Synergistic effect of *Bcl2*, *Myc* and *Ccnd1* transforms mouse primary B cells into malignant cells

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Online Supplementary Design and Methods

Construction of vectors

A schematic diagram of retroviral vectors used in this study is shown in *Online Supplementary Figure S1*. pMXs and pMXs-neo were kindly provided by Dr. T. Kitamura (University of Tokyo, Tokyo, Japan). pMXs-*hygro* was constructed by ligating the SV40-*Hygromycin resistance* gene cassette into the *Sal*I site of pMXs. To construct pMXs-*bsd*, the SV40-*blascitidin resistance* gene cassette was subcloned into the *Sal*I/*Not*I site of pMXs. MSCV-pgk-ires-*GFP* was constructed by ligating the phosphoglycerate kinase (PGK) promoter fragment derived from pSUPER.puro into the *EcoR*I/*Xho*I sites of MSCV-ires-*GFP*.[1] pGCDNsam-ires-*humanized Kusabira-Orange* (huKO)[2] was kindly provided by Dr. M. Onodera (National Research Institute for Child Health and Development, Tokyo, Japan). pMXs-*Bcl2-neo*, pMXs-*Myc-hygro*, pMXs-*Ccnd1-bsd*, MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP*, MSCV-*Ccnd1*-pgk-*Myc*-ires-*GFP*, MSCV-*Bcl2*-pgk-*Ccnd1*-ires-*GFP*, MSCV-*Flag-CCND3*-pgk-*Myc*-ires-*GFP*, MSCV-*Bcl2*-pgk-*Flag-CCND3*-ires-*GFP*, pGCDNsam-*Bcl2*-ires-*huKO*, pGCDNsam-*Myc*-ires-*huKO*, pGCDNsam-*Ccnd1*-ires-*huKO*, pGCDNsam- *Flag-CCND3*-ires-*huKO*, pGCDNsam-*Flag-NRAS*-ires-*huKO*, pGCDNsam-*Flag-RNF14*-ires-*huKO*, pGCDNsam-*Flag-PSAP*-ires-*huKO* and pGCDNsam-*Flag-ASB8*-ires-*huKO* were constructed as detailed in *Online Supplementary Table S1*.

Flow cytometry

Cells were incubated with the appropriate antibodies including biotin-B220 (RA3-6B2; eBioscience), phycoerythrin (PE)-conjugated c-kit (2B8; eBioscience), biotin-c-kit (2B8; eBioscience), biotin-CD19 (MB19–1; eBioscience) and biotin-IgM (II/41; eBioscience). Biotinylated antibodies were then stained with streptavidin-allophycocyanin (APC; eBioscience). Non-specific binding was blocked by pre-incubation with an anti-Fc receptor antibody (2.4G2; BD Pharmingen). Cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences) with FlowJo software (Tree Star). Cells were sorted using a JSAN cell sorter (Bay Bioscience).

Southern blot analysis

Five micrograms of genomic DNA were digested with *Eco*RI restriction enzyme and electrophoresed through a 0.7% agarose gel and processed as described previously.[3] The membranes were washed and then hybridized overnight at 65°C with [α- 32P]dCTP-labeled mouse *JH* probe. Membranes were washed with 2 xSSC/ 0.1% SDS at 25°C and 1 xSSC/ 0.1% SDS at 65°C and finally exposed to BioMax™ MS films (EKC).

Analysis of expression microarray data

Six of the sorted stable integrants in Experiment 1 (Bcl2/Myc/Ccnd1, Ccnd1/Myc/Bcl2, Bcl2/Ccnd1/Myc, Bcl2/Myc/mock, Ccnd1/Myc/mock and Bcl2/Ccnd1/mock) were subjected to microarray analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequent steps for the hybridization to Agilent whole mouse genome oligo microarrays (G4122F; 4X44K; Agilent Technologies) were performed according to standard Agilent protocols. Slides were washed and scanned on an Agilent Micro Array Scanner (Agilent Technologies) and data acquired using Feature extractions version 9.1 (Agilent Technologies). Flagged spots were excluded from the analysis. The raw data were normalized by log conversion (base=2) and z-score calculation [Individual log-changed signal intensity (LS) – average score of all LS / standard deviation of all LS]. Pathway analysis with gene sets (n=880) of canonical pathways obtained from the Broad Institute Molecular Signatures Database (MSigDB; *http://www.broadinstitute.org/gsea/msigdb/index.jsp*) was performed using the Gene Set Enrichment Analysis (GSEA; *http://www.broad.mit.edu/gsea*) pre-ranked analysis program on genes ranked by fold change. A gene set was considered significantly enriched when the nominal *P* value was less than 0.05 and the false discovery rate-q value was less than 0.25. The microarray data obtained in this study will be deposited in ArrayExpress (*http://www.ebi.ac.uk/arrayexpress*) with accession number A-MEXP-3321.

