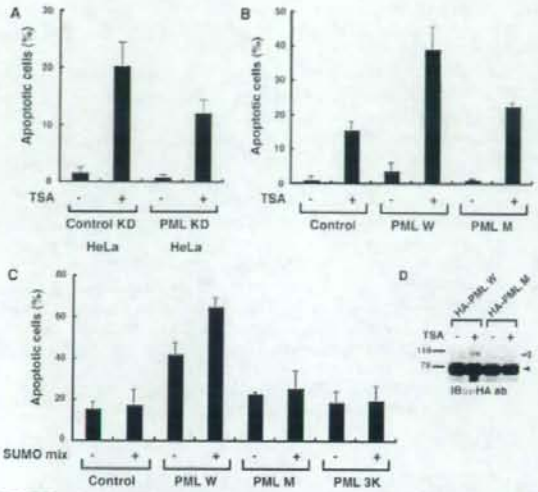

FIGURE 5. **PML acetylation is important for apoptosis induced by TSA.** *A*, PML knockdown reduces TSA-induced apoptosis in HeLa cells. HeLa cells whose PML was stably knocked down (PML KD HeLa) or control cells (Control KD HeLa) were treated with or without 1 μM TSA for 36 h. Apoptotic cells were detected and quantified as described under "Experimental Procedures." The ratios of apoptotic cells are plotted on bar charts. The averages of two independent analyses and standard deviations are shown. *B*, an acetylation-defective mutant of PML displayed impaired ability to mediate TSA-induced apoptosis. PML$^{-/-}$ MEFs were transfected with a bicistronic expression vector for GFP alone (control) or GFP and the indicated PML. GFP$^+$ cells were sorted and treated with or without 1 μM TSA for 48 h. Apoptotic cells were analyzed as in *A*. *C*, coexpression of Ubc9 and SUMO enhances PML-mediated apoptosis in response to TSA. The analyses of apoptosis were performed as in *B* except that cells were cotransfected with expression vectors for SUMO and Ubc9 (SUMO mix) or an empty vector as indicated. *D*, increased PML sumoylation induced by TSA in PML$^{-/-}$ MEFs. PML sumoylation in PML$^{-/-}$ MEFs was examined as in Fig. 4*B*. *IB*, immunoblotting; *ab*, antibody.

also defective in TSA-mediated apoptosis. It should be noted that PML$^{-/-}$ MEF cells are still sensitive to apoptosis by TSA, although at a much reduced level compared with cells expressing PML. There may also be PML-independent apoptotic mechanisms that respond to TSA. This is not surprising, considering that TSA alters the expressions of various genes by the acetylation of histones and many kinds of transcription factors such as p53 (see Introduction). More work will be required to determine the individual contributions of these different actions of TSA on the proliferation, survival, and differentiation of cells.

Acetylation of lysine leads to loss of its positive charge. In some cases, arginine, a positive charged amino acid, and glutamine, a noncharged one, are reported to mimic nonacetylated and acetylated lysine, respectively. We examined whether glutamine substitution at acetylation sites, lysines 487 and 515, had an enhancing effect on PML sumoylation. The PML mutant with glutamine substitution, however, showed impaired sumoylation in HeLa cells similarly to the one with arginine substitution (data not shown). Effects of acetylation other than the loss of positive charge or subtle differences of amino acid structure between lysine and glutamine may be important for PML sumoylation.

Recent studies reveal that increasing numbers of proteins are targeted by both acetylation and sumoylation. However, the correlation between these modifications has hardly been investigated except the cases where both modifications competitively target the same lysine residue such as the cases of HIC1 (hypermethylated in cancer 1) and MEF2 (myocyte enhancer factor 2) (23, 24). This is the first report that suggests acetylation-dependent enhancement of sumoylation. Phosphorylation has been reported to regulate sumoylation. Recently, the classical sumoylation consensus motif (ψKXE) with an adjacent proline-directed phosphorylation motif (SP), ψKXEXXSP motif where ψ is a large hydrophobic residue and X is any residue, has been proposed as a phosphorylation-dependent sumoylation motif (25, 26). Between the two acetylation sites, lysines 487 and 515, lysine 487 is the major acetylation site, and K487R affects sumoylation more than K515R (Fig. 2, *B* and *C*, and data not shown). PML sumoylation is reported to occur at three lysine residues, lysine 65, 160, and 490 (22). It would be interesting to discover whether acetylation at lysine 487 specifically affects sumoylation at any of these lysine residues, especially at the adjacent lysine 490. p53 also has a similar sequence where an acetylated lysine lies adjacent to a sumoylated lysine (supplemental Fig. S9), although the correlation between acetylation and sumoylation has not been investigated (27). K$\psi\psi$KXE might be a motif of acetylation-dependent sumoylation.

In summary, our studies provide evidence for a new post-transcriptional modification of PML and a new mechanism of regulation of PML sumoylation, and establish a novel relationship between PML and TSA-induced apoptosis. This work provides new insights into the regulation of PML function and the control of protein sumoylation. Considering the large number of binding partners of PML and the key contributions of PML to the stability and function of the NBs, PML acetylation is likely to modulate multiple cell activities beyond apoptosis through regulation of recruitment or release of NBs components.

Acknowledgments—We are very grateful to Ryouhei Tanizaki, Yuka Nomura, and Chika Wakamatsu for technical assistance.

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Phosphorylation of Runx1 at Ser249, Ser266, and Ser276 is dispensable for bone marrow hematopoiesis and thymocyte differentiation

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Received 29 December 2007
Available online 6 February 2008

Abstract

Runx1, one of three mammalian runt-domain transcription factor family proteins, is essential for definitive hematopoiesis. Based on transfection assays, phosphorylation of Runx1 at the three serine residues, Ser249, Ser266, and Ser276, was thought to be important for trans-activation activity of Runx1. By using "knock-in" gene targeting, we generated mouse strains expressing mutant Runx1 protein that harbored a combined serine-to-alanine substitution at either of two residues, Ser249/Ser266 or Ser249/Ser276. Either mutation resulted in a lack of major phosphorylated form of Runx1. However, while loss of definitive hematopoiesis and impaired thymocyte differentiation was observed following the loss of Runx1, these phenotypes were rescued in those mice lacking the major phosphorylated form of Runx1. These results not only challenge the predicted regulation of Runx1 activity by phosphorylation at these serine residues, but also reaffirm the effectiveness of "knock-in" mutagenesis as a powerful tool for addressing the physiological relevance of post-translation modifications.

Keywords: Runx1; Phosphorylation; Hematopoiesis; Lymphocyte differentiation; Knock-in mutagenesis; Transcription factor; Post-translational modifications

Runx1 (also known as AML1, CBFα1 or PEBP2αB) belongs to the runt-domain transcription factor family, and functions as α-subunit of the Runx complexes following dimerization with the non-DNA binding obligatory β-subunit Cbfβ [1]. Genetic studies in several species have revealed that Runx complexes engage in a variety of biological activities during the differentiation of many cell types [2–5]. For instances, Runx1 has been shown to be essential for definitive hematopoiesis [6,7]. On the other hand, human *RUNX1/AML1* gene remains the most frequent target of leukemia-associated chromosomal translocation or mutations observed in acute myeloid leukemia (AML) patients [8–10]. It is thus important to identify mechanisms that regulate Runx1 function to further understand the physiological and pathological contribution of Runx1 in hematopoiesis and leukemia, respectively.

In addition to the regulation of their expression levels, the biological activities of transcription factors may be regulated by various post-translational modifications. Runx1 protein has been shown to be phosphorylated by at least two kinase families, mitogen-activated protein kinase (MAPK) and homeodomain-interacting protein kinase (HIPK). Putative amino acid residues destined to be phosphorylated by MAPKs or HIPKs were mapped to the Runx1 protein residues Ser249 and Ser266 or Ser249 and

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doi:10.1016/j.bbrc.2008.01.124

Ser276, respectively [11–13]. In transfection assays, the mutations converting these serine residues to alanine residues diminished the Runx1-mediated activation of reporter genes [12–14]. Given that these serine residues are located in close proximity to the trans-activation domain of Runx1, phosphorylation of Runx1 protein has been postulated to act as an important regulatory mechanism enhancing the trans-activation activity of Runx1.

Consistently, impaired hematopoiesis in mice deficient for both HIPK1 and HIPK2 has suggested a possible involvement of HIPK-mediated Runx1 phosphorylation in Runx1-dependent programming of hematopoiesis [11]. Runx1 has also been shown to regulate thymocyte differentiation [15,16] in part through binding to the TCRβ enhancer (Eβ) [17]. Interestingly phorbol ester-induced activation of Eβ by Runx1 is dependent upon the phosphorylation of several serine/threonine residues including Ser276 [13]. In addition, Runx1 is necessary for the efficient positive selection of $CD4^+CD8^+$ DP thymocytes [16], a process influenced by MAPK pathway downstream of TCR signaling, suggesting that MAPK-dependent phosphorylation of Runx1 plays an important role in Runx1-mediated gene regulation in DP thymocytes. Thus, increasing evidence suggests that phosphorylation at Ser249, Ser266 or Ser276 plays an important role in the regulation of Runx1 activity.

In this study, we introduced two types of mutations into the murine *Runx1* locus in order to target serine-to-alanine substitutions at either of two residues, Ser249/Ser266 or Ser249/Ser276. Unexpectedly, phenotypes resulting from Runx1-deficiency that occurs during hematopoiesis and lymphopoiesis were rescued in those mice, although loss of phosphorylation at the above-mentioned serine residues resulted in a loss of the major phosphorylated form of Runx1. Thus Runx1 protein maintains an activity level sufficient for normal hematopoiesis, even in the absence of major phosphorylation.

Materials and methods

Generation of $Runx1^{PM12A}$ and $Runx1^{PM13A}$ alleles. A genomic DNA fragment used to construct the targeting vectors was obtained from the phage library (Stratagene). Both the 5′ long homology region and the 3′ short homology region were PCR amplified with an LA-PCR kit (TAKARA). The genomic DNA fragments harboring mutations for serine-to-alanine substitutions at the proposed positions were generated by employing overlapping PCR techniques. These three DNA fragments were sequentially ligated into pBluescript vector harboring *neomycin*-resistant gene and HSV-*thymidine kinase* gene in order to generate the targeting vectors. Transfection into E14 ES cells and generation of chimera mice were performed as previously described [15].

