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Generation of mice deficient in RNA-binding motif protein 3 (RBM3) and characterization of its role in innate immune responses and cell growth

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ABSTRACT

The activation of innate immune responses is critical to host defense against microbial infections, wherein nucleic acid-sensing pattern recognition receptors recognize DNA or RNA from viruses or bacteria and activate downstream signaling pathways. In a search for new DNA-sensing molecules that regulate innate immune responses, we identified RNA-binding motif protein 3 (RBM3), whose role has been implicated in the regulation of cell growth. In this study, we generated *Rbm3*-deficient (*Rbm3*$^{-/-}$) mice to study the role of RBM3 in immune responses and cell growth. Despite evidence for its interaction with immunogenic DNA in a cell, no overt phenotypic abnormalities were found in cells from *Rbm3*$^{-/-}$ mice for the DNA-mediated induction of cytokine genes. Interestingly, however, *Rbm3*$^{-/-}$ mouse embryonic fibroblasts (MEFs) showed poorer proliferation rates as compared to control MEFs. Further cell cycle analysis revealed that *Rbm3*$^{-/-}$ MEFs have markedly increased number of G2-phase cells, suggesting a hitherto unknown role of RBM3 in the G2-phase control. Thus, these mutant mice and cells may provide new tools with which to study the mechanisms underlying the regulation of cell cycle and oncogenesis.
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1. Introduction

Nucleic acids from viruses or bacteria potently activate immune responses through nucleic acid-sensing pattern recognition receptors (PRRs), namely, membrane-bound Toll-like receptors (TLRs) such as TLR3, TLR7 and TLR9, and cytosolic receptors, which include retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), DNA-dependent activator of IRFs (DAI) [1–4]. The hallmark of the activation of these receptors is the induction of genes encoding type-I IFN and proinflammatory cytokine gene expression [1–4]; however, the detailed signaling pathways and mechanisms of gene activation following activation of these receptors still remain elusive. Currently, the DNA-sensing system remains less well known than the RNA-sensing system, perhaps suggesting a more complicated system, and there is evidence for an as yet unknown cytosolic DNA sensor(s) that activates the signaling pathway for proinflammatory cytokine genes [3–6]. It has also been known that TLR9 (and also TLR7) needs to translocate from the endoplasmic reticulum to endosomes/lysosomes upon stimulation [7–11], a process which requires Unc-93 homolog B1 (UNC93B1) is involved in the translocation [7,9,11]. How the trafficking signal is activated and regulated still remains to be clarified, and it is therefore possible that another DNA-sensing molecule participates in the regulation of the entire TLR9 signaling process.

To gain new insights into the complexity of the DNA-sensing mechanisms in the cell, we searched for immunogenic DNA-binding proteins and identified RNA-binding motif protein 3 (RBM3). RBM3 was originally described as a nuclear protein with one RNA recognition motif [12]. The *Rbm3* gene is located on chromosome X of mice (also Xp11.23 in humans) and is ubiquitously expressed in a variety of cell types but is expressed at relatively high levels in cancer cell lines [13,14]. Because of its potential role in oncogenesis, RBM3 has been studied in relation to cell growth activity and viability. It has been reported that the suppression of RBM3 expression by siRNA knockdown in cancer cell lines results in an inhibition of cell proliferation and increases susceptibility to anti-cancer drugs, whereas the overexpression of RBM3

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promotes cell proliferation [14,15]. Thus, although these observations are intriguing, the physiological role of RBM3 in cell growth control still remains unclear. To study the role of RBM3 in immunity and cell growth, we newly generated *Rbm3*-deficient (*Rbm3*$^{-/-}$) mice. Our results indicate that RBM3 is not involved in the regulation of nucleic acid-mediated cytokine gene induction, but it does play a critical role in cell cycle regulation. We discuss our findings in terms of the utility of these mutant mice for the analysis of innate immune signaling, cell cycle, and oncogenesis.

2. Materials and methods

2.1. Generation of Rbm3-deficient mice

Genomic DNA containing *Rbm3* gene was amplified by polymerase chain reaction (PCR) from 129/Sv genome DNA. An *Rbm3*-targeting construct that replaces exon 3–6 with a phosphoglycerate kinase promoter-driven neomycin-resistant gene was transfected into E14–1 ES cells. Homologous recombinants were injected into C57BL/6 blastocysts. The resulting chimera mice were intercrossed heterozygous F_1 progenies to obtain *Rbm3*$^{-/-}$ mice as described previously [16]. C57BL/6J mice were purchased from CLEA Japan.

2.2. Screening for CpG-B ODN-binding protein

The screening of CpG-B DNA-binding proteins was performed by ZOEGENE Corporation (Japan) with the cell-free protein display (CFPD) method that is developed on the basis of the technology linking between mRNA and their translated protein through puromycin linker [17]. For this screening, mRNA purified from bone marrow cells cultured with the Flt3-ligand was used. CpG-B DNA-binding proteins were enriched using a column filled with CpG-B DNA-conjugated beads. The binding proteins were assigned by reading the sequence of complementary DNA (cDNA) from mRNA attached to the protein.

