Critical Role of Pcid2 in B Cell Survival through the Regulation of MAD2 Expression

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The mitotic checkpoint is essential for maintaining genomic stability in differentiating B cells undergoing genetic alterations of the Ig gene. In this study, using real-time RT-PCR and in situ RNA hybridization, we demonstrated that MAD2 mRNA export is selectively regulated by Pcid2/Thp1. Pcid2 small interfering RNA induced a cell-cycle abnormality with increased apoptosis and polyploidy, as previously observed in MAD2-knockdown cells. Pcid2 small interfering RNA reduced MAD2 expression, but not the expression of other cell-cycle checkpoint proteins, such as MAD1 and BUBR1, or the cell-cycle–associated proteins, cyclin A, cyclin B1, and cyclin-dependent kinase 1. In mouse B lineage cells, Pcid2 transcripts appeared in a stage-dependent manner at high levels in bone marrow pre-B and immature B cells, and in spleen transitional 1 and follicular B cells, but at lower levels in pro-B, transitional 2, and marginal zone B cells, suggesting a stage-dependent requirement for MAD2 regulation. Cd19-cre–derived targeting of the Pcid2 gene induced a mature B cell deficiency in mice. These findings indicate that Pcid2 is essential for B cell survival through the regulation of MAD2 expression during B cell differentiation. The Journal of Immunology, 2010, 185: 5180–5187.
FO B cells, suggesting that the sensitivity to DNA damage is altered during B cell differentiation in cells undergoing functionally competent genetic alterations.

Materials and Methods

Cell surface staining and sorting

Lymphoid cells from the bone marrow and spleen were stained by FITC-conjugated mAb, PE-conjugated mAb, and biotin-labeled mAb with PerCP-conjugated streptavidin (GE Healthcare, Buckinghamshire, U.K.), and analyzed by FACS Calibur (BD Biosciences, Mountain View, CA) using FlowJo software (Tree Star, Ashland, OR). mAbs were obtained from eBioscience (San Diego, CA) for B220, CD21, CD23, CD24, IgM, IgD, and CD93 (AA4.1). Anti-CD43 mAb was purchased from BD Biosciences. B220+ cells enriched by MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were purified using a JSAN cell sorter (Baybioscience, Kobe, Japan) (15). For each fraction, 1.5 x 10^5 cells were collected with the purity of ~95% by postsorting analysis.

Real-time RT-PCR

Real-time RT-PCR was performed using a LightCycler (Roche Molecular Biochemicals, Basel, Switzerland) with cDNAs after RT reaction using total RNA, oligo-dT primer (Invitrogen, Carlsbad, CA), and SuperScript III (Invitrogen). The data are presented as the relative ratio compared with control GAPDH. Specific oligonucleotide primers and probes of hPcid2, hMAD2, mPcid2, mMAD2, and GAPDH were purchased from Nihon Gene Research Laboratories (Sendai, Japan) (Supplemental Table 1).

Small interfering RNA

Small interfering RNA (siRNA) treatment of HeLa cells was performed with 10 nM Stealth RNAi duplexes (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen).

Cell-cycle analysis

Cells were harvested, washed with ice-cold PBS, resuspended in PBS with 250 μg/ml RNase A, and stained with 2% propidium iodide (PI) solution for 2 h at 4°C. In some experiments, cells were incubated with Colcemid (final 50 ng/ml) for 40 h.

Detection of active caspase 3-positive cells

Cells treated with siRNA were stained with PE-conjugated anti-active caspase 3 mAb (BD Pharmingen, San Diego, CA) and analyzed by FACS Calibur.

Nuclear staining by PI

siRNA-treated cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100, stained with PI (0.5 μg/ml), and observed using a confocal laser microscope.