Immunoblot analysis and antibody generation. Cell lysates were prepared by sonication in a lysis buffer containing 100 mM Tris–HCl (pH 7.4), 300 mM NaCl, 2 mM EDTA, 2% Triton X-100, 0.2% SDS, 0.2% sodium deoxycholate, a protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Pierce). Lysates were separated by 10% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences). Membranes incubated with the first antibody were reacted with HRP-labeled anti-rabbit IgG (Amersham Biosciences), and then visualized with an ECL kit (GE Healthcare). An antibody against the N-terminal end of the distal promoter-derived Runx1 was generated by immunizing rabbits with the peptides, MASDSIFESFPSYPQCFMRDA. The antibody that reacted with Runx1 phosphorylated Ser249 was described previously [11], while that which reacted with Runx1 phosphorylated Ser276 will be described elsewhere by I.K.

Calf intestinal alkaline phosphatase (CIAP) treatment. Immunoprecipitation was performed using a Rabbit IgG TrueBlot Set (eBioscience) according to the manufacturer's protocol. Immunoprecipitated lysates bound to beads were incubated with 1 U/μL CIAP (Promega) for 30 min at 37 °C. After washing twice, samples were boiled with Laemmli sample buffer (Bio-Rad) to release proteins from beads.

Flow cytometry analyses. All antibodies used for flow cytometry were from BD Pharmingen or eBioscience. PE-conjugated αGalCer/CD1d-dimer was kindly provided by Dr. M. Taniguchi (RIKEN, RCAI). For surface staining, cells were incubated for 15–20 min at 4 °C with the corresponding cocktail of fluorescent-labeled antibodies. Data were acquired with a FACS Calibur (BD Biosciences) and were analyzed with FlowJo software (Tree Star Inc.).

Cell preparation. Thymocyte subsets were purified by electrical cell sorting using FACS Vantage (BD Biosciences). $CD43^+$ bone marrow cells and $B220^+$ splenic B cells were prepared using MACS beads (Miltenyi Biotec). For NKT cell preparation, liver cells were suspended in 33% Percoll solution (Amersham Biosciences) and then centrifuged for 20 min at room temperature. Pellets were used as liver mononuclear cells for subsequent study after lysing red blood cells.

Results and discussion

Generation of $Runx1^{PM12A}$ and $Runx1^{PM13A}$ mouse strains

Murine Runx1 protein has been shown to be phosphorylated at Ser249 and Ser266 mainly by Erk [12], while Ser249 and Ser276 residues are phosphorylated by HIPKs [11] (Fig. 1B). We therefore constructed the targeting vectors to introduce mutations leading to serine-to-alanine substitutions at murine Runx1 residues Ser249 and Ser266 (S249/266A) or at Ser249 and Ser276 (S249/276A) (Fig. 1A and B). The constructed mutations of the *Runx1* gene for S249/266A or S249/276A substitution are referred to hereafter as phosphorylation mutation 12A (PM12A) or PM13A, respectively.

After confirming a homologous recombination in ES cells by Southern blot, followed by excision of the *Neo* gene by transient transfection of *Cre* recombinase (Fig. 1A and Supplementary Fig. S1), we generated chimera mice from suitable ES cell clones via transmission of their mutations, thereby establishing the mouse strains $Runx1^{PM12A}$ and $Runx1^{PM13A}$ (Supplementary Fig. S1). Mice homozygous for either mutation were born at the expected frequency rate and grew normally. DNA sequencing confirmed that the mutant animals harbored the constructed mutations (Fig. 1C). Given that Runx1-deficient embryos die around 11.5 dpc by massive hemorrhage [6,7], the normal growth of $Runx1^{PM12A/PM12A}$ and $Runx1^{PM13A/PM13A}$ mice indicates that Runx1 function essential to early mouse development is independent of phosphorylation at residues Ser249, Ser266 or Ser276.

Biochemical characterization of $Runx1^{S249/266A}$ and $Runx1^{S249/276A}$ protein

Using an antibody that recognizes the N-terminal end of Runx1, immunoblot analyses detected Runx1 protein

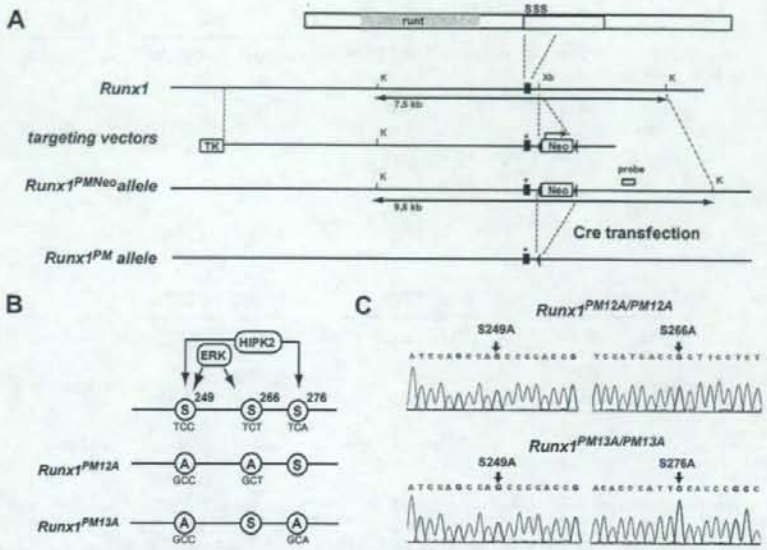

Fig. 1. Generation of mutant mice expressing Runx1$^{S249/266A}$ or Runx1$^{S249/276A}$ mutant Runx1 protein. (A) Structure of the *Runx1* locus, the targeting vectors and the targeted mutant *Runx1* locus. The black box, the black box marked with an asterisk and the triangle represent exon, mutated exon and loxP sequences, respectively. The open boxes marked with TK or Neo indicate *thimidine kinase* genes and *neomycin*-resistant genes, respectively. The DNA fragment used in the Southern blot analysis is indicated as a gray box. Restriction enzyme sites shown are KpnI (K) and XbaI (Xb). (B) Three phosphorylation serine residues of Runx1 and its putative kinases. The constructed DNA mutations *Runx1*^{PM12A} and *Runx1*^{PM13A} for serine-to-alanine substitutions on both Ser249 and Ser266 (S249/266A) or on both Ser249 and Ser276 (S249/276A) residues are indicated, respectively. (C) DNA sequences from mice homozygous for either *Runx1*^{PM12A} or *Runx1*^{PM13A} confirmed that the constructed mutations were correctly targeted into the *Runx1* locus.

expressed at two positions (Fig. 2A). However, only the more rapidly migrating Runx1 protein was detected in thymocytes from *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice (Fig. 2A). An antibody that reacts specifically with Runx1 phosphorylated Ser249 detected Runx1 protein at the position corresponding to the more slowly migrating Runx1 in *wild-type* thymocytes, while there were no detectable signals in the cell lysates of *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice (Fig. 2A). In similar fashion, an antibody against Runx1-derived peptide including phosphorylated Ser276 (anti-p-S^{276} Runx1 antibody) mainly reacted with the more slowly migrating Runx1 in control sample, while no reactive protein was detected in thymocytes from *Runx1*$^{PM13A/PM13A}$ mice (Fig. 2A). The anti-p-S^{276} Runx1 antibody still reacted with Runx1$^{S249/266A}$ protein from *Runx1*$^{PM12A/PM12A}$ mice, indicating that phosphorylation at Ser276 residues is independent from that occurring at the Ser249 and Ser266 residues.

To examine whether the Runx1$^{S249/276A}$ protein is phosphorylated at other residues, we treated *wild-type* Runx1 or Runx1$^{S249/276A}$ protein with calf intestinal alkaline phosphatase (CIAP) prior to immunoblot analyses (Fig. 2B). Disappearance of the more slowly migrating Runx1 protein by CIAP treatment confirmed that Runx1 protein in the upper position undergoes phosphorylation. Furthermore, the position of the CIAP-treated Runx1 protein was slightly lower than that of the more rapidly migrating Runx1. Similar changes in molecular weight following CIAP treatment were observed in Runx1$^{S249/276A}$ protein. These results indicate that Runx1 protein in thymocytes is phosphorylated to differing extents, appearing as two distinct bands (i.e., the major and minor phosphorylated forms) in immunoblot analyses.

Runx1 protein was detected as two bands in CD4$^-$CD8$^-$ DN thymocytes negative for αβTCR expression as well as in CD4$^+$CD8$^+$ DP thymocyte positive for αβTCR expression (Fig. 2C), indicating that natural phosphorylation of Runx1 protein would occur independently from TCR engagement. Runx1 expression in CD43$^+$ bone marrow cells containing hematopoietic stem/progenitors was almost undetectable, although inactivation of the *Runx1* gene affected the differentiation and homeostasis of these cells [6,18]. In contrast, Runx1 expression was easily detected in splenic B220$^+$ B lymphocytes as double bands, the upper one of which was lacked in cells from *Runx1*$^{PM13A/PM13A}$ mice (Fig. 2C). Thus major phosphorylation of Runx1 also occurs in B lymphocytes.