2.3. Pull-down assay

Pull-down assay was performed as described previously [2]. Briefly, 300 μg of Dynabea ds M-280 Streptavidin (Invitrogen) and 60 pmol of 5′-biotinylated CpG-B DNA were mixed and incubated for 15 min at room temperature. After three washes with a buffer [0.5 M Tris–HCl (pH 7.5), 2.5 M NaCl, and 0.5 M EDTA], the Dynabeads-CpG-B DNA complex was equilibrated and resuspended in a lysis buffer [50 mM Tris–HCl (pH8.0), 300 mM NaCl, 20 mM β-glycerophosphate, 2 mM EDTA, 1 mM Na_3VO_4, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM APMSF]. Whole cell lysate (90 μg) was mixed with 30 μl of the complex and incubated for 30 min at room temperature. Pulled-down samples were washed with the buffer three times and subjected to immunoblot analysis.

2.4. Fluorescence microscopy

RAW 264.7 cells and mouse embryonic fibroblasts (MEFs) (1×10^5 cells) were plated on a 15-mm micro cover glass (Matsunami Glass). RAW264.7 cells expressing YFP-tagged RBM3 were analyzed using an Olympus FV-1000 confocal microscope (Olympus). LysoTracker Red was purchased from Invitrogen. MEFs were fixed with PBS containing 4% paraformaldehyde, permealized with 0.2% Triton X, and then stained with antibodies. Primary antibodies for anti-mouse RBM3 (Abgent) and anti-phospho-Histone H3 at Ser10 (H3-pS10) (Millopore) were used. Secondary antibodies for FITC-conjugated donkey anti-mouse IgG antibody and Cy3-conjugated goat anti-rabbit IgG were purchased from Invitrogen. Images were captured using an IL-X71 Applied Precision Deltavision microscope (Olympus) and processed with DeltaVision SoftWorx software (Applied Precision).

2.5. RNA analysis

Total RNA isolation and cDNA synthesis were performed as described previously [2]. mRNA was purified from total RNA using Oligotex-dT30 (TaKaRa). Quantitative real-time PCR (qRT-PCR) analysis was carried out using LightCycler480 and the SYBRGreen system (Roche). The primer sequences for GAPDH, IL-6, IFN-α4, IFN-β, and TNF-α have been described [2]. The following primers for RBM3 were used: 5′-CCTTCACAAACCCAGAGCAT-3′ (sense) and 5′-TAGACCGCCCATACCCATA-3′ (anti-sense). All data are presented as relative expression units after normalization to GAPDH expression level. Additional information is in Supplementary Materials and Methods.

3. Results

3.1. Identification of RBM3 as an immunogenic DNA-associated protein

We first sought to identify proteins involved in immunogenic DNA recognition systems and performed biochemical screening by the cell-free protein display (CFPD) method [17] using purified mRNAs from dendritic cells (DCs) differentiated from bone marrow cells cultured with the Flt3-ligand (Supplementary Fig. S1A). In this screening, we identified RBM3 as the most prominent protein that binds to CpG-B DNA, a TLR9 agonist (Supplementary Fig. S1B). Previously, RBM3 was studied in the context of oncogenesis but not immune regulation [13,14,18]. We further studied the interaction of RBM3 with CpG-B DNA by co-precipitation assay. Protein lysates from HEK293T cells transiently expressing FLAG-tagged RBM3 was subjected to pull-down assay with biotin-labeled CpG-B DNA and streptavidin-conjugated magnetic beads. As shown in Fig. 1A, RBM3 was precipitated with the DNA and this precipitation was inhibited by an excess amount of nonconjugated CpG-B DNA. Thus, these findings indicate that RBM3 indeed binds to immunogenic CpG-B DNA *in vitro*.

It has been reported that RBM3 mainly localizes to the nucleus when cell is at rest, but otherwise shuttles between the cytoplasm and the nucleus [14]. Since CpG-B DNA localizes in the lysosomal compartment in the cytoplasm [8,10], we next examined whether the cellular localization of RBM3 is regulated following CpG-B DNA stimulation. To test this, we stimulated RAW264.7 cells with CpG-B DNA and measured RBM3 protein levels in the nuclear and cytoplasmic fractions. Interestingly, the RBM3 protein level in the cytoplasmic fraction increased after CpG-B DNA stimulation in a time-course-dependent manner, while that in the nuclear fraction decreased (Fig. 1B). Notably, the accumulation of RBM3 in the cytoplasmic fraction was not induced when the cells were stimulated by the TLR4 agonist lipopolysaccharide (LPS), suggesting that the recruitment of RBM3 into the cytoplasm specifically occurs upon CpG-B DNA stimulation (Fig. 1C). We also examined the co-localization of RBM3 with CpG-B DNA by fluorescence microscopy. YFP-tagged RBM3 co-localized with rhodamine-labeled CpG-B DNA in RAW264.7 cells (Fig. 1D) and also merged with LysoTracker, a lysosomes marker (Fig. 1E), suggesting that RBM3 binds to CpG-B DNA in lysosomes, where TLR9 signaling occurs [8,10]. Collectively, these observations suggest the potential involvement of RBM3 in the CpG-B DNA-TLR9 signaling process.

