VWRPY-Motif-Dependent and -Independent Roles of AML1/Runx1 Transcription Factor in Murine Hematopoietic Development

Running title: Functional Dissection of AML1/Runx1

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Abstract

AML1/Runx1 is a frequent target of leukemia-associated gene aberration, and encodes a transcription factor essential for definitive hematopoiesis. We previously reported that the AML1 molecules with trans-activation subdomains retained can rescue in vitro hematopoietic defects of AML1-deficient mouse embryonic stem (ES) cells when expressed by using a knock-in approach. Extending this notion to in vivo conditions, we found that the knock-in ES cell clones with AML1 mutants, which retain trans-activation subdomains but lack a C-terminal repression subdomain including the conserved VWRPY-motif, contribute to hematopoietic tissues in chimera mice. We also found that germline mice homozygous for the mutated AML1 allele, which lacks the VWRPY-motif, have a minimal effect on hematopoietic development, as was observed in control knock-in mice with full-length AML1. On the other hand, reduced cell numbers and deviant CD4-expression were observed during early T-lymphoid ontogeny in the VWRPY-lacking mice, while the contribution to the thymus by the corresponding ES cell clones was inadequate. These findings demonstrate that AML1 with its trans-activating subdomains is both essential and sufficient for hematopoietic development in the context of the entire mouse. In addition, its trans-repression activity, depending on the C-terminal VWRPY-motif, plays a role in early thymocyte development.
Introduction

Vertebrate hematopoietic development is characterized by the sequential appearance of two cell populations, known as primitive and definitive hematopoiesis\(^1\). In mouse, for example, primitive hematopoiesis is first seen in the form of blood islands in the yolk sac of the 7.5-day-old (E7.5) mouse embryo. This cell population is thought to be directly differentiated from hemangioblast, a bi-potent precursor. This hematopoiesis consists predominantly of a large and nucleated erythroid population, which contains embryonic type hemoglobin. In contrast to the restricted and temporal development of this first wave which diminishes at mid-gestation, definitive hematopoiesis originates from the AGM (Aorta-Gonad-Mesonephros) region, where the stem cells with the long-term re-populating ability of multi-lineage hematopoiesis emerge around E9.5, as a result of budding, from the ventral endothelial cells of the great vessels\(^2,3\). The stem cells then migrate into the fetal liver and proliferate to rapidly establish the definitive hematopoiesis of all lineages, including progenitors for T- and B-lymphoid populations. Active sites for definitive hematopoiesis are transferred to bone marrow and spleen prior to birth, and function throughout life within these organs.

These stem cells are equipped with a number of critical transcription factors which play pivotal roles in determining the fate of the cells at discrete developmental stages. Most of these molecules have been identified by either isolating DNA-binding proteins to known cis-regulatory elements of lineage specific genes or cloning the DNA targets of leukemia-associated chromosomal translocations\(^4,5\). Acute Myeloid Leukemia 1: AML1 (also known as runt-related transcription factor 1: Runx1) is a prototype of the latter group. This gene was originally isolated from the breakpoint of the t(8;21)
(q22;q22) reciprocal translocation associated with 40% of cases with acute myelogenous leukemia of the French-British-American classification M2-subtype\textsuperscript{6-8}, and later recognized as one of the most frequent targets of gene aberration associated with human leukemia\textsuperscript{9,10}. AML1 encodes the DNA binding subunit of the core-binding factor transcription complex (CBF; also known as polyomavirus enhancer binding protein 2: PEBP2)\textsuperscript{11,12}. Gene-targeting experiments have demonstrated that AML1 is essential for the early development of definitive hematopoiesis\textsuperscript{13,14}. AML1 is first detectable on E7.5 in the endoderm and in some parts of the extra-embryonic mesoderm, and is expressed in primitive erythrocytes in the yolk sac on E8.0\textsuperscript{15}. Around E10.5, AML1 is expressed in the ventral epithelium of the dorsal aorta and in the vitelline and umbilical arteries within the AGM region\textsuperscript{15}, where hematopoietic cell clusters emerge. AML1-deficient mice lack these hematopoietic clusters, and die \textit{in utero} following complete block of fetal liver hematopoiesis in mid-gestation\textsuperscript{15-18}, underscoring the pivotal role of AML1 in this initial stage of emerging definitive hematopoiesis. In addition, leukemia-associated fusion genes, such as AML1-MTG8 (Myeloid Translocation Gene on Chromosome 8, or Eight Twenty-One [ETO]) formed by the t(8;21) translocation, have been shown to \textit{trans}-dominantly repress this normal AML1 function within the entire animal\textsuperscript{19,20} and thus to contribute to leukemic transformation\textsuperscript{21}.