References

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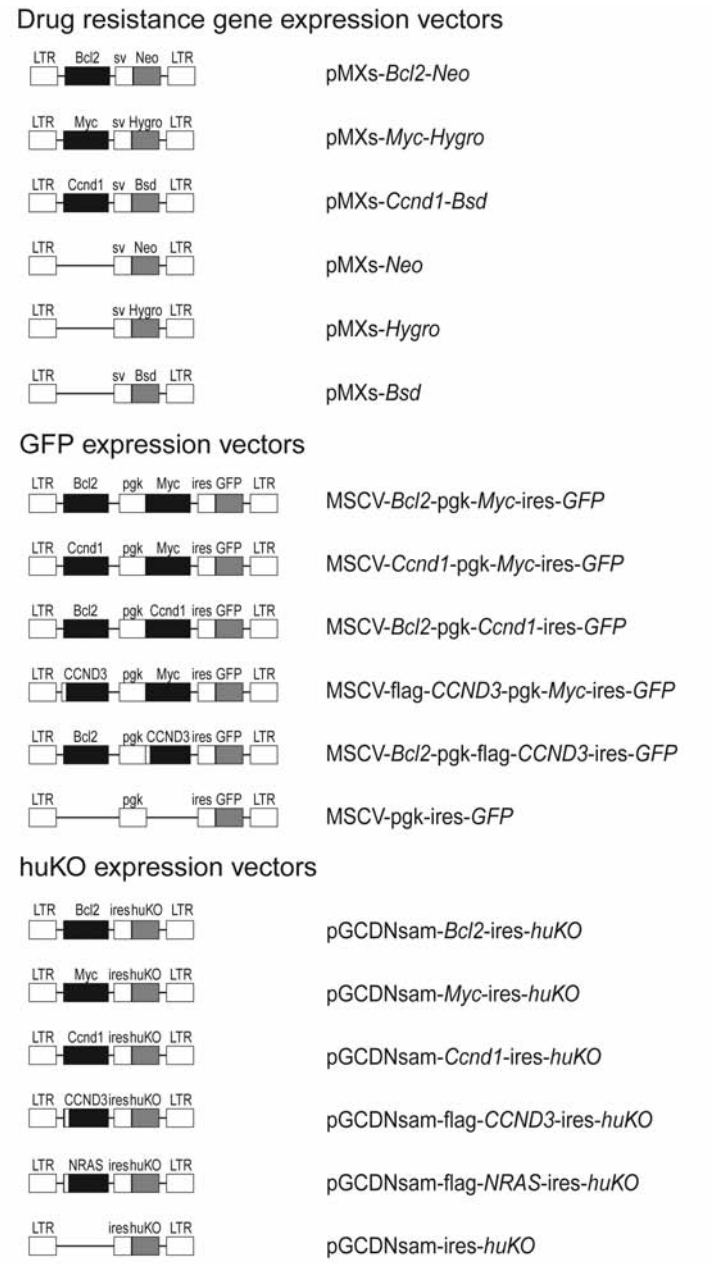
Online Supplementary Table S1. Vector construction.