Collectively, these biochemical results indicate that the Ser249, Ser266, and Ser276 residue are physiological phosphorylation sites. Runx1 protein in lymphocytes is naturally phosphorylated to distinct degrees, thus comprising major and minor phosphorylated forms. Phosphorylation at Ser249 would be essential for sequential

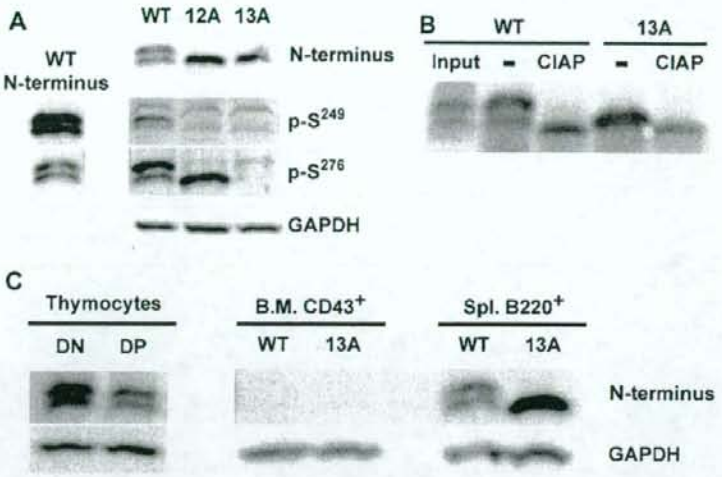

Fig. 2. Loss of the major phosphorylated form of Runx1 in *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice. (A) Immunoblot analyses of total thymocytes from *wild-type* (WT), *Runx1*$^{PM12A/PM12A}$ (12A) and *Runx1*$^{PM13A/PM13A}$ (13A) mice with three different antibodies recognizing either N-terminal end (N-terminus), phosphorylated Ser249 (p-S^{249}) or phosphorylated Ser276 (p-S^{276}) of murine Runx1 protein. Position of WT Runx1 protein detected by anti-N-terminus antibody is indicated as a reference at the left of middle and lower panel. GAPDH expressions are shown as a loading control. (B) Calf intestinal alkaline phosphatase (CIAP) treatment indicated that the most slowly migrating Runx1 constitutes a major phosphorylated form of Runx1, one that is absent in *Runx1*$^{PM13A/PM13A}$ mice. (C) Immunoblot analyses of Runx1 with anti-N-terminus Runx1 in CD4$^-$CD8$^-$ DN thymocytes (DN) and CD4$^+$CD8$^+$ DP thymocytes (DP) from wild-type mice (left panel), as well as CD43$^+$ bone marrow cells (B.M. CD43$^+$) and B220$^+$ splenic cells (Spl. B220$^+$) from wild-type (WT) and *Runx1*$^{PM13A/PM13A}$ (13A) mice.

phosphorylation at other residues when generating the major phosphorylated form of Runx1.

Phosphorylation of Runx1 at Ser249, Ser266, and Ser276 is not essential for early hematopoiesis and myeloid cell differentiation

We next examined the effect of *Runx1*^{PM12A} or *Runx1*^{PM13A} mutation on hematopoietic cell differentiation. In adult bone marrow, hematopoietic stem/progenitor cells and common lymphoid progenitors (CLPs) are included in lineage (Lin)$^-$IL-7Rα^-c-Kit$^+$Sca-1$^+$ cells and Lin$^-$IL-7R$^+$c-Kit^{low}Sca-1^{low} subset, respectively. The percentages of these populations in *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice are compatible with those of *wild-type* mice. Similarly common myeloid progenitors (CMPs, Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRIII/II^{lo}), granulocyte/macrophage progenitors (GMPs, Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRIII/II$^+$) and megakaryocyte/erythroid progenitors (MEPs, Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRIII/II$^{lo/-}$) were normally present in *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice (Fig. 3A). Consistent with normal development of progenitor cells, there were no significant changes in differentiation of megakaryocyte, monocytes, granulocytes or erythroblast in bone marrow (Supplementary Fig. S2). Finally the erythroid or myeloid colony-forming activity of bone marrow cells in methylcellulose medium was not influenced by *Runx1*^{PM12A} or *Runx1*^{PM13A} mutation (Fig. 3B).

These results indicate that phosphorylation of Runx1 at Ser249, Ser266, and Ser276 is nonessential for Runx1 function required in early hematopoiesis. Considering the fact that HIPKs have been shown to be important in early hematopoiesis [11], substrate or interacting molecules other than Runx1 may exist in the HIPK-dependent regulation of early hematopoiesis.

Phosphorylation of Runx1 at Ser249, Ser266, and Ser276 is not essential for thymocyte differentiation

Inactivation of the *Runx1* gene in early thymocytes progenitors by *Lck-Cre* transgene partially inhibited thymocyte development. We therefore examined thymocyte differentiation in *Runx1*$^{PM12A/PM12A}$ and *Runx1* $^{PM13A/PM13A}$ mice. A decrease in the number of thymocytes concomitant with the accumulation of CD4$^-$CD8$^-$ DN thymocytes, as observed in *Runx1*$^{fl/fl}$: *Lck-Cre* mice [15], was not observed in these two mutant mice (Fig. 4A and B). CD4 repression in CD4$^-$CD8$^-$ DN thymocytes is regulated by the intronic *Cd4* silencer, whose activity requires the binding of Runx1 protein [15]. However, CD4 expression levels in TCRβ$^-$CD8$^-$CD25$^+$ populations corresponding to DN3 thymocytes were normal in *Runx1*$^{PM12A/PM12A}$ and *Runx1* $^{PM13A/PM13A}$ mice (Supplementary Fig. S3).

Erk functions downstream of TCR signaling and plays an important role in the selection of CD4$^+$CD8$^+$DP thymocytes [19]. As Runx1 has been shown not only to be a substrate of Erk [12] but also important for positive selec-

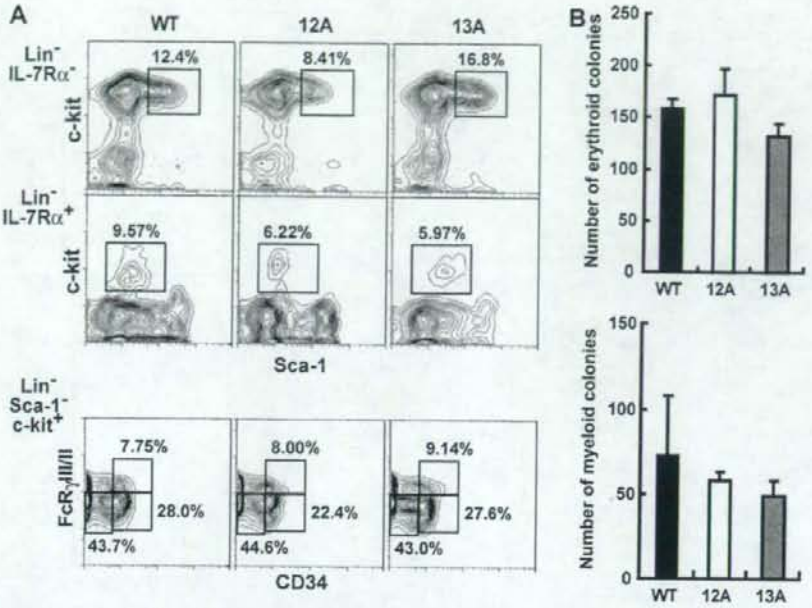

Fig. 3. Phosphorylation of Runx1 at Ser249 and Ser266 or Ser249 and Ser276 is not essential for bone marrow hematopoiesis. (A) Expression patterns of the indicated surface markers in the indicated bone marrow fractions from *wild-type* (WT), *Runx1*$^{PM12A/PM12A}$ (12A) and *Runx1*$^{PM13A/PM13A}$ (13A) mice show normal differentiation of progenitor populations. (B) Normal colony-forming activity in *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice.

tion [16], we examined whether *Runx1*^{PM12A} or *Runx1*^{PM13A} mutation affects the differentiation of $CD4^+CD8^+$DP thymocytes. There were no significant changes detected in the number of $CD4^+CD8^+$DP thymocytes or in the percentage of $TCR\beta^{hi}HSA^{hi}$ post-selection thymocytes (Fig. 4A and B). In addition, decrease in the percentage of mature thymocytes resulting from the loss of Runx1 was not observed either in *Runx1*$^{PM12A/PM12A}$ or *Runx1*$^{PM13A/PM13A}$ mice. The ratio of $CD4^+$ single positive (SP) thymocytes to $CD8^+$ SP thymocytes in mature thymocytes ($TCR\beta^{hi}HSA^{lo}$) was slightly lower in *Runx1*$^{PM13A/PM13A}$ mutant mice, while loss of Runx1 led to a significant decrease in the number of mature CD4-lineage thymocytes [16]. Thus, putative Erk phosphorylation sites are not essential for Runx1-mediated gene regulation, which is important for positive selection and maturation of CD4-lineage thymocytes.

Runx1 is also involved in $CD4^+$ T cell homeostasis, in part by maintaining the expression of IL-7Rα [16]. Although a decrease in the number of $CD4^+$ T cells by loss of Runx1 protein were sufficient enough to reverse the ratio of $CD4^+$ T cells to $CD8^+$ T cells [16], we observed only a modest decrease in $CD4^+$ T cells in the periphery (Fig. 4C and D) and normal IL-7Rα expression levels on $CD4^+$ T cells in *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice (Supplementary Fig. S3)). Finally, while NKT cell development was defective in mice lacking Runx1 in thymus [20], normal NKT cell development was observed in *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice (Fig. 4E).

Using a combined biochemical and genetic approach, our study revealed that the Runx1 protein exists in two phosphorylated forms at least in lymphocytes. Generation of the major phosphorylated form requires the Ser249 residue, suggesting that phosphorylation on this serine residue triggers sequential phosphorylation. It is unclear which kinase(s) is involved in generation these two phosphorylated forms. Since two phosphorylated forms are detected in DN thymocytes, these phosphorylation events could occur independent of TCR engagement. However, it is still possible that Runx1 is phosphorylated by some basic Erk activity that is independent from TCR stimulation. Further studies using Erk1/Erk2 deficient thymocytes would be helpful to address this issue.