AML1 functions both as a transcription activator and a transcription repressor in a context-dependent manner. AML1 protein has a modular structure, and its biochemical properties are mediated by the interaction with functional cofactors through its subdomains. For example, AML1 binds to the enhancer core DNA sequence, TGT/cGGT, which is found in the \textit{cis}-regulatory sequences within a panel of
hematopoiesis-related genes, via the runt domain\textsuperscript{22-24}. This domain consists of 128 amino acid residues localized near the N-terminus with 69\% identity to the Drosophila pair-rule gene known as runt\textsuperscript{25}. The runt domain also serves as the binding site for core-binding factor $\beta$ (CBF$\beta$, also known as PEBP2$\beta$)\textsuperscript{12,26,27}, and this association is essential for AML1’s biologic activity\textsuperscript{28-30}. The trans-activation domain is localized to the region between residues 291 and 371 (451 residues represent one of the “full-length” isoform, AML1b)\textsuperscript{31}, and serves as the binding site for transcription co-activators, such as p300/CBP\textsuperscript{32}. In contrast, the VWRPY-motif, consisting of the five amino acids at the C-terminus, is thought to be involved in transcription repression through its association with a transcription co-repressor, Groucho/Transducin-like Enhancer of Split\textsuperscript{33,34}. The protein-protein interaction through the VWRPY-motif appears to have been evolutionarily conserved\textsuperscript{35,36}, but little is known about its biologic roles in mammals. In addition, it has recently been demonstrated that residues 182 to 211 and 264 to 361 of AML1b also serve as repression subdomains in some cell types\textsuperscript{37}.

We previously showed that the block of definitive hematopoiesis resulting from the loss of AML1 could be replicated \textit{in vitro} by means of a murine embryonic stem (ES) cell differentiation system\textsuperscript{13}. The defect was rescued by re-expressing its cDNA off of an artificial knock-in allele\textsuperscript{38}, thus providing evidence that this phenotype results solely from the absence of this gene. With this experimental system, we were also able to demonstrate that this hematopoietic rescue required the trans-activation domain of AML1 but not the C-terminal trans-repression subdomain\textsuperscript{38}. In order to further define the biologic role(s) mediated through these functional subdomains of AML1, we performed two-step \textit{in vitro} culture experiments and chimera mouse analysis, for which
we used a panel of ES cell clones with C-terminal deletion mutants of AML1. In addition, the knock-in approach was used to generate genome-manipulated mouse lines which express the AML1 molecule lacking the VWRPY-motif. Our results indicate that transcriptionally active AML1 is essential for the early development of definitive hematopoiesis in the entire animal and that trans-repression activity via the VWRPY-motif of the molecule plays a role in the early development of T-cell lineage.
Materials and Methods

ES cell clones

All replacement-type vectors were constructed based on a 12-kb genomic DNA fragment encompassing exon 4 of AML1, which corresponds to the middle of the runt domain as described elsewhere\textsuperscript{38}). Wild-type or one of the C-terminal deleted cDNAs (Fig 1A) was inserted into exon 4 so as to keep the reading frame open, and polyadenylation (A) signal sequences of the rabbit globin gene and a puromycin resistance cassette for the positive selection were inserted at the 3’ end of the artificial exon 4. A poly-(A)-less diphtheria toxin-A suicide cassette was added at the utmost 3’-end of the construct for the negative selection (Fig 1B and Fig 3A). C-terminal deletion mutants used for the vectors were constructed by inserting artificial stop codons into the mouse AML1b cDNA at residue 293, 320, 390, or 446, and the resulting mutant molecules were named \(\Delta_{293}, \Delta_{320}, \Delta_{390}, \) and \(\Delta_{446}\) (Fig 1A). Each targeting vector was linealized and transfected into one million of the ES cells of either the wild-type (E14 or its derivatives) (Fig 3A) or AML1-deficient genotype (Fig 1B), and puromycin-resistant clones were selected. Homologous-recombinant clones were then serially assessed by means of Southern blot analysis with 5’- and 3’-outside probes as previously described\textsuperscript{38}).

Two-step culture analyses for hematopoietic differentiation of the ES cell clones.

ES cells were first induced to form embryoid bodies in semi-solid methylcellulose culture media as described by Keller et al\textsuperscript{39} with modifications\textsuperscript{38}). Grown embryoid bodies were then disrupted by collagenase treatment, and \(10^5\) recovered single cells were replated in secondary methylcellulose cultures supplemented with either human
erythropoietin (EPO; purchased from Kirin Brewery Co., Tokyo, Japan) alone for primitive erythroid colonies, or a combination of human EPO, human G-CSF (Kirin), murine GM-CSF, murine SCF, and murine IL-3 (all from Genzyme Corporation, Cambridge, MA, USA), which allows for growth of progenitors of definitive origin as previously described\(^{38}\).

**Generation of chimeric and germline mice**

ES cells were injected into the blastocysts of E3.5 obtained from C57BL6-strain mice as described elsewhere\(^{13}\) by using an inverted microscope (IX-70; Olympus, Tokyo, Japan) equipped with micro-manipulators (Narishige, Tokyo, Japan). Manipulated blastocysts were then transferred into the uterus of pseudopregnant mother mice of the ICR strain. The resultant chimeric mice were then crossed with C57BL6-strain mice to obtain ES cell-derived offspring. Transmission of the mutated alleles and genotype of the progeny were determined by Southern blot analysis as previously described\(^{13,20,38}\). All procedures for mouse experiments performed in this study were approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine.

**Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis for AML1 expression**

Semi-quantitative RT-PCR analysis to evaluate the expression of exogenous AML1 cDNA was performed as previously described\(^{40}\). 5 \(\mu\)g of total RNA isolated from each of the tissue samples was used as the template for the random hexamer-primed RT reaction with M-MLV reverse-transcriptase (Invitrogen Co., Carlsbad, CA,
USA), and serially diluted cDNA samples were assessed by PCR for a house keeping
gene, *hypoxantin-guanine phosphoribosyl transferase (HPRT)*, to yield a standard
representing the mRNA level within the samples (the primers were 5'-
GCTGGTGAAAAGGACCTCTCG-3' for sense and 5'-
CCACAGGACTAGAACACCTGC-3' for anti-sense orientation). The equivalent
dilutions of the cDNA samples were then analyzed for the *AML1* message of a 292-bp
fragment with a primer pair which encompassed the intron 3: forward, 5'-
CCAGCAAGCTGAGGAGCGGCG-3'; and reverse, 5'-
CCGACAAACCTGAGGTCGTTG-3'\(^{(40)}\).

**Glucose phosphate isomerase (GPI) isoenzyme analysis**

Organ or tissue samples obtained from chimera mice at the age of five weeks were
homogenized, and the appropriately diluted supernatants were electrophoresed on
cellulose acetate membranes (Helena Laboratories, Beaumont, TX, USA) so as to
separate GPI isozymes (GPI-A from ES cells: the 129 strain; GPI-B from the host: the
C57BL6 strain) according to their respective electrophoretic mobility (Fig 2A). The
amount of each isozyme was then assayed by an on-the-membrane enzyme reaction as
described elsewhere\(^{13,38}\). In parallel experiments, a panel of mixtures of 129-strain cells
and C57BL6-strain cells in pre-determined serial proportions (by increments of 10%)
were analyzed to obtain a standard for determining the relative amount for the two
isoforms in the samples of interest.

**Flow cytometric analysis**

Flow cytometric analysis was performed with conventional methods described
elsewhere\textsuperscript{20}). The dissected thymus or spleen was mechanically disaggregated into single cells in RPMI1640 medium (Invitrogen) containing 5\% FCS, and were examined for live cells by trypan-blue dye-exclusion test. Appropriate aliquots of the cells were then re-suspended in PBS containing 3\% FCS and 0.05\% sodium azide. This was followed by incubation with each of the appropriately diluted monoclonal antibodies (mo-Abs) on ice for 60min. The following fluorochrome-conjugated Abs were used: anti-CD3 conjugated with FITC (Beckman Coulter Inc., Fullerton, CA, USA), anti-CD4 with PE or with Per-CP, anti-CD8a with FITC or with APC, anti-CD25 with FITC, anti-CD44 with PE, anti-B220 with PE, and anti-TCR\(\beta\) with FITC (all from BD Biosciences, San Jose, CA, USA). After two washings, samples were analyzed on a FACSCalibur flowcytometer (BD Biosciences). Isotyping-matched Abs conjugated with the appropriate fluorochrome (BD Biosciences) at the same protein concentrations were used as negative controls for all experiments.

**Hematopoietic progenitor assay for fetal liver cells**

Fetal liver of E12.5 embryos was dissected under a stereoscopic microscope (SZX-12; Olympus), and then disaggregated by passing it through a 26-G needle. In a triplicate experiment, 5000 cells were plated in a 1-ml mixture of IMDM with 1.2\% methylcellulose, supplemented with a combination of colony-stimulating factors as described for the two-step culture procedure\textsuperscript{13,38}. Colonies were counted under an inverted microscope (CK-2; Olympus), and the total number of the progenitors was estimated in proportion to the total number of cells obtained from each liver.
Results

**AML1 transcription factor without its C-terminal repression subdomain rescues definitive myeloid lineages in vitro**

AML1 is essential for the early development of definitive hematopoiesis but not for primitive erythropoiesis\(^{13,14}\). The hematopoietic defect resulting from the loss of AML1 could be replicated *in vitro* with the murine embryonic stem (ES) cell differentiation system, and the phenotype was rescued by re-expressing its cDNA by using a knock-in approach as previously reported\(^{38}\). When definitive hematopoiesis is judged by the emergence of macrophages differentiated from the ES cell-derived embryoid bodies, the AML1 molecule with its *trans*-activation subdomain appears to be sufficient for this rescue\(^{38}\). In contrast, the C-terminal repression subdomain including conserved VWRPY-motif does not appear indispensible for rescue\(^{38}\). In order to determine whether individual hematopoietic lineages showed any preference for rescue by specific deletion mutants, we subjected the constituent cells of the EBs to a second-step culture in the presence of the appropriate colony-stimulating factors. *AML1*-deficient ES cell clones, which carry the knocked-in AML1 mutants that retain the *trans*-activating domain (wild-type AML1, Δ446 or Δ390) (Fig 1A and B), could rescue all lineages examined, including definitive erythroid (Ery-D), myeloid, and mixed lineage with erythroid (E-Mix) (Fig 1C). In contrast, the mutants which lacked this domain (mutants Δ293 and Δ320) could not rescue any of the lineages (Fig 1C). In addition, examination of cytospin preparations by May-Grünewald-Giemsa staining showed that the appearance of the constituent cells from the colonies of definitive origin grown from knock-in clones was indistinguishable from that of the control heterozygous ES clones (not shown). Thus, *in vitro* hematopoietic rescue of individual
definitive lineages requires the trans-activating domain of AML1, but the C-terminal repression subdomain does not appear to be essential.

