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Cyclin D3 Governs Clonal Expansion of Dark Zone Germinal Center B Cells

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SUMMARY

Germinal center (GC) B cells surge in their proliferative capacity, which poses a direct risk for B cell malignancies. G1- to S-phase transition is dependent on the expression and stability of D-type cyclins. We show that cyclin D3 expression specifically regulates dark zone (DZ) GC B cell proliferation. B cell receptor (BCR) stimulation of GC B cells downregulates cyclin D3 but induces c-Myc, which subsequently requires cyclin D3 to exert GC expansion. Control of DZ proliferation requires degradation of cyclin D3, which is dependent on phosphorylation of residue Thr283 and can be bypassed by cyclin $D3^{T283A}$ hyperstabilization as observed in B cell lymphoma. Thereby, selected GC B cells in the light zone potentially require disengagement from BCR signaling to accumulate cyclin D3 and undergo clonal expansion in the DZ.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

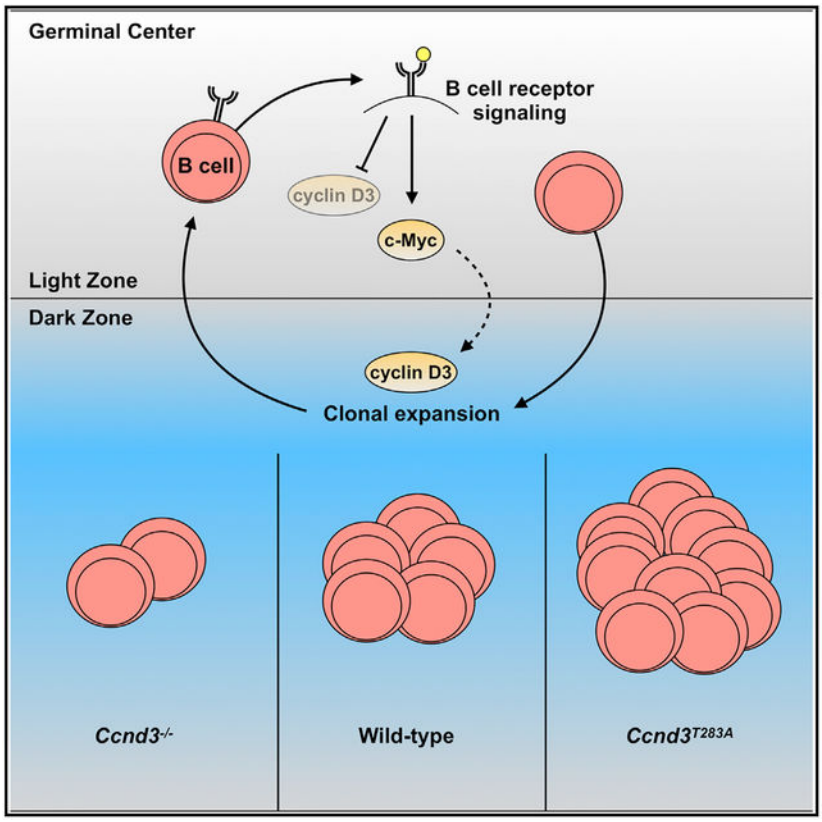
P.R.R. designed research, carried out the majority of experiments, interpreted data, and wrote the manuscript. R.C.R. conceived initial project idea, interpreted data, and secured funding. C.C. provided technical assistance with experiments, and Z.Z. helped with *Foxo1*$^{fl/fl(T24A)}$ experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.108403.

DECLARATION OF INTERESTS

The authors declare no competing interests.



In Brief

Mutations of cyclin D3 occur in B cell lymphomas, which derive from highly proliferating germinal center (GC) B cells. Ramezani-Rad et al. show that cyclin D3 in GC B cells is controlled by B cell receptor signaling and is required for proliferation of dark zone GC B cells.

INTRODUCTION

The human immune system is the warden of local and global cellular fitness, protecting the health of bodily functions. B cells act at the forefront of providing lifelong immunity to pathogens through memory B cells and the secretion of protective antibodies by plasma cells (Akkaya et al., 2019). More than a hundred immunoglobulin (Ig) gene segments stochastically assemble a membrane-bound antibody in the form of a B cell receptor (BCR) during B cell development (Schatz and Ji, 2011). These genetic recombination events generate a broad spectrum of BCR specificity to virtually any antigen. Only B cells that express a functional non-autoreactive BCR are tolerated to survive (Nemazee, 2017). After successful completion, B cells egress the bone marrow expressing two Ig isotypes, namely IgM and IgD. Both isotypes are expressed through alternative splicing and possess identical specificity. Upon antigen encounter through their BCR, these naive resting B cells are activated and subsequently seed the germinal center (GC) to engage in an immune response. Clonal expansion is pivotal for the functional response of GC B cells, yielding optimal numbers of few selected cells. The pathways and molecules controlling proliferative

responses are essential for understanding GC biology, and this knowledge is elementary to modulate immune responses for effective vaccine strategies, control autoimmune disorders, and treat B cell malignancies.

The D-type cyclins (cyclin D1, D2, and D3) facilitate the earliest step of commitment in G1- to S-phase of the cell cycle in all mammalian tissues. The D-type cyclins are best characterized for regulating the catalytic activity of cyclin-dependent kinases 4 and 6 (CDK4/6), which leads to the degradation of retinoblastoma protein (RB) to drive S-phase entry (Sherr, 1995). In B cells, cyclins D2 and D3 are central mediators of proliferative responses with partially redundant, but also distinct, roles among B cell stages. Early B cell development in the bone marrow specifically requires cyclin D3 for proliferative expansion of pre-B cells (Cooper et al., 2006), whereas in mature B cells cyclin D2 participates in proliferative responses (Solvason et al., 2000). In GC B cells, cyclin D3 is crucial for GC development (Cato et al., 2011; Peled et al., 2010); however, the specific regulatory pathways and contribution of cyclin D3 in GC B cells remain elusive. We show cyclin D3 expression is restricted to the dark zone (DZ) of GC B cells mediating clonal expansion and is downregulated by BCR signaling (occurring in light zone [LZ] GC B cells). Thereby, cyclin D3 is the driving force of DZ GC expansion, making it a critical factor during immune responses and in B cell lymphomas.

