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Roles of AML1/RUNX1 in T-cell malignancy induced by loss of p53

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***AML1/RUNX1* is a frequent target of chromosome translocations and mutations in myeloid and B-cell leukemias, and upregulation of AML1 is also observed in some cases of T-cell leukemias and lymphomas. This study shows that the incidence of thymic lymphoma in p53-null mice is less frequent in the *Aml1*$^{+/-}$ than in the *Aml1*$^{+/+}$ background. AML1 is upregulated in p53-null mouse bone-marrow cells and embryonic fibroblasts. In the steady state, p53 binds to and inhibits the distal *AML1* promoter. When the cells are exposed to stresses, p53 is released from the distal *AML1* promoter, resulting in upregulation of *AML1*. Overexpression of AML1 stimulates T-lymphocyte proliferation. These results suggest that upregulation of AML1 induced by loss of p53 promotes lymphoid-cell proliferation, thereby inducing lymphoma development. (*Cancer Sci* 2013; 104: 1033–1038)**

AML1, also known as the RUNX1 transcription factor, is encoded by one of the most frequently mutated genes in acute leukemia. The *AML1* gene is disrupted by translocations in leukemia.[1,2] AML1 is also inactivated by point mutations within its DNA-binding domain in both acute myeloid leukemia (AML)[3] and myelodysplastic syndromes (MDS).[4] *AML1* haploinsufficiency underlies familial platelet disorder, an inherited leukemia predisposition syndrome.[5] On the other hand, amplification of the locus containing *AML1* and overexpression of AML1 are observed in some cases of leukemias and lymphomas,[6–10] suggesting that an increased dosage of normal *AML1* may contribute to leukemogenesis.

The p53 tumor suppressor functions in the most important checkpoint-control pathway that prevents human cancer.[11] Although hematopoiesis in p53-null mice proceeds apparently normally, many studies have implicated p53 in the proliferation, differentiation, apoptosis, and aging of hematopoietic cells.[12–16] Moreover, p53 deletions and mutations are detected at high frequencies in blast crisis chronic myelogenous leukemia and acute leukemia, as well as in almost 50% of adult T-cell leukemias.[17,18] In the present study, we aimed to elucidate the relationship between AML1 and p53 in T-cell malignancy. Because the most frequent tumor to develop in p53-null mice is T-cell lymphoma,[19] we studied the role of AML1 in cancer development using the p53-null mouse as an animal model system of T-cell acute lymphoblastic leukemia (T-ALL).

Materials and Methods

Mice. *Aml1*$^{+/-}$ mice (a gift from T. Okuda) were mated to *p53*$^{+/-}$ mice (a gift from H. Koseki) to generate *Aml1*$^{+/-}$ *p53*$^{+/-}$ male mice. The mice were then mated to *p53*$^{+/-}$ female mice to generate *Aml1*$^{+/+}$ *p53*$^{-/-}$ or *Aml1*$^{+/-}$ *p53*$^{-/-}$ mice. For *in vitro* T-cell proliferation experiments, *Aml1*$^{fl/fl}$ mice[20] were mated to tamoxifen-inducible *ERT2-Cre* knock-in mice (Artemis Pharmaceuticals GmbH, Cologne, Germany)[21] to generate *Aml1*$^{fl/fl}$ *ERT2-Cre* mice. Active Cre-recombinase was induced by the addition of 50 nM 4-hydroxytamoxifen into the culture medium. The background of the mice used in this study is C57BL/6. The mice were kept at the Division of Animal Laboratory (National Cancer Center, Tokyo, Japan), according to institutional guidelines.

Quantitative RT-PCR. Total RNA extraction, reverse transcription, and quantitative PCR were performed using ISOGEN (Wako Chemical, Osaka, Japan); the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA); and TaqMan probes for *RUNX1* (Hs00231079_m1), *RUNX3* (Hs00231709_m1), *TP53* (Hs01034249_m1), *Runx1* (Mm00486762_m1), *Runx2* (Mm0003003491_m1), *Runx3* (Mm00490666_m1), *Trp53* (Mm01731290_g1), *Pre T-cell antigen receptor alpha* (Mm00478363_m1), *Fas* (Mm00433237), and *18S* rRNA (Hs99999901_s1), purchased from (Applied Biosystems). Expression levels were normalized to those of *18S* rRNA.