Although both *Runx1*$^{PM12A/PM12A}$ and *Runx1*$^{PM13A/PM13A}$ mice not only were devoid of phosphorylation on Ser249, Ser266, and Ser276, but also lacked major phosphorylated forms of Runx1, hematopoiesis and lymphocytes development in those mice remained normal. These observations counter findings made in previous studies involving phosphorylation-dependent trans-activation activity of Runx1 in transfection assay. The maximum trans-activation activity detectable in a transfection assay may be dependent upon the phosphorylation of Runx1. However, the latter is unlikely to be essential to minimum Runx1 activity that is sufficient for normal hematopoiesis in *Runx1*$^{PM12A/PM12A}$

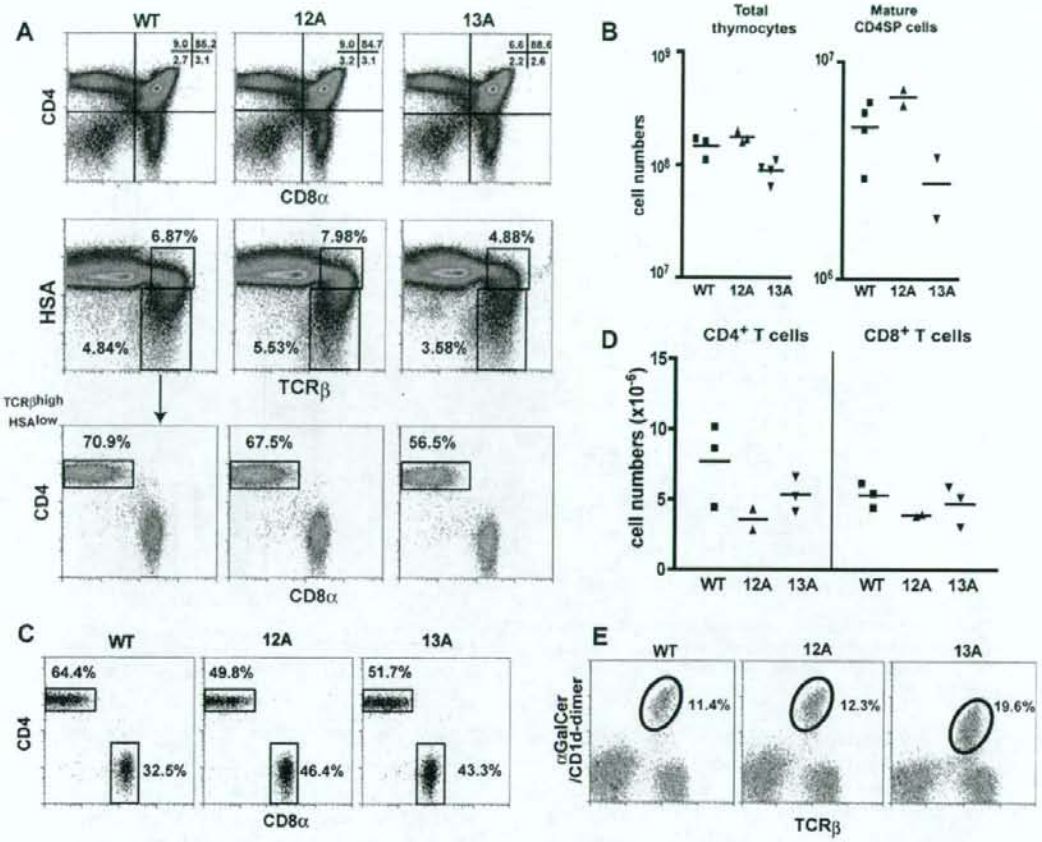

Fig. 4. Phosphorylation of Runx1 at Ser249 and Ser266 or Ser249 and Ser276 is not essential for T cell development. (A) Expression patterns of the indicated surface markers in total thymocytes or mature ($TCR\beta^{hi}HSA^{lo}$) thymocytes from *wild-type* (WT), $Runx1^{PM12A/PM12A}$ (12A) and $Runx1^{PM13A/PM13A}$ (13A) mice. (B) Cell numbers of total thymocytes and mature CD4 SP thymocytes. (C) CD4 and CD8 expression profile in $TCR\beta^{+}$ splenocytes. (D) Cell numbers of $CD4^{+}$ and $CD8^{+}$ splenic T cells from the indicated genotyped mice. (E) Normal NKT cell development in $Runx1^{PM12A/PM12A}$ (12A) and $Runx1^{PM13A/PM13A}$ (13A) mice.

and $Runx1^{PM13A/PM13A}$ mice. It is therefore possible that a competitive repopulation assay in host mice would reveal the masked effects of $Runx1^{PM12A}$ or $Runx1^{PM13A}$ mutation. Alternatively, as Runx1 and Runx3 have redundant functions each other during thymocyte differentiation [16], the effect of $Runx1^{PM12A}$ and $Runx1^{PM13A}$ mutations would become apparent under a Ruxn3-deficient background.

At any rate, to address the physiological relevance of post-translational modifications, gene targeting-based "knock-in" mutagenesis would likely be the most reliable approach. Our results in this study cannot rule out the possibility that phosphorylation events on unidentified residues may play important roles in regulating Runx1 function. Indeed recent studies have reported that phosphorylation of Runx1 by cyclin-dependent kinase on residues other than Ser249, Ser266, and Ser276 was important for trans-activation of Runx1 protein [21]. Further mutation-based approaches are needed to identify those residues that are involved in the key post-translational modifications of Runx1.

Acknowledgments

We thank S. Mochizuki for injecting the ES clones, and M. Taniguchi for providing the αGalCer/CD1d-dimer. This work was supported by grants from PRESTO, JST.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.01.124.

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Leukemia (2008) 22, 273–280

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ORIGINAL ARTICLE

Phosphorylation of PML is essential for activation of C/EBPε and PU.1 to accelerate granulocytic differentiation

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Promyelocytic leukemia (PML) is a nuclear protein that functions as a regulator of transcription, cell proliferation, apoptosis and myeloid cell differentiation. PML is subjected to post-translational modifications such as sumoylation and phosphorylation. However, the physiological significance of these modifications, especially for myeloid cell differentiation, remains unclear. In this report, we found that four serine residues in the PML C-terminal region are highly phosphorylated in a myeloid cell line. Wild-type PML accelerated G-CSF-induced granulocytic differentiation, but a phosphorylation-deficient PML mutant failed. PML interacted with C/EBPε, a transcription factor essential for granulopoiesis, activated C/EBPε-mediated transcription in concert with p300 and accelerated C/EBPε-induced granulocytic differentiation. Phosphorylation of PML was required for stimulating C/EBPε-dependent transcription and accelerating C/EBPε-induced granulocytic differentiation. We also found that PML phosphorylation was required for stimulation of PU.1-dependent transcription and acceleration of PU.1-induced granulocytic differentiation. These results suggest that phosphorylation plays essential roles in the regulation of PML to accelerate granulocytic differentiation through multiple pathways.

Leukemia (2008) **22**, 273–280; doi:10.1038/sj.leu.2405024; published online 8 November 2007
Keywords: PML; phosphorylation; C/EBPε; PU.1; granulocytic differentiation

Introduction

Promyelocytic leukemia (PML) is a nuclear protein that plays a role in growth suppression, apoptosis, premature senescence and myeloid cell differentiation. PML concentrates in speckled subnuclear structures, termed PML nuclear bodies (NBs)/ND10/PODs, together with many other proteins, including Sp100, p53, pRb, Daxx and p300/CBP.[1] These facts suggest that PML plays a role in transcriptional regulation. The *PML* gene is involved in the chromosomal translocation t(15;17) and fuses to the retinoic acid receptor α (*RARα*) gene in the majority of cases of acute promyelocytic leukemia (APL), which is characterized by disruption of NBs into abnormal microspeckle structures.[2] In APL, the fusion gene product PML-RARα has been thought to block granulopoiesis by dominant-negative inhibition of both PML and RARα functions. PML is important for terminal differentiation of granulocytes, as shown by impaired granulopoiesis in PML-deficient mice.[3] Although PML plays a role in granulopoiesis, at least in part, by its modulation of the retinoic acid pathway,[3] it does not fully explain the role of PML in granulopoiesis, suggesting that other PML actions should be considered for myelopoiesis in the physiological condition.[4]

PML function is regulated by at least two distinct modifications, specifically, phosphorylation and sumoylation. Sumoylation is required for NB formation and enhancement of PML-dependent apoptosis.[5] Phosphorylation of PML is induced by ATR or Chk1/2 after DNA damage and it regulates p53-dependent and -independent apoptosis.[6,7] extracellular signal-regulated kinases (ERK)-mediated phosphorylation of PML increases sumoylation and enhances apoptosis in response to arsenic trioxde.[8] CK2-mediated phosphorylation leads to ubiquitin-dependent degradation of PML.[9] Thus, these two modifications are important for regulating PML-dependent apoptosis and PML stability. We previously reported that PML sumoylation might have an impact on granulocytic differentiation,[10] but the role of PML phosphorylation in regulating granulocytic differentiation has not yet been addressed.

Granulopoiesis is tightly controlled by lineage-specific transcription factors. CCAAT/enhancer-binding protein ε (C/EBPε) is expressed exclusively in granuloid cells and is essential for terminal differentiation of committed granulocyte progenitors.[10] Although C/EBPε can activate or repress target genes depending on its associated protein,[11] the essential partner in terminal granulocytic differentiation remains to be explored. PU.1 is also expressed exclusively in hematopoietic cells, and it is indispensable for the terminal differentiation of myeloid cells.[12] Recently, we reported that PML promotes the association of PU.1 with p300 to form the active transcriptional complex,[13] but the regulatory mechanism of their interaction remains to be elucidated.

L-G is an interleukin-3 (IL-3)-dependent myeloid cell line that can be differentiated into mature granulocytes in response to granulocyte-colony stimulating factor (G-CSF).[14] We found that PML is highly phosphorylated in L-G cells and the phosphorylation of PML is essential for accelerating G-CSF-induced granulocytic differentiation. We also found that PML associates with C/EBPε. PML activated C/EBPε-mediated transcription in cooperation with p300 and accelerated C/EBPε-induced granulocytic differentiation in a phosphorylation-dependent manner. These effects of phosphorylation on the PML-dependent regulation of granulopoiesis and transcription were also observed in the case of PU.1 regulation. Taken together, these findings suggest an essential role of PML phosphorylation in transcriptional regulation during the terminal differentiation of granulocytes.

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Received 20 June 2007; revised 25 September 2007; accepted 8 October 2007; published online 8 November 2007

Materials and methods

Plasmids

The expression vectors for PML isoform IV, pLPCX-HA-PML and pLPCX-FLAG-PML, pMT-PU.1 and pLNCX–PU.1 were described previously.[10,13] C/EBPε cDNA encoding a 32-kDa protein was generated as described previously[15] and subcloned into pHM6, pLNCX and pMT vectors. Phosphorylation-deficit PML-4A or phosphorylation-mimic PML-4D mutants were generated by site-specific mutagenesis with overlapping extension PCR. Four serine residues at codons 505, 518, 527 and 530 were substituted to alanines or aspartic acids (TCC508SerGC C508Ala, -GAC505Asp; TCA518Ser-GCA518Ala, -GAC518Asp; AGC527Ser-GCC527Ala, -GAC527Asp; AGC530Ser-GCC503Ala, -GAC530Asp), respectively. The construction of sumoylation-deficient mutant PML-3R has been previously described.[10] A PML-dSP mutant lacking the serine- and proline-rich (SP) region (aa 502–554) was generated by appropriate restriction enzymes and PCR. All constructs were verified by DNA sequencing.

