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MOZ increases p53 acetylation and premature senescence through its complex formation with PML

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Monocytic leukemia zinc finger (MOZ)/KAT6A is a MOZ, Ybf2/Sas3, Sas2, Tip60 (MYST)-type histone acetyltransferase that functions as a coactivator for acute myeloid leukemia 1 protein (AML1)- and Ets family transcription factor PU.1-dependent transcription. We previously reported that MOZ directly interacts with p53 and is essential for p53-dependent selective regulation of p21 expression. We show here that MOZ is an acetyltransferase of p53 at K120 and K382 and colocalizes with p53 in promyelocytic leukemia (PML) nuclear bodies following cellular stress. The MOZ–PML–p53 interaction enhances MOZ-mediated acetylation of p53, and this ternary complex enhances p53-dependent p21 expression. Moreover, we identified an Akt/protein kinase B recognition sequence in the PML-binding domain of MOZ protein. Akt-mediated phosphorylation of MOZ at T369 has a negative effect on complex formation between PML and MOZ. As a result of PML-mediated suppression of Akt, the increased PML–MOZ interaction enhances p21 expression and induces p53-dependent premature senescence upon forced PML expression. Our research demonstrates that MOZ controls p53 acetylation and transcriptional activity via association with PML.

protein modification | DNA damage

The p53 protein functions as a key regulator of pathways mediating cellular responses by inducing myriad target genes that regulate diverse cellular processes including cell-cycle arrest, apoptosis, and genomic stability (1–4). Regulation of p53 transcriptional activities is crucial for genotoxic stress because of the varieties of cellular responses that are mediated by p53, which, in some cases, can be mutually exclusive (e.g., arrest and apoptosis) (5). p53 has been detected in discrete nuclear speckles, known as promyelocytic leukemia nuclear bodies (PML-NBs), in which CREB binding protein (CBP)/p300, Tip60, and pRB are also found (6–8).

PML was originally identified as a $t(15, 17)$ chromosomal translocation partner with the retinoic acid receptor-α (RARα) in acute promyelocytic leukemia, in which fusion genes encoding the PML-RARα fusion protein are generated (9–11). The ability of PML to interact with activators such as CBP/p300 in the nuclear body suggests that PML could modulate transcription through its ability to stabilize complexes of cofactors (12, 13). Overexpression of PML, γ-irradiation of cells, or oncogenic signals such as Ras overexpression can recruit p53 into PML-NBs (14, 15). The resulting ternary p53–PML–CBP complex then promotes the acetylation of p53 and harmonically coordinates critical tumor-suppressive functions such as apoptosis, senescence, and growth arrest (16, 17). Unlike bona fide coactivators such as CBP and p300, PML does not possess intrinsic histone acetylase activity. PML-mediated recruitment of both coactivators promotes p53 modification, such as acetylation and phosphorylation (16, 17). Acetylation levels of p53 are known to be significantly enhanced in response to stress and are involved in p53 activation and stabilization (18, 19). Given that $p300^{-/-}$ mouse embryonic fibroblasts (MEFs) retain the ability to respond to UV irradiation by stabilization of p53 and induction of p21, it is possible that other cofactors may compensate for p300 function in $p300^{-/-}$ MEFs (20).

Recent studies have shown PML to be a component of the PI3K-signaling network, negatively regulating the nuclear content of phosphorylated Akt/protein kinase B via its dephosphorylation by protein phosphatase 2A (PP2A) (21). The PI3K-Akt pathway has also been well documented to delay the p53-mediated response (22). The central role of Akt in this process is illustrated by the physical association of Akt with murine double minute 2 (MDM2), which inhibits p21 expression through its phosphorylation and activation of MDM2 and subsequent MDM2-mediated ubiquitination of p53 (23, 24). Recent studies suggest that Mdm2 mediates transcriptional repression by forming a protein complex with p53 on the promoter of specific p53-responsive genes (25), whereas accumulating observations suggest that degradation-independent mechanisms are also crucial for MDM2 in controlling p53 activities (26). Regardless, it is clear that the molecular mechanisms by which p53 activity is controlled are complex and how such mechanisms function in opposition to the Akt-signaling network remain to be elucidated.

Monocytic leukemia zinc finger (MOZ) protein is a MYST [MOZ, Ybf2/Something about silencing protein 3 (Sas3), Something about silencing protein 2 (Sas2), Tip60/KAT5]-type histone acetyltransferase (HAT) that functions as a coactivator for AML1- and p53-dependent transcription (27–29). $MOZ^{-/-}$ mice die at around embryonic day 15 (30). In serially passaged $MOZ^{-/-}$ MEFs, DNA damage-induced expression of p21 and ensuing cell-cycle arrest is profoundly impaired, suggesting that the p53 pathway may be altered in $MOZ^{-/-}$ MEFs (27). MOZ is involved in leukemia-associated chromosome rearrangements such as $t(8, 16)$ (p11;p13) (31), $t(8, 22)$ (32), and inv(8) (33), which result in fusion to the transcription coactivators CBP, p300, and transcriptional intermediary factor 2 (TIF2), respectively. Recently, we reported that MOZ directly interacts with p53 through its modification and selective activation of p21 but not Bcl-2 associated x protein (Bax) (27). Although MOZ stimulates p53-mediated transcription of the p21 gene selectively to induce cell-cycle arrest, neither the precise mechanisms of p53 transcriptional activation by MOZ nor the interactions between MOZ and p53 are fully understood.

In this report, we demonstrate that MOZ interacts with PML and recruits it into PML-NBs. A ternary p53–PML–MOZ interaction promotes the acetylation of p53 at K120 and K382 and subsequent p53-induced transcription of the p21 gene. Moreover, the MOZ amino acid sequence contains an Akt substrate motif, and phosphorylation of MOZ by Akt at T369 has a negative effect on its complex formation with PML. PML–MOZ complex formation, which is increased through PML-mediated Akt kinase activity suppression, induces acetylation of p53 and, consequently, p21 expression and p53-dependent premature senescence. These findings reveal a second mechanism that is distinct from Akt phosphorylation and activation of Mdm2, by which the Akt pathway counteracts p53 and prevents it from activating its downstream target genes.

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Results

MOZ Interacts with PML in PML-NBs. PML is critical for the proper activation of p53 transcriptional activity (15, 16). Upon exposure to DNA damage (e.g., Ras activation, UV radiation), p53 becomes associated with PML-NBs. To investigate whether MOZ colocalizes with PML in PML-NBs, we exposed MCF-7 (breast cancer cell line) cells to UV radiation. In untreated cells, MOZ exhibited both speckled and diffuse nuclear staining in both the nucleoplasm and the nucleolus. When cells were exposed to UV radiation, both PML and MOZ showed a high degree of colocalization in PML-NBs (Fig. 1*A* and Fig. S1*A*). Coimmunoprecipitation analysis confirmed that both PML–MOZ and p53–MOZ interactions were increased after UV radiation, but not after treatment with As_2O_3 (Fig. 1*B* and Fig. S1 *B* and *C*). These results suggest that UV radiation enhances p53–PML–MOZ ternary complex formation.