NAME	Original Vector	Restriction enzyme sites	Fragment	Template	S primer AS primer
pMXs-*Bcl2-neo*	pMXs-neo	*Eco*RI/*Not*I	PCR-amplified	mouse spleen cDNA	5'-GGAA15'-TGCGGCCGCCATGGTACATCATTGATAATGCATA-3'
pMXs-*Myc-hygro*	pMXs-*hygro*.	*Eco*RI/NotI	PCR-amplified	pGEM-T-mouse-Myc	5'-GGAA15'-TGCGGCCGCTTAGGTCAGTTTATGCACCAGAGTT-3'
pMXs-*Ccnd1-bsd*	pSKII	*Eco*RV/HincII	PCR-amplified	mouse spleen cDNA	5'-GGAA15'-TGCGGCCGCTTTGCGGGTGCCACTACTTGGTGGC-3'
	pMXs-*bsd*	*Eco*RI	*Eco*RI-digested	pSKII-*Ccnd1*	
MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP*	MSCV-pgk-ires-*GFP*	*Eco*RI (Klenow filled)	*Eco*RI/*Not*I-digested	pMXs-*Bcl2-neo*	
	MSCV-pgk-ires-*GFP*	*Xho*I (Klenow filled)	*Eco*RI/*Not*I-digested	pMXs-*Myc-hygro*	
MSCV-*Ccnd1*-pgk-*Myc*-ires-*GFP*	MSCV-pgk-ires-*GFP*	*Eco*RI (Klenow filled)	*Eco*RI-digested	pMXs-*Ccnd1-bsd*	
	MSCV-pgk-ires-*GFP*	*Xho*I (Klenow filled)	*Eco*RI/*Not*I-digested	pMXs-*Myc-hygro*	
MSCV-*Bcl2*-pgk-*Ccnd1*-ires-*GFP*	MSCV-pgk-ires-*GFP*	*Eco*RI (Klenow filled)	*Eco*RI/*Not*I-digested	pMXs-*Bcl2-neo*	
	MSCV-pgk-ires-*GFP*	*Xho*I (Klenow filled)	*Eco*RI-digested	pMXs-*Ccnd1-bsd*	
MSCV-Flag-*CCND3*-pgk-*Myc*-ires-GFP	MSCV-pgk-ires-*GFP*	*Eco*RI (Klenow filled)	*Eco*RI-digested	pMXs-Flag-*CCND3-bsd*	
	MSCV-pgk-ires-*GFP*	*Xho*I (Klenow filled)	*Eco*RI/*Not*I-digested	pMXs-*Myc-hygro*	
MSCV-*Bcl2*-pgk-Flag-*CCND3*-ires-*GFP*	MSCV-pgk-ires-*GFP*	*Eco*RI (Klenow filled)	*Eco*RI/*Not*I-digested	pMXs-*Bcl2-neo*	
	MSCV-pgk-ires-*GFP*	*Xho*I (Klenow filled)	*Eco*RI-digested	pMXs-Flag-*CCND3-bsd*	
pGCDNsam-*Bcl2*-ires-*huKO*	pGCDNsam-ires-huKO	*Sna*BI	*Eco*RI/*Not*I-digested	pMXs-*Bcl2-neo*	
pGCDNsam-*Myc*-ires-*huKO*	pGCDNsam-ires-huKO	*Sna*BI	*Eco*RI/*Not*I-digested	pMXs-*Myc-hygro*	
pGCDNsam-*Ccnd1*-ires-*huKO*	pGCDNsam-ires-huKO	*Sna*BI	*Eco*RI-digested	pMXs-*Ccnd1-bsd*	
pGCDNsam- Flag-*CCND3*-ires-*huKO*	pGCDNsam-ires-huKO	*Sna*BI	*Eco*RI-digested	pMXs-Flag-*CCND3-bsd*	
pGCDNsam-Flag-*NRAS* -ires-*huKO*	pcDNA3-Flag	*Xba*I/*Apa*I	PCR-amplified	SU-DHL-6 cDNA	5'-GCTC15'-TTGGGCCCTTACATCACCACACATGGCAATCC-3'
	pGCDNsam-ires-huKO	*Sna*BI	PCR-amplified	pcDNA3-Flag-*NRAS* (Q61K)	5'-GGAA15'-ATAGTTTAGCGGCCGCTTACATCACCACACATGGCAATCC-3'
pGCDNsam-Flag-*RNF14*-ires-*huKO*	pcDNA3-Flag	*Xba*I/*Apa*I	PCR-amplified	SU-DHL-6 cDNA	5'-CTAGC5'-TTGGGCCCCTAGTCTTCTACCTCATCTTCCCAA-3'
	pGCDNsam-ires-huKO	*Not*I	PCR-amplified	pcDNA3-Flag-*RNF14*	5'-ATAAG5'-TAGTTTAGCGGCCGCCTAGTCTTCTACCTCATCTTCCCAA-3'
pGCDNsam-Flag-*PSAP*-ires-*huKO*	pcDNA3-Flag	*Xba*I	PCR-amplified	SU-DHL-6 cDNA	5'-GCTC15'-TGCTCTAGAAGCTTCTAGTTCCACACATGGCGTTTGC-3'
	pGCDNsam-ires-huKO	*Sna*BI	*Hind*III-digested	pcDNA3-Flag-*PSAP*	
pGCDNsam-Flag-*ASB8*-ires-*huKO*	pcDNA3-Flag	*Xba*I/*Eco*RI	PCR-amplified	SU-DHL-6 cDNA	5'-TGCTC5'-GGAATTCGGATCCTATTCTAAAAGTAACAGGTATTCCTTCAAAGAAG-3'
	pGCDNsam-ires-huKO	*Bam*HI	*Bam*HI-digested	pcDNA3-Flag-*ASB8*	