Chromatin immunoprecipitation. ChIP analysis was performed as described,[22] using primary antibodies specific for p53 (sc-6243 and DO1) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or antibodies for Histone H3 (ab1791), Histone H3 K4Me3 (ab8580), or Histone H3 K9Ac (ab4441) purchased from Abcam (Cambridge, MA, USA). Quantitative real-time PCR was performed on precipitated DNA using primers designed by PRIMER EXPRESS software (Applied Biosystems). The values relative to input were determined using a standard curve, and the relative quantification method was as described in ABI User Bulletin #2. The ChIP primer sequences corresponding to the mouse *Aml1* or human *AML1* distal promoter regions are available upon request.

T-cell proliferation assay. Number of living cells was determined by measuring ATP production using the Cell Titer Glo assay kit (Promega, Fitchburg, WI, USA) and a GLOMAX microplate luminometer (Promega).

Results

Dosage of *Aml1/Runx1* is critical for development of T-cell lymphoma in p53-null mice. Loss of the p53 allele and amplification or overexpression of the *AML1* gene is frequently observed in human lymphoblastic leukemia or lymphoma. Consistent with this, thymic lymphoma is the most frequent malignant disease in p53-null mice.[19] To analyze the effect of *Aml1* gene dosage on the generation of lymphoma, we generated *Aml1*$^{+/-}$ mice in a *p53*$^{-/-}$ background. We were unable to produce *Aml1*$^{-/-}$ *p53*$^{-/-}$ mice because the *Aml1*$^{-/-}$ mice died at embryonic days E11.5–E12.5, as reported.[23] We then compared the frequency of spontaneously occurring

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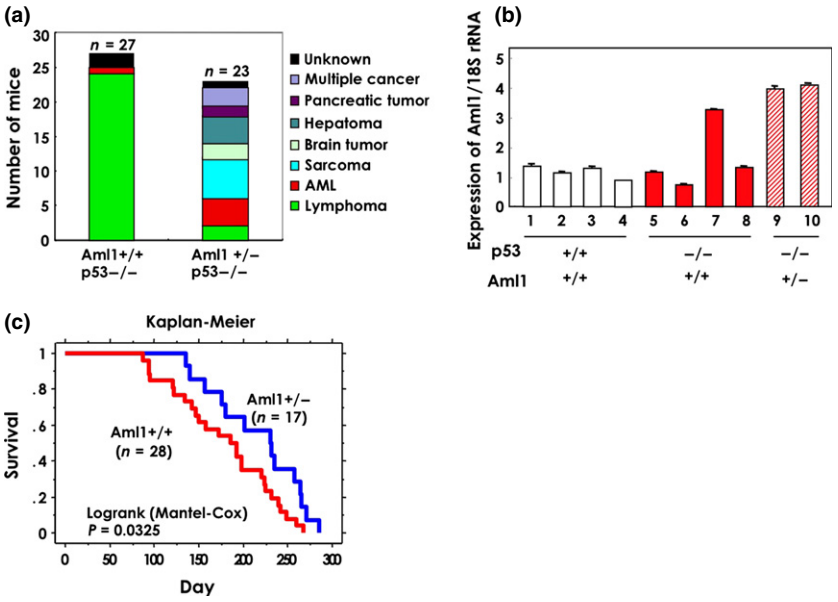

Fig. 1. *Aml1* haploinsufficiency in p53-null mice prevents the development of T-cell lymphoma. (a) Spontaneous onset of malignant transformation in p53-null mice of *Aml1*$^{+/+}$ and *Aml1*$^{+/-}$ genotypes. The majority of lymphomas developed in *Aml1*$^{+/+}$ mice were T-cell lymphomas (for details, see Table S1). (b) The level of *Aml1* transcripts in T-cell lymphoma. Total RNA of thymus from six mice with lymphoma (red bar, filled: *Aml1*$^{+/+}$ *p53*$^{-/-}$, red bar, diagonal stripes: *Aml1*$^{+/-}$ *p53*$^{-/-}$) and four normal controls (white) was isolated and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed as described in Experimental Procedures. The data shown are the means ± standard error of the mean (SEM). (c) Event-free survival curves for *p53*$^{-/-}$ *Aml1*$^{+/+}$ mice (n = 28) and *p53*$^{-/-}$ *Aml1*$^{+/-}$ mice (n = 17). Mice carrying sarcomas (n = 6) were not included in this analysis, because these mice were killed at the onset of disease. Statistical analysis was performed using the StatView software.