To identify the PML-interacting domain in MOZ, the interactions between various deletion mutants of MOZ and PML were examined by immunoprecipitation (IP) immunoblotting. Coimmunoprecipitation experiments using these deletion mutants identified two PML-interacting regions (amino acids spanning 144–664 and 1517–1741) in MOZ (Fig. 1 *C* and *D* and Fig. S2*A*). To determine the region of PML required for binding to MOZ, a pulldown assay using anti-FLAG epitope peptide antibody (see *SI Materials and Methods*) was performed using a series of PML deletion mutants. Results from these studies suggest that PML possesses one MOZ-interacting coiled-coil domain within amino acids 229–360 (Fig. 1*E* and Fig. S2*B*).

To confirm that the interaction between PML and MOZ is direct, we performed a Far-Western blot analysis. In brief, full-length PML protein was translated in reticulocyte lysates, and then proteins were separated by SDS/PAGE, followed by transfer to nitrocellulose. Following denaturation and renaturation, proteins on the membrane were incubated with sf9 insect cells-purified MOZ proteins, and bound MOZ was detected by immunoblotting analysis. As shown in Fig. 1*F*, PML directly bound to MOZ in vitro. As negative controls, BSA and the polypeptides present in reticulocyte extracts did not interact with MOZ. Therefore, we conclude that PML is a bona fide interacting partner for MOZ.

PML-MOZ Association Enhances MOZ-Mediated p53 Acetylation and p21 Expression. PML directly interacts with HATs, including CBP, p300, and Tip60, and PML-NBs serve as sites for posttranslational modifications of p53 (17, 34). MOZ is a MYST-type HAT, like Tip60 and MOF (35, 36), and the MYST domain of MOZ has 69% homology with that of Tip60. We therefore hypothesized that MOZ may play an important role in acetylation of p53.

In fact, an in vitro acetylation assay showed that MOZ could acetylate p53 at K120 and K382 (Fig. 2*A* and Fig. S3). Furthermore, knockdown of MOZ expression partially inhibited irradiation-dependent acetylation of p53 at K120 and K382, but not at K373 (Fig. 2*B*). Therefore, MOZ displays aspects of both Tip60 and p300 in acetylation of p53. To further confirm that MOZ acetylates p53 at K120 and K382, we generated an acetylase-deficient MOZ variant (AD-MOZ: Q654E/G657E), in which the acetyl-CoA–binding sequences are mutated to glutamine, mimicking the MYST domain of a previously characterized acetylase-deficient mutant of Tip60 (37) (Fig. 2*C*). Immunoblotting showed that wild-type (WT) MOZ, but not AD-MOZ, could acetylate itself and p53 at K382 (Fig. S4 *A* and *B*). Because we observed that MOZ is recruited into PML-NBs like CBP/p300, we wanted to determine whether the PML–MOZ interaction could also be enhanced by MOZ-mediated acetylation of p53. To this end, we performed immunoblotting with antibodies that specifically recognize acetylated-p53 at K120 and K382 and showed that PML promotes MOZ-mediated p53 acetylation (Fig. 2*D*).

To test whether PML also affects transcriptional activation of the p53–MOZ complex, we used reporter plasmids under the control of promoters for p53 target genes such as p21 and bax in $MOZ^{-/-}$ MEFs. Whereas PML stimulated p53-mediated activation of p21-luc in wild-type MEFs, p21-luc induction was