RESULTS

cyclin D3 Is Required for GC B Cell Proliferation in the DZ

To investigate the loss of cyclin D3 in GC B cells, we crossed conditional cyclin D3 (*Ccnd3*$^{fl/fl}$) mice (Choi et al., 2012) with Cγ1-Cre (*Ighg1*$^{Cre/+}$) mice (Casola et al., 2006). *Ccnd3*$^{fl/fl}$ Cγ1-Cre and *Ccnd3*$^{+/+}$ mice were immunized with sheep red blood cells (SRBCs) to induce GC responses and were analyzed after 7 days. The frequency of GC B cells was drastically reduced in *Ccnd3*$^{fl/fl}$ Cγ1-Cre mice (Figures 1A and 1B). Contrary to wild-type GC B cells, the majority of *Ccnd3*$^{fl/fl}$ Cγ1-Cre GC B cells were present in the LZ (CXCR4^{lo}CD86^{hi}), with much lower quantities of DZ GC B cells (CXCR4^{hi}CD86^{lo}) (Figures 1C and 1D). Administration of bromodeoxyuridine (BrdU) for a brief pulse during the GC response highlighted the reduced proliferation of *Ccnd3*$^{fl/fl}$ Cγ1-Cre GC B cells through lower BrdU incorporation into these cells (Figure 1E, top). Among the BrdU^{+} GC B cells, *Ccnd3*$^{fl/fl}$ Cγ1-Cre GC B cells had much lower DZ GC B cell proliferation compared with wild-type controls and most of the BrdU incorporation was present in GC B cells of the LZ (Figure 1E, bottom).

Mutant cyclin D3 Exceeds Normal Limits of DZ GC B Cells

Gain-of-function mutations in *CCND3* are found in particularly aggressive forms of B cell non-Hodgkin lymphoma (B-NHL) (Ramezani-Rad and Rickert, 2017). The majority of these mutations are heterozygous and affect the PEST domain of cyclin D3 (Richter et al., 2012; Schmitz et al., 2012). In particular, phosphorylation of Thr283 in cyclin D3 is crucial for proteasomal degradation and the T283A mutation effectively prevents degradation, leading to the hyperstabilization of cyclin D3 (Naderi et al., 2004). We CRISPR-engineered knockin mice carrying cyclin D3^{T283A} in the native *Ccnd3* locus by microinjecting C57BL/6 zygotes

with *Cas9* mRNA, *Ccnd3* single guide RNA (sgRNA), and a donor single-stranded oligodeoxynucleotide (ssODN) template with a single point mutation for $Ccnd3^{T283A}$ (Figure 2A). Of 35 pups, ~40% showed editing in the *Ccnd3* locus; however, one mouse success-fully repaired the CRISPR-induced DNA damage by homologous recombination and presented with substitution of adenine (A) for guanine (G) (ACT to GCT), which resulted in a heterozygous $Ccnd3^{T283A/+}$ mouse (Figures S2A and 2B). Animals with $Ccnd3^{T283A/+}$ were comparable in appearance to wild-type animals; however, they did not breed very effectively and occurred at a much lower Mendelian frequency (Figure S2B). The B splenocytes from $Ccnd3^{T283A/+}$ mice had much greater cyclin D3 protein levels compared with wild-type mice(Figure2C). To investigate the effects of this cyclin D3 mutant during the GC response, we reconstituted C57BL/6 recipient mice with bone marrow from either $Ccnd3^{+/+}$ or $Ccnd3^{T283A/+}$ mice. Total B splenocytes were slightly reduced, showing lower follicular B cell ($IgD^{hi}IgM^{lo}$) frequencies (Figure S2C). However, the GC B cell frequency was significantly upregulated in $Ccnd3^{T283A/+}$ mice after SRBC immunization (Figures 2D and 2E). Notably, DZ GC B cells were vastly enriched in $Ccnd3^{T283A/+}$ mice (Figures 2F and 2G). Similar results were obtained in mixed bone marrow chimeras using μMT mice (Kitamura et al., 1991) with either $Ccnd3^{+/+}$ or $Ccnd3^{T283A/+}$ mice (Figures S2D–S2G). Furthermore, $Ccnd3^{T283A/+}$ mice showed a modest increase in antibody responses (Figure 2H). Immunofluorescence of splenic follicles showed normal architecture of the spleen and the GC reaction (Figure 2I).

cyclin D3 Is Modulated by BCR Signaling in GC B Cells

The regulatory pathways that control cyclin D3, specifically in the GC, are not well described and critical for the understanding of GC B cell dynamics. GC stimuli (CD40, interleukin-4 [IL-4], and BAFF) induced higher proliferation in B cells from mutant $Ccnd3^{T283A/+}$ mice compared with wild-type controls, whereas cyclin D3-deficient B cells ($Ccnd3^{fl/fl}$ crossed to $Mb1^{Cre/+}$ [Hobeika et al., 2006]) proliferated much lower than both (Figure 3A). Interestingly, BCR stimulation (via IgM) also increased proliferation in $Ccnd3^{T283A/+}$ mice, but it was comparable in wild-type and $Ccnd3^{fl/fl}$ $Mb1^{Cre/+}$ mice, highlighting the specific requirement of cyclin D3 during CD40-driven responses. We hypothesized that cyclin D3 expression is functionally restricted in DZ GC B cells and potentially inhibited in LZ GC B cells. During LZ GC B cell selection, cells bearing a functional BCR compete for antigen (BCR signaling) and T follicular helper cell signals (CD40 signaling; but also IL-4 and IL-21) (Mesin et al., 2016). Positively selected GC B cells induce transient and restricted c-Myc expression in the LZ (Calado et al., 2012; Dominguez-Sola et al., 2012). To study the control of the signaling patterns in the small population of selected LZ GC B cells, we generated *in-vitro*-derived GC B cells (Nojima et al., 2011). For this, B splenocytes were propagated in the presence of IL-4 and on a feeder layer providing CD40L and BAFF (40LB cells). After 5 days in culture, B cells upregulated GC markers (GL7 and FAS) and underwent class switching, expressing cells with either IgM or IgG1 (Figure S3A). BCR stimulation of *in-vitro*-derived GC B cells robustly induced c-Myc protein and sharply downregulated cyclin D3 (Figure 3B). In contrast to cyclin D3, BCR stimulation induced cyclin D2 (Figure 3C), which is a known target of c-Myc (Bouchard et al., 1999) and is specifically enriched in c-Myc-expressing GC B cells by transcript (Calado et al., 2012; Dominguez-Sola et al., 2012). Interestingly, we found higher