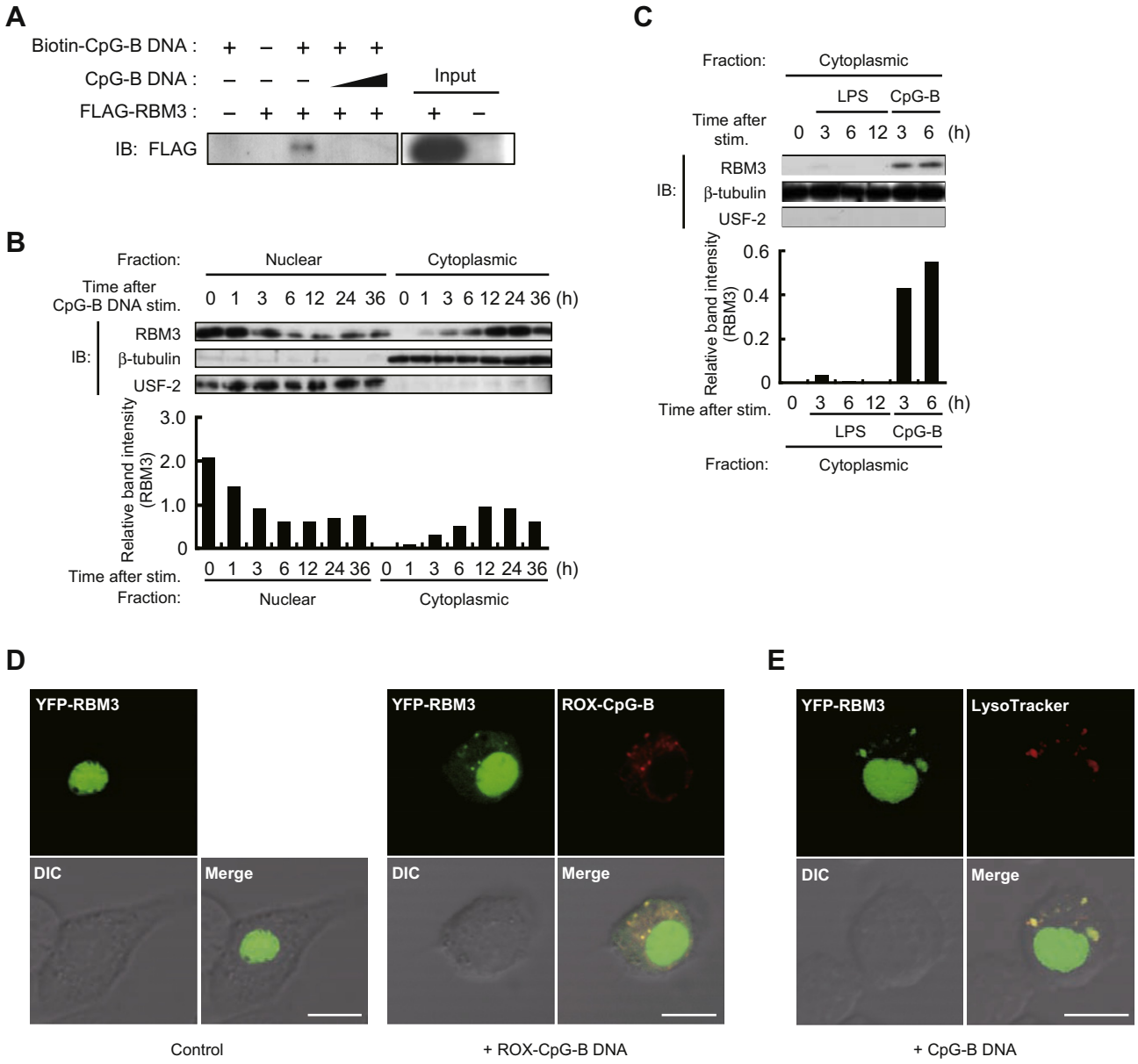

Fig. 1. Identification of RBM3 as CpG-B DNA-binding protein. (A) Binding of RBM3 to CpG-B DNA. Pull-down assay was performed using biotin-conjugated CpG-B DNA, streptavidin-conjugated magnet beads, and cell lysates from HEK293T cells expressing FLAG-tagged RBM3. Whole cell lysates (20 μg) were loaded as expression control (Input) of FLAG-tagged RBM3. As a competitor, nonbiotinylated CpG-B DNA (15 or 30 μM) was added. (B, C) RAW264.7 cells were stimulated with CpG-B DNA (1 μM) or LPS (100 ng/ml) for the indicated periods. Protein (20 μg) from the nuclear or cytoplasmic fraction was subjected to immunoblot analysis. β-tubulin and USF-2 are shown as the cytoplasmic and nuclear fraction markers, respectively. The band intensity of the RBM3 protein is also shown in the lower panel. (D) RAW264.7 cells expressing YFP-tagged RBM3 (green) were left unstimulated (left panels) or stimulated with rhodamine-conjugated CpG-B DNA (ROX-CpG-B; red) (1 μM) for 1 h (right panels). Fluorescence images were observed by a confocal microscope. (E) RAW264.7 cells expressing YFP-tagged RBM3 (green) were stimulated with CpG-B DNA (1 μM) for 1 h, and then incubated with LysoTracker (red) in the last 20 min of culture. Fluorescence images were observed by a confocal microscope. Scale bars indicate 10 μm. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

3.2. Generation of Rbm3-deficient mice

The above observations prompted us to examine the role of RBM3 in CpG-B DNA-mediated innate immune responses and cell growth control by generating mice deficient in *Rbm3* (*Rbm3*$^{-/-}$ mice). The mouse *Rbm3* gene comprises seven exons spanning about 8 kb on chromosome X (Fig. 2A). In the generation of *Rbm3*$^{-/-}$ mice, we employed the standard homologous recombination procedure to disrupt *Rbm3* by deleting the exons 4 and 5, and part of exons 3 and 6 (Fig. 2A). The absence of RBM3 was confirmed by Southern blot, RNA blot, and immunoblot analysis of *Rbm3*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Fig. 2B). *Rbm3*$^{-/-}$ mice fertilized normally and showed no significant differences in size and behavior compared with the wild-type (WT) littermates (Fig. 2C). When lymphocyte populations were analyzed in the thymus and spleen of *Rbm3*$^{-/-}$ mice, no significant abnormalities were found neither in CD4$^+$ or CD8$^+$ T cells in the thymus and spleen, nor in conventional DCs (cDCs) (CD11c$^+$B220$^-$), plasmacytoid DCs (pDCs) (CD11c^{int}B220$^+$), NK cells (CD3$^-$NK1.1$^+$), or NKT cells (CD3$^+$NK1.1$^+$) in the spleen (Fig. 2D). These findings suggest that the loss of RBM3 does not affect the development of mice and differentiation of immune cells.