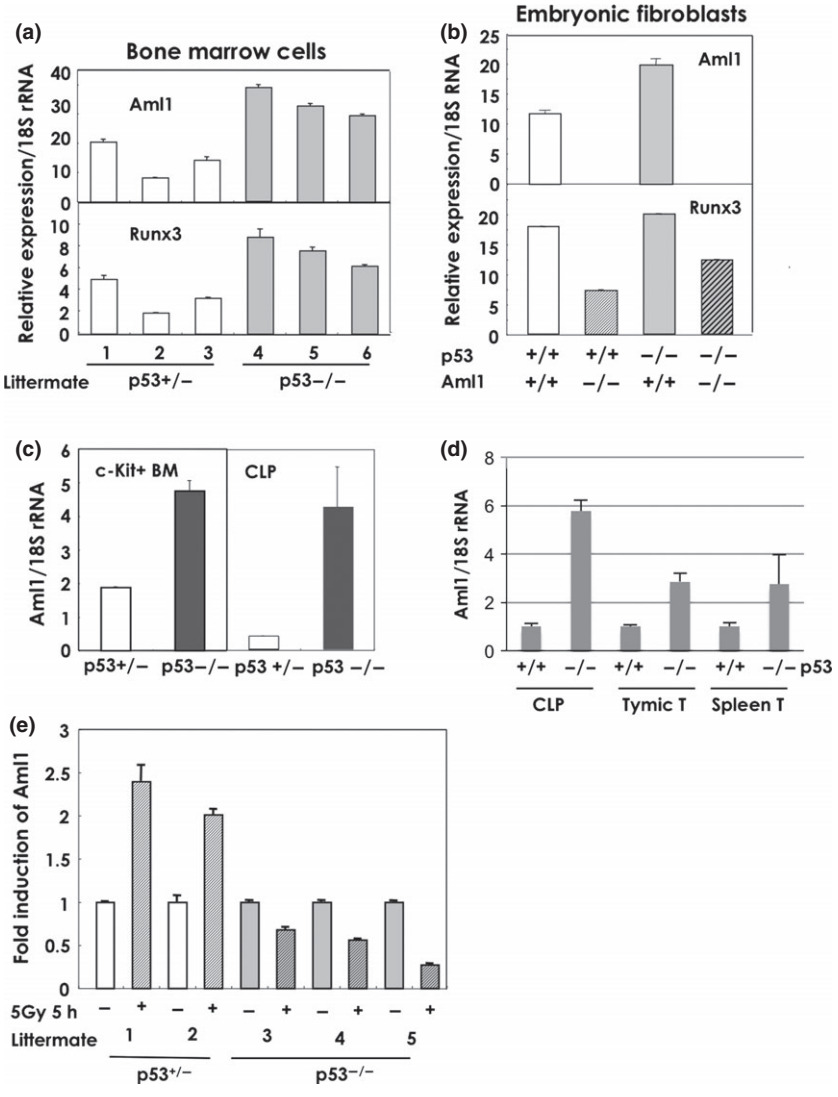

Fig. 2. AML1 is overexpressed in cells with p53-null mutations and is induced by stress. (a) *Aml1* is overexpressed in bone-marrow cells from p53-null mice in the steady state. The level of *Aml1* transcripts in bone marrow cells from littermate mice, generated by mating a p53$^{+/-}$ female and a p53$^{-/-}$ male mouse, was measured. Total RNA was prepared and subjected to quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *Aml1*and *Runx3*. 1–3: p53$^{+/-}$ littermates, 4–6: p53$^{-/-}$ littermates. (b) *Aml1* is overexpressed in mouse embryonic fibroblast (MEF) cells from p53-null mice The level of *Aml1* and *Runx3* transcripts in the MEFs of the indicated phenotype was measured by qRT-PCR using total RNA of cells at the time of passage 2 as described in Experimental Procedures. MEF cells were isolated from littermate E11.5 embryos that were generated by crossing *Aml1*$^{+/-}$ male and *Aml1*$^{+/-}$ female mice of p53 wild type background. The data shown are the means ± standard error of the mean (SEM). (c) *Aml1* is overexpressed in bone marrow cells from p53-null mice. The level of *Aml1* transcripts, and control *18S* rRNA, in c-Kit^{+} bone marrow cells and in the bone marrow CLP (c-Kit^{low}, Sca1^{+}, IL-7Ra^{+}, Lin^{-}) fraction of littermate mice, generated by mating a p53$^{+/-}$ female and a p53$^{-/-}$ male mouse. Total RNA was prepared and subjected to qRT-PCR for assay of *Aml1* and *18S* rRNA. The data shown are the means ± SEM. (d) *Aml1* is overexpressed in p53-null cells. Total RNA was isolated from c-Kit^{+} bone marrow (BM) cells, CLP, thymocytes, CD4+ splenic T cells, and MEFs, all of which were prepared from wild-type and *p53*$^{-/-}$ littermates. The level of *Aml1* transcripts was analyzed. The data shown are means ± SEM. (e) *Aml1* expression is also induced by IR in mouse cells. The level of *Aml1* transcripts was measured in bone-marrow cells from littermate mice, generated by mating a *p53*$^{+/-}$ female and a *p53*$^{-/-}$ male mouse. The cells were exposed to 5 Gy of IR and cultured for 5 h. Total RNA was prepared and subjected to semi-quantitative RT-PCR for analysis of *Aml1*. 1, 2: *p53*$^{+/-}$ littermates, 3–5: *p53*$^{-/-}$ littermates.