cyclin D2 protein levels in B cells from mice with ectopic expression of c-MYC ($^{LSL}MYC/MYC$ [Calado et al., 2012] crossed to $Cd19^{Cre/+}$ [Rickert et al., 1997]), particularly via IgM/CD40 stimulation (Figure S3C). Also, BCR stimulation of *in-vitro*-derived GC B cells downregulated FOXO1, which is antagonized by phosphatidylinositol 3-kinase (PI3K)-AKT signaling in LZ GC B cells (Dominguez-Sola et al., 2015; Sander et al., 2015). Consistently, phosphorylation of Akt^{S473} was induced upon BCR signaling (Figure 3C). In order to validate the regulation of cyclin D3 in bona fide GC B cells, we purified untouched GC B cells from immunized mice, which largely expressed BCRs with κ light chains (Figure S3B). BCR stimulation of GC B cells induced c-Myc and downregulated cyclin D3 (Figures 3D and 3E). FOXO1 levels did not appear to be downregulated; however, phosphorylation of $FOXO1^{T24}$ increased, which is important for nuclear export and subsequent degradation. Since FOXO1 has been implicated to regulate cyclin D3 in GC B cells (Dominguez-Sola et al., 2015), we tested whether FOXO1 mediates cyclin D3 expression. The B splenocytes from nuclear trapped FOXO1 ($Foxo1^{fl/fl(T24A)}$) mice (Zhu et al., 2019) crossed to $Cd19^{Cre/+}$ mice were cultured with various stimulants. Notably, cyclin D3 protein expression was upregulated in $Foxo1^{fl/fl(T24A)}$ $Cd19^{Cre/+}$ cells compared with controls (Figure 3F).

c-Myc Requires cyclin D3 for Proliferative Expansion of GC B Cells

Our results indicated that cyclin D3 is the major D-type cyclin that controls GC B cell proliferation. However, the specific protein dosage of c-Myc dictates the relative proliferative expansion of DZ GC B cells (Finkin et al., 2019). To test the relevance of cyclin D3 in this process, we posited the question whether ectopic c-MYC protein can overcome the proliferative defects of cyclin D3-deficient GC B cells. For this, we crossed $Ccnd3^{fl/fl}$ Cγ1-Cre mice to ^{LSL}MYC mice to ablate cyclin D3 and induce c-MYC simultaneously in ongoing GC responses. When we immunized these mice with SRBCs, we found no difference in the GC frequency compared with cyclin D3 deficiency alone ($Ccnd3^{fl/fl}$ Cγ1-Cre) (Figures 4A and 4B). Also, the zonal ratio between DZ and LZ GC B cells was not changed in these mice (Figure 4C). However, c-MYC control mice ($Ccnd3^{+/+}$ ^{LSL}MYC Cγ1-Cre) had higher GC B cells compared with mice without c-MYC ($Ccnd3^{+/+}$ Cγ1-Cre), which was consistent with earlier reports (Sander et al., 2012). Furthermore, c-MYC-induced hCD2 reporter expression was significantly lower in $Ccnd3^{fl/fl}$ ^{LSL}MYC Cγ1-Cre mice than in $Ccnd3^{+/+}$ ^{LSL}MYC Cγ1-Cre mice, highlighting Cre-mediated induction of c-MYC in these GC B cells, but lower accumulation of $c\text{-}MYC^{+}$ cells upon cyclin D3 loss (Figure 4D).

DISCUSSION

cyclin D3 Is the Preferred and Indispensable D-Type cyclin during GC Expansion

Our data indicate that cyclin D3 is crucial for DZ GC B cell proliferation. Although cyclins D1, D2, and D3 can bind interchangeably to CDK4/6, it has been shown that substrate specificities are quite different in cyclin D3:CDK6 compared with cyclin D1:CDK4 complexes (Anders et al., 2011; Sarcevic et al., 1997). In human GC B cells, it has been shown that cyclin D3 mostly functions with CDK6, whereas in murine B cells this appears to be via CDK4 (Tanguay et al., 2001; Wagner et al., 1998). Furthermore, kinase-

independent roles of D-type cyclins highlight unique features among its members, including cyclin D1 regulating DNA repair through interaction with Rad51 (Jirawatnotai et al., 2011; Musgrove et al., 2011). Although high homology among D-type cyclins exists, specific cyclin D2 expression under the control of the *Ccnd3* locus still fails to compensate for the loss of cyclin D3 during B and T lymphopoiesis (Sawai et al., 2012), suggesting that non-overlapping roles appear to occur due to distinct regions in the protein rather than expression patterns. However, cyclin D3 can compensate for the loss of cyclin D2 in B cells (Lam et al., 2000). Thereby, cyclin D3 appears to show functional redundancy with cyclin D2 but possesses unique roles irreplaceable by cyclin D2 in B cells.

BCR Signaling Impedes FOXO1-cyclin D3 Signaling in GC B Cells

We show that BCR signaling in GC B cells, which typically occurs in LZ GC B cells, controls cyclin D3 protein (Figures 3D and 3E). We have previously shown that GSK3 is involved in the control of cyclin D3 protein in GC B cells (Cato et al., 2011). However, this role might be less prominent in LZ GC B cells as AKT appears to inhibit GSK3 (phospho-GSK3β^{S9}), which overlaps with c-Myc-expressing cells in GC B cells (Jellusova et al., 2017). AKT also mediates the nuclear export and degradation of FOXO1 via phosphorylation of Thr24 in GC B cells (Luo et al., 2018), which is consistent with our results (Figure 3E). Nuclear trapped FOXO1^{T24A} mutant showed higher levels of cyclin D3 protein (Figure 3F) and transcripts (Figure S3D) in stimulated B cells. FOXO1 has been shown to regulate cyclin D3 expression in malignant B cells (Gehringer et al., 2019; Wang et al., 2018); however, several studies did not observe proliferative defects in GC B cells in the absence of FOXO1 (Dengler et al., 2008; Dominguez-Sola et al., 2015; Sander et al., 2015). Although Inoue et al. (2017) showed lower proliferation in FOXO1-deficient GC B cells, this effect was enhanced in competition to wild-type cells and more modest alone. It is not clear how proliferation in FOXO1-deficient GC B cells is controlled. Our results suggest that cyclin D3 expression in the DZ is in part regulated by FOXO1, which is inhibited by BCR signaling in the LZ. However, additional factors downstream of BCR signaling might be involved in the regulation of cyclin D3. The regulation of cyclin D3 in LZ GC B cells may act as a brake to DZ-to-LZ transitioning cells for proper selection, as DZ GC cells could arrive to the LZ being in late G1 phase (Stewart et al., 2018), but it requires further investigations to clarify the functional relevance of this process.

c-Myc Requires cyclin D3 Signaling to Relay DZ GC B Cell Expansion

Enforced levels of c-MYC could not overcome the expansion deficits of cyclin D3-deficient GC B cells (Figure 4). During GC B cells selection, the expression of c-Myc is integral and loss of c-Myc leads to the collapse of the GC B cell reaction (Calado et al., 2012). The expression of c-Myc is restricted to LZ GC B cells, and it has been shown that c-Myc induces AP4, which persists after c-Myc decay and regulates cyclin D3 expression (Chou et al., 2016). However, several findings indicate that GC B cells can start S phase in the LZ before entering the DZ for extended expansion (Gitlin et al., 2014; Hauser et al., 2007; Victora et al., 2010). It is possible that c-Myc initiates this step via cyclin D2, which then displaces the positively selected cell from BCR signaling, permitting FOXO1 accumulation to induce CXCR4 for DZ entry and cyclin D3 expression for DZ expansion. Overall, our

findings indicate the dependency of c-Myc on cyclin D3 to relay the positive expansion signal from the LZ to the DZ.