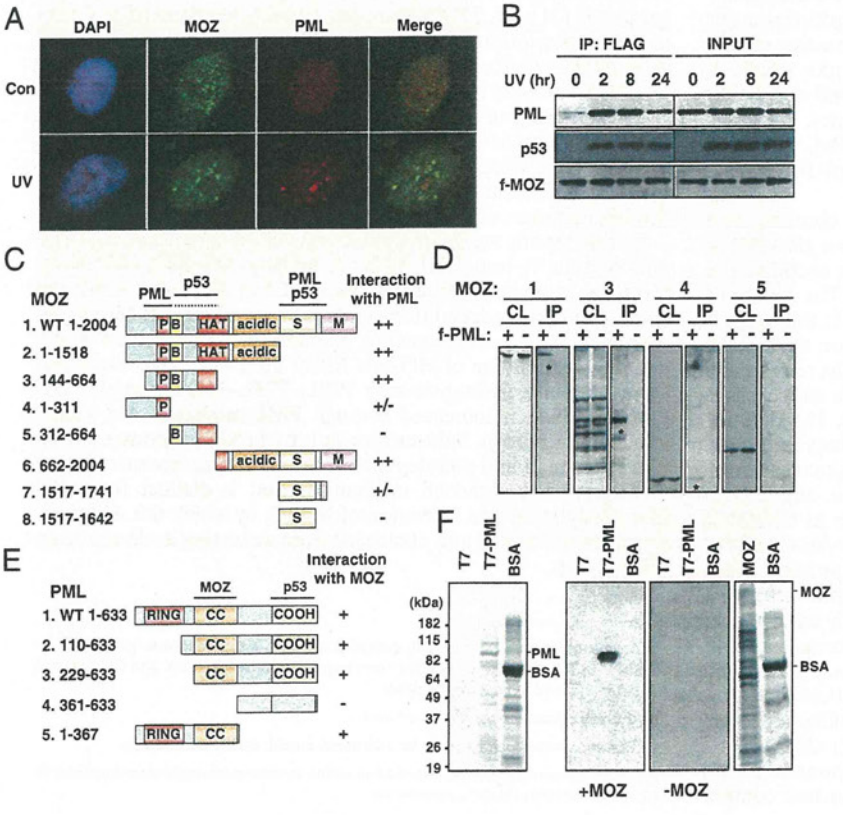

Fig. 1. MOZ is recruited into promyelocytic leukemia nuclear bodies after DNA damage. (*A*) Colocalization of MOZ with PML. MCF-7 cells were irradiated with UV (30 J/m^2) and then processed for immunofluorescence. (*B*) MOZ interacts with PML and p53. MCF7 cells were cotransfected with HA-epitope tagged PML and FLAG-epitope tagged MOZ and then subjected to UV irradiation (30 J/m^2) followed by harvesting at indicated times. The cell lysates were immunoprecipitated with anti-FLAG beads and subjected to immunoblotting. (*C*) Schematic representation of the structure of MOZ deletion mutants. The plant homeo domain (PHD) finger domain (P), the basic domain (B), the HAT domain, the serine-rich region (S), the methionine-rich region (M), and the regions required for interaction with PML (bars) and for strong interaction with PML (dotted bar) are indicated. (*D*) BOSC23 cells were transfected with HA-MOZ deletion mutants along with FLAG-PML. Cell lysates (CL) were immunoprecipitated with anti-FLAG beads and were subjected to immunoblotting. (*E*) Schematic representation of the structure of PML deletion mutants. The region required for interaction with MOZ is indicated (bar). (*F*) PML can interact with MOZ in vitro as indicated by Far-Western analysis. FLAG-MOZ protein purified from sf9 (*Right*) and HAPML immunoprecipitated from reticulocyte lysate (*Left*) were subjected to SDS/PAGE and stained by Coomassie blue. Mock (T7) and PML (T7-PML) proteins were resolved and transferred to a nitrocellulose membrane, which was then incubated with 1 μg/mL of purified FLAG-MOZ (*Center*) and detected by immunoblotting.

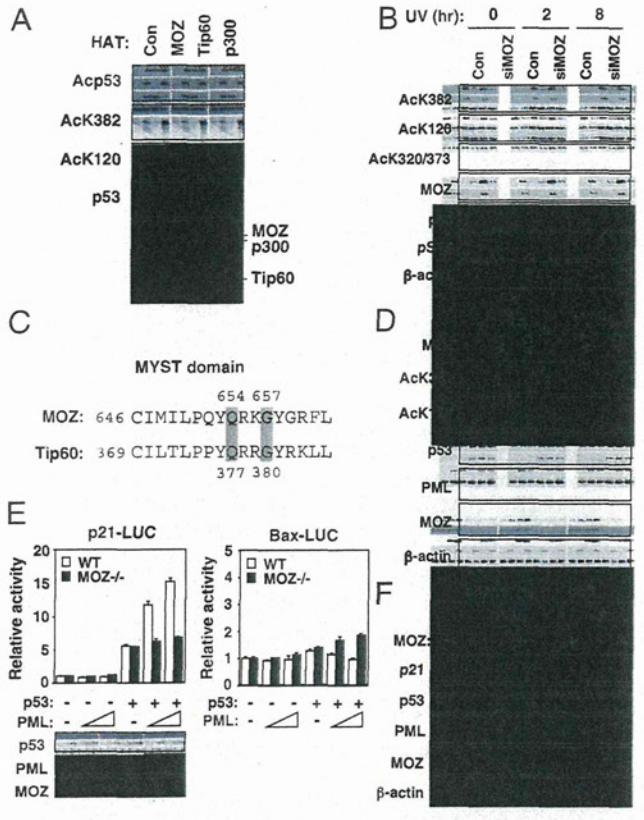

Fig. 2. MOZ acetylates p53 at K382 and K120. (*A*) In vitro acetylation of p53 by MOZ, Tip60, and p300. Baculovirus-expressed p53, MOZ, Tip60, and p300 were assayed for acetylation activity of p53. The acetylation samples were detected by immunoblotting with antibodies against Acp53, AcK382, AcK120, and p53 (*Upper*). MOZ, Tip60, and p300 proteins were visualized by silver staining (*Lower*). (*B*) Inhibition of MOZ blocks p53 acetylation. The siRNA-treated MCF-7 cells were irradiated with 30 J/m^2 of UV for indicated times and were subjected to immunoblotting. (*C*) MYST domain sequences of MOZ and Tip60. (*D*) PML enhances MOZ acetylation of p53. BOSC23 cells were transfected with indicated FLAG-p53 along with either HA-MOZ or PML and were subjected to immunoblotting. (*E*) PML enhances MOZ-mediated p21-luc but not Bax-luc reporter activity. Either wild-type (WT) or MOZ$^{-/-}$ MEFs were transfected with p21-luc (*Left*) or Bax-luc (*Right*), p53, PML, and pRL-CMV as a transfection control. (*F*) PML enhances MOZ-mediated p21 expression in a p53-dependent manner. Either HCT116 p53$^{+/+}$ or p53$^{-/-}$ cells were cotransfected with FLAG-MOZ along with HA-PML and were subjected to immunoblotting.

suppressed in MOZ$^{-/-}$ MEFs. On the other hand, induction of bax-luc was slightly induced in MOZ$^{-/-}$ MEFs compared with wild-type MEFs (Fig. 2*E*). This supports the likelihood that MOZ is a key component of the regulation of p53 by PML.

Although further luciferase and immunoblotting demonstrated that wild-type MOZ strongly enhanced *p21* gene transcription and *p21* expression in the presence of PML, AD-MOZ partially suppressed the expression of *p21* compared with wild-type MOZ, suggesting that MOZ-mediated acetylation of p53 is important but not sufficient for MOZ-mediated expression of p21 (Fig. 2*F* and Fig. S5). A MOZ-CBP fusion gene, produced by the *t*(8, 16) translocation, is associated with acute monocytic leukemia. We tested the effects of the MOZ-CBP chimera and found that MOZ-CBP suppressed p53-dependent transcriptional activation of p21 (Fig. S5). These results indicate that MOZ associates with PML and that the PML–MOZ complex induces p21 expression.

Akt Phosphorylates MOZ and Inhibits Its Interaction with PML. Analysis of the human MOZ sequence revealed one site (T369) that conforms to the Akt phosphorylation consensus site (Fig. 3*A*). The Akt phosphorylation motif (R-X-R-X-X-S/T-B, where "X" represents any amino acid and "B" represents a hydrophobic residue), has been refined to include amino acids that contribute to its 3D structure (38). We found that the Akt phosphorylation motif is also conserved in human-, mouse-, rat-MOZ, and RXRXXT in human MORF/MYST4, suggesting its possible functional importance. The presence of putative Akt phosphorylation sites in MOZ led us to determine whether MOZ is a substrate for Akt.

In fact, we observed that recombinant Akt was sufficient for inducing phosphorylation of wild-type MOZ in vitro, but not T369A-MOZ (Fig. 3*B*). We also confirmed that wild-type Akt, but not dominant negative (DN) Akt, was capable of inducing MOZ phosphorylation in vitro (Fig. 3*C*). To further examine whether Akt can phosphorylate MOZ at T369, we generated a polyclonal antibody that specifically recognizes MOZ phosphorylated at that site (pT369-MOZ). By immunoblotting, this antibody detected MOZ that had been phosphorylated at T369 by myristoylated active Akt (Myr) while it partially cross-reacted with nonphosphorylated MOZ in a manner similar to a pAkt-substrate antibody that recognized phosphorylated MOZ (Fig. 3*D*). These results support the possibility that Akt mediates MOZ phosphorylation at T369 in vivo and in vitro.