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tumors in *Aml1*$^{+/+}$ *p53*$^{-/-}$ mice and *Aml1*$^{+/-}$ *p53*$^{-/-}$ mice. Within a period of 40 weeks after birth, the majority of malignant disease (89%) in *Aml1*$^{+/+}$ *p53*$^{-/-}$ mice was T-cell lymphoma. However, in *Aml1*$^{+/-}$ *p53*$^{-/-}$ mice, a wide range of malignancies was observed, with sarcoma being the major malignancy (26%) (Fig. 1a, Table S1); by contrast, only two cases of lymphoma (9%) were observed in *Aml1*$^{+/-}$ *p53*$^{-/-}$ mice. In those lymphomas, the levels of *Aml1* mRNA were higher than those in normal T cells (Fig. 1b). Fluorescence-activated cell sorting and RT-PCR analyses revealed that

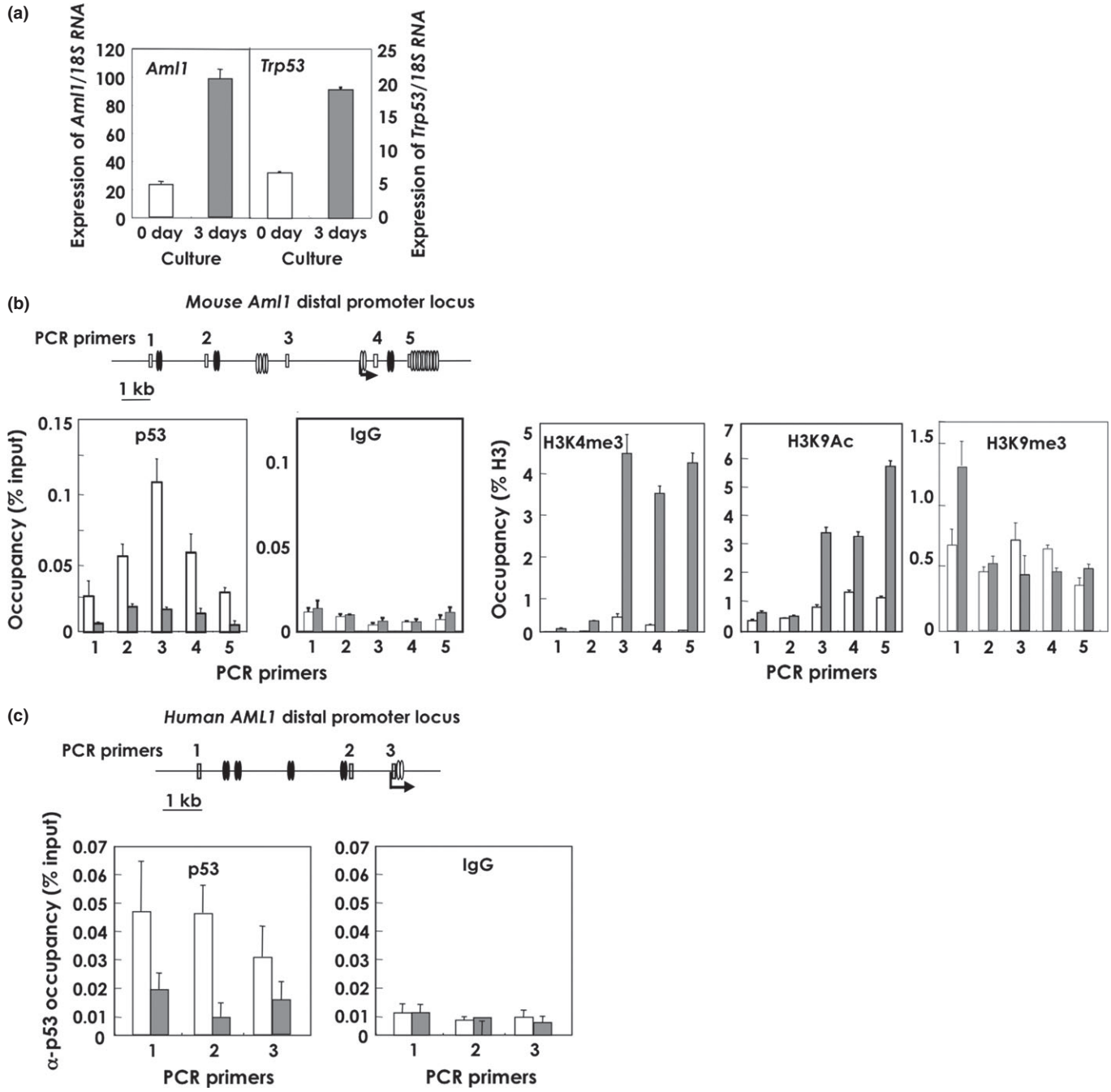

Fig. 3. p53 binds to the *AML1* promoter of hematopoietic cells at steady state, and the binding is reduced after exposure to stresses. (a) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of the *Aml1* and *Trp53* transcripts in mouse bone-marrow c-Kit$^+$ cells before (0 day) or after (3 day) 3-day *in vitro* culture with cytokines (SCF and IL-3). The data shown are means ± standard error of the mean (SEM). (b) Upper panel: Mouse *Aml1* distal promoter regions contain potential AML1- and p53-binding motifs. The primers designed for ChIP assay of the mouse *Aml1* distal promoter region are indicated by numbers. The arrow indicates the transcription start site. Oval doublets represent two separate DNA motifs within 20 bp (black: p53-binding candidate motifs, white: AML1-binding motifs). Lower panel: ChIP assays for the detection of p53 binding and chromatin modification (methylation: H3K4me3; acetylation: H3K9Ac), as indicated in the figures. White bars: uncultured mouse bone-marrow c-Kit$^+$ cells; gray bars: c-Kit$^+$ cells after a 3-day culture. ChIP primers, corresponding to bases within the DP2 region of the *Aml1* promoter as shown in a, were used. The data