*In vivo* rescue for myeloid lineages requires transcriptionally active AML1, while C-terminal repression subdomains may have a function in thymus and/or liver

To define whether the trans-activating subdomain is required for *in vivo* hematopoietic development as was observed in the *in vitro* experiments, we next evaluated the ability of ES cell clones to contribute to tissue formation in chimera mice. We generated chimera mice by injecting each of the knock-in ES cell clones into blastocysts obtained from a wild-type (C57BL6) host strain, and contribution of the ES cells to tissue formation was assessed by means of glucose phosphate isomerase (GPI) isoenzyme analysis (Fig 2A). For each mutation at least four five-week-old chimera mice were examined. ∆446 and ∆390 mutants, which retain the activation subdomain but lack C-terminal repression subdomains, always contributed to myeloid tissues, including bone marrow, peripheral blood, and spleen, as was seen in the case of the full-length *AML1b* cDNA knock-in clone (Fig 2B and C). In contrast, ∆320 and ∆293 mutants did not contribute to the hematopoietic tissues (Fig 2B and C), indicating that the activation subdomain is important for the establishment of *in vivo* hematopoiesis and that the C-terminal repression subdomain including the VWRPY-motif is not required.

ES cell clones bearing the ∆320 or ∆293 mutant, which did not contribute to haematopoietic tissues, did not contribute to the liver either (Fig 2B and C). In addition, the rescued ES clones with the ∆446 or ∆390 mutant, which did contribute to myeloid tissues in the chimera mice, contributed less to the formation of the liver (Fig 2B and C),
suggesting that the cells with the AML1 function intact were preferentially used to
generate the liver. Similarly, rescued ES cell clones with the Δ446 or Δ390 molecule
tended to contribute less to the thymus than to the myeloid tissues (Fig 2C), indicating
that AML1 may play a role in thymus development via its C-terminal VWRPY-motif.

**Generation of germline mice bearing the knock-in allele of full-length or Δ446
cDNA for AML1**

For the next step, we examined whether the findings from ES cell-based
experiments apply to the entire animal. For this purpose, we generated mouse lines
which carry a knock-in allele of either the full-length or the Δ446 cDNA of the AML1
molecule in their germline. As outlined in the Materials and Methods section and
illustrated in Fig 3, we introduced an exogenous cDNA into exon 4 of the AML1 gene
locus by homologous recombination in wild-type mouse ES cell lines. These artificial
alleles were designed to express 3’-sequences of AML1b cDNA downstream from its
exon 4 under control of the endogenous cis-elements of the transcription. Two
independent mouse lines were established for each germline knock-in mutation (Table
1), and the phenotype of a mutation was recognized when it was observed in both lines.
Assessment by means of semi-quantitative RT-PCR analysis showed that the expression
level of the knocked-in gene in thymus or spleen was indistinguishable from that of the
endogenous gene (Fig 3C). The two mutant alleles were segregated according to
Mendelian inheritance (Table 1), and the homozygous mice carrying either of the
knocked-in cDNAs (AML1<sup>WT/WT</sup> or AML1<sup>Δ446/Δ446</sup>) and kept under specific pathogen-
free conditions were fertile and appeared healthy in comparison to the wild-type control
mice. Thus, the knock-in procedure resulted in generating mice which expressed
exogenous AML1 cDNA of either full-length or of the Δ446 mutant, and both artificial alleles could rescue these mice from the embryonic death which had been observed in the simple targeted-disruption\textsuperscript{13,14} of this gene.

Genetically modified mice of the AML1\textsuperscript{WT/WT} or AML1\textsuperscript{Δ446/Δ446} genotype showed minimal abnormality in myeloid hematopoiesis

The homozygous mice had a tendency to have fewer white blood cells and lower platelet counts in comparison with those observed in the control wild-type or heterozygous littermates regardless of which knock-in allele, AML1\textsuperscript{WT/WT} or AML1\textsuperscript{Δ446/Δ446}, they carried. However, these difference were not statistically significant (Fig 3D). In addition, red blood cell count, hemoglobin concentration, and hematocrit of the mutant mice were indistinguishable from those of control animals (Fig 3D). In agreement with these observations, histological examinations revealed that the mutant mice had a normal white blood cell differential as well as indistinguishable architecture and cellularity of hematopoietic bone marrow as compared with controls (negative data, not shown).

The biologic effect of AML1 on early hematopoietic development depends on the dosage. Mice heterozygous for the inactivating mutation of AML1 have been found to feature precocious appearance of hematopoietic stem cells in the yolk sac\textsuperscript{41} and a reduction by about half of the hematopoietic progenitor cells in the fetal liver\textsuperscript{14}. To analyze fetal liver hematopoiesis in the knock-in animals, we cultured fetal liver cells obtained from the litter-embryos of intercrossed heterozygotes at E12.5. In the presence of a combination of colony-stimulating factors which support the growth of hematopoietic progenitors of definitive origin, a total of around 2-3 x 10\textsuperscript{4} colonies of
various cell-lineages per liver were detected in wild-type littermates. In contrast, the average number of progenitors for the mutant mice was approximately 20,000 for $AML1^{WT/WT}$ and 19,000 for $AML1^{\Delta446/\Delta446}$, for an efficiency of 60-70% of that seen in wild-type littermates (Table 2). The mutant mice thus showed a tendency to have fewer progenitors, but the difference was not statistically significant.