Because T369 of MOZ is located within the PML-binding domain, we further tested whether this phosphorylation affects binding between MOZ and PML or p53 (Fig. 1*C*). Coimmunoprecipitation

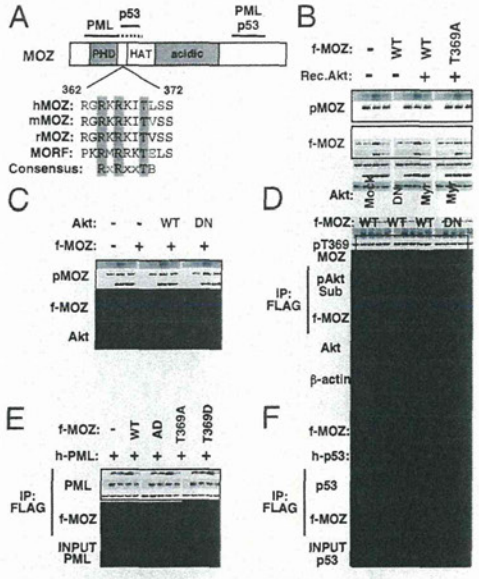

Fig. 3. Akt-mediated phosphorylation of MOZ suppresses the interaction between PML and MOZ. (*A*) Structure of the consensus Akt phosphorylation motif in MOZ. Sequences of human-, mouse-, rat-MOZ, and MORF are shown for comparison. (*B*) Recombinant Akt phosphorylates MOZ in vitro. FLAG-tagged wild-type (WT) and T369A (TA) of MOZ proteins were immunoprecipitated with anti-FLAG beads from transfected BOSC23 cells. MOZ proteins were incubated with recombinant Akt (Rec. Akt) in the presence of [γ-^{32}P] ATP. (*C*) FLAG-tagged WT and dominant negative (DN) Akt and MOZ proteins were immunoprecipitated with anti-FLAG beads from transfected BOSC23 cells. MOZ proteins were incubated with WT- or DN-Akt in the presence of [γ-^{32}P]ATP. (*D*) Akt phosphorylates MOZ at T369. BOSC23 cells were cotransfected with FLAG-tagged WT- or TA-MOZ along with myristoylated (Myr)- or DN-Akt. Cell lysates were immunoprecipitated with anti-FLAG beads and were subjected to immunoblotting using antibodies for phospho-T369 (pT369-MOZ), phospho-Akt substrate (pAkt-Sub), FLAG (MOZ), Akt, and β-actin. (*E* and *F*) Akt-mediated phosphorylation of MOZ suppresses the PML–MOZ interaction but not the p53–MOZ interaction. BOSC23 cells were cotransfected with FLAG-tagged WT, AD, T369A, and T369D of MOZ along with HA-PML (*E*) or HA-p53 (*F*). Cell lysates were immunoprecipitated with anti-FLAG beads and subjected to immunoblotting. Ten percent of input lysates were used for input control.

analysis using phosphorylation-deficient MOZ (T369A) demonstrated that it had increased interaction with PML (Fig. 3*E*). Interestingly, these mutations did not affect MOZ-p53 binding (Fig. 3*F*). These results indicate that Akt phosphorylates T369 of MOZ to inhibit its interaction with PML.

Phosphorylation of MOZ at T369 Is Important for Negative Regulation of p53 Acetylation. Because the interaction between PML and MOZ is likely dependent on the phosphorylation state of MOZ at T369, we determined whether Akt-mediated phosphorylation of MOZ inhibits its acetylation activity. An IP immunoblotting showed that T369A-MOZ strongly acetylates p53 at K382 and K120 in transfected cells, unlike the phosphorylation-mimicking mutant T369D-MOZ (Fig. 4*A*). To examine the T368A-MOZ acetylation activity per se, we next performed an in vitro HAT activity assay using histone peptides as substrates. In contrast to Fig. 4*A*, T369A-MOZ showed only slightly increased acetylation of histone H3 and H4 peptides compared with T369D-MOZ (Fig. 4*B*). These results are consistent with results showing that wild-type or T369 mutant forms of MOZ displayed equivalent abilities to self-acetylate and that the acetylation activity of MOZ was enhanced in the presence of PML (Fig. 4*C*). Thus, our data indicate that phosphorylation of MOZ at T369 is important for the negative regulation of acetylation of p53 in the presence of PML, whereas such phosphorylation is not important for its acetylation activity per se.

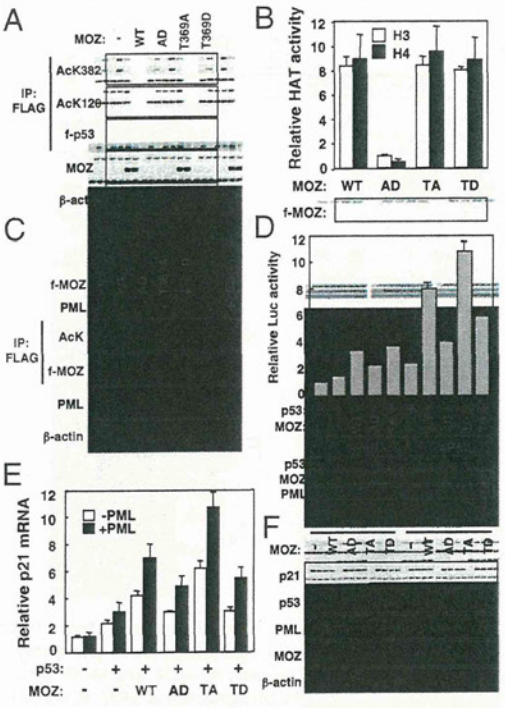

Fig. 4. Phosphorylation of MOZ is important for negative regulation of p53 acetylation. (*A*) BOSC23 cells were cotransfected with HA-tagged WT, AD, T369A or T369D along with FLAG-p53. Cell lysates were purified with anti-FLAG beads and were subjected to immunoblotting using antibodies for AcK382, AcK120, FLAG (f-p53), HA (MOZ), and β-actin. (*B*) Phosphorylation of MOZ has no effect on HAT activity. FLAG-tagged WT, AD, T369A, and T369D MOZ were purified with anti-FLAG beads from transfected BOSC23 cells. The acetylase activities of mutant MOZ were measured as described in *Materials and Methods*. (*C*) BOSC23 cells were cotransfected with FLAG-tagged WT, AD, T369A, or T369D of MOZ with HA-PML. Cell lysates were immunoprecipitated with anti-FLAG beads and subjected to immunoblotting. (*D*) The PML–MOZ interaction stimulates p53-mediated p21 reporter activity. H1299 cells were cotransfected with p21-luc, p53, and PML along with WT, AD, T369A, or T369D of MOZ. (*E* and *F*) The PML–MOZ interaction stimulates p53-dependent p21 induction and expression. H1299 cells were cotransfected with p53 and PML along with HA-tagged WT, AD, T369A, and T369D of MOZ. The harvested cells were subjected to real-time PCR (*E*) and to immunoblotting.

