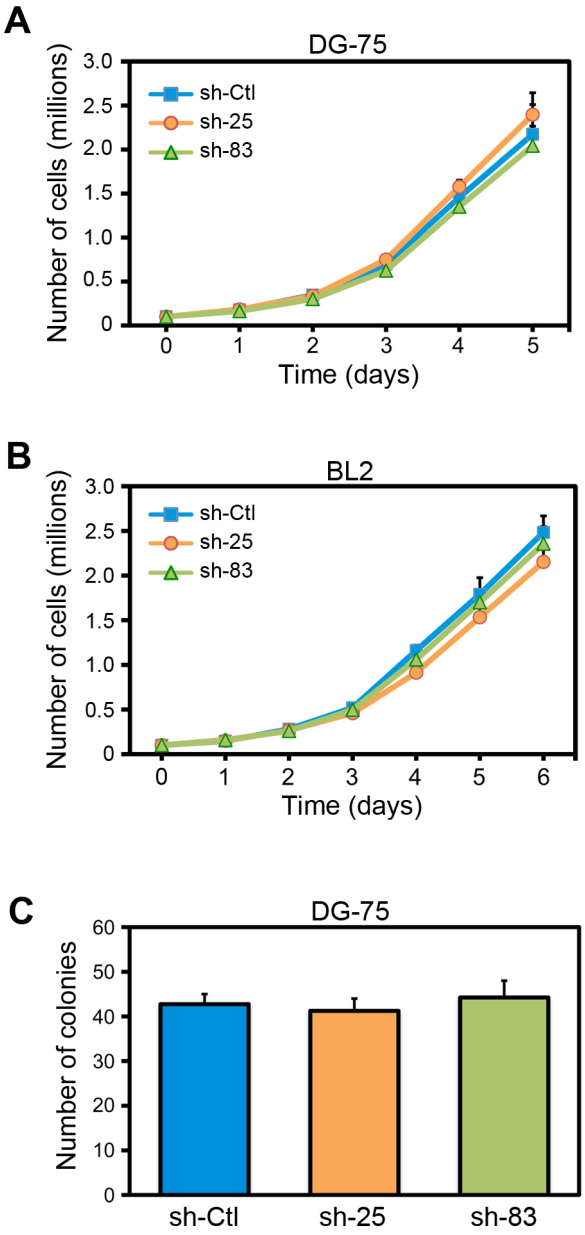
Supplementary Figure S4. Production of S99, a novel anti-CDCA7 polyclonal antibody. (**A**) Diagram of CDCA7 isoforms showing the presence of leucine zipper (LZ) and zinc finger (ZF) regions, the CDCA7-1-specific sequence (blue box) and the approximate location of the peptide used for rabbit immunization (green line). (**B**) Protein extracts from HEK-293-T cells non-transfected (NT) or transfected with empty plasmid or plasmids encoding HA-tagged versions of CDCA7-1 or CDCA7-2 were assessed by immunoblot analysis with either a mouse anti-HA monoclonal antibody or our anti-CDCA7 rabbit serum S99. Beta-actin was used as loading control. (**C**) Protein extracts from BL (DG-75 and Ramos) and LCL (JY and X50-7) cell lines were analyzed by immunoblotting with S99 anti-CDCA7 antibody before (S99) or after neutralization with the immunizing peptide (S99 (neutralized)). Beta-actin was used as loading control.