Construction of stable clones and retrovirus

First, 1×10^7 L-G cells were electroporated with pMT-C/EBPε or pMT–PU.1 plasmid, and stable clones were selected with 1 μg/ml of G418. Expression of C/EBPε or PU.1 was induced by adding 100 μM $ZnSO_4$ to the medium containing IL-3. Wild-type PML or its mutants were transduced by retrovirus infection as described previously,[10] and stable infectants were selected by 1 μg/ml of puromycin.

Identification of phosphorylation sites in the PML protein

FLAG–PML proteins purified from L-G cells were subjected to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis as previously described.[10] Phosphopeptides were identified using TurboSEQUEST software.

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting analysis were performed as previously described.[10]

Antibodies

Primary antibodies used in this study were as follows: anti-FLAG (M2, Sigma, St Louis, MO, USA), anti-HA (3F10, Roche, Mannheim, Germany), anti-human C/EBPε (C-22, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human PML (1B9, MBL, Nagoya, Japan; H238, Santa Cruz Biotechnology), anti-human p300 (NM11, BD Bioscience, San Jose, CA, USA), anti-human PU.1 (T-21, Santa Cruz Biotechnology) and anti-mouse TFIIB (C-18, Santa Cruz Biotechnology).

Cells, in vitro phosphatase treatment, immunofluorescence, luciferase reporter assay, quantitive reverse transcription PCR (qRT-PCR)

Technical details are available in Supplementary Information.

Results

Identification of phosphorylation sites in PML protein

The primary structure of PML predicts putative phosphorylation sites within the N-terminal proline-rich (Pro) region and the C-terminal serine- and proline-rich (SP) region.[16] We first investigated the post-translational modification of PML stably expressed in L-G cells (Figure 1a). Western blot analysis showed that PML migrates with variable electrophoretic mobility. Four distinct bands were observed after the treatment of PML proteins with alkaline phosphatase (CIAP), indicating that PML is modified by phosphorylation as well as sumoylation in L-G cells.

To determine phosphorylation sites, exogenously expressed PML was purified from L-G cells and analyzed by LC/MS/MS. Four serines at codons 505, 518, 527 and 530 in the SP region of PML were identified as phosphorylation sites (Figure 1b). A mutant in which these serines were substituted to alanines (PML-4A) migrated to a similar position to that of phosphatase-treated wild-type PML, indicating that the four serine residues were mainly phosphorylated in L-G cells (compare Figures 1a and c).

Phosphorylation and sumoylation of PML are essential for acceleration of G-CSF-induced granulocytic differentiation

To elucidate the significance of PML phosphorylation and sumoylation in granulocytic differentiation, we also constructed phosphorylation-mimic PML-4D mutant with substitutions of serines 505, 518, 527 and 530 by aspartic acids, sumoylation-deficient PML-3R mutant with substitutions of lysines 65, 160 and 490 by arginines, or PML-dSP mutant with a deletion of the SP region containing the phosphorylation sites (Figure 1b). Then, we introduced these mutants as well as wild-type and PML-4A into L-G cells by retrovirus infection and tested their effects on the differentiation of L-G cells. Equivalent levels of wild-type and mutant PML proteins were expressed in L-G cells (Figure 1c). In the presence of IL-3, all of these infectants remained in immature myeloblasts (Figure 2a). After treatment with G-CSF for 5 days, an increased population of mature granulocytes was observed in PML-WT and PML-4D infectants when compared with vector-transduced cells (Figures 2a and b). However, the majority of PML-4A, -dSP and -3R infectants still remained at the myelocyte or metamyelocyte stage and only a small population of mature granulocytes was observed. To objectively evaluate the effects of PML mutants on cell differentiation, we used qRT-PCR to quantify the expression of neutrophil gelatinase (*NG*), a gene encoding a secondary granule protein which is upregulated in mature granulocytes (Figure 2c). Compared to vector-transduced cells, PML-WT and -4D, but not PML-4A, -dSP and -3R, enhanced the increase in expression of *NG* after treatment with G-CSF. These results indicate that, in addition to sumoylation, phosphorylation in the SP region is essential for PML to accelerate G-CSF-induced granulocytic differentiation.

PML associates with C/EBPε

Since PML is a transcriptional coregulator, the above results suggest that phosphorylation and sumoylation may be crucial for its regulatory action on some transcription factors involved in granulocytic differentiation. It has been demonstrated that C/EBPε functions during the G-CSF-induced granulocytic differentiation.[17] To examine the interaction between PML and C/EBPε, co-immunoprecipitation assays were performed. FLAG-PML and HA-C/EBPε were transiently coexpressed in Bosc23 cells, and immunoprecipitants with anti-FLAG antibody were analyzed by western blot with anti-HA antibody, showing co-precipitation of C/EBPε with PML (Figure 3a). Reciprocally, HA-PML was also co-precipitated with FLAG-C/EBPε. In HL60

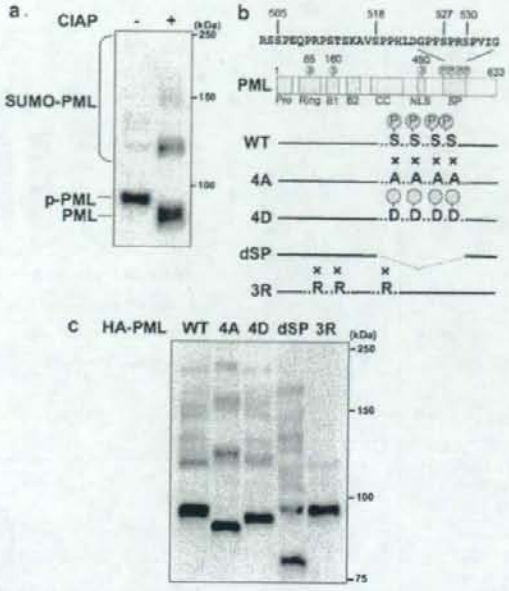

Figure 1 Covalent modifications of promyelocytic leukemia (PML) in granulocyte precursor cells. (**a**) Phosphorylation and sumoylation of PML in L-G cells. Stably expressed FLAG-PML was immunoprecipitated from the lysate of L-G cells, treated with (+) or without (−) CIAP and then analyzed by western blot with anti-FLAG antibody. Sumoylated, phosphorylated and unmodified PML are indicated. (**b**) Schematic diagrams of PML and PML mutants. Sites of phosphorylation and sumoylation are shown. Pro, proline-rich region; Ring, RING finger domain; B1 and B2, B boxes; CC, coiled-coil domain; NLS, nuclear localization signal; SP, serine- and proline-rich region. (**c**) Expression of each PML protein in stable L-G infectants. Total cell lysates from each PML infectant were analyzed by western blot with anti-HA antibody.

cells, endogenous PML and p300 were co-precipitated with C/EBPε whose expression was immediately increased after differentiation induced by all-*trans* retinoic acid (ATRA) treatment (Figure 3b). Notably, the amount of p300 that co-precipitated with C/EBPε was significantly increased within 2 days, demonstrating an accumulation of p300 in the C/EBPε/PML complex. To further confirm the association of C/EBPε and PML, HA–C/EBPε and PML were coexpressed in NIH3T3 cells, and double immunofluorescent staining was performed using anti-HA or anti-PML antibodies (Figure 3c). Without co-transfection of PML, C/EBPε dispersed throughout nuclei. When PML was coexpressed, C/EBPε accumulated in small dot-like structures, which coincided with NBs. Taken together, these results indicate that PML interacts with C/EBPε.

Essential role of PML phosphorylation for regulating C/EBPε activity

We generated an L-G/pMT-C/EBPε cell line, in which C/EBPε expression could be induced by exposure to $ZnSO_4$. The L-G/pMT-C/EBPε cells differentiated into mature granulocytes with segmented nuclei even in the presence of IL-3 within 6 days after exposure to $ZnSO_4$ (data not shown). To examine the effects of PML and its modifications on the C/EBPε-induced granulocytic differentiation, the cells were further infected with retroviruses encoding PML constructs or control vector, and then C/EBPε expression was induced (Figure 4a). The induced C/EBPε expression suppressed cell proliferation, which was enhanced by coexpression of PML-WT (Figure 4b). Compared to vector-transduced cells, an increased population of mature granulocytes was observed 4 days after PML-WT infectants were treated with $ZnSO_4$ (Figures 4c and d). Similarly, PML-4D inhibited cell proliferation and accelerated cell differentiation, but neither PML-4A nor -dSP did. Unexpectedly, PML-3R inhibited cell proliferation and accelerated cell differentiation as strongly as PML-WT. The increased expression of *NG* after $ZnSO_4$ treatment was enhanced by PML-WT, -4D and -3R, but not by PML-4A and -dSP (Figure 4e). A similar result was observed for the expression of lactoferrin (*LTF*), a gene that encodes a protein that is present in the secondary granules and is directly activated by C/EBPε.[18] These results indicate that PML accelerates C/EBPε-induced granulocytic differentiation and that phosphorylation, but not sumoylation, of PML is required for the effect.