Online Supplementary Table S2. SEE ONLINE EXCEL FILE

Online Supplementary Figure S1. Constructed retroviral vectors.



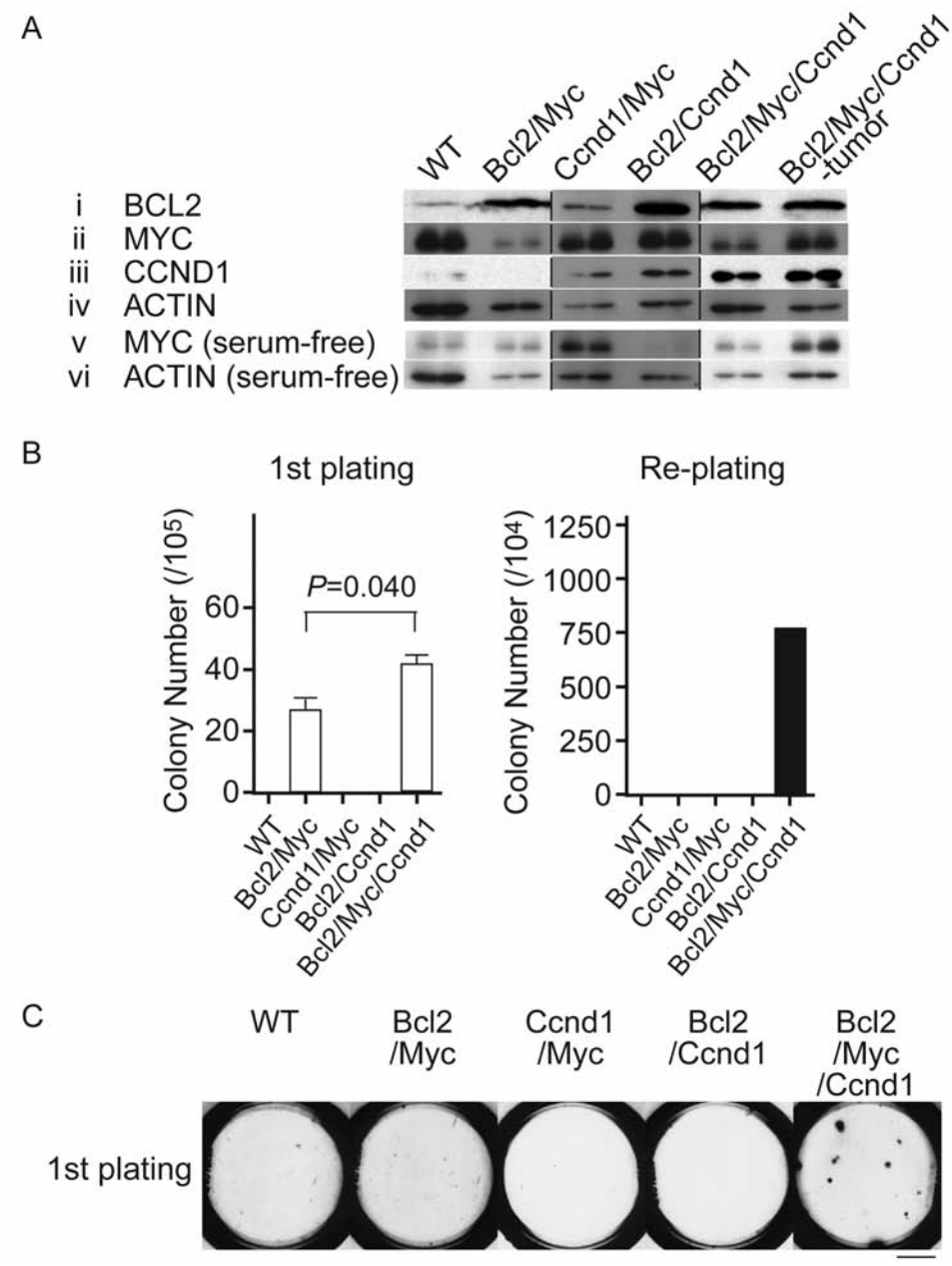
Schematic diagram of retroviral vectors used in this study. The combination of retroviral vectors used for establishing the various stable integrants are as follows.