These results indicate that, as in the case of the knock-in expression of full-length $AML1$ cDNA, the expression of the exogenous $AML1$ cDNA of the $\Delta446$ mutant, which retains its trans-activation subdomains but lacks the VWRPY-motif, is sufficient to support myeloid hematopoiesis throughout the life of the entire mouse.

$AML1^{\Delta446/\Delta446}$ mice have smaller thymus

Further examination showed that the thymus obtained from $AML1^{\Delta446/\Delta446}$ mice was smaller than that from wild-type or heterozygous littermates or that from age-matched homozygotes with the full-length cDNA knock-in allele, $AML1^{WT/WT}$. This difference was detectable at birth or when they were 4 days old, and became more prominent at the age of 5 weeks when this organ normally reaches its largest size. This size-difference became less prominent in adult mice, however (Fig 4A). The average number of cells in the thymus from $AML1^{\Delta446/\Delta446}$ mice was almost half of that in the thymus from controls, for a statistically significant difference (Fig 4B).

Despite this quantitative difference, the histological findings for the thymus from $AML1^{\Delta446/\Delta446}$ mice, obtained from microscopic examination of hematoxylin-eosin-stained sections, were not significantly different from those for control animals (Fig 4C). Flow cytometric analysis showed a lower CD4:CD8 cell ratio of the splenic T-lymphocytes for both $AML1^{\Delta446/\Delta446}$ and $AML1^{WT/WT}$ mice than for the wild-type mice.
(Fig 4C), as was previously reported for mice heterozygous for an inactivated AML1 allele\(^2\). However, the proportion of CD4- and CD8-expressing thymocytes in both knock-in strains was indistinguishable from that found in wild-type control mice (Fig 4C), in contrast to the findings for haplo-insufficient mice which showed reduced cell numbers for thymic single-positive cell populations\(^2\). The thymocytes from \(AML1^{\Delta 446/\Delta 446}\) mice expressed the CD3 antigen, which suggests that mature T-cell receptors are expressed on the surface of the cells (not shown). Furthermore, there was no increase in TUNEL-(terminal deoxynucleotidyl transferase-mediated nick-end-labeling)-positive apoptotic cells in the thymocytes of four-day-old or five-week-old \(AML1^{\Delta 446/\Delta 446}\) mice in comparison with that of control littermates (negative data, not shown).

Together with the findings obtained from chimera mouse analysis, these results indicate that AML1 plays an important \textit{in vivo} role, somehow mediated by the C-terminal repression subdomain, in determining the number of cells in the developing thymus, most likely, in a cell-autonomous fashion.

**\(\Delta 446\)-mutation affects CD4-expression in early thymocyte development**

Early T-cell development is a multi-step process in the thymus, where hematopoietic stem cells migrate and are instructed and selected to eventually constitute mature peripheral T-cell populations of either CD4-positive helper cells or of CD8-positive cytotoxic cell lineage. The developmental stages are characterized by specific surface marker expression: in the very early stage the thymocytes are negative for CD4 or CD8, and are thus called a double-negative (DN) cell population. Upon successful rearrangement of one allele of the \textit{T-cell receptor} \(\beta\) gene locus (\(\beta\)-selection),
DN cells then express both CD4 and CD8 antigens to become a double-positive (DP) cell population. Finally, DP cells lose the expression of either antigen to form mature single-positive (SP) cell populations. In addition, progression of thymocytes through the DN stage can be further categorized according to the expression of CD25 or CD44. The four stages of DN development, with increasing levels of maturity, are called DN1 (CD44\(^+\)CD25\(^-\)), DN2 (CD44\(^+\)CD25\(^+\)), DN3 (CD44\(^-\)CD25\(^+\)) and DN4 (CD44\(^-\)CD25\(^-\)), and \(\beta\)-selection occurs in DN3 cells\(^{43}\). We analyzed the thymus of the mutant mice in the late gestational stage, and found that differences in the size of the thymus of the \(\Delta446\) embryo compared with that of control littermates were detectable as early as E17.5. In addition, the CD4 single-positive fraction in the mutant embryos was higher than that in the wild-type littermate-embryos on E17.5 and E18.5 (Fig 5). It should be noted that these phenomena were not observed in \(AML1^{WT/WT}\) embryos (Fig 5). Nevertheless, the \(AML1^{\Delta446/\Delta446}\) embryos regained the normal CD4-CD8 differential at birth or thereafter (Fig 4C), and there was no apparent block in the transition between the DN3 and DN4 stages in \(AML1^{\Delta446/\Delta446}\) embryos (Fig 5).
Discussion