We also examined whether the PML mutations affected the interaction and colocalization of PML with C/EBPε and p300 (supplementary figure). However, neither mutation affected these interactions and colocalizations. To test the effect of these modifications on C/EBPε-dependent transcription, we performed a luciferase reporter assay by co-transfecting plasmids for C/EBPε, p300 and wild-type or mutant PML together with a luciferase reporter containing the G-CSF receptor promoter (G-CSFR-luc), which contains a binding site for C/EBP family members (Figure 4f). While p300 alone modestly stimulated the transcriptional activity of C/EBPε, the coexpression of PML-WT further enhanced the C/EBPε-mediated transcription. PML-4D and -3R also stimulated transcription. However, PML-4A was

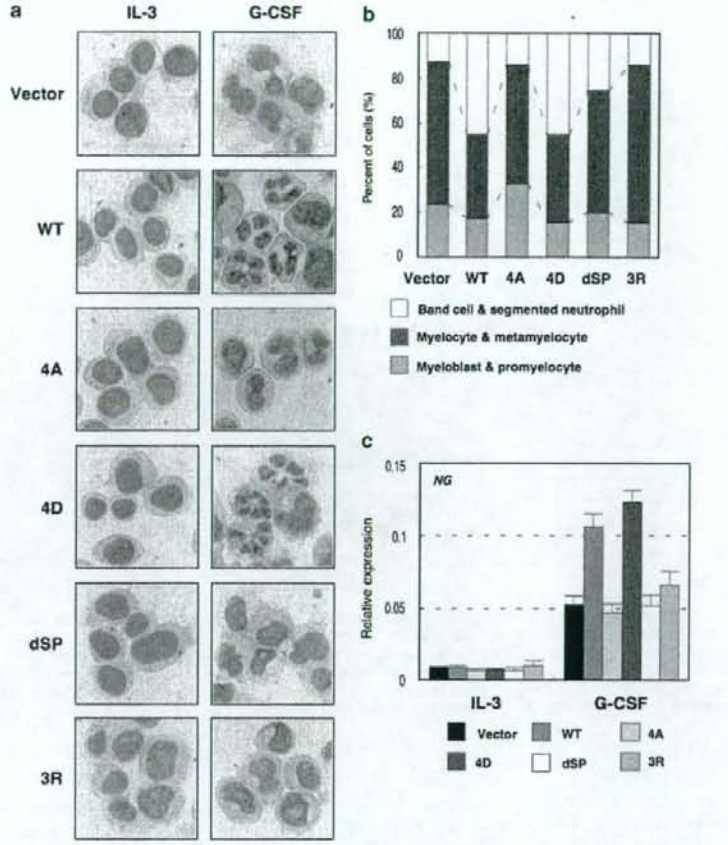

Figure 2 Phosphorylation and sumoylation of PML are essential for accelerating granulocyte-colony stimulating factor (G-CSF)-induced granulocytic differentiation. (**a**) Morphological evaluation of differentiation of L-G promyelocytic leukemia (PML) infectants treated with G-CSF for 5 days. (**b**) Differential count of L-G PML infectants after 5 days of treatment with G-CSF. (**c**) Comparison of secondary granule protein expression. Expression of neutrophil gelatinase (*NG*) in L-G PML infectants cultured in the presence of interleukin-3 (IL-3)- or G-CSF (for 3 days) was quantified by real time quantitative reverse transcription PCR (qRT-PCR). Data represent means ± s.d. of triplicate determinations of a representative experiment.

less potent, and PML-dSP was completely silent on the C/EBPε/p300-mediated transcription. It is particularly noteworthy that these effects of PML-WT and PML mutants on the C/EBPε-mediated transcription were correlated with their abilities to accelerate C/EBPε-induced granulocytic differentiation, suggesting that the activation of C/EBPε transcription by the phosphorylated, but not the sumoylated, form of PML plays an important role in granulopoiesis.

Requirement of phosphorylation for PML-dependent regulation of PU.1

Recently, we demonstrated that the transcriptional activity of PU.1 is also positively regulated by interaction with PML.[12] Therefore, we investigated the roles of PML modifications in PU.1-mediated transcription. A reporter assay showed that PML-WT, -4D and -3R activated PU.1-dependent transcription while PML-4A and -dSP did not (Figure 5a). To analyze the effects of PML modifications on PU.1-induced differentiation, we transduced PML constructs into L-G/pMT-PU.1 cells and then induced differentiation by $ZnSO_4$ treatment to express PU.1 (Figure 5b). PML-WT, -4D and -3R suppressed proliferation and accelerated granulocytic differentiation, whereas PML-4A did not (Figures 5c–e). The expression of *NG* was further increased in PML-WT, -4D and -3R infectants, but not PML-4A infectants, after treatment with $ZnSO_4$ (Figure 5f). These results indicate that PU.1-mediated transcription and granulocytic differentiation are also regulated by phosphorylated PML.

Discussion

PML accelerates granulocytic differentiation

One role of PML in terminal myeloid differentiation has been demonstrated in PML-deficient mice, which experience

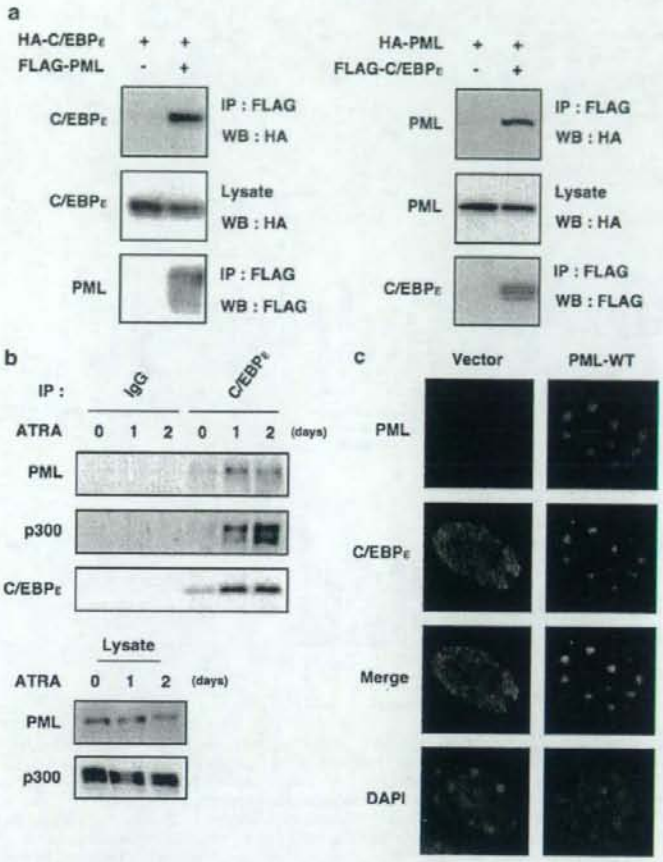

Figure 3 *In vivo* association of promyelocytic leukemia (PML) and C/EBPε. (**a**) Co-immunoprecipitation of PML and CCAAT/enhancer-binding protein e (C/EBPε). HA-C/EBPε and FLAG-PML were coexpressed in BOSC23 cells. Total expression (middle) or co-precipitated (top) C/EBPε was detected by western blot with anti-HA antibody. Immunoprecipitated PML was also analyzed with an anti-FLAG antibody (bottom) (left). A reciprocal experiment was also performed (right). (**b**) Association of endogenous PML and p300 to C/EBPε in HL60 cells. Cell lysates from HL60 cells treated with all-trans retinoic acid (ATRA) for the indicated days were immunoprecipitated with an anti-C/EBPε antibody and analyzed by western blot with anti-PML (top), anti-p300 (middle) and anti-C/EBPε antibodies (bottom),(upper panel). Levels of total PML and p300 in cell lysates were also analyzed (lower panel). (**c**) Colocalization of PML and C/EBPε within nuclear bodies (NBs). NIH3T3 cells were co-transfected with an expression vector for HA-C/EBPε together with either empty vector or FLAG–PML. C/EBPε was stained with anti-HA and FITC-labeled anti-rat antibodies. PML was stained with anti-PML and Texas red-labeled anti-rabbit antibodies. Nuclei were counterstained by 4′,6-diarnidino-2-phenylindole (DAPI).

impaired granulopoiesis.[3,13] In the present study, we found that PML accelerates G-CSF-induced granulocytic differentiation. A previous study[17] and our results (data not shown) demonstrate that G-CSF stimulation induces the expression of C/EBPε followed by granulocytic differentiation. These findings prompted us to determine whether PML regulates C/EBPε transcriptional activity to accelerate granulocytic differentiation. The current data illustrate that PML interacts with C/EBPε to activate its transcriptional activity and accelerates the granulocytic differentiation induced by overexpression of C/EBPε. Previously, we found that PML also accelerates PU.1-induced granulocytic differentiation.[13] Thus, PML appears to contribute to the regulation of granulopoiesis through interactions with C/EBPε and PU.1.

Phosphorylation of PML in myeloid cells

It has been suggested that the functions of PML are regulated at least in part by phosphorylation and sumoylation.[5–9] However, the role of PML phosphorylation in myeloid cell differentiation has not previously been addressed. In the present study, we found that four serine residues within the SP region of PML are highly phosphorylated in L-G cells. PML also contains several other serine residues in the N- and C-terminal regions that have been reported to be phosphorylated by ERK or CK2.[8,9] However, we did not detect these modifications by LC/MS/MS. Furthermore, alanine mutations of the phosphorylation sites did not affect the electrophoretic mobility of PML in L-G cells (data not shown). Thus, the SP region of PML is the main target of phosphorylation in L-G myeloid cells. While the upstream