Drug selection method
Bcl2/Myc/Ccnd1: pMXs-*Bcl2-neo*, pMXs-*Myc-hygro* and pMXs-*Ccnd1-bsd*.
Bcl2/Ccnd1: pMXs-*Bcl2-neo* and pMXs-*Ccnd1-bsd*.
Bcl2/Myc: pMXs-*Bcl2-neo* and pMXs-*Myc-hygro*.
Ccnd1/Myc: pMXs-*Ccnd1-bsd* and pMXs-*Myc-hygro*.

Cell sorting method
Bcl2/Ccnd1/Myc: MSCV-*Bcl2*-pgk-*Ccnd1*-ires-*GFP* and pGCDNsam-*Myc*-ires-*huKO*.
Bcl2/Myc/Ccnd1: MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP* and pGCDNsam-*Ccnd1*-ires-*huKO*.
Ccnd1/Myc/Bcl2: MSCV-*Ccnd1*-pgk-*Myc*-ires-*GFP* and pGCDNsam-*Bcl2*-ires-*huKO*.
Bcl2/Ccnd1/mock: MSCV-*Bcl2*-pgk-*Ccnd1*-ires-*GFP* and pGCDNsam-ires-*huKO*.
Bcl2/Myc/mock: MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP* and pGCDNsam-ires-*huKO*.
Ccnd1/Myc/mock: MSCV-*Ccnd1*-pgk-*Myc*-ires-*GFP* and pGCDNsam-ires-*huKO*.
WT/mock: MSCV-pgk-ires-*GFP* and pGCDNsam-ires-*huKO*.
Bcl2/CCND3/Myc: MSCV-*Bcl2*-pgk-Flag-CCND3-ires-*GFP* and pGCDNsam-*Myc*-ires-*huKO*.
Bcl2/Myc/CCND3: MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP* and pGCDNsam-Flag-CCND3-ires-*huKO*.
CCND3/Myc/Bcl2: MSCV-Flag-CCND3-pgk-*Myc*-ires-*GFP* and pGCDNsam-*Bcl2*-ires-*huKO*.
Bcl2/CCND3/mock: MSCV-*Bcl2*-pgk-Flag-CCND3-ires-*GFP* and pGCDNsam-ires-*huKO*.
CCND3/Myc/mock: MSCV-Flag-CCND3-pgk-*Myc*-ires-*GFP* and pGCDNsam-ires-*huKO*.
Bcl2/Myc/NRAS: MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP* and pGCDNsam-Flag-NRAS-ires-*huKO*.

LTR: long terminal repeat; sv: SV40 promoter; pgk: phosphoglycerate kinase promoter; ires: internal ribosomal entry site; *neo*: neomycin resistance gene; *hygro*: hygromycin resistance gene; *bsd*: blasticidin resistance gene; *GFP*: green fluorescent protein gene; *huKO*: humanized Kusabira -Orange gene.