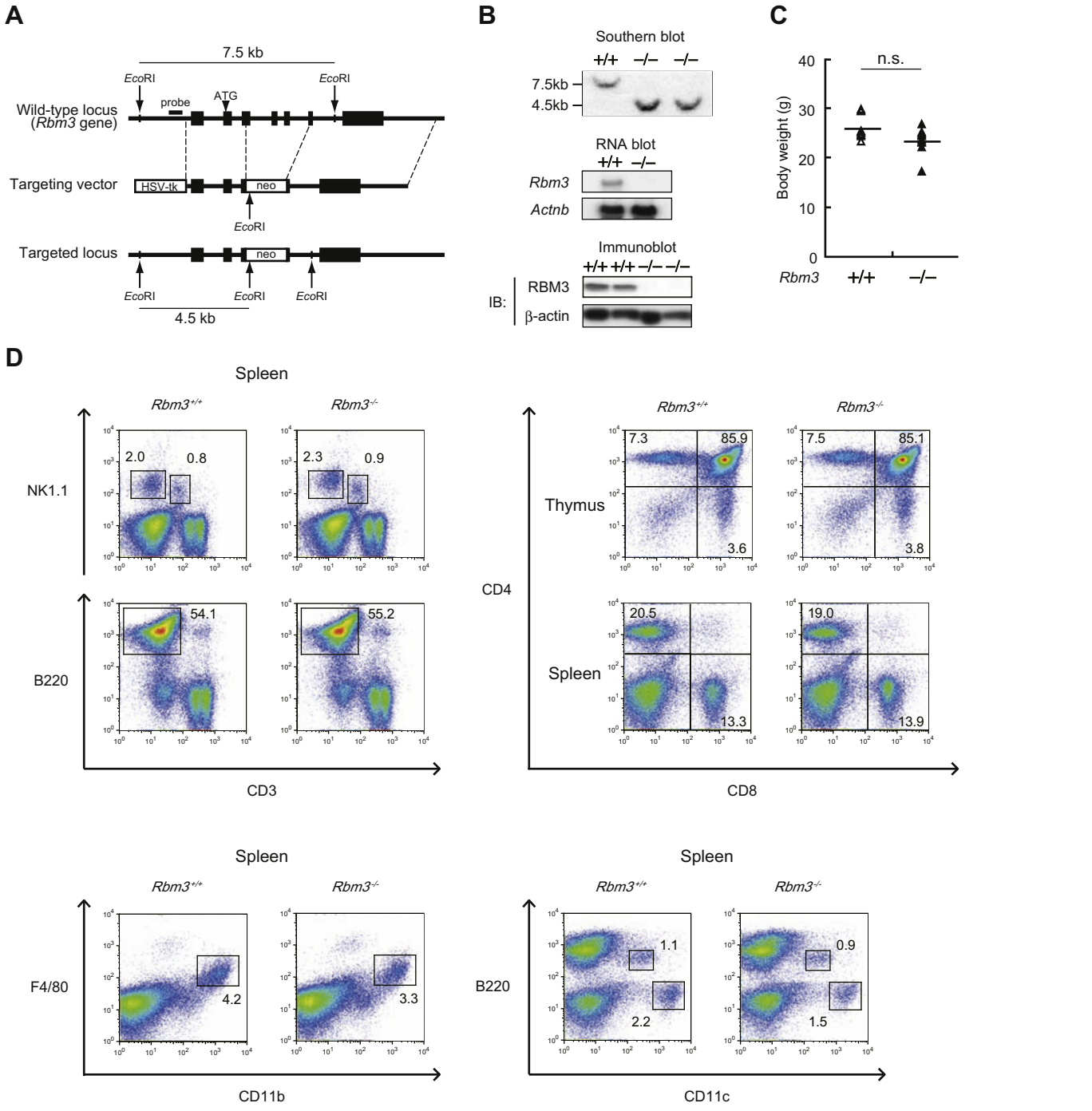

Fig. 2. Generation of *Rbm3*-deficienct mice. (A) Partial restriction map of the wild-type (WT) locus and targeting strategy for *Rbm3* disruption. The region containing exons 4 and 5 and part of exons 3 and 6 were replaced with a pgk-neo cassette. The herpes simplex virus thymidine kinase (HSV-tk) cassette was attached at the 5′end of the targeting vector as a negative selection marker. The probe for genomic Southern blot analysis is indicated by a thick bar. (B) Southern blot analysis of *Eco*RI-digested genomic DNA isolated from WT (+/+) and homozygous mutant (-/-) mice (upper panel). RNA blot analysis was performed using total RNA (5 μg) from WT (+/+) or homozygous mutant (-/-) MEFs (middle panel). Whole cell extracts (30 μg) from WT (+/+) or homozygous mutant (-/-) MEFs were subjected to immunoblot analysis to detect the RBM3 protein (lower panel). (C) The body weight of male *Rbm3*$^{-/-}$ and WT littermates was determined at 8 weeks of age ($n = 8$). (D) Single cell suspensions were prepared from the thymus and spleen from 8-week-old WT and *Rbm3*$^{-/-}$ mice and stained with the indicated combination of the following fluorochrome-conjugated antibodies: anti-CD8 phycoerythrin (PE), anti-CD4 allophycocyanin (APC), anti-CD3 fluorescein isothiocyanate (FITC), anti-B220 PE, anti-NK1.1 PE, anti-F4/80 FITC, anti-CD11b PE, and anti-CD11c APC antibodies. The numbers represent the percentage of cells contained in each region.

3.3. Role of RBM3 in nucleic acid-mediated innate immune responses

Because RBM3 associates with CpG-B DNA, we next examined the effect of RBM3 deficiency on CpG-B DNA-mediated innate immune responses. When cDCs differentiated from the bone marrow cells of *Rbm3*$^{-/-}$ mice with GM-CSF were stimulated with CpG-B DNA, IL-6 and TNF-α mRNAs were equally induced as compared with the WT cells (Fig. 3A). Consistently, the induction of these cytokines by CpG-B DNA at various doses was essentially similar between *Rbm3*$^{-/-}$ and WT cDCs as monitored by ELISA (Supplementary Fig. S2). Since pDCs produce type-I IFNs at high levels upon the activation of TLR9 [19], we also examined *Rbm3*$^{-/-}$ and WT pDCs for