Further luciferase analysis and real-time PCR analysis upon coexpression of MOZ with PML revealed that T369A-MOZ strongly enhanced the expression of p21, whereas T369D-MOZ partially suppressed the expression of p21 compared with T369A-MOZ (Fig. 4 *D* and *E*). These data are consistent with p21 expression analyzed by immunoblotting (Fig. 4*F*). Taken together, these findings indicate that suppression of Akt-mediated phosphorylation at T369 of MOZ is critical for MOZ recruitment into PML-NBs and its acetylation of p53. We conclude that the PI3K-Akt pathway-mediated suppression of p21 is not only through Akt phosphorylation and stabilization of MDM2, but also through blocking the recruitment of MOZ into PML-NBs and the acetylation of p53.

Loss of MOZ Contributes to Resistance to PML-Induced Senescence. To characterize PML expression-induced cell-cycle arrest and senescence, we infected wild-type and MOZ$^{-/-}$ primary mouse embryonic fibroblasts (pMEFs) with a pLNCX retroviral vector expressing PML and Ha-Ras Ha-Ras (v-Ha-ras Harvey rat sarcoma viral oncogene homolog, V12), cultured them for 3 d under puromycin selection, and counted cells for the next 5 d. Wild-type pMEFs expressing PML as well as oncogenic Ha-Ras (V12) ceased to proliferate at subconfluent densities (Fig. 5*A*). Conversely, in MOZ$^{-/-}$ pMEFs, ectopic PML expression did not lead to cessation of proliferation, although RasV12-expressing MOZ$^{-/-}$ MEFs exhibited slowed proliferation (Fig. 5*B*). The morphology of MOZ$^{-/-}$ pMEFs differed from senescent wild-type pMEFs, even after infection of these cells with retroviruses containing PML or Ras (Fig. S6).

We next examined the cell-cycle distribution of pMEFs arrested by retroviral-mediated PML expression by following BrdU incorporation. Wild-type pMEFs did not incorporate BrdU (Fig. 5 *C* and *D*) and became positive for senescence-associated β-galactosidase (SA β-gal) staining, a marker of cellular senescence (Fig. 5*E* and Fig. S7). Consistent with Fig. 5*B*, forced PML expression in MOZ$^{-/-}$ pMEFs did not alter BrdU incorporation (Fig. 5 *C* and *D*). The frequency of SA β-gal–positive cells was markedly lower in PML-expressing MOZ$^{-/-}$ pMEF cells than in wild-type pMEFs (Fig. 5*E* and Fig. S7). These results indicate that loss of MOZ contributes to resistance to PML-induced premature senescence.

Based on the above findings, we examined p53 modifications and p21 expression in WT and MOZ$^{-/-}$ MEFs expressing either RasV12 or PML. Although both p53 and p16 are vital components of the intracellular senescence pathway (39), PML does not affect p16 expression (17), consistent with our observed results (Fig. 5*F*). Although ectopically expressed Ras or PML induced equivalent levels of p53 in both wild-type and MOZ$^{-/-}$ MEFs, their expression led to the appearance of more nonphosphorylated MOZ in wild-type cells, which corresponded to increased acetylation of p53 at K117 and K379 (equivalent to human p53 K120 and K382, respectively). In line with this, p21 expression was induced in wild-type pMEFs, but not in MOZ$^{-/-}$ pMEFs after PML expression despite similar p53 transcriptional levels in wild-type and MOZ$^{-/-}$ pMEFs (Fig. 5*F* and Fig. S8). This suggests that, through PML-mediated suppression of the Akt pathway, increased PML–MOZ complex formation enhances p21 expression, which mediates p53-dependent premature senescence upon PML overexpression. In short, MOZ is crucial for p53-mediated p21 expression induced by PML expression and the ensuing PML-induced senescence.

Discussion

Several factors, such as Tip60, hematopoietic zinc finger (Hzf), and human cellular apoptosis susceptibility protein (hCAS/CSE1L) are involved in the selection of p53 target genes (35, 40, 41). However, the molecular mechanisms by which p53 "chooses" cell-cycle arrest or apoptosis are not fully understood. Here we discovered that the MYST-type HAT MOZ interacts with PML and is recruited into PML-NBs. Significantly, we confirmed that the