shown are means ± SEM. (c) Upper panel: Primers designed for the ChIP assay of the human *AML1* distal promoter region are indicated as numbers. Lower panel: ChIP assay for detection of anti-p53 antibody binding. The data shown are means ± SEM. White bars: MOLT4 cells without IR treatment; gray bars: MOLT4 cells 2 h after IR (10 Gy) treatment. Oval doublets represent two separate DNA motifs within 20 bp (black: p53-binding candidate motifs; white: AML1-binding motifs).

lymphoma cells derived from *Aml1*$^{+/+}$ *p53*$^{-/-}$ and *Aml1*$^{+/-}$ *p53*$^{-/-}$ mice were positive for T-cell receptor, CD4 and CD8 (Fig. S1a,b). Event-free survival was significantly different between the two genotypes ($P = 0.0325$) (Fig. 1c). These results suggest that expression levels of AML1 affect the incidence of malignancies in p53-null mice.

AML1 is upregulated in cells with p53 aberrations. To determine whether the level of *Aml1* transcripts correlates with the presence or absence of p53, we compared expression of *Aml1* in various types of cells from littermate mice of the *p53*$^{+/-}$ and *p53*$^{-/-}$ genotypes. We observed increased expression of *Aml1* in total bone-marrow (BM) cells, c-Kit^{+} BM cells, common lymphoid progenitors (CLPs), splenic T cells, and thymic T cells, as well as in *p53*$^{-/-}$ mouse embryonic fibroblast (MEF) cells (Fig. 2a–d). Considering that the major function of p53 is regulation of stress-response genes, we tested the effect of stress on the expression of *Aml1* in BM cells from another littermate mouse. *Aml1* is induced by ionizing radiation (IR) in mice with normal *p53*, but this induction was not observed in *p53*$^{-/-}$ cells (Fig. 2e). Levels of *Aml1* expression were increased by p53 shRNA treatment (Fig. S2). Together, these data strongly suggest that *Aml1* is transcriptionally regulated by p53 both in the steady state and following genotoxic stress.