cyclin D3 Is Functionally Relevant in B Cell Malignancies

Our study highlights that control of cyclin D3 is required for proper proliferation of GC B cells but also can act as an efficient brake to halt improper GC expansion. The normal biology of GC B cells has profound implications for B cell malignancies, in particular for GC-derived lymphomas. Among patients with diffuse large B cell lymphoma (DLBCL), ~3.3% (33/1,001) have *CCND3* mutation, which occur frequently in exon 5, leading to a premature stop or missense mutations affecting Thr283 or nearby residues (Reddy et al., 2017). DLBCL patients with oncogenic NOTCH pathway aberration appear to have the highest enrichment for *CCND3* mutations (Schmitz et al., 2018). In Burkitt lymphoma (BL), all subtypes are characterized by *MYC* deregulation, but *CCND3* mutations predominantly occur in the sporadic BL subtype (Panea et al., 2019; Schmitz et al., 2012). Given its unique role in B cell biology and wide involvement in B cell malignancies, it is worthwhile to understand how cyclin D3 is specifically involved in the pathogenesis of B cell lymphomas. We CRISPR-engineered mutant mice with hyper-stabilized cyclin D3, which have increased GC B cell frequencies (Figure 2). In our mouse cohort, we also maintained mice under continuous antigenic challenge for up to a year, but we did not observe malignant transformation of GC B cells (data not shown). Interestingly, enforced cyclin D3 expression can induce lymphomagenesis in combination with other oncogenic factors. Mice reconstituted with hematopoietic progenitor cells from VavP-*BCL2* mice, which are prone to lymphomagenesis, have shortened disease onset when transduced with cyclin $D3^{T283A}$ (Oricchio et al., 2014). In *in-vitro*-derived GC B cells, four factors (i.e., c-MYC, TCF3, AKT, and TCL1A) in addition to cyclin $D3^{T283A}$ induced the occurrence of tumors (Arita et al., 2014). In human B cells, cyclin D3 in combination with gains in BCL2, BCL6, and dominant-negative p53 lead to tumor formation (Caeser et al., 2019). These reports and our results highlight the potential requirement of other factors for cyclin D3 (or cyclin $D3^{T283A}$) during lymphomagenesis to exert an oncogenic function. Beyond lymphoma, cyclin $D3^{T283A}$ occurrence in B cells may also contribute to the pathogenesis of autoimmune disorders (Singh et al., 2020). Overall, cyclin D3 plays a crucial and unique role during normal GC biology and its control is critical to prevent GC-derived diseases.

STAR★METHODS

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Parham Ramezani-Rad (prad@sbp.edu).

Materials Availability—All unique reagents generated in this study are available from the Lead Contact and may require completion of a Materials Transfer Agreement.

Data and Code Availability—This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—*Ccnd3*$^{fl/fl}$, *Foxo1*$^{fl/fl(T24A)}$, Cγ1-Cre, *Mb1*^{Cre}, ^{LSL}*MYC*, μMT and *Cd19*^{Cre} were all kept on a C57BL/6 background. Mouse crossings are indicated in the figures and text. The following activity of Cre recombinase occurs in these mouse lines: *Mb1*^{Cre} (pro-B cells), *Cd19*^{Cre} (pro-B to mature B cells) and Cγ1-Cre (upon onset of germ-line Cγ1 transcription in activated B cells/GC B cells). Female and male experimental animals over 6 weeks of age were age-matched without gender bias. Animals were housed under specific-pathogen-free conditions and a 12 h light/12 h dark cycle in the animal facility of SBP. Experimental procedures were in accordance with IACUC regulations.

CRISPR-engineered knockin mice—To generate knockin mice with a cyclin D3 missense mutation (Thr to Ala substitution at residue 283; T283A) in the native *Ccnd3* locus, appropriate guides with close proximity to exon 7 in the murine *Ccnd3* locus were first selected with Feng Zhang's lab CRISPR Design tool (discontinued https://zlab.bio/guide-design-resources). The guide with the lowest off-target binding using Cas-OFFinder (http://www.rgenome.net/cas-offinder) was selected. The complete *Ccnd3* sgRNA (spacer and scaffold) was transcribed with HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). A donor template with a single point mutation for *Ccnd3*^{T283A} (substitution of adenine (A) for guanine (G): **A**CT to **G**CT) in the center and homologous flanking arms (to the native *Ccnd3* locus) was synthesized as a single-stranded oligodeoxynucleotide (ssODN) (Integrated DNA Technologies). A master mixture of *Cas9* mRNA (50ng/μl) (Thermo Fisher Scientific), *Ccnd3* sgRNA (25ng/μl) and *Ccnd3*^{T283A} ssODN (100ng/μl) was prepared and microinjected into the cytoplasm of zygotes from superovulated female C57BL/6 mice and cultured until the two-cell stage to be then implanted in the oviduct of pseudopregnant female mice. Resulting pups were screened for editing efficiency by PCR amplification of the genomic region and direct sequencing of amplicons. Potential mosaicism or further validation was confirmed by sequencing of individual amplicons using the Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher Scientific).

Cell culture—B splenocytes were MACS-purified, collecting the negative fraction of CD43 Microbeads (Miltenyi Biotec). GC B cells were MACS-purified, collecting the negative fraction of CD43 Microbeads, CD38 (90) Biotin (Thermo Fisher Scientific), CD11c (N418) Biotin (Thermo Fisher Scientific) and anti-Biotin Microbeads (Miltenyi Biotec). Cells were cultured in RPMI 1640 (Corning) supplemented with 10% FBS (MilliporeSigma and Thermo Fisher Scientific), 1x Penicillin-Streptomycin, 1x MEM Nonessential Amino Acids (Corning), 1mM Sodium Pyruvate, 2mM GlutaMax, and 55 μM 2-Mercaptoethanol (Thermo Fisher Scientific). Cells were stimulated with the following reagents: 10μg/ml goat anti-mouse IgM F(ab')$_2$, 10μg/ml goat anti-mouse IgG F(ab')$_2$ (Jackson ImmunoResearch Labs), 10μg/ml goat anti-mouse κ F(ab')$_2$ (SouthernBiotech), 10ng/ml mouse rBaff (R&D Systems), 5μg/ml rat anti-mouse CD40 (1C10) and 10ng/ml mouse rIL-4 (Thermo Fisher Scientific). For *in vitro*-derived GC B cell cultures, B splenocytes were cultured on 40LB cells (3T3 fibroblasts expressing CD40L and secreting BAFF) with addition of 1ng/ml mouse rIL-4.