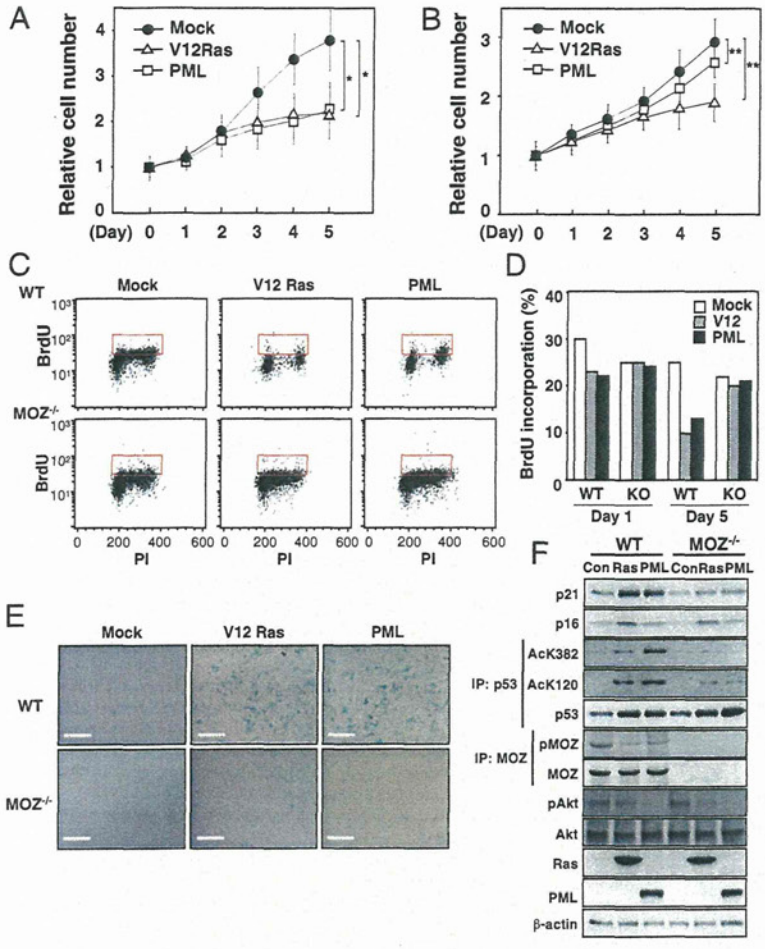

Fig. 5. MOZ-knockout cells display resistance to PML-induced senescence. (*A* and *B*) Growth curves of wild-type (*A*) and MOZ$^{-/-}$ (*B*) pMEFs infected with vector (Mock), RasV12, or PML. Day 0 is the first day after puromycin selection. Error bars represent the standard deviation for three independent littermate experiments. *$P < 0.02$, **$P > 0.03$. (*C*) Flow cytometry analysis of pMEFs. pMEFs in growth phase were stained with an anti-BrdU antibody and propidium iodide at 5 d after selection. (*D*) Histogram comparing BrdU incorporation of RasV12- and PML-infected pMEFs at 1 or 5 d after selection. (*E*) SA β-gal staining of WT or MOZ$^{-/-}$ pMEFs infected with pLNCX (Mock), RasV12, or PML at 5 d after selection. (Scale bars: 50 μm.) (*F*) MOZ$^{-/-}$ cells are resistant to PML-induced p21 expression. The infected WT or MOZ$^{-/-}$ pMEFs were harvested at 5 d after selection. The cells were partially subjected to immunoprecipitation with anti-p53 (IP: p53) and anti-MOZ (IP: MOZ) and were subjected to immunoblotting using indicated antibodies.

PML–MOZ interaction promotes the MOZ-mediated acetylation of p53 at K120 and K382, which makes MOZ distinct from other KATs that can target only one of these residues and p53-mediated expression of p21 (Fig. S9).

It has been reported that p53 can be acetylated at K373, K382, K164 (catalyzed by p300/CBP), K320 (PCAF/KAT2B), and K120 (Tip60) (42, 43). Although p53 mice engineered to lack acetylatable lysines within their C termini are viable and phenotypically normal, p53-mediated transcriptional activation upon DNA damage is partially impaired in the ES cells and thymocytes of these mice (44). In addition, the p53 7KR mutation, where the seven C-terminal lysines were changed to arginine, significantly contributes to hematopoietic stem cell homeostasis and mouse radiosensitivity (45). Thus, it is possible that other cofactors or additional acetylation sites of p53 may compensate for the loss of p53 acetylation at the C terminus. In this report, we found that MOZ is an acetylation regulator of p53. In contrast to normal MOZ and CBP, the leukemia-associated MOZ-CBP fusion protein inhibited p53-mediated transcription (Fig. S5). These results suggest that inhibition of MOZ-p53–mediated transcription might be involved in pathogenesis of tumors and leukemia.

Our previous study revealed that MOZ-deficient MEFs exhibit impaired p21 expression and fail to arrest in G1 phase in response to DNA damage (27). Evidence of the significance of lysine modifications in the functions of the DNA-binding region comes from studies showing that Tip60/KAT5 and hMOF/KAT8, members of the MYST family of acetyltransferases, acetylate K120 of p53 followed by its accumulation on Puma and Bax promoters (35) and that K120/K164R double-mutant mice display reduced induction of *p21* and *puma* (43). In addition, K120 acetylation of p53 exhibited specific DNA binding and discriminated among response elements at effective physiological salt concentration (46). The ability of MOZ to simultaneously acetylate both K120 and K382 might be responsible for the difference of target genes from previous reports. It might be also possible that MOZ acetylation of p53 occurs in a different subcellular compartment, such as PML-NBs, from that of Tip60/hMOF. Thus, the DNA-binding domain acetylation by MOZ appears to play a role in selective gene regulation.

Akt kinase activity is frequently elevated in several high-grade, late-stage cancers (47), and a somatic constitutively active Akt mutant has been identified in human breast, colorectal, and ovarian cancers (48). In fact, the role of Akt in the p53-mediated response was shown to involve Akt suppression of p21 expression through phosphorylation and activation of MDM2 and subsequent MDM2-mediated ubiquitination of p53 (23, 24, 49). Here we demonstrate that Akt works by a second process in which Akt phosphorylation of MOZ at T369 within its PML interaction region blocks its complex formation with PML (Fig. 3*A*). A mutant MOZ (T369A) displays enhanced acetylation of p53 at K120 and K382 with ensuing increased expression of p21, indicating that phosphorylation at T369 of MOZ is important for the acetylation of p53, but not for its acetylation activity per se (Fig. 4 *A* and *C*). Suppressing Akt-mediated phosphorylation of MOZ at T369 is likely very important for the recruitment of MOZ into PML-NBs and the subsequent acetylation of p53.

In this report, we demonstrate that forced PML expression in MOZ$^{-/-}$ primary MEFs somewhat suppresses either G1 arrest or senescence (Fig. 5*B*). PML expression in MOZ$^{-/-}$ MEFs only slightly alters BrdU incorporation (Fig. 5 *C* and *D*), but the frequency of SA β-gal–positive senescent cells is markedly lower (Fig. 5*E* and Fig. S7). These results indicate that loss of MOZ contrib-

utes to resistance to PML-induced G1 arrest and premature senescence. In contrast, $MOZ^{-/-}$ primary MEFs exhibit a high proportion of cells in S phase but lower BrdU incorporation after forced PML expression. This is also true for serially passaged MEFs after DNA damage, suggesting that MOZ might be involved in DNA replication. These results are consistent with a report that depletion of inhibitor of growth family, member 5 (ING5), which comprises a subunit of the ING5-MOZ stoichiometric HAT complex, renders cells unable to complete S phase, allowing only a few cells to proceed to the G2/M phase. Together, these results suggest that ING5–MOZ complexes are essential for DNA replication, not only for initiation but also for replication fork movement (50). Recently, it has been reported that Pten-loss–induced cellular senescence, where the activation of the PI3K-Akt pathway is the key mechanism triggering a p53-dependent senescence, represents a senescence response that is distinct from oncogene-induced senescence (51). Further studies are required to investigate whether the sequential phenomenon—down-regulation of Akt activity and MOZ acetylation of p53—occurs in the PML-NB subcellular compartment.

Materials and Methods

Plasmids expressing FLAG-tagged and HA-tagged human MOZ, CBP, MOZ-CBP, and p300 were cloned into the pLNCX vector (Clontech). Human cDNA encoding FLAG- and HA-tagged PML isoform IV and HA-tagged V12 Ras were cloned into pLPCX retrovirus vectors. Myristoylated active, FLAG-tagged, wild-type, or dominant negative Akt1 were described previously (52). Details of other methods used in this article, including cell culture, antisense oligonucleotides, baculovirus protein expression and purification, flow cytometry, SA β-gal assay, immunofluorescence, immunoprecipitation, immunoblotting, Far-Western blotting, real-time PCR, luciferase assay, in vitro acetylation assay, and in vitro kinase assay are described in *SI Materials and Methods*.