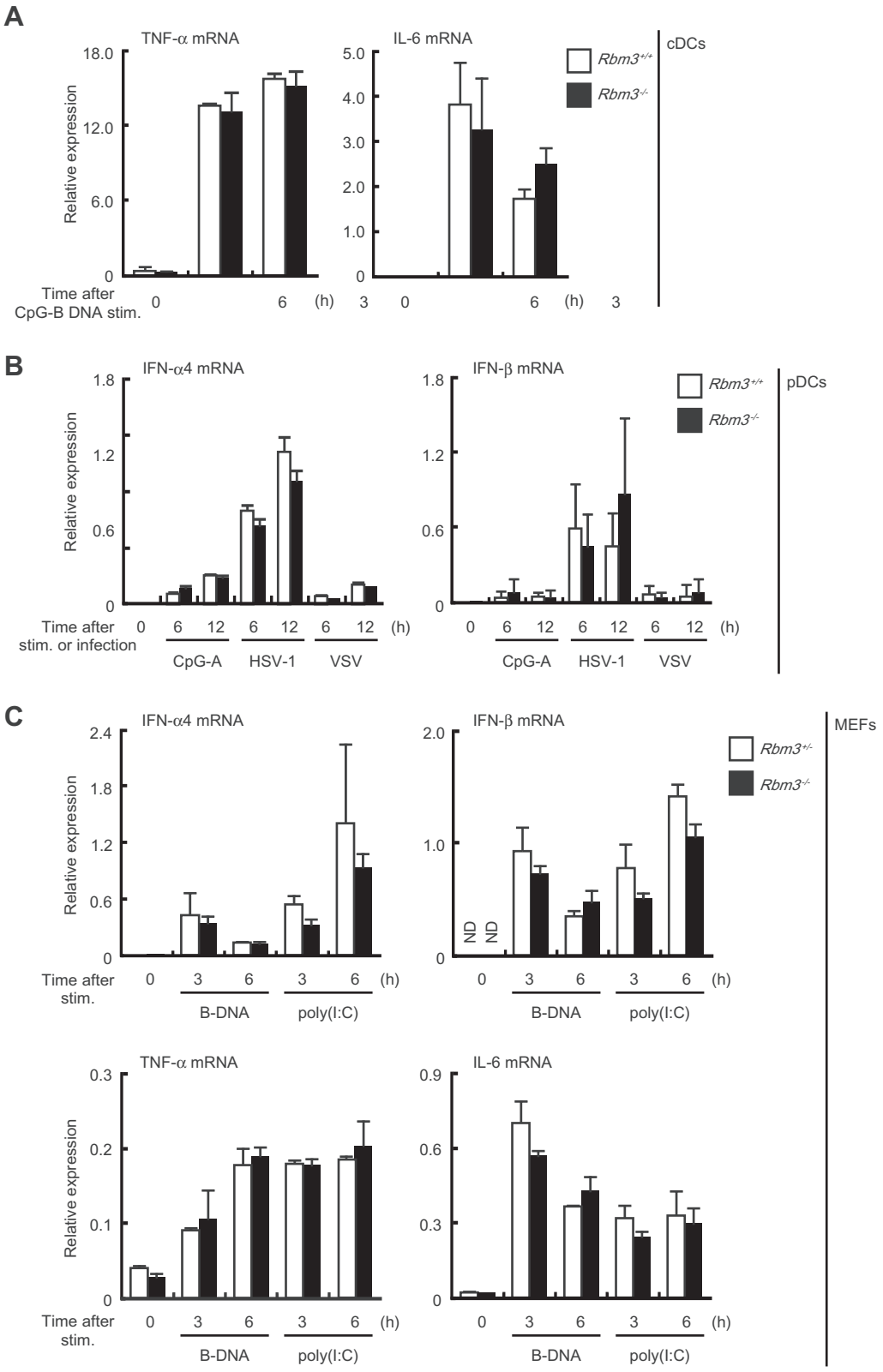

Fig. 3. Effect of RBM3 deficiency on nucleic acid-mediated innate immune responses. (A) Bone marrow-derived cDCs from wild-type (WT) and *Rbm3*$^{-/-}$ mice were stimulated with CpG-B DNA (1 μM) for the indicated time periods. TNF-α and IL-6 mRNA expression levels were examined by qRT-PCR. (B) Bone marrow-derived pDCs from WT and *Rbm3*$^{-/-}$ mice were stimulated with CpG-A DNA (1 μM) or infected with HSV-1 (m.o.i. of 1) or VSV (m.o.i. of 1) for the indicated time periods. Type-I IFN mRNA expression levels were analyzed by qRT-PCR. (C) WT and *Rbm3*$^{-/-}$ MEFs were stimulated with B-DNA (1 μg/ml) or poly(I:C) (1 μg/ml) for the indicated time periods. mRNA expression levels of the indicated cytokine genes were measured by qRT-PCR. Data in all panels are presented as means and s.d. ($n = 3$). ND, not detected.

the induction of type-I IFN mRNAs. As shown in Fig. 3B, the induction of type-I IFN mRNAs following the stimulation by CpG-A DNA or by infection with herpes simplex virus type 1 (HSV-1), which also activates TLR9 [20], were normally observed in *Rbm3*$^{-/-}$ pDCs

enriched in bone marrow cells cultured with the Flt3-ligand *in vitro*. As expected, type-I IFN induction by TLR7 activation by vesicular stomatitis virus (VSV) [21] was also normal (Fig. 3B). We further examined the contribution of RBM3 to the activation of innate immune responses by other nucleic acid-sensing receptors. We stimulated *Rbm3*$^{-/-}$ MEFs with cytosolically delivered poly(dA-dT)·poly(dT-dA) (B-DNA) [22] or poly(I:C) and examined cytokine gene induction. As shown in Fig. 3C, the activation of type-I IFNs and proinflammatory cytokine genes was equally observed in both *Rbm3*$^{-/-}$ and control MEFs. Thus, these findings show that RBM3 deficiency does not affect cytokine gene induction by nucleic acid-sensing PRRs (see Section 4).