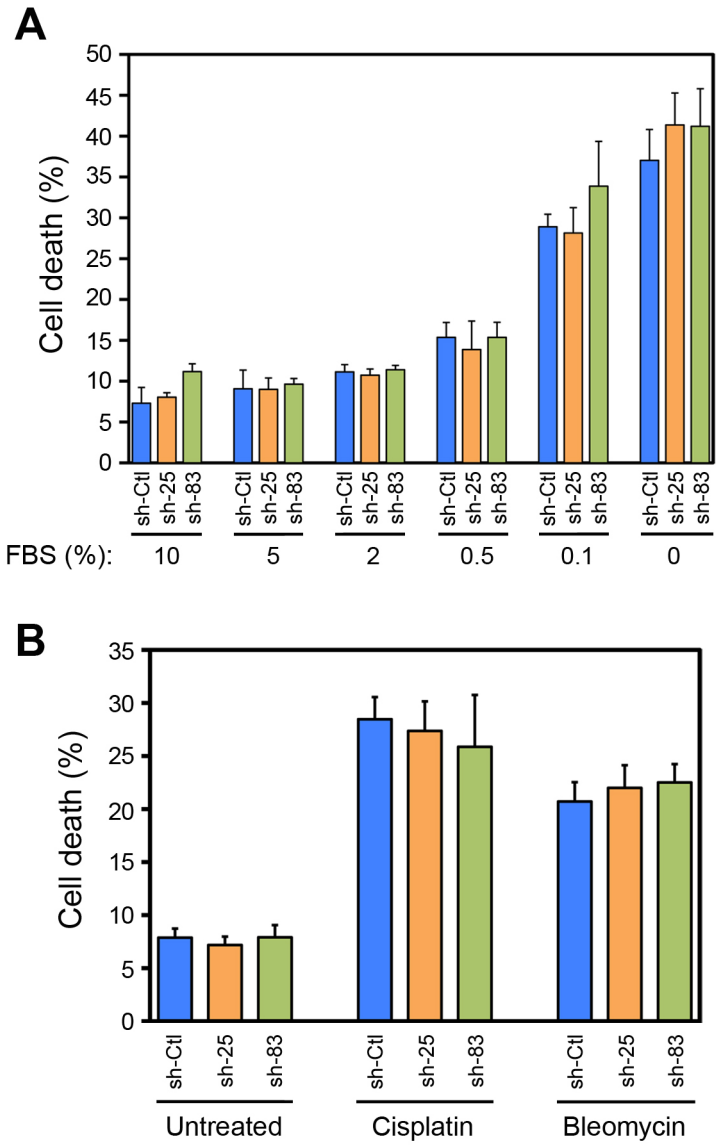
Supplementary Figure S5. *CDCA7* mRNA expression is down-regulated by specific shRNAs. DG-75 BL cells were transduced with lentivirus encoding a non-targeting shRNA (sh-Ctl) or shRNAs targeting *CDCA7* (sh-25, sh-40, sh-56, and sh-83) and then selected in the presence of puromycin >5 days. Total *CDCA7* (both isoforms), *CDCA7-1* and *CDCA7-2* mRNA levels were determined in non-transduced DG-75 cells (NT) or in DG-75 cells expressing the indicated shRNAs by qPCR analysis employing TaqMan assays either common to both isoforms or isoform-specific. Data were normalized with *ACTB* and are shown relative to sh-Ctl. A representative experiment is shown.



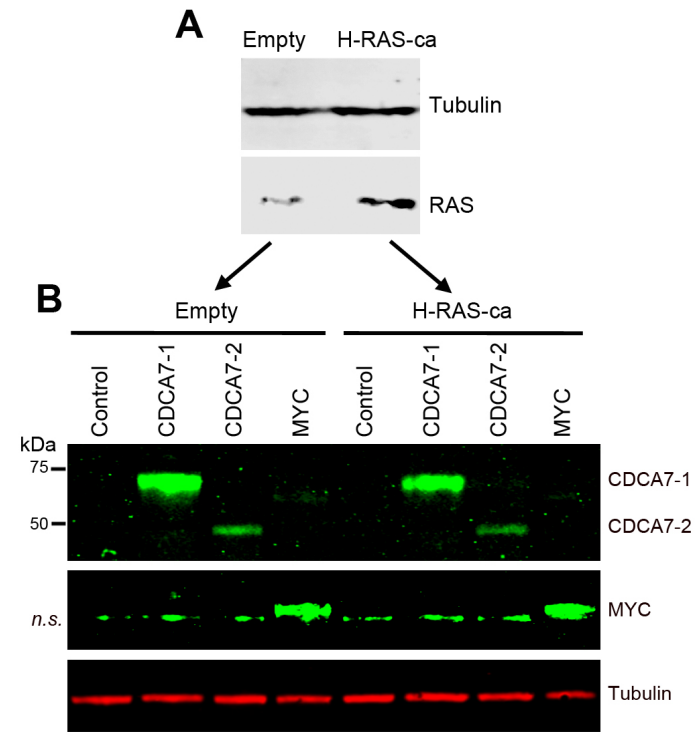
Supplementary Figure S6. CDCA7 silencing does not affect the cell cycle of BL cells grown in liquid culture. DG-75 (n=3), BL2 (n=3), and Ramos (n=2) cells were transduced with lentivirus encoding the indicated shRNAs and then selected in the presence of puromycin > 5 days. Representative cell cycle profiles of these cells are shown.