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B Cell Receptor-ERK1/2 Signal Cancels PAX5-Dependent Repression of BLIMP1 through PAX5 Phosphorylation: A Mechanism of Antigen-Triggering Plasma Cell Differentiation

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B Cell Receptor-ERK1/2 Signal Cancels PAX5-Dependent Repression of BLIMP1 through PAX5 Phosphorylation: A Mechanism of Antigen-Triggering Plasma Cell Differentiation

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Plasma cell differentiation is initiated by Ag stimulation of BCR. Until BCR stimulation, B lymphocyte-induced maturation protein 1 (BLIMP1), a master regulator of plasma cell differentiation, is suppressed by PAX5, which is a key transcriptional repressor for maintaining B cell identity. After BCR stimulation, upregulation of BLIMP1 and subsequent suppression of PAX5 by BLIMP1 are observed and thought to be the trigger of plasma cell differentiation; however, the trigger that derepresses BLIMP1 expression is yet to be revealed. In this study, we demonstrated PAX5 phosphorylation by ERK1/2, the main component of the BCR signal. Transcriptional repression on *BLIMP1* promoter by PAX5 was canceled by PAX5 phosphorylation. BCR stimulation induced ERK1/2 activation, phosphorylation of endogenous PAX5, and upregulation of BLIMP1 mRNA expression in B cells. These phenomena were inhibited by MEK1 inhibitor or the phosphorylation-defective mutation of PAX5. These data imply that PAX5 phosphorylation by the BCR signal is the initial event in plasma cell differentiation. ***The Journal of Immunology*****, 2012, 188: 6127–6134.**

PAX5 is a member of the highly conserved paired-box (PAX) domain family of transcription factors. PAX5 is exclusively expressed from the pro-B to mature B cell stages and is downregulated during terminal differentiation into plasma cells (1). PAX5 is not only indispensable for B-lineage commitment; its continuous expression is essential for maintaining the identity of B cells (2–4). PAX5 functions as both a transcriptional activator of B lineage-specific genes and a repressor of B lineage-inappropriate genes (1) [i.e., it activates *CD19* (5), *CD79A* (6), and B cell linker protein (7) and represses CSF1 receptor (8), *Notch1* (9), and FMS-like tyrosine kinase 3 (10)]. In addition, it checks the initiation of plasma cell differentiation and the terminal differentiation of B cells by repressing B lymphocyte-induced maturation protein 1 (*BLIMP1*) and X box-binding protein 1 (11, 12).

BCR signal plays important roles in the activation, survival, and differentiation of B lymphocytes. The initial event after BCR engagement is the activation of Lyn and Syk. These kinases trigger a complex network of signaling pathways downstream of the receptor, including the Ras-Raf-MEK-ERK1/2 pathway, the Vav-cell division cycle 42-JNK pathway, and the NF-κB pathway (13). The resulting signals quickly reach the nucleus and alter gene expression. The ultimate effects on B cells are profound and vary depending on the maturation state of the cell and on additional signals that the cell receives. For germinal center B (GCB) cells, BCR signal after encountering Ag is known to initiate PAX5 downregulation, BLIMP1 upregulation, and eventually, plasma cell differentiation (14, 15). In these cells, PAX5 suppresses BLIMP1 expression and checks plasma cell differentiation. After BCR stimulation by Ag, BLIMP1 repression by PAX5 is abolished, and once BLIMP1 is expressed, it suppresses PAX5. Eventually, PAX5 is replaced by BLIMP1, which initiates plasma cell differentiation (14). The abolition of PAX5-mediated repression was thought to be the first event to initiate plasma cell differentiation (16); however, the mechanism is still unknown. In this study, we demonstrated that PAX5 was phosphorylated by ERK1/2 in vitro and in vivo at serines 189 and 283. This phosphorylation attenuated the transcriptional repression of BLIMP1 by PAX5. Finally, BCR stimulation induced the phosphorylation of ERK1/2 and PAX5, as well as BLIMP1 mRNA expression in B cells, which were inhibited by MEK1 inhibitor or the phosphorylation-defective mutation of PAX5. These data imply that PAX5 phosphorylation by the BCR signal is an initial event in plasma cell differentiation.

Materials and Methods

Cells, Abs, and reagents

Burkitt lymphoma cell line Ramos cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Anti-HA Ab, anti-ERK2 Ab, and anti-PAX5 Ab (C-20) for immunoblotting, and anti-PAX5 Ab (N-19) for the supershift assay in EMSA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human IgM Ab and anti-mouse IgM Ab for BCR stimulation and anti–phospho-ERK1/2 Ab were from Abcam (Cambridge, U.K.), Jackson ImmunoResearch Laboratories (West Grove, PA), and Cell Signaling (Beverly, MA), respectively. U0126 was obtained from Calbiochem (San Diego, CA).

Plasmids

PAX5/pCDNA, the expression vector for PAX5, was described previously (17). PAX5/pGEX and PAX5(1-279)/pGEX, expression vectors for GST-

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The online version of this article contains supplemental material.

Abbreviations used in this article: BLIMP1, B lymphocyte-induced maturation protein 1; GCB, germinal center B; siRNA, small interfering RNA; UTR, untranslated region.

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fused full-length and partial PAX5, as well as PAX5/pBGJR, were made by subcloning PAX5 cDNA digested from PAX5/pCDNA with appropriate restriction enzymes into pGEX 5X-1 vector (Pharmacia, Uppsala, Sweden) and pBGJR, a lentivirus expression vector kindly provided by Dr. Stefano Rivella (Memorial Sloan-Kettering Cancer Center). Mutations for serine/threonine-to-alanine substitutions were introduced into PAX5/pCDNA using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All clones were subjected to sequence analysis to confirm the introduction of the correct mutations and to exclude PCR artifacts. The expression vector for HA-tagged CA-MEK1, HA–CA-MEK1/pCMV, was described previously (18). The −1921 to +138 region (relative to the translation start site) of the *BLIMP1* promoter containing putative NF-κB- and PAX5-binding sequences was amplified by PCR, according to a previous report (19), and subcloned into pGL4.20 (Promega, Madison, WI). Two specific primer genes, 5′-TAACAGTGAGTTGATTCACTGGC-3′ (sense) and 5′-CTCGGCGGTCCCTCCTCG-3′ (antisense), were selected on the basis of the sequence of the human *BLIMP1* genomic DNA (accession number AL358952, http://www.ncbi.nlm.nih.gov/nuccore/AI358952). This reporter gene was designated as BLIMP1-luc/pGL4. Expression vectors for NF-κB p50 and p65 were purchased from Addgene (Cambridge, MA).