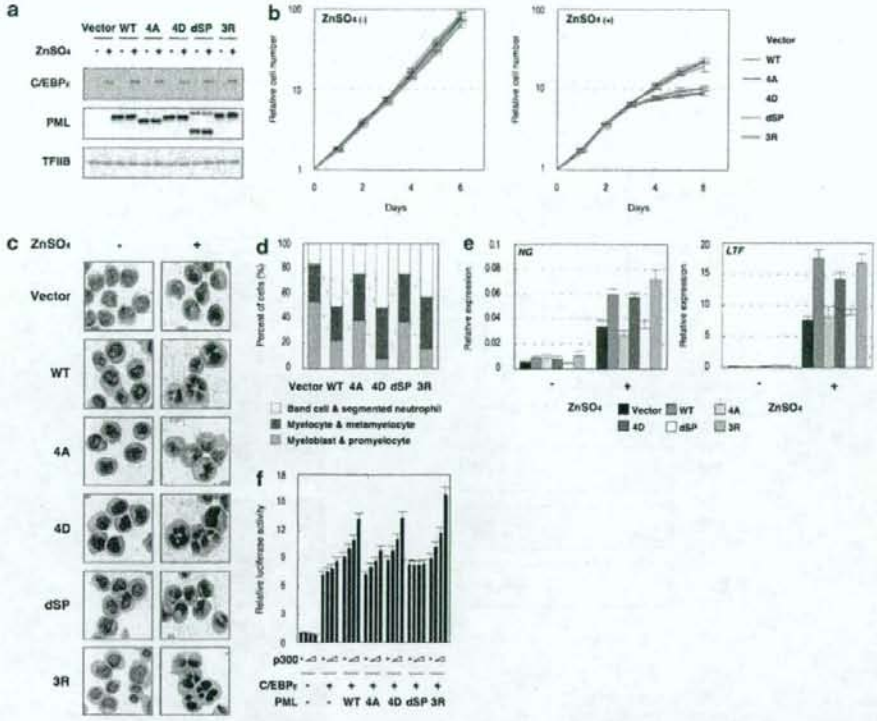

Figure 4 Effects of promyelocytic leukemia (PML) and PML phosphorylation on CCAAT/enhancer-binding protein e (C/EBPε)-induced granulocytic differentiation. (**a**) The expression of C/EBPε and PML in L-G/pMT-C/EBPε cells. Cells were cultured in the absence (−) or presence (+, for 24 h) of $ZnSO_4$. Total cell lysates were analyzed by western blot with anti-C/EBPε, -HA and -TFIIB antibodies. (**b**) Growth suppression of L-G/pMT-C/EBPε infectants by phosphorylated PML. Cells were cultured in the absence (left) or presence (right) of $ZnSO_4$. The relative number of viable cells is shown. The error bars represent the s.d. (**c**) Morphological evaluation of L-G/pMT-C/EBPε infectants cultured in the absence (−) or presence (+, for 4 days) of $ZnSO_4$. (**d**) Differential count of L-G/pMT-C/EBPε infectants. Cells were evaluated after 4 days of treatment with $ZnSO_4$. (**e**) Comparison of secondary granule protein expression. The expression of neutrophil gelatinase (*NG*) and lactoferrin (*LTF*) in L-G/pMT-C/EBPε infectants cultured in the absence (−) or presence (+, for 3 days) of $ZnSO_4$ was quantified by real time quantitative reverse transcription PCR (qRT-PCR). (**f**) Requirement of PML phosphorylation for cooperative activation of C/EBPε-mediated transcription with p300. NIH3T3 cells were transfected with the G-CSFR-luc reporter gene together with the indicated plasmids. The error bars represent the s.d.

kinase that phosphorylates PML during differentiation of L-G cells is unknown, kinases such as ERK and HIPK2, which phosphorylate serine residues within PxSP or SP sequences, interact with PML.[8,19,20] Since the overexpression of these kinases increases the phosphorylation of PML,[8,19] it is possible that they are involved in the phosphorylation of PML during the differentiation of L-G cells.

Role of PML modifications in granulocytic differentiation

In C/EBPε-induced granulocytic differentiation, we showed that the phosphorylation of PML is required for the acceleration of cell differentiation and the further increase in the expression of secondary granule protein gene including *LTF*, the product of a C/EBPε target gene. Although the mechanism by which PML regulates transcription is not sufficiently understood, it has been shown that PML promotes the interaction between transcription factors and coregulators such as p300.[10,13] In the present study, we found that p300 accumulates in the C/EBPε/PML complex during granulocytic differentiation. Despite the phosphorylation-independent association and colocalization of PML with C/EBPε and p300, the phosphorylation of PML is required for the synergistic effect of PML and p300 on the activation of C/EBPε-dependent transcription. Therefore, the phosphorylation of PML contributes to the acceleration of granulocytic differentiation, at least in part, by enhancing the effect of p300 on C/EBPε-dependent transcription.

The role of PML sumoylation in granulopoiesis remains unclear. In the present study, sumoylation of PML was not required for the acceleration of C/EBPε- and PU.1-induced granulocytic differentiation; however, sumoylation was required for induction by G-CSF, which suggests that the sumoylation of PML may contribute to the regulation of factors other than C/EBPε and PU.1 to accelerate G-CSF-induced granulocytic differentiation. These results suggest that G-CSF signaling induces cell differentiation through multiple PML-regulated pathways.

We conclude that both phosphorylation and sumoylation are essential for the ability of PML to accelerate granulocytic

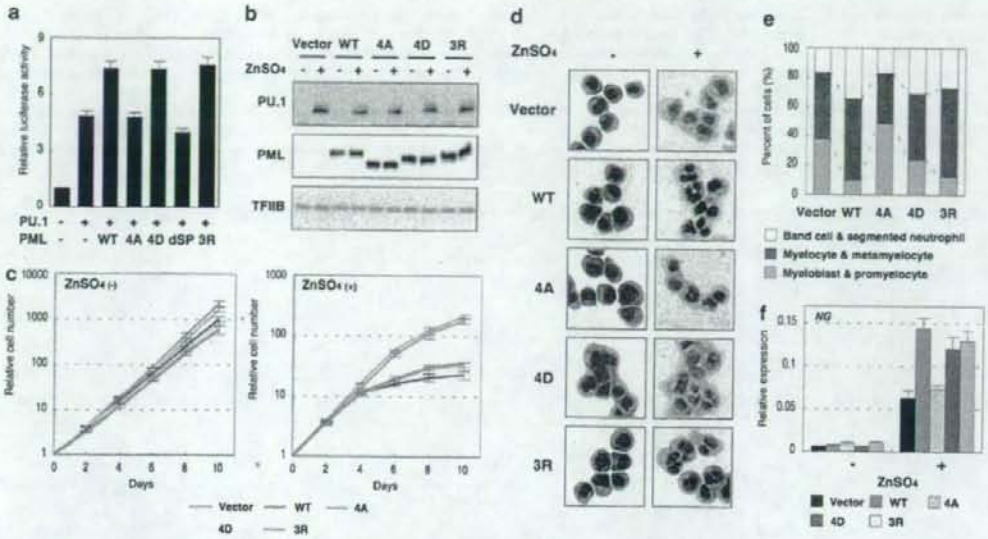

Figure 5 Effects of promyelocytic leukemia (PML) phosphorylation on PU.1-induced granulocytic differentiation. (**a**) Requirement of PML phosphorylation for activation of PU.1-mediated transcription. NIH3T3 cells were transfected with the C/EBPε-luc reporter gene together with indicated plasmids. (**b**) The expression of PU.1 and PML in L-G/pMT–PU.1 cells. Cells were cultured in the absence (−) or presence (+, for 24 h) of $ZnSO_4$. Total cell lysates were analyzed by western blot with anti-PU.1, -HA and -TFIIB antibodies. (**c**) Growth suppression of L-G/pMT–PU.1 infectants by phosphorylated PML. Cells were cultured in the absence (left) or presence (right) of $ZnSO_4$. The relative number of viable cells is shown. (**d**) Morphological evaluation of L-G/pMT–PU.1 infectants cultured in the absence (−) or presence (+, for 6 days) of $ZnSO_4$. (**e**) Differential count of L-G/pMT–PU.1 infectants. Cells were evaluated after 6 days of treatment with $ZnSO_4$. (**f**) Comparison of secondary granule protein expression. Expression of neutrophil gelatinase (*NG*) in L-G/pMT–PU.1 infectants cultured in the absence (−) or presence (+, for 3 days) of $ZnSO_4$ was quantified by real time quantitative reverse transcription PCR (qRT-PCR).

differentiation. Elucidating the regulatory mechanism of these modifications may help the development of therapeutic agents that induce differentiation of leukemia cells.

Acknowledgements

We thank Ms Yukiko Aikawa and Noriko Aikawa (National Cancer Center Research Institute) for technical assistance. This research was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from the Leukemia Study Group of the Ministry of Health, Labour and Welfare of Japan.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Int J Hematol (2008) 87:19–24
DOI 10.1007/s12185-007-0004-0

Chromatin regulation by AML1 complex

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Received: 1 August 2007 / Accepted: 1 September 2007 / Published online: 27 November 2007

Abstract The *AML1* gene is the most frequent target of chromosomal translocations in acute leukemias. AML1 is essential for definitive hematopoiesis and regulates transcription of its target genes by binding to the specific DNA sequence. AML1 forms large multiprotein complexes including CBFβ as a "core component" as well as several classes of chromatin modulators such as p300/CBP, MOZ, PML and HIPK2 as "regulatory complex". In this review, we describe the mechanisms by which AML1 complex regulates gene transcription and hematopoiesis, and its disruption by the leukemia-associated chromosomal translocations that affect genes for components of AML1 complex in view of deregulation of chromatin structure.

Keywords Chromatin · Leukemia · AML1 complex · MOZ · HIPK2

1 Introduction

The *AML1* (*CBFA2/PEBP2B/RUNX1*) gene is the most frequent target of leukemia-associated chromosome translocations [22]. The *AML1* gene was originally identified at the breakpoint of chromosome 21 in t(8;21)(q22;q22) translocation found in the FAB M2 subtype of acute myeloid leukemia (AML) [25]. Thereafter, *AML1* has been reported to be disrupted by other translocations such as t(3;21)(q26;q22) (AML1-EV11/EAP/MDS1) [24, 27], t(16;21)(q24;q22) (AML1-MTG16) [6] and t(12;21)(p13;q22) (TEL-AML1) [7, 32]. The somatic mutations of *AML1* have been also found in familial platelet disorder associated with a predisposition to leukemia, or sporadic cases of AML and myelodysplastic syndrome (MDS) [9, 13, 29, 30]. AML1 heterodimerizes with CBFβ to form the core binding factor (CBF), which binds to the specific DNA sequence PyGTPyGGT to activate or repress the expression of a number of hematopoietic genes dependent of each promoter context. Both AML1 and CBFβ are essential for the development of all definitive hematopoiesis lineages [26, 28, 33, 35, 36]. AML1 is also required for differentiation of megakaryocytes and lymphocytes [10]. Those findings indicate the critical roles of AML1 in both normal hematopoiesis and leukemogenesis.