METHOD DETAILS

Immunization—Mice were immunized i.p. or i.v. with 100μl of PBS-washed citrated SRBCs (Colorado Serum Company) or i.p. with 50μg NP_{20}-CGG (Biosearch Technologies) plus Imject Alum Adjuvant (ThermoFisher Scientific) in 100μl PBS. For SRBC-specific antibody titers, serum was collected on indicated days and bound to SRBCs and detected with IgM (II/41) APC (Thermo Fisher Scientific) and IgG1 (A85–1) PE (BD Biosciences) by flow cytometry. For BrdU pulse experiments, mice were injected i.p. with 2mg BrdU in 200μl PBS and sacrificed 2 h later. Incorporation of BrdU was detected using the BrdU Flow Kit (BD Biosciences) according to manufacturer's instructions and BrdU (BU20A) PE (Thermo Fisher Scientific).

Flow cytometry—Single cell suspensions from tissues were ACK lysed and stained in 1% FBS in PBS containing 0.05% sodium azide. Cells were gated according to size and granularity based on FSC-A and SSC-A. Doublets were excluded by FSC-A versus FSC-H gating. Non-antigen-specific binding was blocked with CD16/CD32 (2.4G2) (BD Biosciences). The following antibodies were used for staining: B220 (RA3–6B2) APC-eFluor780, IgM (II/41) APC, IgD (11–26c) FITC, CD86 (PO3.1) PE, hCD2 (RPA-2.10) APC (Thermo Fisher Scientific), CXCR4 (L276F12) APC, CD86 (GL1) PerCP Cy5.5 (BioLegend), κ (187.1) FITC, CD19 (1D3) APC-Cy7, FAS (Jo2) PE-Cy7, GL7 FITC and IgG1 (A85–1) FITC (BD Biosciences). Cell proliferation was analyzed using the eBioscience Cell Proliferation Dye eFluor670 (Thermo Fisher Scientific). Samples were acquired on a FACSCanto (BD Biosciences) and analyzed with Flowjo (Becton, Dickinson and Company).

Immunofluorescence—Spleens embedded in Tissue TEK O.C.T. (Sakura Finetek) were sectioned on a Microtome Cryostat HM 505 E (Microm). Sections were fixed with acetone and blocked with 5% FBS in PBS. Fluorescent images were acquired on an Axio Imager.M1 (Zeiss) microscope equipped with an Orca-ER (Hamamatsu) camera. SlideBook (3i) was used as the imaging software. Gimp (GNU Image Manipulation Program) was used for image editing. The following antibodies/reagents were used: B220 (RA3–6B2) APC, CD21/CD35 (4E3) FITC (Thermo Fisher Scientific), PNA Biotin (Vector Laboratories) and Streptavidin-Cy3 (Jackson ImmunoResearch Labs).

qRT-PCR—RNA was extracted with RNeasy Mini Kit (QIAGEN), cDNA was synthesized with iScript cDNA Synthesis Kit and reactions were run with iTaq Universal SYBR green (Bio-Rad) on a LightCycler 96 (Roche). The following program was used: preincubation at 95°C for 5 mins, 2-step amplification of 40 cycles at 95°C for 10 s and 60°C for 40 s.

Immunoblotting—Cells were lysed either in CelLytic M (Millipore Sigma) with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology) or in 1% SDS buffer. Immunoblotting was performed following standard procedures. PVDF membranes were blocked with 5% Milk in TBST and probed with primary antibodies overnight on a rotating platform at 4°C. The following antibodies were used: c-Myc (D84C12), cyclin D2 (D52F9), cyclin D3 (DCS22), FOXO1 (C29H4), phospho-$FOXO1^{T24}$, phospho-Akt^{S473} (D9E), β-actin (13E5) (Cell Signaling Technology) and cyclin D2 (Santa Cruz Biotechnology). The

following horseradish-peroxidase-coupled antibodies were used as secondaries: donkey anti-rabbit IgG and goat anti-mouse IgG (Jackson ImmunoResearch Labs). Protein signal was detected by film or on a ChemiDoc Imaging System (Bio-Rad).

QUANTIFICATION AND STATISTICAL ANALYSIS

The sample size (n) indicated in the figure legends defines the number of mice used for each experimental group. The statistical analysis was performed by Prism 8 (Graphpad Software). The *p* value was calculated using an unpaired t test and is shown in the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Dark zone germinal center (GC) B cells are diminished upon cyclin D3 loss
- Hyperstabilized cyclin $D3^{T283A}$ mutant drives dark zone GC B cell expansion
- B cell receptor signaling in GC B cells suppresses cyclin D3
- c-Myc requires cyclin D3 for GC B cell expansion