3.4. Critical involvement of RBM3 in cell cycle regulation

Since RBM3 is implicated in the regulation of cell growth, we examined whether RBM3 deficiency affects the proliferation of MEFs. As shown in Fig. 4A, *Rbm3*$^{-/-}$ MEFs showed a markedly delayed cell proliferation. The phosphorylation of extracellular signal-regulated kinase (ERK), one of the hallmarks of cellular events for the initiation of cell growth [23], remained unaffected in *Rbm3*$^{-/-}$ MEFs, which suggests that growth factor-mediated signaling remains unaffected in the absence of RBM3 (Fig. 4B). When we examined the cell cycle state of *Rbm3*$^{-/-}$ MEFs by flow cytometry, an interesting picture has emerged; *Rbm3*$^{-/-}$ MEFs showed an increased number of cells in the G2/M-phase compared with control cells (Fig. 4C). Furthermore, immunofluorescence microscopy revealed that *Rbm3*$^{-/-}$ MEFs show a smaller number of histone H3 phosphorylated at Ser10 (H3-pS10)-positive cells than control cells (Fig. 4D), as well as condensed chromosome-containing cells (data not shown), indicating the decrease in the number of M-phase cells [24]. Collectively, these observations indicate that RBM3 deficiency affects cell cycle progression in the G2-phase.

4. Discussion

In this paper, we report on the generation of mice deficient in RBM3 (*Rbm3*$^{-/-}$ mice) and our initial results on the role of this protein in the context of innate immune responses and cell cycle regulation. We newly identified RBM3 as a binding protein for CpG-B DNA that activates TLR9 (Fig. 1A) and adduced evidence that RBM3 accumulates in lysosomes, where TLR9 signaling is reported to occur [8,10], upon CpG-B DNA stimulation. However, our analysis of cDCs and pDCs from these mice revealed that the TLR9-mediated induction of cytokines remained unaffected in the absence of RBM3 (Fig. 3A, B, and Supplementary Fig. S2). Although RBM3 may not participate in the induction of innate immune responses, our results suggesting the CpG-B DNA stimulation-specific 'shuttling' of RBM3 from the nucleus to the cytoplasm and RBM3-CpG-B DNA interaction in lysosomes (Fig. 1B, D, and E) are intriguing, hence, it is not excluded that a redundant mechanism is operational, that is, a related molecule(s) may compensate for the absence of RBM3. It will also be interesting to study whether *Rbm3*$^{-/-}$ mice normally respond to infectious pathogens, such as bacteria, that activate