FIGURE 1. Expression levels of Pcid2 transcripts at various stages of B lineage cell differentiation. B lineage cells were purified from bone marrow (BM) and spleen (SPL) using a B cell selection kit and further sorted using Hardy’s differentiation strategy with surface markers for B220, CD43, IgM, IgD, AA4.1, CD21, and CD23. After purifying RNAs from 1.5 x 10^5 cells per each fraction, Pcid2 transcription was measured by real-time RT-PCR based on control mRNA expression of GAPDH. The expression level of Pcid2/GAPDH was compared with that of pro-B cells. All experiments were repeated three times and the results were reproducible. Imm. B, immature B cells.
**Immunoblotting**

siRNA-treated cells were harvested, lysed with Tris/Nonidet P-40/EDTA buffer, and analyzed by SDS-PAGE for immunoblotting (11). The mAbs against MAD2 (BD Transduction Laboratories, San Diego, CA), MAD1 (Santa Cruz Biotechnology, Santa Cruz, CA), BUBR1 (MBL, Nagoya, Japan), BUB3 (BD Transduction Laboratories), CENP-E (Abcam, Cambridge, MA), p55CDC (Santa Cruz Biotechnology), cyclin A (Santa Cruz Biotechnology), cyclin B1 (Thermo Fisher Scientific, Fremont, CA), CDK1 (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich, St. Louis, MO) were used in combination with HRP-conjugated secondary Abs.

**Preparation of cytoplasmic and nuclear RNAs**

Cells were suspended in ice-cold lysis buffer and incubated for 5 min on ice (12), and fractionated by centrifugation at 2500 rpm for 5 min to obtain the nuclear pellet and at 15,000 rpm for 20 min to obtain the cytoplasmic supernatant. The RNA was extracted by using TRIzol (Invitrogen).

**In situ RNA hybridization**

The cDNAs of the coding regions of hMAD2 (261–618 nt) and hBUB3 (340–642 nt) were cloned into the pGEM-3Z vector (16). Anti-sense and sense (control) probes with digoxigenin were generated using an RNA labeling in vitro transcription kit (Roche Diagnostics, West Sussex, U.K.). Cells were fixed with 4% paraformaldehyde/PBS, acetylated, and then hybridized with probes for 16 h at 50˚C. After washing with SSC, the slides were incubated with sheep anti-digoxigenin Ab (Roche Diagnostics), followed by indocarbocyanine-conjugated rabbit anti-sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and stained with Hoechst33258.

**Establishment of B cell-specific Pcid2-deficient mice**

A targeting vector was prepared using pMCDT-A(A+T/pau) by inserting the neomycin-resistance gene at the SphI site of Pcid2 genomic DNA from the phage library. LoxP sites were introduced at both ends of the neo gene and the 5' HindIII site of exon I. Homologous recombinants after transfection into TT2 embryonic stem cells were obtained by PCR and Southern

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**FIGURE 2.** Cell-cycle abnormality caused by Pcid2-knockdown. A, Generation of apoptotic and hyperploid cells by Pcid2-knockdown. HeLa cells were treated with siRNAs for 24 h and analyzed by PI staining at days 2 and 6. Polyploid or sub-G1 apoptotic cells were counted. B, Active caspase 3 was induced by Pcid2-knockdown. Scatter plot showing cells stained with a PE-conjugated anti-active caspase 3 mAb (horizontal axis) and a forward scatter plot (vertical axis), and analyzed by gating the active caspase 3-positive cells. C, The appearance of multinucleated cells by Pcid2-knockdown. PI staining was performed and analyzed by confocal laser microscopy. Original magnification ×400. D, Impairment of the spindle assembly checkpoint in Pcid2-knockdown cells. After 40 h of Colcemid treatment, most control siRNA-treated cells accumulated at the G2/M phase with tetraploid DNA content (4N), whereas in Pcid2 siRNA-treated cells the number of hyperploid cells (≥8N) increased significantly. All experiments were repeated three times and the results were reproducible.
blot analysis. B cell-specific targeting by crossing with Cd19-cre knockin mice was used to generate Pcid2 flox/+ (Pcid2 ^lox−/lox−) mice (17).