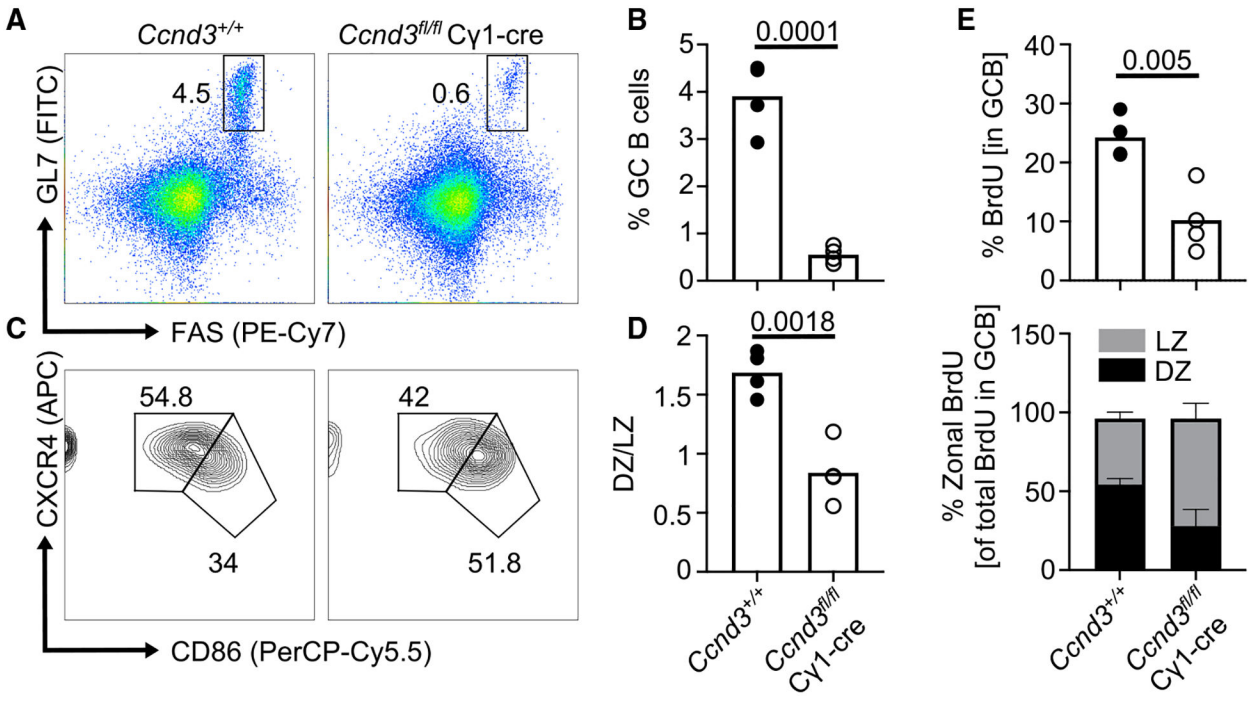

Figure 1. Loss of cyclin D3 in GC B Cells Reduces Proliferation in the DZ

(A) Deletion of cyclin D3 (*Ccnd3*) in GC B cells. *Ccnd3*$^{fl/fl}$ Cγ1-Cre mice were compared with *Ccnd3*$^{+/+}$ mice. Splenocytes after SRBC immunization on day 7 were analyzed. Single cells were gated on B cells (B220^{+}) to determine GC B cell frequency based on GL7^{+}FAS^{+} expression. See also Figures S1A and S1B.

(B) Quantification of GC B cell frequency (shown in A) (n = 4).

(C) Zonal distribution of GC B cells (shown in A) based on relative expression of CXCR4^{hi}CD86^{lo} (DZ) and CXCR4^{lo}CD86^{hi} (LZ).

(D) Quantification of respective DZ/LZ ratio of GC B cells (shown in C) (n = 4).

(E) Administration of BrdU for 2 h on day 10 after SRBC immunization. (Top) Total frequency of BrdU incorporation is shown in GC B cells (B220^{+}GL7^{+}FAS^{+}). (Bottom) Distribution of total BrdU^{+} GC B cells of control and knockout mice in the DZ and LZ. Error bars indicate mean + SD (n = 4). See also Figure S1C.

Data are representative of two to three independent experiments.

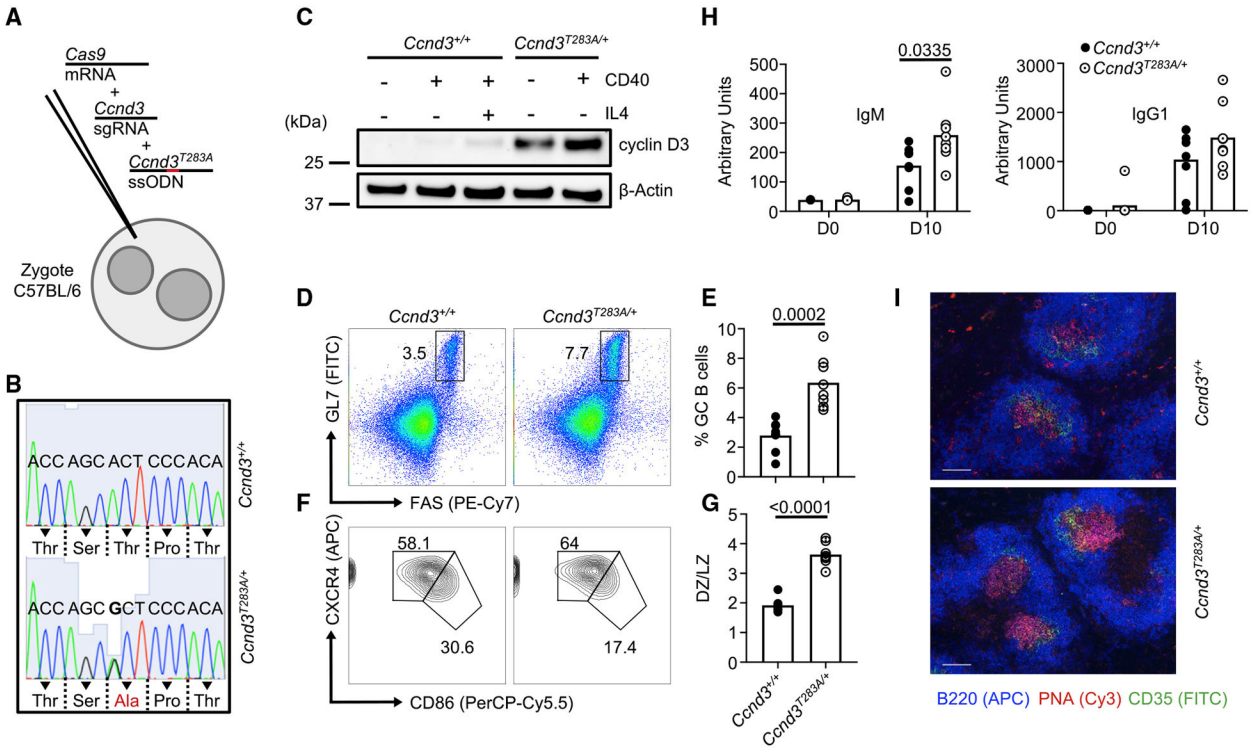

Figure 2. CRISPR-Engineered Hyperstabilized cyclin D3 Mutant Mice Increase DZ Expansion

(A) Generation of *Ccnd3*$^{T283A/+}$ mice was achieved by microinjection of *Cas9* mRNA, *Ccnd3* sgRNA, and *Ccnd3*^{T283A} ssODN into zygotes from superovulated C57BL/6 female mice.

(B) DNA sequencing chromatogram shows the point mutation and resulting amino acid change in a heterozygous animal for *Ccnd3*$^{T283A/+}$.

(C) Assessment of cyclin D3 protein levels in *Ccnd3*$^{T283A/+}$ mice. The B splenocytes from *Ccnd3*$^{T283A/+}$ or *Ccnd3*$^{+/+}$ mice were cultured in the presence of indicated stimulants for 16 h, and cell lysates were probed for cyclin D3 (and β-actin as a loading control). Data are representative of two independent experiments.

(D) Irradiated (10 Gy) C57BL/6 recipient mice were reconstituted with bone marrow from *Ccnd3*$^{+/+}$ or *Ccnd3*$^{T283A/+}$ mice. After 6 weeks, mice were immunized with SRBCs and analyzed 10 days later. Single cells were gated on B splenocytes (B220^{+}) to determine GC B cell frequency based on GL7^{+}FAS^{+} expression.

(E) Quantification of (D) GC B cells in *Ccnd3*$^{T283A/+}$ mice compared with *Ccnd3*$^{+/+}$ control mice (n = 8).