***AML1* is regulated by p53.** There are two promoters in the *AML1* gene.[24] The distal promoter is more active than the proximal promoter in hematopoietic stem cells and developing T cells.[25] To determine whether p53 binds to the distal promoter region of *Aml1*, we performed chromatin immunoprecipitation (ChIP) using c-Kit^{+} cells from mouse BM. For this purpose, we used PCR primers corresponding to regions ranging from −10 kb upstream to +4 kb downstream of the *Aml1* transcription start site (Fig. 3b). When c-Kit^{+} cells were cultured *in vitro*, the expression of *Aml1* was induced (Fig. 3a). Binding of p53 to the *Aml1* promoter could be detected in ChIP analysis before, but not after, cultivation (Fig. 3b). ChIP analysis of histone H3 trimethylated at lysine 4 (H3K4Me3) and histone H3 acetylated at lysine 9 (H3K9Ac), both of which are hallmarks of active gene transcription, indicated that activating modifications of chromatin increased in proportion to the *Aml1* mRNA level. H3 trimethylated at lysine 9 (H3K9Me3), which is a repressive mark, was not altered by the cultivation. These results indicated that, at steady state immediately following the isolation of c-Kit^{+} cells from BM, p53 was preferentially bound close to and downstream of the transcription start site. However, after a 3-day culture of c-Kit^{+} cells, significant binding of p53 was no longer detected at any locus tested (Fig. 3b), even though the expression of p53 was high at this time. At steady state, the *Aml1* distal promoter region appeared to be inactive, as demonstrated by the relatively lower levels of H3K4Me3 and H3K9Ac occupancy revealed by ChIP analysis. Conversely, levels of H3K4Me3 and H3K9Ac increased at the downstream region of the *Aml1* promoter (primers no. 3–5) after a 3-day culture (Fig. 3b and Fig. S3). Occupancy of the *Aml1* promoter by p53 was also decreased following irradiation (Fig. 3c). The results of these ChIP experiments indicate that the activity of the *Aml1* promoter is inhibited by binding of p53 under normal conditions, and that this inhibition was abolished under conditions of stress. In a reporter assay, the *Aml1* promoter was inhibited by wild-type p53 and activated by the p53 R175H mutant, which is defective in binding to DNA (Fig. 4). These results indicate that p53 inhibits transcription of the *Aml1* gene. The p53 R175H mutant may function in a dominant-negative manner.

Depletion of AML1 blocks T-cell proliferation. To determine whether the expression level of AML1 affects proliferation of T cells, we mated mutant animals carrying *lox*P-flanked (*Aml1*^{fl}) alleles[20] with *ERT2-Cre* knock-in mice[21] to generate *Aml1*$^{fl/fl}$ *ERT2-Cre* mice. In these mice, the *Aml1*^{fl} allele

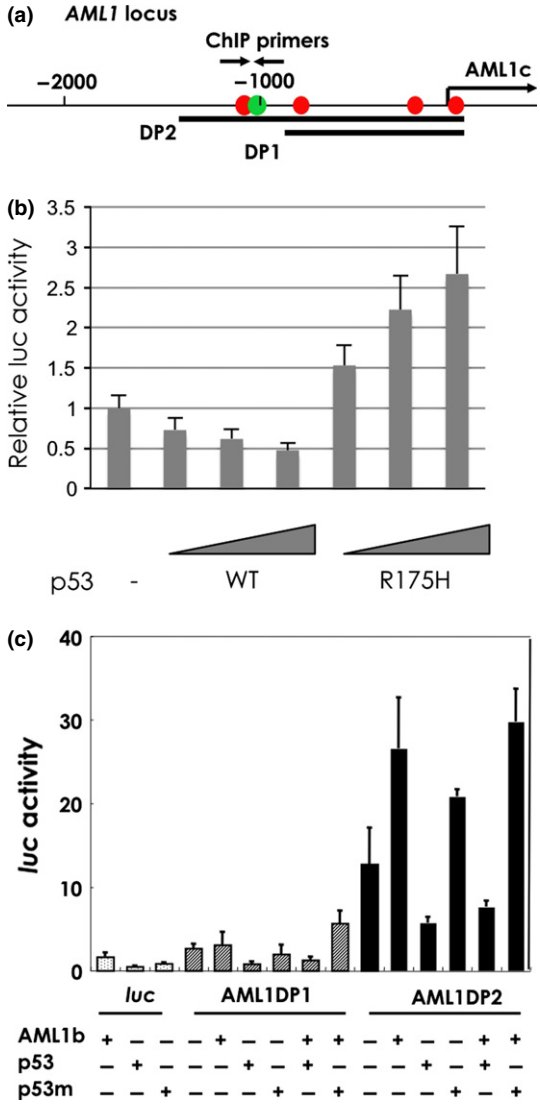

Fig. 4. p53 inactivates *AML1* promoter. (a) The indicated promoter regions of 900 bp (DP1) and 1400 bp (DP2) were cloned into *luc* reporter plasmids. The arrow labeled AML1c indicates the transcription start site. The opposing arrows indicate the positions of the primers used for ChIP analysis. (b, c) AML1 promoter is repressed by p53. SaOs2 cells were transfected with expression vector for wild-type p53, p53R175H and/or AML1b together with a human *AML1* distal promoter–luciferase (*luc*) reporter containing DP1 or DP2. Data shown are relative values of firefly luciferase activity normalized to *Renilla* luciferase activity. The data shown are the means ± standard error of the mean (SEM).