**Immunohistochemistry**

Spleens from Pcid2 ^lox−/lox− and B cell-specific Pcid2-deficient (B-Pcid2 ^−/−) mice were snap frozen, and cryosections (8 μm thick) fixed with acetone were incubated with rat anti-B220 or anti-monooy and macrophage 1 mAb (Seropec, Dusseldorf, Germany) with Alexa 488-conjugated goat anti-rat IgG (Invitrogen). For two-color staining, sections were incubated with biotin-labeled anti-IgM or anti-Thy1.2 mAb with Alexa 594-conjugated streptavidin (Invitrogen). The slides mounted in 2.5% 1,4-diazabicyclo[2.2.2]octan/80% glycerol were observed using a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan).

**Introduction of cre recombinase into Pcid2 ^lox/fl −/− Cells**

The cre recombinase gene was inserted into a pPB-IRE-SEQ retroviral vector (Strategene, La Jolla, CA), which was then transferred into PLAT-E ecotropic retrovirus packaging cells (from Dr. T. Kitamura, University of Tokyo, Tokyo, Japan) by FuGENE 6 (Roche Diagnostics) to recover retroviruses at day 2. LPS-stimulated spleen B cells from Pcid2 ^lox/fl −/− mice were infected with the retroviruses with polybrene 8 μg/ml; Sigma) for 2 d. The GFP+ cells were sorted using JSAN, and the apoptotic cells were analyzed next day using PE-conjugated Annexin V (BD Biosciences).

**Statistical analysis**

Differences examined by Student t test were considered significant when p < 0.05.

**Results**

**Stage-dependent expression of Pcid2 transcripts during B lineage cell differentiation**

The expression of Pcid2 transcripts (2.4 kb) varies among various tissues and organs. Quantitative measurement of Pcid2 transcripts showed differences among the stages of B lineage cell differentiation (Fig. 1). From normal mice, bone marrow cells were first gated with IgM/B220 and then with CD43/B220 to obtain pro-B (B220^high/IgM^ high CD43^ high) fraction and pre-B cells (B220^high/IgM^ intermediate CD43^ intermediate) fraction, or with IgM/IgD for immature B cells (B220^ high IgM^ high IgD^ high) fraction of B lineage differentiation (Fig. 1, top left), and spleen cells were sorted with B220/AA4.1 to obtain T1 and T2 (by IgM/IgD expression), and FO and MZ (by CD21/CD23 expression) B cells (Fig. 1, top right). In comparison with pro-B (1.0) cells, pre-B (13.5) and immature B (18.0) cells had greater levels of Pcid2/GAPDH, and markedly greater levels of Pcid2/GAPDH were observed in T1 (29.0) and FO (30.0) B cells, but not in T2 (6.5) and MZ (4.5) B cells during differentiation (Fig. 1, bottom). These results suggest that the regulation of Pcid2 is important for maintaining B cells during the transition from early precursor cells to mature surface Igα+ B cells through multiple genetic alterations of Ig genes.

**Effect of Pcid2-knockdown on cell cycling**

We first examined the effect of Pcid2 knockdown on cell cycling in HeLa cells. The Pcid2-knockdown strategy by siRNA succeeded in reducing the Pcid2 transcript levels (Supplemental Fig. 1A), which resulted in a decrease in the Pcid2 protein in a reporter system using a stable cDNA transfectant expressing a tagged-Pcid2 protein (Supplemental Fig. 1B). Based on PI staining, Pcid2-knockdown induced abnormalities in cell cycling, particularly at day 6, with an increase in apoptotic (sub-G1) and hyperploid cells (Fig. 2A). The flow cytometric profile of Pcid2-knockdown cells was different from that of ganp-knockdown cells (12). The phenotype was similar to that caused by the mitotic catastrophe or im-