Supplementary Figure S7. CDCA7 silencing does not affect BL cell growth in liquid culture. Growth curves of (**A**) DG75 and (**B**) BL2 cells transduced with lentiviruses encoding the indicated shRNAs. Data are mean + s.e.m. (n=3). (**C**) DG-75 cells transduced with lentiviruses encoding the indicated shRNAs were plated into 96 replicate wells at a dilution, as calculated from the cell count, which would result in a mean of 0.5 cells per well. The plates were cultured for 14 days and were then scored for the presence of colonies. The number of colonies/plate is shown as mean + s.e.m. (n=3).



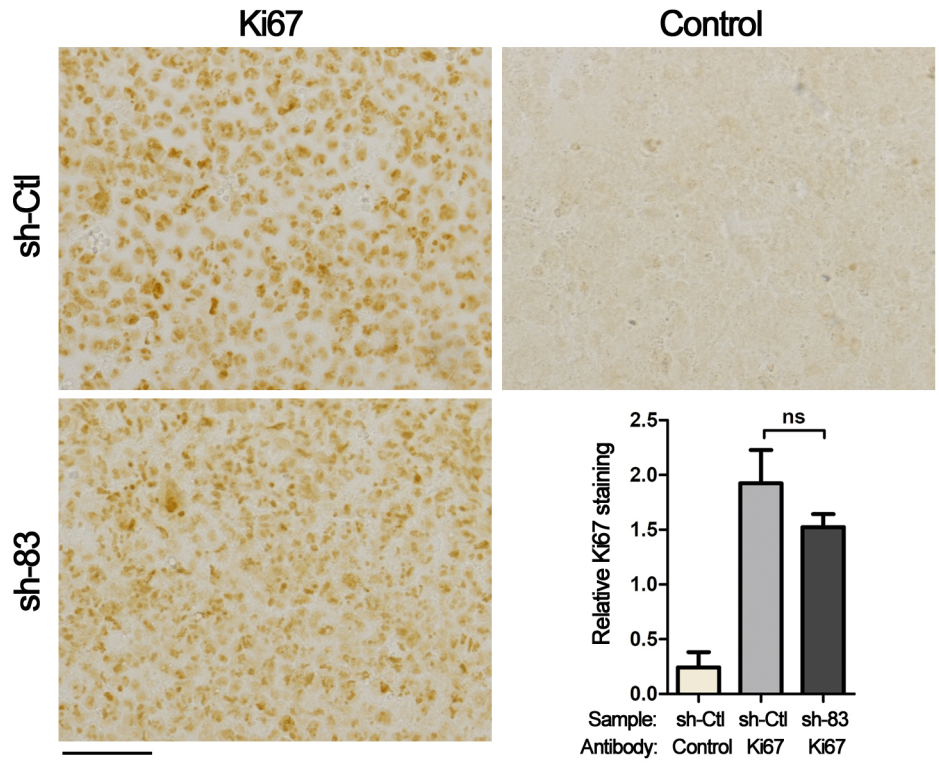
Supplementary Figure S8. *CDCA7* silencing does not affect BL cell viability in liquid culture. DG-75 cells were transduced with lentivirus encoding the indicated shRNAs and cultured (**A**) in RPMI medium supplemented with the indicated concentrations of fetal bovine serum (FBS) or (**B**) in RPMI + 10% FBS together with Cisplatin (25 µg/ml) or Bleomycin (50 U/ml). Cell death, as determined by Trypan blue staining, was analyzed (A) 48h or (B) 72h after cell plating. Data are shown as mean + s.e.m. (n=3).