It has been established that AML1 plays a pivotal role in definitive hematopoiesis since the loss of this gene in mouse results in mid-gestational death due to a block of fetal liver hematopoiesis\textsuperscript{13,14}. We previously demonstrated that the hematopoietic defect can be replicated by using ES cells in an \textit{in vitro} differentiation system and that this \textit{in vitro} phenotype could be rescued by the re-expression of \textit{AML1} cDNA from a knock-in allele\textsuperscript{38}. In the study presented here, we focused on the biologic properties of AML1 mediated through its C-terminal subdomains as a result of hematopoietic rescue of \textit{AML1}-deficiency at single cell, tissue, and entire animal levels. The findings for the C-terminal deletion mutants indicate that the expression of the AML1 molecule with its trans-activation subdomains retained is both necessary and sufficient for the \textit{in vitro} differentiation of ES cells into definitive hematopoietic cells of all myeloid lineages and for \textit{in vivo} contribution of ES cells to myeloid tissues in chimeric mice. These results further support our earlier observations in embryoid body experiments\textsuperscript{38}. In addition, our results provide genetic proof that the AML1 molecule with its trans-activation subdomains retained is sufficient for hematopoietic rescue within the entire animal. The germline mice that homozygously carry knock-in alleles that express either full-length or C-terminally deleted cDNA which lacks a \textit{trans}-repression subdomain including the VWRPY-motif, were found to be viable and show minimal abnormalities in myeloid hematopoiesis throughout their life.

The VWRPY-motif localized at the C-terminus is one of the functional subdomains for the \textit{trans}-repression activity of AML1 molecule, which has been posited as the binding site for the transcription co-repressor Groucho/TLE\textsuperscript{34-36}. The orthologue of AML1 in \textit{Drosophila}, runt, has been demonstrated to function as a
transcriptional repressor for its target, the even-skipped gene, and also to function in the maintenance of the repressed state for another target, the engrailed gene. Both these functions are realized in a VWRPY-motif/Groucho binding-dependent fashion to mediate the segmentation process of the blastoderm\textsuperscript{33,44}. Although the trans-repression property of AML1 via the VWRPY-motif is genetically conserved\textsuperscript{35,36}, the biologic importance of this activity in mammals has not yet been analyzed in detail. In this study, we observed that mice lacking this VWRPY-motif of AML1 demonstrated less thymus cellularity and more CD4 single-positive cells than seen in controls, including heterozygous or wild-type littermates and age-matched mice homozygous for full-length AML1 cDNA. In addition, ES cell clones carrying AML1 mutants without the C-terminal trans-repression subdomains were found to make an inadequate contribution to the thymus in chimera mice. These findings indicate that mammalian AML1 does have biologic functions specific to this trans-repression subdomain in T-cell ontogeny.

Data available so far strongly suggest that the observed AML1’s function is cell-autonomous. Nevertheless, further studies, such as complementation assay experiments involving T-cell depleted Rag2-mutant\textsuperscript{45,46} mice transplanted with VWRPY-mutant mice-derived bone marrow cells, should result in a more specific characterization of the thymus manifestation of the mutation and contribute to defining the nature of the activity in greater detail.

A very recent study described T-cell-specific conditional disruption of AML1\textsuperscript{47}. A genetically modified mouse line was generated which carries both floxed exon 4 of the AML1 gene locus and the transgene of lck-promoter-driven-Cre. The fetal liver cells from these mice were then used to complement the T-cell depletion of Rag2-null mice\textsuperscript{45}. The thymocytes from these mice are thus lacking in whole AML1 activity in
the early DN stages of the T-cell ontogeny. Subsequently, a reduction in the number of thymocytes and premature CD4-expression due to the loss of appropriate repression of this gene \(^\text{48}\) were noted in the thymus during late gestation \(^\text{47}\). In our study, the \(AML1^{\Delta446/\Delta446}\) embryos showed a reduction in cell number and an increase in CD4 single-positive cell population during early thymus development, both of which were observed when AML1 was conditionally disrupted in thymus \(^\text{47}\). The data available did not allow us to draw a conclusion regarding the cellular mechanism of AML1, that is, whether the premature CD4-expression occurred due to the loss of appropriate repression of this gene or CD8-expression was reversed in the double-positive cell population. Nevertheless, the results presented here at least indicate that these phenomena are mediated by AML1’s \textit{trans}-repression activity, which depends on its VWRPY-motif. In contrast to the T-cell-specific AML1-disruption, which also showed an impaired \(\beta\)-selection process leading to a block in the DN3 stage during the late gestation and abnormal CD8 expression in young adults \(^\text{47}\), \(AML1^{\Delta446/\Delta446}\) was found to have no effect on these stages. These results suggest that these biologic functions are mediated by the activity of AML1, and that this activity is independent from the VWRPY-motif.

The contribution to the liver by ES cells with AML1 without the C-terminal repression subdomain was found to be inadequate in the chimera mouse analysis, while the \(AML1^{\Delta446/\Delta446}\) mice developed a functioning liver. In addition, even though the contribution to the liver by ES cells, lacking entire AML1 activity, was even less \(^\text{13,29,38}\), the \(AML1\)-knockout embryo does develop a liver at least up to mid-gestation \(^\text{13,14}\). It is obvious that ES cells with an intact AML1 function are preferentially used to form the liver, and conditional liver-specific disruption of this gene should make it clear whether
this gene is also involved in liver development.