![FIGURE 3](http://www.jimmunol.org/DownloadedFrom) Selective decrease of MAD2 in Pcid2-knockdown cells. A, The expression levels of various mitotic checkpoint proteins (MAD2, MAD1, BUB1, BUB3, CENP-E, and p55CDC) and cell-cycle-associated proteins (cyclin A, cyclin B, and CDK1) in Pcid2-knockdown cells. siRNA-treated cells were cultured for 72 h and analyzed by immunoblotting. β-actin was used as a loading control. B, MAD2 transcripts in Pcid2-knockdown cells. The levels of MAD2 transcripts normalized to GAPDH mRNA were compared between control and Pcid2 siRNA-treated cells using real-time RT-PCR. The relative expression level (%) is shown based on the control siRNA-treated cells. Each group shows the results of three samples per experiment and repeated four times. C, The expression levels of MAD2 transcripts in the nuclei (N) or the cytoplasm (C) were measured in control or Pcid2 siRNA-treated cells by real-time RT-PCR. Cytoplasmic and nuclear RNAs were extracted from cells after treatment with siRNA for 60 h. Each group shows the results of three samples per experiment and repeated four times. D, In situ RNA hybridization of MAD2 mRNA. MAD2 mRNA was detected in siRNA-treated cells with the digoxigenin-conjugated specific RNA probe; then DNA was visualized with Hoechst33258 staining. Arrows indicate the focal accumulation of MAD2 mRNA in the cytoplasm of control siRNA-treated cells. The control BUB3 expression was not impaired in Pcid2-knockdown cells. All experiments were repeated three times and the results were reproducible. Original magnification ×400. Scale bars, 5 μm.
paired mitotic checkpoint function observed in MAD2-knockdown cells (18). Pcid2-knockdown cells had increased caspase 3 activity (Fig. 2B), leading to apoptosis and impaired spindle assembly checkpoint with multinucleated cells (Fig. 2C). Colcemid increased the generation of aneuploid cells carrying with 4N (Fig. 2D), indicating that the spindle assembly checkpoint was impaired in Pcid2-knockdown cells. This abnormal cell cycling was also consistently observed when we used another sequence set of Pcid2 siRNA (data not shown).

Decrease in MAD2 transcript in Pcid2-knockdown cell cytoplasm

Pcid2-knockdown markedly suppressed MAD2 expression levels but did not affect the expression of MAD1, BUBR1, BUB3, CENP-E, p55CDC, cyclin A, cyclin B1, and CDK1 in comparison with the β-actin control (Fig. 3A). Pcid2-knockdown significantly reduced the MAD2 expression level in comparison with control cells (p < 0.003; Fig. 3B). These results indicated that Pcid2 is

**FIGURE 4.** Establishment of B-Pcid2−/− mice. A, Targeting Pcid2 gene in B cells. Exon I (Ex I) was deleted in B cells by crossing Cd19-cre knockin mice and the floxed mice of Ex I with the Pcid2 gene. B, The mice with the hetero-targeted allele (3.5-kb band after EcoRI/XbaI digestion) in addition to the 9.0-kb wild-type allele was confirmed by Southern blot analysis with Probe B. C, Transcription of Pcid2 was impaired in pre-B and immature B cells, but not in pro-B cells, in the bone marrow of B-Pcid2−/− mice. RNA was purified after cell sorting as in Fig. 1. Each group shows the results of three samples per experiment and repeated four times. E, EcoRI; H, HindIII; Xb, XbaI.

**FIGURE 5.** Severe reduction of mature B cells in the spleen of B-Pcid2−/− mouse. Spleen B cells initially express CD19 and differentiate into IgM+IgD+ immature, IgM+IgD− mature, CD21loCD24hi (T1), CD21hi CD24hi (T2/MZ), CD21−/−CD24lo (FO), CD21loCD23hi (FO), and CD21hiCD23lo (MZ) B cells. The numbers of B lineage cells per spleen were calculated as B220+ (B cells), B220− (non-B cells) (left), and the various stages in the spleen (right), and indicated that mature B cell survival was impaired in B-Pcid2−/− mice. Each group shows the results of two samples per experiment and repeated four times.