Transient transfection, lentivirus infection, immunoblotting, immunofluorescence, EMSA, luciferase assay, and in vitro kination assay

Transient transfection, lentivirus infection, immunoblotting, immunofluorescence, EMSA, luciferase assay, and in vitro kination assay were performed as described previously (17, 18, 20).

PAX5 knockdown

The small interfering RNA (siRNA) targeting the 5′-GACTATCCATCCATCATAA-3′ sequence in the 3′ untranslated region (UTR) of *PAX5* was purchased from Sigma-Aldrich (St. Louis, MO) and introduced into Ramos cells with nucleofector (Lonza, Wuppertal, Germany), according to the manufacturer's instructions.

Phosphate-affinity SDS-PAGE

Phosphate-affinity SDS-PAGE was performed similarly to SDS-PAGE, except that Phos-tag acrylamide-containing acrylamide gel was used. Phos-tag acrylamide was obtained from Wako Laboratory Chemicals (Osaka, Japan). In this system, Phos-tag acrylamide binds to phosphorylated amino acids during electrophoresis and slows the migration of phosphorylated proteins, according to the number of phosphorylation sites. Autoradiography or immunoblotting following electrophoresis can detect the phosphorylation of the target protein as band shifts.

Mouse spleen cell isolation

Mouse spleen cells were collected from 10-wk-old BALB/c mice and were used for immunoblotting analysis to detect PAX5 phosphorylation. Mouse spleen B cells were purified from the spleen cells using CD45R (B220) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and subjected to RT-PCR to detect BLIMP1 expression. These cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 U/ml streptomycin.

Real-time PCR

Total RNA was purified using the QIAamp RNA Blood Mini Kit (QIAGEN, Hilden, Germany), and reverse transcription was performed with random hexamers using the Superscript III First Strand kit (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed by standard procedures using TaqMan Universal PCR Master Mix; quantitative PCR primers for human BLIMP1 (Hs00153357), mouse Blimp1 (Mm00476128), GAPDH, or ACTB; and the ABI Prism 7000 Sequence Detection System. All of these reagents, primers, and equipment were from Applied Biosystems (Foster City, CA). Each reaction was performed in duplicate, and results were normalized by GAPDH or ACTB expression.

PAX5 DNA sequencing of clinical samples

Lymph nodes or other tissues containing tumor cells were collected with informed consent from 85 patients diagnosed with diffuse large B cell lymphoma. The sequencing study was approved by the institutional review board of Nagoya University Graduate School of Medicine. Genomic DNA was extracted from those samples with the QIAamp DNA Micro Kit (QIAGEN), according to the manufacturer's instructions. Pyrosequencing was performed for analysis of DNA mutation surrounding codons 189 and 283, according to the manufacturer's instructions. Briefly, a 125-bp sequence of exon 5, including 189 codon, or a 128-bp sequence of exon 7, including 283 codon, was amplified by PCR using biotin-tagged primers. The biotinylated PCR strands were immobilized and purified by streptavidin-Sepharose beads, denatured, and added to annealing buffer containing 250 nM sequencing primer. Sequencing was carried out with the PyroMark Q96 ID system (QIAGEN). DNA sequences corresponding to codons 181–199 and codons 273–292 were analyzed using two sequence primers. Primers used in this analysis are described in Supplemental Table I.

Results

PAX5 is phosphorylated by ERK2 in vitro

It was recently reported that ERK1/2 signal was a key initiation signal for BLIMP1 expression and plasma cell differentiation of GCB cells (21); however, the detailed mechanism of ERK1/2 signal that induces BLIMP1 expression is still unknown. Combined with the open question about the initial event in plasma cell differentiation, these findings gave rise to the speculation that PAX5 phosphorylation by ERK1/2 negatively affected BLIMP1 repression by PAX5 and is the trigger of plasma cell differentiation. To test whether PAX5 can be phosphorylated in response to ERK1/2 signal, we performed an in vitro kinase assay using GST-PAX5 as a substrate. rERK2 efficiently phosphorylated full-length PAX5 and the N-terminal region of PAX5, whereas JNK did not (Supplemental Fig. 1A). Inspection of the PAX5 sequence revealed the presence of eight ERK1/2 consensus sites, S/T-P. Therefore, we introduced a series of alanine substitutions at these sites to map the actual phosphorylation sites by ERK2 and to check the phosphorylation status with the phosphate-affinity SDS-PAGE system. In this system, PAX5 phosphorylation by ERK2 was detected as two shifted bands (Fig. 1A), suggesting that PAX5 had two phosphorylation sites. Substitutions of alanine at codons 189, 283, and 285 (combined mutation), as well as at 283, caused the disappearance of one of the shifted bands, whereas other substitutions, including at codon 285, did not affect the shifted bands (Fig. 1A, Supplemental Fig. 1B, 1C), indicating that the phosphorylation sites were serines 189 and 283. Consistently, all shifted bands disappeared by combined substitution at codons 189 and 283 (Fig. 1A).

PAX5 phosphorylation occurred in vivo through ERK1/2 signaling

We next examined whether PAX5 phosphorylation occurred by the activation of ERK1/2 in vivo. Coexpression of the constitutively active mutant of MEK1 (CA-MEK1, an upstream activator of endogenous ERK1/2) in 293T cells caused similar PAX5 phosphorylation as in vitro (i.e., two shifted bands were observed in phosphate-affinity SDS-PAGE and disappeared by the mutation at ERK2 phosphorylation sites determined in vitro) (Fig. 1B). These results indicated that ERK1/2 could also phosphorylate PAX5 in vivo. We demonstrated the schema of the PAX5 structure, pointing out the phosphorylation sites. The amino acid sequences surrounding the phosphorylation sites are conserved evolutionarily, except in zebrafish (Fig. 1C).

Next, we attempted to determine whether endogenous PAX5 was phosphorylated by BCR stimulation. We stimulated BCR by anti-IgM Ab in Ramos cells, a cell line of Burkitt lymphoma, which is thought to be a tumor of GCB cells. Strikingly, BCR stimulation induced strong ERK1/2 phosphorylation and PAX5 phosphorylation in Ramos cells. This phosphorylation was inhibited by the MEK1 inhibitor, U0126, indicating the mediation of PAX5 phosphorylation by ERK1/2 signal (Fig. 2A). Furthermore, to examine whether BCR signal-induced PAX5 phosphorylation