Consistent with our observation that AML1 performs biologic functions in above-mentioned sites through its C-terminal VWRPY-motif, the expression of its associated co-repressor, TLE, has been reported to also occur in those tissues. The *Drosophila* orthologue, Groucho protein, which plays critical roles in multiple developmental processes by acting as a dedicated transcriptional co-repressor for a number of transcription regulators, including Hairy, Dorsal, engrailed, Tcf, as well as Runx, is encoded by a single gene and ubiquitously expressed during early development of the fly\(^{49,50}\). In contrast, at least six homologues have so far been identified in mammals: TLE1 through TLE6 in human and *Groucho*-related genes (Grg)1 through Grg6 in mice\(^{51-56}\). TLE/Grg1, 2, 3, 4, and 6 are thought to be full-length molecules with all five functional subdomains, whereas TLE/Grg5 is a truncated form which retains only the two N-terminal domains \(^{50,52,55}\). The expression profiles of the TLE/Grg molecules have been extensively analyzed and reportedly occur in unique but overlapping patterns during embryonic development. The sites where they are highly expressed include the central nervous system, somites, and proliferating epithelial tissues which undergo mesenchymal induction \(^{51-58}\). Expression of the genes was also detected in liver and thymus of adult \(^{51,56-58}\). Of note is the fact that a histological study using *in situ* hybridization analyses showed Grg3 to be highly expressed in thymocytes at E16.5 \(^{50}\), which coincides with the developing stages of the thymus when the *AML1\(^{∆446/∆446}\)* mice manifested the phenotype. This observation may explain the stage-specific effect of the VWRPY mutation. However, so far only TLE/Grg1 and TLE/Grg2 have been experimentally proven to function as co-repressors for AML1 \(^{35,36,59}\) whereas no evidence has been provided yet that other TLE/Grg family members could function in a
similar fashion. Further analysis of the temporal and spatial expression patterns of the TLE/Grp family members in mouse development as well as their functional relationship with AML1 should make a significant contribution to a better understanding of the molecular basis for the $AML1^{\Delta446/\Delta446}$ phenotype.

The $AML1$ gene locus is one of the most frequent targets of human leukemia-associated gene aberrations, and the leukemic oncoprotein is believed to be formed by two mechanisms. One is a chromosomal translocation which results in the formation of chimera proteins, such as AML1-MTG8 (ETO), AML1-Evi1, or Tel-AML1$^{9,10}$. These molecules acquire a strong ability to trans-dominantly repress AML1’s transcriptional activities$^{60-65}$. The other mechanism is a genetic mutation often found within the runt domain sequences$^{66-69}$. The mechanisms of leukemogenesis caused by these point mutations of $AML1$ remain for the most part unknown. The experimental system with a knock-in approach employed in this study to express exogenous mutant AML1 in germline mice by a knock-in approach is expected to become a valuable tool for probing the molecular basis for leukemogenesis mediated by subtle mutations in the $AML1$ gene locus which have been found in the human leukemia and related disorders.

To summarize, genetical dissection of the biologic function along with its C-terminal functional subdomain-structure of AML1 allowed us to identify their respective roles as C-terminal repression subdomain-dependent and -independent. In addition, we successfully developed an experimental system which enabled us to directly analyze the genetic consequences of AML1 mutations in the context of the entire animal. These findings and genetic tools can be expected to provide new insights into the molecular basis of hematopoietic regulation as well as leukemogenesis caused by alterations in the $AML1$ gene.
Acknowledgements

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References


16) de Bruijn MF, Speck NA, Peeters MC, Dzierzak E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J. 2000; 19: 2465-2474.


26) Ogawa E, Inuzuka M, Maruyama M, et al. Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel *Drosophila* runt-


38) Okuda T, Takeda K, Fujita Y, et al. Biological characteristics of the leukemia-


50) Chen G, Courey AJ. Groucho/TLE family proteins and transcriptional repression.


Legends for Figures

Fig. 1. Two-step culture experiments for *in vitro* hematopoietic rescue of knock-in ES cell clones

(A) Structure of the subdomain of AML1 molecule and C-terminal deletion mutants used for the knock-in experiments. Numerals indicate positions of the residues. Runt: the runt domain; AD: *trans*-activation domain; ID: inhibitory domain; VWRPY: VWRPY-motif. (B) Targeted-insertion of the cDNA into *AML1*-deficient ES cells, whose disrupted alleles are indicated by insertions of hygromycin-resistant (hygr) and neomycin-resistant (neo) cassettes at exon 4. Replacement-type vector for the targeted-insertion and the schema of the resultant knock-in allele are shown at the bottom. pA: poly-adenylation signal sequences; puro: puromycin-resistance cassette. (C) The capability for *in vitro* differentiation of the knock-in ES cell clones for each of the *AML1* mutant cDNAs in a representative two-step re-plating culture experiment (see Materials and Methods). ES cells were cultured to form embryoid bodies (EBs). Hematopoietic progenitors of individual lineages developed within EBs were then analyzed by the second step culture on day-6 for primitive erythroid (Ery-P), and on day-10 for those of definitive origin, including definitive erythroid (Ery-D), granulocyte-macrophage and macrophage (Myeloid), and mixed lineages including erythroid (E-Mix). Columns indicate numbers of progenitors per $10^5$ disaggregated cells in culture.