**FIGURE 5.** Severe reduction of mature B cells in the spleen of B-Pcid2−/− mouse. B cells in the spleen of B-Pcid2−/− mouse. Spleen B cells initially express CD19 and differentiate into IgM+IgD+ immature, IgM+IgD− mature, CD21loCD24hi (T1), CD21hi CD24hi (T2/MZ), CD21−/−CD24lo (FO), CD21loCD23hi (FO), and CD21hiCD23lo (MZ) B cells. The numbers of B lineage cells per spleen were calculated as B220+ (B cells), B220− (non-B cells) (left), and the various stages in the spleen (right), and indicated that mature B cell survival was impaired in B-Pcid2−/− mice. Each group shows the results of two samples per experiment and repeated four times.
selectively required for expression of the mitotic checkpoint protein MAD2. *Thp1*-deficient yeast cells have severely impaired mRNA nuclear export (14). To examine the effect of *Pcid2*-knockdown, we measured the *MAD2* transcript levels in cytoplasmic and nuclear fractions. Compared with the control, *Pcid2*-knockdown significantly reduced the level (60%; *p* < 0.001) of cytoplasmic *MAD2* transcript with an apparent decrease in the relative level based on the cytoplasmic to nuclear ratio (5.6 to 2.3; Fig. 3C). This finding was supported by in situ RNA hybridization, which showed a marked decrease in *MAD2* mRNA in the cytoplasm compared with that in the nucleus (Fig. 3D). In control siRNA-treated cells, *MAD2* appeared to accumulate at perinuclear foci in the cytoplasm in the merged image (Fig. 3D, arrows), but neither a dense accumulation in the cytoplasm nor the MAD2 signal in cells was detected by *Pcid2*-knockdown. Expression of the control *BUB3* mRNA was comparable in both cells.

We examined alternative explanations that would account for *MAD2* downregulation in *Pcid2*-knockdown cells. An inhibitor of proteasome protein degradation, MG132, did not affect *MAD2* expression, ruling out the involvement of ubiquitin ligase-dependent degradation (data not shown). Expression of the upstream transcription factor for the *MAD2* gene, REST (19), was not affected by *Pcid2*-knockdown (Supplemental Fig. 2). Thus, *Pcid2* is essential for the expression of the *MAD2* transcript, presumably through the regulation of mRNA metabolism.

**Pcid2 is critical for B cell survival in vivo**

We investigated whether *Pcid2* is required for B cell survival during differentiation. A lack of SAC3 does not affect yeast cell survival and only marginally affects the growth rate (20). *B-Pcid2*<sup>-/-</sup> mice were prepared by conditional gene targeting using *Cd19-cre* and floxed *Pcid2* genes (Fig. 4A,4B). Real-time RT-PCR revealed a marked reduction in *Pcid2* transcripts in pre-B and immature B cells in *B-Pcid2*<sup>-/-</sup> mice (Fig. 4C). In Fig. 5, *Pcid2*-knockout B cells had severe defects in the number of spleen B cells in the T1, T2, FO, and MZ stages compared with those of control mice (right). Most of the mature stage B cells were severely affected, notably displaying severe decrease of FO B cells. Immunohistochemistry showed a marked reduction of B220<sup>+</sup> (green) cells particularly at FO regions in the spleen (Fig. 6b), displaying smaller size FO regions surrounded by MOMA1<sup>+</sup> (green) cells (Fig. 6d) in comparison with the control *Pcid2*<sup>fl/+</sup> spleen (Fig. 6a, 6c). Finally, we examined the effect of *Pcid2* depletion in B cells by retrovirus introduction of cre recombinase. Spleen B cells from

**FIGURE 6.** Formation of follicles in the spleen. Splenic sections were double-immunostained with B220 (green) and Thy1 (red) (a, b), or MOMA1 (green) and IgM (red) (c, d). Alexa 488-conjugated goat anti-rat IgG and Alexa 549-conjugated streptavidin were used as secondary reagents. MOMA1<sup>+</sup> cells demarcated the smaller lymphoid follicles in B-*Pcid2*<sup>-/-</sup> mice. Original magnification ×400. Scale bar, 300 μm.