(F) Zonal distribution of GC B cells (shown in D) based on relative expression of CXCR4^{hi}CD86^{lo} (DZ) and CXCR4^{lo}CD86^{hi} (LZ).

(G) Quantification of (F) GC B cells respective DZ/LZ ratio (n = 8).

(H) Antibody titers of SRBC-specific IgM or IgG1 in sera from *Ccnd3*$^{+/+}$ or *Ccnd3*$^{T283A/+}$ mice (n = 8).

(I) Immunofluorescence of GC reaction in *Ccnd3*$^{T283A/+}$ and *Ccnd3*$^{+/+}$ mice. Mice were immunized with NP-CGG and analyzed on day 14. Splenic follicles with B cells (B220^{+})

and GC B cells (PNA^+) are shown. CD35 expression on follicular dendritic cells depicts the LZ of the GC. Scale bar, 100 μm. Data are representative of three independent experiments.

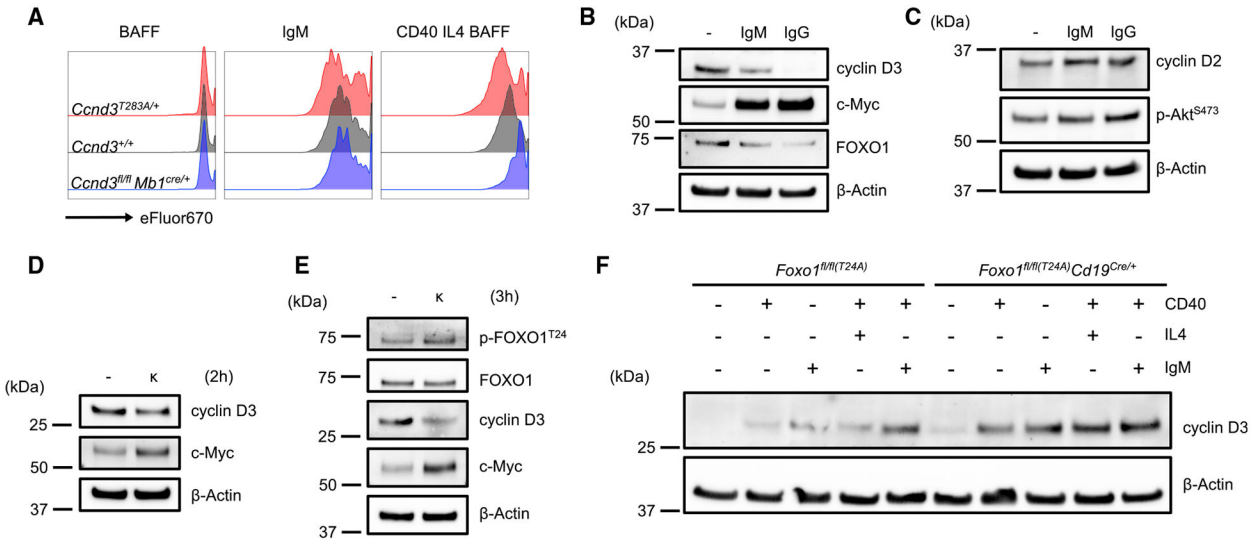

Figure 3. BCR Signaling Downregulates cyclin D3 in GC B Cells

(A) The B splenocytes from *Ccnd3*$^{T283A/+}$, *Ccnd3*$^{fl/fl}$ *Mb1*$^{Cre/+}$, or *Ccnd3*$^{+/+}$ mice were cultured in the presence of indicated stimulants for 3 days. Proliferation of cells was assessed through dilution of eFluor670 in daughter cells. Data are representative of three independent experiments.

(B) Wild-type B splenocytes were cultured for 5 days on 40LB cells plus IL-4. The resulting *in-vitro*-derived GC B cells were stimulated with anti-IgM or anti-IgG for 4 h and lysed. Cell lysates were probed for c-Myc, cyclin D3, and FOXO1 (β-actin serves as loading control). Data are representative of three independent experiments.

(C) Cell lysates from *in-vitro*-derived GC B cells (generated as described in B) were probed for cyclin D2 and phospho-Akt^{S473} (β-actin serves as loading control). Data are representative of two to three independent experiments.

(D and E) Wild-type mice were immunized with SRBCs, and GC B cells were purified on day 7. GC B cells were stimulated with anti-κ for 2 h (D) or 3 h (E) and lysed. Cell lysates were probed for c-Myc, cyclin D3, FOXO1, and phospho-FOXO1^{T24} (β-actin serves as loading control). Data are representative of three independent experiments.

(F) The B splenocytes from *Foxo1*$^{fl/fl(T24A)}$ *Cd19*$^{Cre/+}$ mice and corresponding controls were cultured in the presence of indicated stimulants for 24 h. Cell lysates were probed for cyclin D3 (β-actin serves as loading control). Data are representative of three independent experiments.

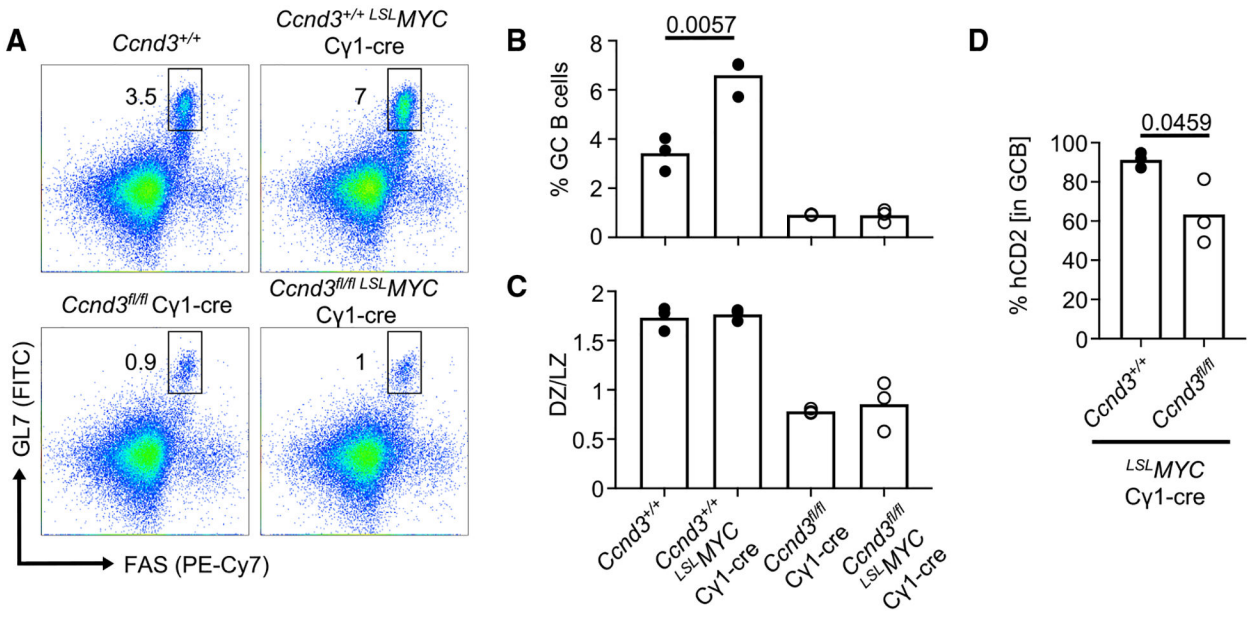

Figure 4. Enforced c-MYC Expression in GC B Cells Does Not Overcome cyclin D3 Deficiency