could be effectively deleted *in vitro* in hematopoietic cells by the addition of 4-hydroxytamoxifen (4-OHT), which activates ERT-Cre recombinase (Fig. 5a). After 5 days of 4-OHT treatment, the *Aml1* gene was deleted from almost all cells (Fig. 5b), and the *Aml1* transcript was also almost completely absent, as determined by RT-PCR (Fig. 5c). At this time, the expression level of Runx3, which might compensate for loss of AML1 function, was not altered (Fig. S4). We then examined T-cell proliferation in these *Aml1*$^{fl/fl}$ *ERT2-Cre* mice. Splenic CD4 cells were isolated from the *Aml1*$^{fl/fl}$ *ERT2-Cre* mice and cultured in the presence of IL-2 after stimulation with anti-CD3e. Ten days after the addition of 4-OHT, we compared the number of live cells from *Aml1*$^{fl/fl}$ *ERT2-Cre* and *Aml1*$^{w/w}$ *ERT2-Cre* mice. The proliferation of T-cells from

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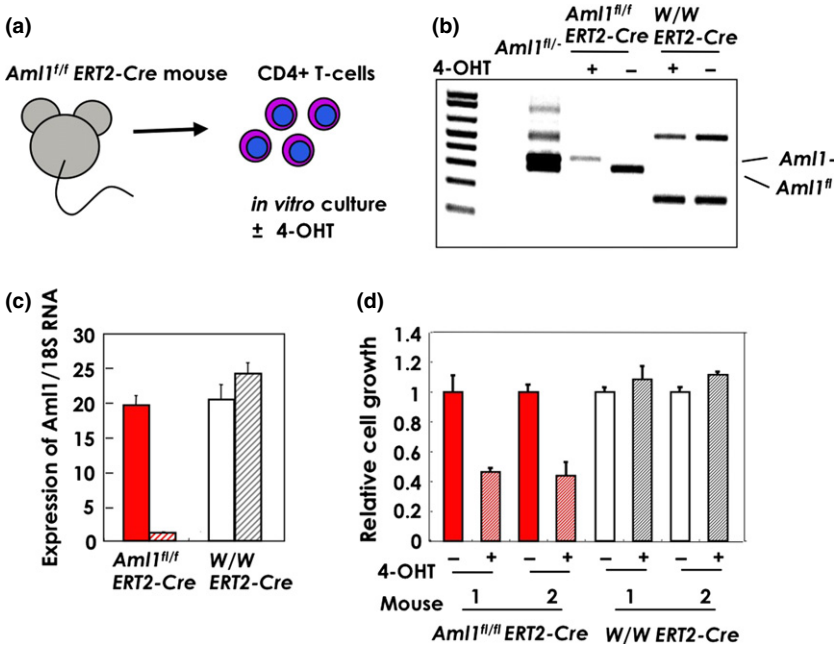

Fig. 5. Depletion of AML1 inhibits the growth of CD4 positive T-cells. (a) Splenic T-cells of *Aml1* $^{fl/fl}$ *ERT2-Cre* mice were isolated using an antibody against CD4 and cultured for 5 days with or without 4-hydroxytamoxifen (4-OHT) in the presence of IL-2. (b) Genotype of *Aml1* was analyzed after 5-day culture with or without 4-OHT in the presence of IL-2. Primers used for genotyping were as follows: f2: ACAAAACCTAGGTGTACCAGGAGAACAAGT, r1: GTCTACTCCTTGCCTCAGAAAACAAAAAC, fl20: CCCT GAAGACAGGAGAAGTTTCCA. (c) Expression of *Aml1* was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and correlated to *18S* rRNA levels after 5-day culture with or without 4-OHT in the presence of IL-2. (d) T-cells with or without the Aml1 allele were cultured in the presence of IL-2 for 10 days. The growth rate (live cells) was measured by the production of adenosine triphosphate (ATP). ATP production of cells from *Aml1* $^{fl/fl}$ *ERT2-Cre* mice (red bars, diagonal stripe) treated with 4-OHT was less than that of untreated cells (red bars, filled). The drug was not toxic to wild-type cells (white and gray diagonal stripes: treated and non-treated, respectively).

which *Aml1* was deleted was clearly lower than that of T-cells from *Aml1* $^{w/w}$ *ERT2-Cre* mice (Fig. 5d). This result is consistent with a previous report showing that conditional deletion of *Aml1* results in decreased numbers of T cells.[20] These observations suggest an important role for AML1 in T-cell proliferation.