**FIGURE 7.** Effect of *Pcid2* depletion in B cells. Spleen B cells from *Pcid2*<sup>fl/fl</sup> mice were stimulated with LPS for 24 h and infected with retroviruses carrying cre recombinase. A and B, Mouse *Pcid2* and *MAD2* transcripts were measured by real-time RT-PCR. RNA was purified after cell sorting as in Fig. 1. C, Apoptotic cells were compared by Annexin V staining. D, Cell-cycle analysis by PI staining. Subdiploid cells were shown as apoptotic cells, and the polyploid cells were shown as indicated. E, Expression level of *MAD2* transcripts in pre-B cells of the bone marrow from *Pcid2*<sup>fl/+</sup> and *B-Pcid2*<sup>-/-</sup> mice.
numbers of bone marrow and spleen cells from MAD2+/−/ rapid proliferating cells, such as blood vessel cells. The absolute important for protecting or promoting the replication fidelity of assembly checkpoint, and the interaction between ER onset (28), suggesting that ER checkpoint molecule (MAD2, MAD1, BUBR1, or BUBR3) causes and in the delay of anaphase. The loss of a spindle assembly point is involved in the mitotic arrest caused by spindle poisons and in the delay of anaphase. The loss of a spindle assembly checkpoint molecule (MAD2, MAD1, BUBR1, or BUBR3) causes the embryonic death in mice. An increased incidence of constitutive tumors was revealed in MAD2-haplodeficient mice (MAD2−/−) or MAD1−/− mice but not in BUBR1−/− or BUBR3−/− mice (21). The deletion of one MAD2 allele results in a defective mitotic checkpoint in mammalian cells with an increased rate of chromosome missegregation (22).

Other aspects of MAD2 protein have been demonstrated. MAD2 interacts with various signal transduction molecules, such as the insulin receptor (23), TNF-α convertase (24), estrogen receptor (ER) (25), common β-chain of GM-CSF receptor (26), and c-Kit (27). ERβ and MAD2 interact in the absence or presence of estradiol (25). MAD2 protein function is essential for the timing of anaphase onset (28), suggesting that ERβ is a component of the spindle assembly checkpoint, and the interaction between ERβ and MAD2 is important for protecting or promoting the replication fidelity of rapidly proliferating cells, such as blood vessel cells. The absolute numbers of bone marrow and spleen cells from MAD2−/− are decreased, and the cycling status of hematopoietic progenitor cells is slower in their synergistic response to stem cell factor and GM-CSF (27). Mantel and Broxmeyer (29) suggested that MAD2, interacting with c-Kit, is involved in cell-cycle regulation governing the proper sequential progression of mitotic events involving chromosome alignment, segregation, and timing of mitotic events, particularly in rapidly proliferating hematopoietic cells.

The mode of recruitment to the kinetochore is different between MAD2 and BUB1/BUBR1 (10). Although MAD2 is not responsive to spindle tension compared with BUB1 and BUBR1, MAD2 is sensitive to microtubule attachment to the kinetochore, suggesting that mammalian cells contain at least two discrete checkpoints at metaphase that are regulated by different sets of checkpoint proteins. Previous experiments demonstrated that MAD1 and MAD2 are bound to nuclear basket structures of nuclear pores embedded in the nuclear envelope in the interphase (30). The mRNA export factor, RAEl/GLE2 (RNA export 1/ GLFG [glycline-leucine-phenylalanine-glycine] lethal), and the spindle assembly checkpoint proteins, BUB1 and BUBR1, share a GLE2p-hinging sequence (GLEBS)-like motif, similar to the nuclear complex protein NUP98 (31). Although whether the nuclear basket-bound MAD1 and MAD2 proteins play roles in mRNA export remains to be determined, our study provides evidence that these checkpoint proteins associate with both kinetochores and nuclear pores.