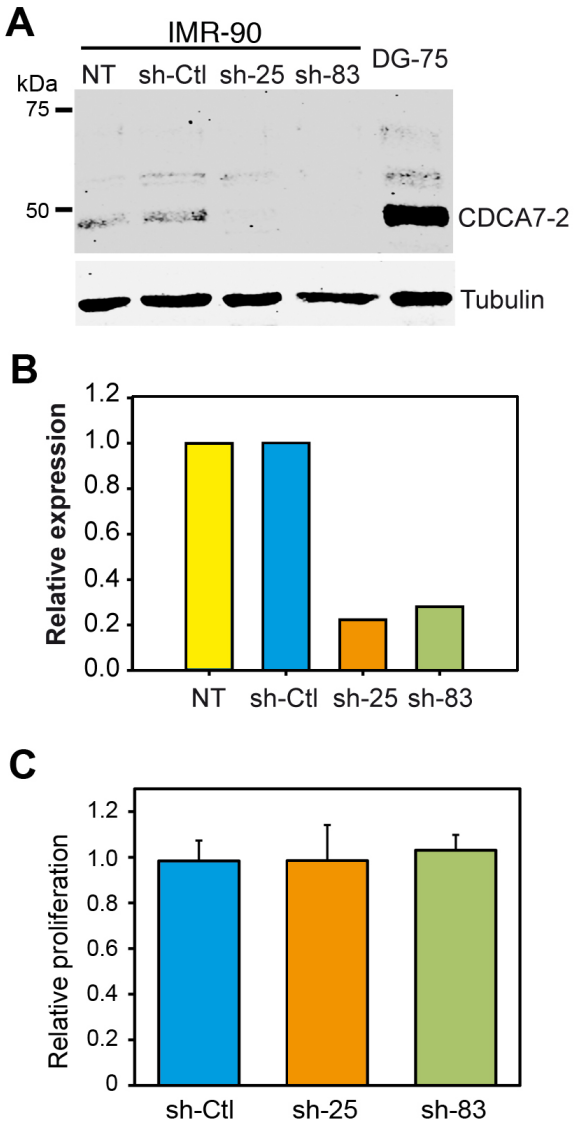
C

		Experiment 1	Experiment 2
JY	Control	0	0
	CDCA7-1	0	0
	CDCA7-2	0	0
	MYC	0	0
	RAS-ca + Control	0	0
	RAS-ca + CDCA7-1	0	0
	RAS-ca + CDCA7-2	0	0
	RAS-ca + MYC	0	0
	DG-75	44	34