(A) Ectopic expression of c-MYC in *Ccnd3*$^{fl/fl}$ Cγ1-Cre GC B cells compared with *Ccnd3*$^{+/+}$ Cγ1-Cre controls. Splenocytes after SRBC immunization on day 10 were analyzed. Single cells were gated on B cells (B220^{+}) to determine GC B cell frequency based on GL7^{+}FAS^{+} expression.

(B) Quantification of (A) overall GC B cell frequency (n = 3).

(C) Quantification of respective DZ/LZ ratio of GC B cells based on relative expression of CXCR4^{hi}CD86^{lo} (DZ) and CXCR4^{lo}CD86^{hi} (LZ) (n = 3). See also Figure S4A.

(D) Reporter expression of hCD2 in *Ccnd3*$^{fl/fl\ LSL}$*MYC* Cγ1-Cre was compared with *Ccnd3*$^{+/+\ LSL}$*MYC* Cγ1-Cre (n = 3). See also Figure S4B. Data are representative of two to three independent experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE
Antibodies
Goat anti-mouse IgM F(ab')$_2$
Goat anti-mouse IgG F(ab')$_2$
Goat anti-mouse κ F(ab')$_2$
Rat anti-mouse CD40 (clone 1C10)
Rat anti-CD16/CD32 (clone: 2.4G2)
Rat anti-B220 (clone: RA3–6B2) APC-eFluor780
Rat anti-CD19 (clone: 1D3) APC-Cy7
Rat anti-IgM (clone: II/41) APC
Rat anti-IgD (clone: 11–26c) FITC
Rat anti-κ (clone: 187.1) FITC
Rat anti-CXCR4 (clone: L276F12) APC
Rat anti-CD86 (clone: GL1) PerCP Cy5.5
Rat anti-CD86 (clone: PO3.1) PE
Armenian hamster anti-FAS (clone: Jo2) PE-Cy7
Rat anti-mouse T- and B Cell Activation Antigen (clone: GL7) FITC
Mouse anti-BrdU (clone: BU20A) PE
Rat anti-IgG1 (clone: A85–1) FITC
Rat anti-IgG1 (clone: A85–1) PE
Rat anti-B220 (clone: RA3–6B2) APC
Rat anti-CD21/CD35 (clone: 4E3) FITC
Mouse anti-hCD2 (clone: RPA-2.10) APC
Rabbit anti-c-Myc (clone: D84C12)
Rabbit anti-cyclin D2 (clone: D52F9)
Mouse anti-cyclin D3 (clone: DCS22)

REAGENT or RESOURCE
Rabbit anti-FOXO1 (clone: C29H4)
Rabbit anti-phospho-FOXO1/FOXO3a (T24/T32)
Rabbit anti-phospho-Akt (S473) (clone: D9E)
Rabbit anti-β-Actin (clone: 13E5)
Rabbit anti-cyclin D2
Donkey anti-rabbit IgG HRP
Goat anti-mouse IgG HRP
Rat anti-CD43 MicroBeads
Rat anti-CD38 (clone: 90) Biotin
Armenian hamster anti-CD11c (clone: N418) Biotin
Mouse anti-Biotin MicroBeads
Biological Samples
Citrated sheep red blood cells
Chemicals, Peptides, and Recombinant Proteins
Mouse rBaff
Mouse rIL-4
Cell Proliferation Dye eFluor670
NP_{20}-CGG
Imject Alum Adjuvant
PNA Biotin
Streptavidin-Cy3
Critical Commercial Assays
BrdU Flow Kit
Zero Blunt TOPO PCR Cloning Kit
HiScribe T7 Quick High Yield RNA Synthesis Kit
iScript cDNA Synthesis Kit
iTaq Universal SYBR green
Experimental Models: Cell Lines

REAGENT or RESOURCE
Mouse: 40LB cells
Experimental Models: Organisms/Strains
Mouse: *Ccnd3*$^{fl/fl}$: Ccnd3$^{tm2.1Pisc}$
Mouse: *Ccnd3*^{T283A}
Mouse: Cγ1-Cre: B6.129P2(Cg)-Ighg1$^{tm1(cre)Cgn}$/J
Mouse: *Foxo1*$^{fl/fl(T24A)}$
Mouse: *Mb1*^{Cre}: B6.C(Cg)-Cd79a$^{tm1(cre)Reth}$/EhobJ
Mouse: ^{LSL}*MYC*: C57BL/6N-Gt(ROSA) 26Sor$^{tm13(CAG-MYC,-CD2*)Rsky}$/J
Mouse: μMT: B6.129S2-Ighm^{tm1Cgn}/J
Mouse: *Cd19*^{Cre}: B6.129P2(C)-Cd19$^{tm1(cre)Cgn}$/J
Oligonucleotides
Ccnd3 sgRNA template: GCTAGAGCCCCGGGGGGCTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTC
Ccnd3^{T283A} ssODN donor template: GAGAGCCTCAGGGAAGCTGCTCAGACAGCCCCCAGCCCAGTGCCCAAAGCCCCCCGGGGCTCTAGCAGCCAGGGGCCCAGTCAGACCAGCGCTCCCACAGATGT
Cas9 mRNA
Primer: *Ccnd3* locus for sequencing Forward: TGAAGGACACTTGGCATGTAGA
Primer: *Ccnd3* locus for sequencing Reverse: GAGGGACTCAAAAGAAATGCTG
Primer: *Actb* for qRT-PCR Forward: GGCTGTATTCCC CTCCATCG
Primer: *Actb* for qRT-PCR Reverse: CCAGTTGGTAAC AATGCCATGT
Primer: *Ccnd3* for qRT-PCR Forward: CAGAGTTTACT CCGCCTGGA
Primer: *Ccnd3* for qRT-PCR Reverse: AGCCAGAGGG AAGACATCCT
Software and Algorithms
FlowJo 10
Prism 8
GIMP