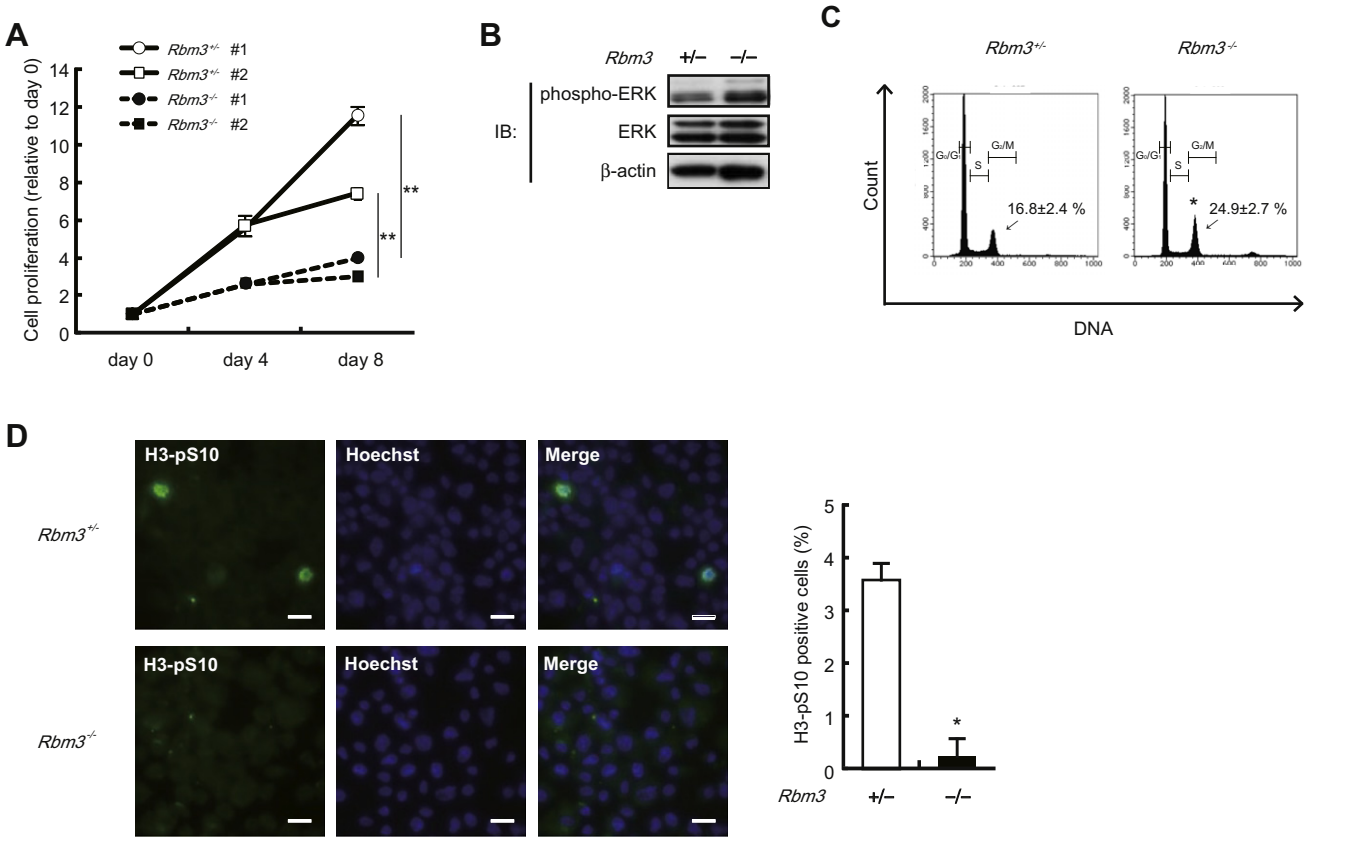

Fig. 4. Role of RBM3 in cell cycle control. (A) *Rbm3*$^{-/-}$ MEFs (1×10^5 cells) and control cells were seeded on six-well plates (day 0). Every 4 days, cells from each well were trypsinized and counted. Proliferation rate relative to day 0 is shown. (B) Whole cell lysates (20 μg) from *Rbm3*$^{-/-}$ MEFs and control cells were subjected to immunoblot analysis with antibodies for phospho-ERK, ERK, and β-actin. (C) *Rbm3*$^{-/-}$ MEFs and control cells were fixed and stained with propidium iodide and then analyzed by flow cytometry. Plots reflect cell number *versus* DNA content. Proportions of cells in G_0/G_1-, S-, and G2/M-phases of the cell cycle are shown. The data shown are representative of three independent experiments. (D) *Rbm3*$^{-/-}$ MEFs and control cells were fixed and stained with anti-phosphohistone H3 at Ser10 (H3-pS10) antibody and Hoechst. Fluorescence images were observed by a confocal microscope (left panel). The percentages of H3-pS10-positive cells among observed cells (200 cells) are also shown (right panel). Double asterisks, $P < 0.01$; single asterisk, $0.01 < P < 0.05$ compared with control MEFs. Data are presented as means and s.d. ($n = 3$). Scale bars indicate 10 μm.

TLR9 [1,3]. Therefore, further analysis is obviously required to clarify the role of RBM3 in the regulation of innate immune responses *in vitro* and *in vivo*.

Rbm3$^{-/-}$ mice fertilized well with normal cellularity of immune organs, although reports have suggested that RBM3 functions in spermatogenesis and cell growth [25,26]. Interestingly, we found a delayed proliferation and an increased G2/M-phase cell population in the *Rbm3*$^{-/-}$ MEFs (Fig. 4A and B). Since we could not find any differences in the proliferation of GM-CSF cultured bone marrow cells from *Rbm3*$^{-/-}$ mice (data not shown), RBM3 may have a cell-type specific function in cell growth