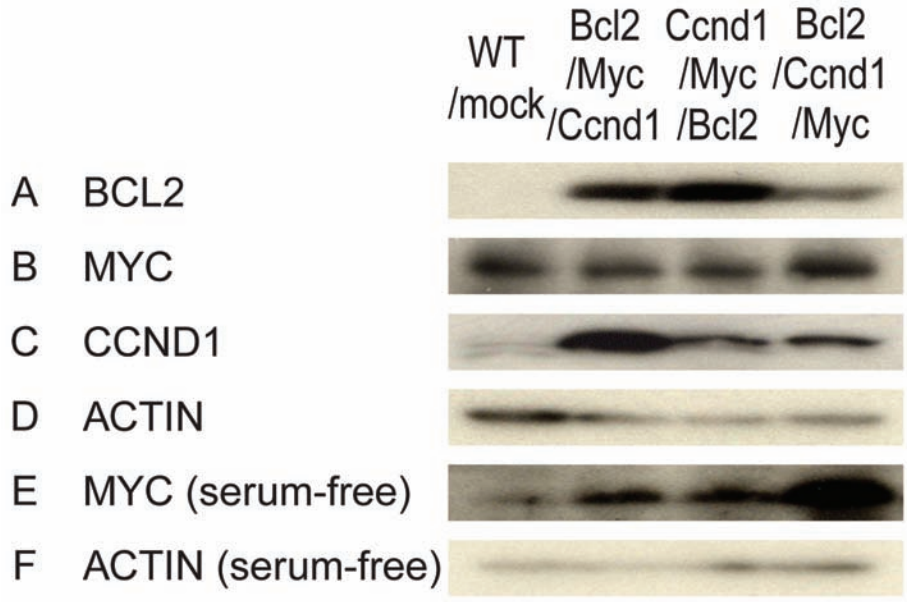
Online Supplementary Figure S2. Western blot analysis and colony-forming assays with drug selected stable integrants in EXP1 in Figure 1A.



(A) Western blot analysis of various stable integrants established with the drug selection method to determine the expression of BCL2, MYC and CCND1 proteins. (i-iv) Protein expression of BCL2, MYC, CCND1 and ACTIN of stable integrants established with the drug selection method. (v and vi) Protein expression of MYC and ACTIN of stable integrants established with the drug selection method cultured under serum-free condition for 8 h to evaluate deregulated exogenous MYC expression. Western blot analysis was performed as previously described with minor modifications.[4] Extracted proteins were loaded onto a SDS-PAGE gel. Protein expression of BCL2, MYC, CCND1 and ACTIN was detected using anti-mouse BCL-2 hamster monoclonal antibody (3F11; PharMingen), anti-MYC rabbit polyclonal antibody (#9402; Cell Signaling Technology), anti-CCND1 mouse monoclonal antibody (clone 5D4; IBL), and anti-ACTIN mouse monoclonal antibody (clone AC-40, Sigma). A goat anti-hamster Ig-horseradish peroxidase conjugate (Caltag Laboratories), goat anti-rabbit Ig-horseradish peroxidase conjugate (GE Healthcare) or a goat anti-mouse Ig-horseradish peroxidase conjugate (GE Healthcare) was used as the secondary antibody. (B) Stable integrants were examined for colony-forming ability. In the first plating, Bcl2/Myc/Ccnd1 cells formed more colonies compared to the other cells (left panel). On re-plating, Bcl2/Myc/Ccnd1 cells showed enhanced colony-forming capability, while that of Bcl2/Myc was limited. Colonies were counted on day 14. Data are presented as the mean ± s.e.m. (triplicate). *P* values are two-sided (Student's t test). (C) Photograph of representative colonies of various stable integrants in the first plating. Bcl2/Myc/Ccnd1 cell colonies were larger than those of Bcl2/Myc cells. Scale bar represents 10 mm.

WT: wild-type pro B-cells; Bcl2/Myc: stable integrant serially infected with pMXs-*Bcl2-neo* and pMXs-*Myc-hygro*, and purified by serial selection with neomycin and hygromycin; Ccnd1/Myc: stable integrant serially infected with pMXs-*Ccnd1-bsd* and pMXs-*Myc-hygro*, and purified by serial selection with blasticidin and hygromycin; Bcl2/Ccnd1: stable integrant serially infected with pMXs-*Bcl2-neo* and pMXs-*Ccnd1-bsd* and purified by serial selection with neomycin and blasticidin; Bcl2/*Myc/Ccnd1*: stable integrant serially infected with pMXs-*Bcl2-neo*, pMXs-*Myc-hygro* and pMXs-*Ccnd1-bsd* and purified by serial selection with neomycin, hygromycin and blasticidin. Bcl2/Myc/Ccnd1-tumor: tumor cells derived from neoplastic lymph node of a SCID mouse transplanted with the Bcl2/Myc/Ccnd1 cells.