Histone tails undergo covalent modifications such as acetylation, phosphorylation, methylation, ubiquitination and sumoylation. These modifications regulate the histone structure and the histone–DNA interaction as well as the higher order chromatin structure. Furthermore, the histone interplays each other, and represent an evolutionarily conserved "histone code" that marks binding sites for effecter proteins, thereby plays a fundamental role in transcriptional regulation [15]. Some histone acetyltransferases (HATs), such as p300 and the closely related CREB-binding protein (CBP) have been shown to function as transcriptional coactivators to play central roles in transcriptional control in response to a diverse range of physiological stimuli. They interact with specific DNA-binding transacting factors, and modulate the transcriptional activity of specific promoters by regulating local histone acetylation [8].

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In this review, we focus on the current achievement for regulation of AML1 complex and provide the new insight into its regulatory components essential for chromatin structure dynamics. In addition to the *AML1* gene, some of AML1-interacting transcriptional cofactors or chromatin modifiers are also frequent targets of leukemia-associated chromosome translocations. We also discuss the mechanisms of leukemogenesis in view of perturbing both global and promoter-specific alterations in AML1-regulating chromatin structure.

2 Identification of AML1 complex

AML1/CBFβ heterodimer regulates the expression of target genes that are important for hematopoiesis. AML1 functions as a large multiprotein complex. LC/MS/MS analysis of partially purified from myeloid progenitor cells, and subsequent western blot analysis demonstrate that several classes of chromatin modifying proteins are identified to specifically interact with AML1b, those include p300/CBP HATs, monocytic leukemia zinc finger protein (MOZ) and promyelocytic leukemia protein (PML) as well as homeodomain-interacting protein kinase 2 (HIPK2) [18, 20]. Since the amount of these co-purified proteins was relatively small compared with that of CBFβ, we suppose that AML1b forms 'regulatory' complexes with these proteins as well as a 'core' complex with CBFβ. In the following sections, we will overview that AML1 complex is fine-tuned by protein-to-protein interactions as a chromatin regulatory factory, and discuss a new role of AML1 to serve as a regulatory factor for HATs beyond its conventional roles as a transcriptional activator.

2.1 MOZ, a potent coactivator for AML1, is essential for maintenance of hematopoietic stem cells

MOZ, a MYST family histone acetyltransferase, was first isolated as a gene involved in chromosome translocaton t(8;16) associated with the M4/M5 subtype of AML. MOZ acts as a transcriptional coactivator for AML1 [20]. Two types of mice with mutations in MOZ gene have been reported. Mice with a null muation in MOZ (*MOZ−/−*) die around embryonic day 15 (E15) [16]. In the *MOZ−/−* E14.5 embryos, hematopoietic stem cells, lineage-committed progenitors, and B lineage cells are severely reduced. Maturation arrest in erythroid lineage cells, and increased population of myeloid lineage cells are also observed. Expression of thrombopoietin receptor (c-Mpl), HoxA9, and c-Kit is down regulated in *MOZ−/−* fetal liver cells. Those cells cannot reconstitute hematopoiesis of recipients after transplantation. These findings suggest that MOZ is required for maintenance of hematopoietic stem cells, and plays a role in the differentiation of erythroid and myeloid cells. Mice with a truncation of the MOZ gene (MOZd/d) die at birth [34]. Defects in the hematopoietic stem cell compartment and reduction in the number of progenitors of all lineages were also observed in the mutant mice. However, blood cell lineage commitment was unaffected in MOZd/d mice.

The C-terminal region of MOZ, which contains serine-, proline- and methionine-rich regions, is required for both interaction with AML1 and robust stimulation for AML1-mediated transcription. In addition, the N-terminal region and a part of the acidic region are required for co-activation of AML1-mediated transcription. Homology searches indicated that the N-terminal region of MOZ has a similar motif (H1/5) seen in linker histones such as histone H1 and H5. Deletion analysis indicated that the H1/5-like domain is essential for co-activation and involved in nuclear localization. Since the H1/5 domain of histone H5 is implicated in nucleosome binding [31], MOZ might directly regulate chromatin structure by interaction with nucleosome. The HAT domain of MOZ is, unexpectedly, dispensable for AML1-mediated transcription. MOZ can acetylate AML1 in vitro, but its biological significance remains to be seen.

2.2 HIPK2 integrates the activity of AML1 complex

We also identified HIPK2 as a component of AML1 complex. The HIPK family belongs to nuclear serine/threonine kinases, and three paralogs (HIPK1, 2 and 3) have been so far reported. HIPK2 interacts with and phosphorylates targets, including homeoproteins, p53, CtBP1 and Myb, suggesting that HIPKs play critical roles in transcriptional regulation.

HIPK2 phopshorylates AML1b at Ser249 and Ser276 residues as well as p300 [1]. Interestingly, wild type AML1b enhances HIPK2-induced phosphorylation of p300, whereas the phosphorylation-deficient mutant AML1-S249/276A does not. These findings suggest that phosphorylation of AML1 is prerequisite for the HIPK2-mediated phosphorylation of p300 and subsequent transcription activation. Since AML1 interacts with both p300 and HIPK2, AML1 would be a 'scaffold' for the physical interaction between the enzyme HIPK2 and its substrate p300, and function as a regulatory subunit for HIPK2 to control p300 phosphorylation, local histone acetylation and transcription of target genes. Another interesting finding is that HIPK-induced phosphorylation of p300 is commonly regulated by a certain sets of transcription factors that cooperate with AML1 such as PU.1, c-MYB, c-JUN and c-FOS, but not by other transcription factors including as IRF3, p53, RAR and ATF2 in spite of their ability to

interact with p300. Thus phosphorylation of p300 by HIPK2 is a common pathway in AML1-related transcriptional activation essential for myeloid development. *Hipk1/2* double-deficient (*HIPK1−/−HIPK2−/−*) mice died between E9.5 and E12.5 [14], and exhibits defects in vasculogenesis, angiogenesis, and primitive and definitive hematopoiesis with developmental abnormalities similar to those observed in p300- and CBP-deficient mice [1]. In addition, highly phosphorylated forms of p300 are scarcely detected in those embryos, suggesting that both HIPK1 and HIPK2 regulate phosphorylation and function of p300 during embryonic development.

MOZ is also phosphorylated by HIPK2 [1]. Although phosphorylation of MOZ is not efficiently induced by either AML1 or HIPK2 alone, coexpression of both proteins efficiently induces phosphorylation of MOZ. Thus interaction between these three molecules is likely to be required for efficient MOZ phosphorylation. Regulation of AML1/HIPK2/MOZ seems inter-dependent during myeloid terminal differentiation, since phosphorylation of AML1 is correlated with MOZ phosphorylation as well as increased amounts of AML1/MOZ complex, and AML1 preferentially interacts with the highly phosphorylated form of MOZ. Thus, HIPK2 stabilizes the AML1/MOZ complex by phosphorylating MOZ. Taken together with these findings, we propose a model that HIPKs also play a key role in regulating the transcription of target genes by both stabilizing and activating AML1/MOZ/p300 complex. In addition, HIPK2 may regulate AML1 activity by phosphorylating TLE corepressor complex to dissociate it from AML1 complex [12].

3 The AML1 complex: target of chromosome translocation in leukemia

AML1 complex components including core CBFβ and associated cofactors MOZ, p300/CBP are frequently targeted by chromosomal rearrangements in human leukemias [11, 19, 22]. Since rearrangements of genes for AML1-interacting co-repressors such as mSin3A and TLE/Groucho have not yet been found, active AML1 complex should be the target of leukemia-associated chromosome aberrations. These facts suggest that altered expression of genes regulated by AML1 complex might be important for leukemic transformation. In fact, AML1-mediated transcription is inhibited by leukemia-associated fusion proteins such as AML1-MTG8 (ETO), TEL-AML1, AML1-EVI1 and CBF-MYH11; and those are excellently reviewed elsewhere [23]. In the following sections, we will review the recent advance of molecular mechanisms of selected AML1 complex-related chimeras affect the chromatin structures of its target genes and thus causes deregulated gene expression in leukemia.

3.1 AML1–ETO inhibits AML1-mediated transcription

The translocation t(8;21)(q22;q22) results in the expression of the fusion protein AML1–ETO(MTG8). With its strong capability to recruit mSin3A/histone deacetylases (HDAC) corepressor complex through the ETO domain, AML1–ETO binding to target gene promoters correlates with changes in the histone modification pattern. Follows et al. [5] reported that AML1–ETO is a part of an extended transcription factor complex binding to the regulatory region of the M-CSFR gene, and that it alters equilibrium of HATs and HDACs recruitment. Liu et al. [21] reported that DNA methyltransferase 1 (DNMT1) is also part of the transcriptional repressor complex recruited by AML1–ETO to silence the target genes and marks Lys9 of histone H3 tail with higher level methylation instead of acetylation. It is noteworthy that AML-ETO induces the strong DNase I-hypersensitivity within the target gene, in contrast to the PML-RARA-mediated epigenetic silencing mechanism [4]. Those findings imply that AML-ETO-mediated repression requires its continuous presence on the target sites, providing a therapeutic relevance for the combination of HDAC and DNMT inhibitors as a novel therapeutic approach for t(8;21) AML (Fig. 1).

3.2 MOZ–CBP inhibits AML1-mediated transcription

MOZ–CBP and MOZ–p300 leukemogenic fusion proteins are generated by the t(8;16) and t(8;22) translocations associated with monocytic AML (Fig. 2). MOZ–CBP inhibits AML1-mediated transcription, whereas MOZ and CBP activate AML1-mediated transcription [20]. N-terminal part of MOZ retains the histone acetyltransferase domain, PHD-type zinc-finger motif, and histone H1/5-like domain but lacks the C-terminal transactivation domain, and fuses to largely intact CBP. Since MOZ–CBP retains the transrepression domain of MOZ as well as binding sites

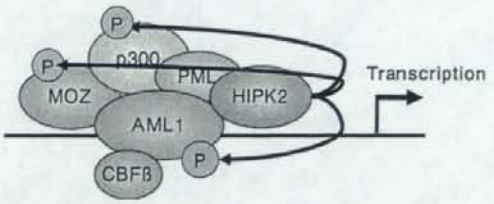

Fig. 1 A model for HIPK2 action on AML1/MOZ complex. Phosphorylation of p300/CBP HATs is essential for their activity. AML1 acts both as a sequence-specific transacting factor and a scaffold to intermediate the association of HIPK2 and HATs. Phosphorylation of AML1 by HIPK2 is prerequisite for its action