activity. Although previous reports have suggested that the expression of RBM3 increases cyclooxygenase-2 (COX2) and vascular endothelial growth factor (VEGF) mRNA expression levels through the stabilization of these mRNAs which then promotes cell growth [14,27], no significant difference in the expression levels of these mRNA was observed between *Rbm3*$^{-/-}$ MEFs and control cells (data not shown). Therefore, we surmise that the defect in growth of *Rbm3*$^{-/-}$ MEFs is not due to the decreased expression of these molecules. Since our fluorescence microscopy showed a smaller number of H3-pS10-positive cells in the M-phase among *Rbm3*$^{-/-}$ MEFs (Fig. 4D), RBM3 may have a specific role in the regulation of the G2-phase rather than maintenance of overall mRNA stability as reported previously [28]. Congruent with this notion is that the expression levels of cyclin B and cell division cycle 2 (cdc2) proteins, which are critical regulators in the G2/M-phase transition state [29], remain unaffected by the deficiency of RBM3 (data not shown). Therefore, we surmise that RBM3 may function in a facet related to an as yet unknown mechanism underlying the G2/M-phase regulation. Since RBM3 is expressed highly in many cancer cell lines and is thought to function as an oncogene [18], clarification of the precise mechanism by which RBM3 regulates cell growth is an interesting issue that should be addressed in future studies.

In conclusion, the generation of *Rbm3*$^{-/-}$ mice and the initial characterization of *Rbm3*$^{-/-}$ cells may provide new tools with which the intricate regulatory mechanisms underlying the immune responses, cell cycle, and oncogenesis will be further studied.

Note added in proof

Currently, we have no evidence for an interaction of RBM3 with TLR9 in CpG-B stimulated cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.038.

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