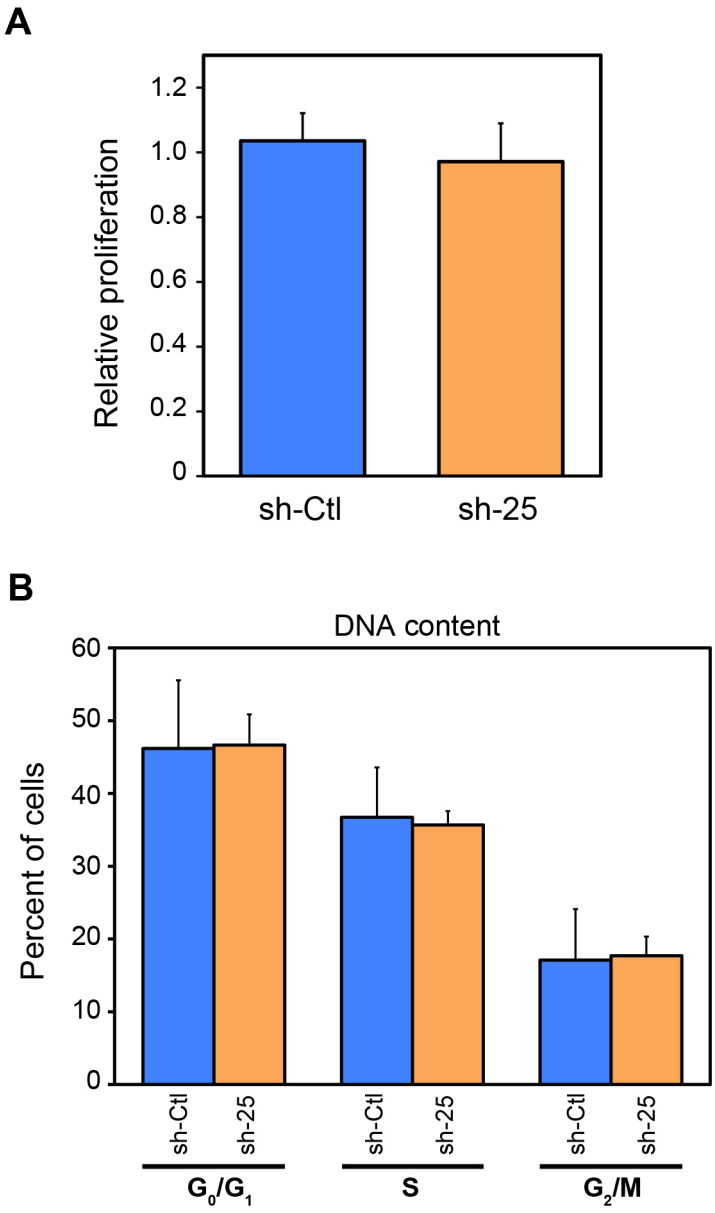
Supplementary Figure S9. Forced CDCA7 expression in non-malignant B cells does not induce anchorage-independent growth. JY cells transduced with pBabe-Hygro-based retroviruses encoding constitutively active H-RAS^{T12,R59} (H-RAS-ca) or empty vector (Empty) were selected in the presence of Hygromicin. Each of these cells was then transduced with a control lentivirus (Control) or with lentivirus encoding MYC or HA-tagged versions of CDCA7-1 or CDCA7-2. (**A**) Immunoblot analysis of RAS and Tubulin in the indicated cells. (**B**) HA (top panel), MYC (middle panel), and Tubulin (bottom panel) immunoblot analysis of the indicated cells. (**C**) JY cells transduced as indicated and DG-75 cells (used as positive control) were seeded in soft agar and the number of colonies was quantified after 3 weeks. Data from two independent experiments are shown.



Supplementary Figure S10. Ki67 levels in lymphoma tumors are not regulated by CDCA7. DG-75 cells transduced with a control shRNA (sh-Ctl) or a *CDCA7*-specific shRNA (sh-83) were inoculated into NOD-SCID mice. Tumors formed by these cells were stained with a Rabbit anti-mouse antibody (Control) or with anti-Ki67 antibody. Representative images of Control and Ki67 immunohistochemistry in sections of these tumors and relative percentage of stained area in these tumors (mean + s.e.m) are shown. Bar, 50 µm. ns, not significant by Student t-test.



Supplementary Figure S11. CDCA7 silencing does not inhibit proliferation of human primary diploid fibroblasts. IMR-90 human primary diploid fibroblasts were transduced with lentivirus encoding the indicated shRNAs. (**A**) Representative immunoblot analysis of CDCA7 expression in these cells. Tubulin is shown as loading control. (**B**) qPCR analysis of *CDCA7* mRNA expression in non-transduced IMR-90 fibroblasts (NT) or in IMR-90 fibroblasts expressing the indicated shRNAs. Expression was normalized by *ACTB* and is shown relative to that found in non-transduced cells. A representative experiment (n=3) is shown. (**C**) Relative Edu incorporation analysis of IMR-90 cells expressing the indicated shRNAs. Columns show normalized percentage of Edu incorporation in each of these cells as mean±s.e.m (n=3).



Supplementary Figure S12. CDCA7 silencing does not affect Toledo cell cycle and proliferation in liquid culture. Cell cycle analysis of Toledo cells transduced with lentivirus encoding the indicated shRNAs and then selected in the presence of puromycin >5 days. Columns show the percentage of each of these cells in the indicated cell cycle phases as mean+s.e.m (n=3).