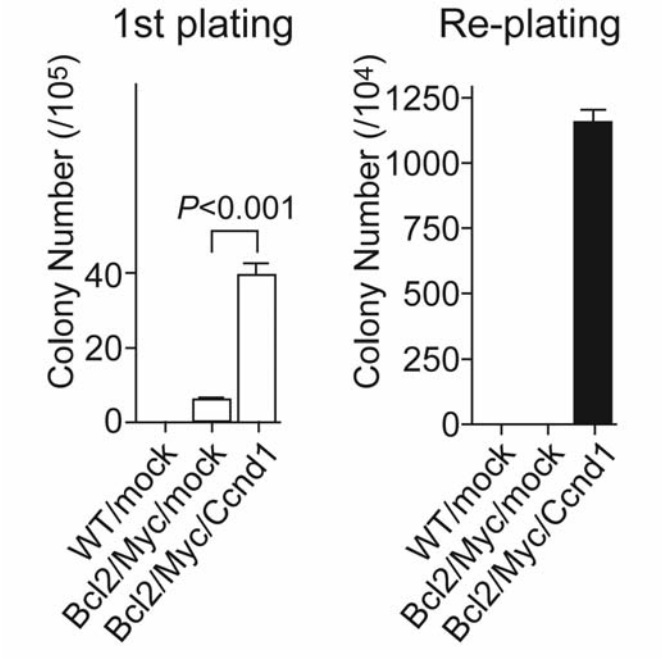
Online Supplementary Figure S3. Western blot analysis of stable integrants established using the cell sorting method in EXP1.



Western blot analysis of various stable integrants established using the cell sorting method to determine the expression of BCL2, MYC and CCND1 proteins. (A-D) Protein expression of BCL2, MYC, CCND1 and ACTIN of stable integrants established using the cell sorting method. (E and F) Protein expression of MYC and ACTIN of stable integrants established using the cell sorting method cultured under serum-free condition for 8 h to evaluate deregulated exogenous MYC expression.

WT/mock: stable integrant infected with MSCV-pgk-ires-*GFP* and pGCDNsam-ires-*huKO* and sorted for GFP and huKO expression; Bcl2/Myc/Ccnd1: stable integrant infected with MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP* and pGCDNsam-*Ccnd1*-ires-*huKO* and sorted for GFP and huKO expression; Ccnd1/Myc/Bcl2: stable integrant infected with MSCV-*Ccnd1*-pgk-*Myc*-ires-*GFP* and pGCDNsam-*Bcl2*-ires-*huKO* and sorted for GFP and huKO expression; Bcl2/Ccnd1/Myc: stable integrant infected with MSCV-*Bcl2*-pgk-*Ccnd1*-ires-*GFP* and pGCDNsam-*Myc*-ires-*huKO* and sorted for GFP and huKO expression.