Discussion

RUNX family members function as oncogenes when they are ectopically overexpressed.[26–29] In this study, we elucidated a novel relationship between AML1 and p53 *in vivo* using mice that carried mutations in one or both genes, and we present genetic evidence for a mechanism underlying the overexpression of AML1.

The lower frequency of onset of T-cell lymphoma in association with the *Aml1* $^{+/-}$ *p53* $^{-/-}$ genotype may indicate that the presence of two copies of the *Aml1* gene are needed for T-cell transformation and/or proliferation, and that one allele of *Aml1* confers no growth advantage to malignant cells. Because we did not examine the expression level of *Aml1* at the early stage of T-cell transformation, we cannot fully resolve the importance of AML1 in T-cell malignancy. Moreover, a genetic defect in p53 affects the regulation of a large number of genes. Therefore, *Aml1* hemizygosity might not be the factor that directly prevents malignant transformation in a p53-null background.

Aml1 might function as an oncogene in a lymphoma that develops due to a defect in *p53*. Consistent with this idea, expression levels of *Aml1* were higher in *p53* $^{-/-}$ lymphocytes than those observed in wild type cells (Fig. 2c,d). However, expression levels of *Aml1* were not constantly high in lymphomas (Fig. 1b). These results suggest that high levels of *Aml1* expression might be important for development, but not for maintenance, of lymphomas. The mechanism of this enhanced expression of *Aml1* remains unclear, although the data support the hypothesis that a high level of *Aml1* is critical for lymphoma development. In addition, the depletion or deletion of *Aml1* induced the expression of *Fas* (Fig. S5), suggesting that AML1 plays some roles in cell survival, as reported.[30] In the early stages of lymphoma development, anti-apoptotic regulation by AML1 might also be important.

The primary role of p53 in the cell-cycle checkpoint in hematopoietic cells is probably to repress stem cell division, thereby maintaining the quiescent status of these cells in the steady state.[16] Another recent report also indicated that the reduced p53 activity of *p53* $^{+/-}$ mice is associated with higher numbers of proliferating hematopoietic stem and progenitor cells in older mice.[15] Furthermore, a p53-null genotype promotes expansion of long-term proliferating stem cells in BM and activates HSCs.[15,16] However, the target of p53 that induces HSC proliferation is unknown. Here we showed that AML1 is at least one of the targets of p53 in hematopoietic stem or progenitor cells. AML1 plays a positive role in cell-cycle progression in mouse myeloid cells.[31,32] Thus, the data in this study indicates that p53 may repress AML1 in HSCs in the steady state, but induce AML1 once HSCs are exposed to stress.

The mechanism by which p53 represses *AML1* gene expression may differ from the known mechanisms by which p53 regulates other genes. Our results support a novel mechanism of p53 gene regulation, in which p53 binding to the target-gene locus represses transcription, but release of p53 activates transcription. The ability of p53 to respond to stress is usually associated with modification of p53 by phosphorylation, acetylation, or sumoylation.[33,34] However, it is difficult to use this model to explain our results concerning the stress response of p53 and *AML1* transcription, because p53 normally represses the *AML1* gene but activates it when the cell is exposed to various stresses. We also observed constitutive activation of *AML1* gene in cells with a p53-null genotype. It is likely that, once activated (and probably modified), p53 dissociates from the repressor complex on the *Aml1* gene locus.

In future studies, it will be important to analyze the AML1 level in human T-ALL specimens caused by p53 deficiency or mutation. AML1 has important functions not only in hematopoiesis, but also in neurogenesis and skeletal muscle.[23,35,36] Therefore, a broad-based study of the relationship between the two important transcription factors p53 and AML1 in various malignant diseases could provide information that would aid in understanding how these tumors are generated. If the same results are obtained in human hematopoietic stem cells as in mouse cells, then it may be possible to design drugs that inhibit AML1 activity for use in treating T-cell malignancy.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phenotypes of lymphomas.

Fig. S2. Effects of p53 shRNA on AML1 expression.

Fig. S3. Active chromatin modifications in the distal promoter regions of the mouse and human *AML1* genes.

Fig. S4. Effect of AML1 depletion on the *Runx3* gene expression.

Fig. S5. Effect of AML1 depletion on *Fas* gene expression.

Table S1. Type of diseases and latency seen in p53-null mice.

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