**Discussion**

In mammalian somatic cells, the MAD2-dependent spindle checkpoint is involved in the mitotic arrest caused by spindle poisons and in the delay of anaphase. The loss of a spindle assembly checkpoint molecule (MAD2, MAD1, BUBR1, or BUBR3) causes the embryonic death in mice. An increased incidence of constitutive tumors was revealed in MAD2-haplodeficient mice (MAD2−/−) or MAD1−/− mice but not in BUBR1−/− or BUBR3−/− mice (21). The deletion of one MAD2 allele results in a defective mitotic checkpoint in mammalian cells with an increased rate of chromosome missegregation (22).

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B cells are continuously generated in the bone marrow throughout the mammalian life span, and this generation is absolutely dependent on the increase in the number of B cells and the expression of the unique BCR after genetic alterations in the Ig genes (2–8). The major genetic alteration occurs in pro-B cells undergoing D-J rearrangement of the IgH chain gene and later at pre-B cells undergoing V-J rearrangement of the IgL chain gene. The checkpoint around the pre-B cells prohibits the transition of self-reactive B cell clones into peripheral lymphoid organs by clonal deletion, receptor editing, or clonal anergy (32). Nearly half of the newly generated B cell clones are estimated to react with self-Ags (33). The expression of RAG proteins edits the IgV region in such B cells and changes the specificity of the BCR with dsDNA breaks. MAD2 might be highly required for pre-B cells.

Immature B cells appear in the peripheral lymphoid organs as T1 cells, in which the expression of RAG proteins seems not to be completely shutdown (34). The early migratory T1 cells might encounter exogenous Ags, which would induce rapid proliferation because of costimulatory signals provided in the peripheral lymphoid organs. Pcid2 transcription is at greater levels in these stages of B lineage differentiation, and presumably maintains the level of MAD2 expression required for the proliferation of B lineage cells, similar to hematopoietic progenitor cells stimulated synergistically with stem cell factor and GM-CSF. B cells accumulated in the follicles undergo cell proliferation at an extraordinarily high rate. The FO B cells express a high level of activation-induced cytidine deaminase, which induces cytidine deamination leading to genetic alterations of V region SHM and CSR of the Ig gene. The FO B cells might require higher Pcid2 expression to ensure the cell-cycle transition through the mitotic checkpoint for cells with DNA alterations. In contrast, T2 cells and MZ B cells do not express similarly high levels of the Pcid2 transcript.

These findings demonstrate that mitotic checkpoint function might be differentially regulated at different stages of B-lineage cell differentiation with genetic alterations at Ig genes. Pcid2 is critical for the selective regulation of MAD2. No changes occurred at other mitotic checkpoint proteins in the Pcid2-knockdown cells, indicating that MAD2 regulation by Pcid2 might be a key event at the mitotic checkpoint in B cells, whose genomic alterations occur during differentiation in a stage-dependent manner. These results help to elucidate the molecular mechanism underlying the regulation of Ig gene diversification during clonal expansion, activation of Ag-specific B cells, and the maintenance of memory B cells.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


**Supplementary Table 1: Sequences of primers**

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<td>GCCATTCAGAGATAGGGCAGT</td>
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<tr>
<td>GAPDH acceptor probe</td>
<td>CATCAAGAGGGGAGGACAGCATCT</td>
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Supplementary Figure 1. Reduced expression of Pcid2 by Pcid2-knockdown. A, HeLa cells were treated with siRNA and incubated for 48 h. The levels of Pcid2 transcripts by the ratio with GAPDH were compared between control and Pcid2 siRNA-treated cells. GAPDH was used as an internal control. B, HeLa cells, stably expressing Flag-Pcid2, were treated with control or Pcid2 siRNA. After 60 h, the lysates were subjected to SDS-PAGE and immunoblotting by anti-Flag mAb. β-actin was used as a loading control.
Supplementary Figure 2. Effect of Pcid2-knockdown on the expression of REST, a transcriptional repressor for MAD2 transcription. HeLa cells were treated with control or Pcid2 siRNA. After 60 h, cell lysates were subjected to SDS-PAGE and immunoblotting with anti-REST polyclonal Ab (Millipore) as in Fig. 3A. β-actin was used as a loading control.