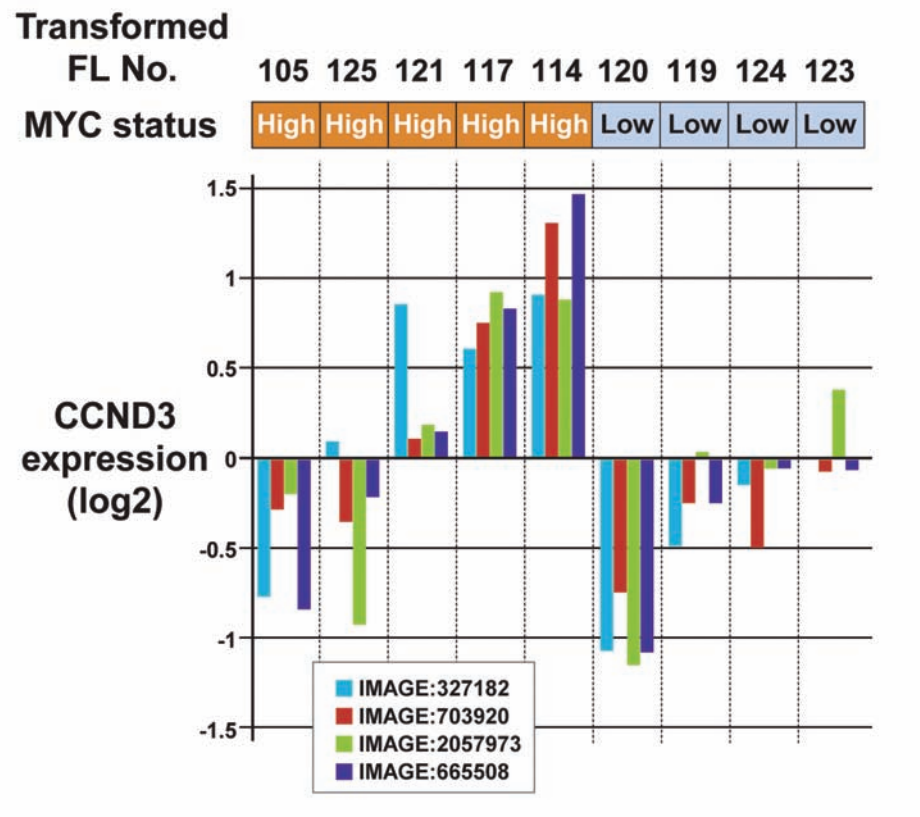
Online Supplementary Figure S4. Confirmatory colony-forming assays with independently established stable integrants using the drug selection method (EXP2).



Stable integrants independently established using the drug selection method were examined for colony-forming ability. In the first plating, Bcl2/Myc/Ccnd1 cells formed more colonies compared to other cells (left panel). On re-plating, Bcl2/Myc/Ccnd1 cells showed enhanced colony-forming capability, unlike the case with Bcl2/Myc/mock cells (right panel). Colonies were counted on day 14. Data are presented as the mean ± s.e.m. (triplicate). *P* values are two-sided (Student's t test).

WT/mock: Stable integrant serially infected with pMXs-*neo*, pMXs-*hygro* and pMXs-*bsd*, and purified by serial selection with neomycin, hygromycin and blasticidin. Bcl2/Myc/mock: stable integrant serially infected with pMXs-*Bcl2-neo*, pMXs-*Myc-hygro* and pMXs-*bsd*, and purified by serial selection with neomycin, hygromycin and blasticidin. Bcl2/Myc/Ccnd1: stable integrant serially infected with pMXs-*Bcl2-neo*, pMXs-*Myc-hygro* and pMXs-*Ccnd1-bsd*, and purified by serial selection with neomycin, hygromycin and blasticidin.

Online Supplementary Figure S5. *CCND3* expression in histologically transformed cases of follicular lymphoma.



Case numbers and *MYC* status with *CCND3* expression levels of cases of histologically transformed human follicular lymphoma (FL). Expression values were normalized by subtraction of the average of all nine cases for each probe. "IMAGE" indicates probe identifiers. "MYC status" indicates the expression level of *MYC* and genes regulated by *MYC* and is assigned as either "High" or "Low" according to the report by Lossos *et al.*[9]

In an effort to study the clinical relevance of the synergistic effect of *BCL2*, *MYC* and *CCND3*, we analyzed microarray gene expression data of cases of human FL [Lossos *et al.* Gene Expression Omnibus (GEO) database accession n.GSE3458]. FL is characterized by the presence of a *BCL2* translocation. Twenty-five to 45% of FL cases show histological transformation to a more aggressive lymphoma which is associated with rapid disease progression. Additional chromosomal translocations and/or increased expression of *MYC* are found in some cases of transformed FL.[5-9]

In this study, we analyzed the microarray gene expression data of histologically transformed human FL cases generated by Lossos *et al.*[9] We found increased expression of *CCND3* in three of the five cases with increased expression of *MYC* and genes regulated by *MYC*. On the other hand, we could not find increased expression of *CCND3* in any case with decreased expression of *MYC* and genes regulated by *MYC*. We defined the thresholds for markedly and moderately increased expression of *CCND3* as 0.5 and 0.2 of average expression of four probes (IMAGE: 327182, 703920, 2057973 and 665508), respectively.

This new finding suggested that *CCND3* might cooperate with *BCL2* and *MYC* and contribute to the histological transformation of some FL cases. These data also demonstrated the potential of our *in vitro* screening method for finding cooperative oncogenes with clinical relevance.