Fig. 2. Glucose phosphate isomerase (GPI) isozyme analysis for the contribution by the knock-in ES clones to tissue in the chimera mice

(A) Schematic representation of the procedure for GPI analysis (see Materials
and Methods). (B) Representative results of the assay for chimeric mice generated with knock-in clones for each of the AML1 mutant cDNAs. Lysates from tissues were separated by electrophoresis and stained for GPI activity. Contribution by ES cells is indicated by the GPI-A isoform, and that by host-derived cells by the GPI-B isoform. Results shown are for brain (Br), kidney (K), thymus (T), liver (L), spleen (Sp), bone marrow (BM), and peripheral blood (PB), with ES cells as GPI-A controls. (C) Quantification of the tissue contribution by the knock-in clones is indicated by columns with bars signifying standard deviation. Seven chimera mice were analyzed for full-length AML1 (451), four each for ∆446, ∆390, ∆320, and five for ∆293 knock-in clones.

Fig.3. Procedure for the generation of germline mice carrying knock-in AML1 allele

(A) Procedure for the targeted-insertion (knock-in) of full-length AML1 cDNA or of the ∆446 mutant into wild-type ES cells by homologous recombination. (B) Southern blot analysis of the knock-in allele using XbaI-digested genomic DNA obtained from a representative litter of crossed ∆446-heterozygotes. Lanes 1, 5, and 6: wild-type (+/+); lanes 4, 7, and 10: homozygote (∆446/∆446); remaining lanes: heterozygote (+/∆446). (C) Semi-quantitative RT-PCR for comparison of the expression of AML1 with that for the HPRT gene in wild-type germline mice (+/+), those homozygous for full-length cDNA (WT/WT), and those homozygous for ∆446 (∆446/∆446) genotypes (see Materials and Methods). Results for two independent mice for each genotype are shown. Serially diluted cDNA pools were analyzed. Lanes a, b, and c: 5^2, 5^3, and 5^4 dilutions, respectively. (D) Peripheral blood cell counts for the
mutant mice in comparison to those for the matched-sibling control mice are indicated by columns. Bars signify standard deviations.

**Fig. 4. Thymus size reduction found in born \textit{AML1}^{\Delta446/\Delta446} mice**

(A) Macroscopic appearance of the dissected thymus from representative litters resulting from inter-crossing of mutant mice heterozygous for either full-length cDNA knock-in (+/WT) or \(\Delta446\) mutant knock-in (+/\(\Delta446\)) at the age of 4 days, 5 weeks, and 15 weeks. Homozygous mutants are indicated by “M” in the photos. (B) Numbers of cells per one lobe of the thymus from littermate-mice of each genotype are indicated by columns with bars for standard deviation. The numbers of mice examined are indicated in parentheses at the bottom. Statistically significant differences are shown as \(p\)-values. (C) Microscopic appearance of hematoxylin-eosin stained sections of the thymus are illustrated in the top panels, and results of flow cytometric analysis of the CD4 and CD8 expression for thymocytes and splenic T-cells are shown below.

**Fig. 5. Effects of \(\Delta446\)-mutation on CD4 and CD8 expression during T-cell ontogeny**

The embryonic thymi from wild-type (\textit{AML1}^{+/+}), \textit{AML1}^{WT/WT}, and \textit{AML1}^{\Delta446/\Delta446} genotypes at several developmental stages, including E16.5, E17.5, and E18.5, were analyzed for CD4 and CD8 expression by flow cytometric analysis. \(\Delta446\) mutation resulted in an increase in the CD4 single-positive cell population as indicated. Total numbers of thymus cells are shown above the quadrant graphs. Double-negative populations at E18.5 were further analyzed by four-color analysis for CD25 and CD44 expression as shown in the panels on the right. Thymi from the \(\Delta446\)-mutant showed no
difference in DN stage progression in comparison to those from controls.
Table 1 Genotypes of Born Litters by Intercrossing Heterozygous Parents

<table>
<thead>
<tr>
<th>Clone #</th>
<th>AML1+/WT-Crossing</th>
<th>AML1+/∆446-Crossing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>+/WT</td>
</tr>
<tr>
<td>164</td>
<td>46</td>
<td>68</td>
</tr>
<tr>
<td>734</td>
<td>26</td>
<td>72</td>
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</tbody>
</table>

+: wild-type allele for AML1 gene, WT: knock-in allele with full-length (451 residues) cDNA of AML1 gene, ∆446: knock-in allele with D446-deletion mutant cDNA of AML1 gene
Table 2  Hematopoietic Progenitors Detected in Fetal Livers of Knock-in Embryos at E12.5

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>BFU-E**</th>
<th>CFU-GM</th>
<th>CFU-Mix</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>n=11</td>
<td>5±1 (1725)</td>
<td>73±20 (25524)</td>
<td>17±3 (5635)</td>
</tr>
<tr>
<td>WT/WT</td>
<td>n=11</td>
<td>7±1 (1219)</td>
<td>79±19 (14684)</td>
<td>20±4 (3706)</td>
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<tr>
<td>+/-</td>
<td>n=11</td>
<td>6±2 (1693)</td>
<td>75±27 (23464)</td>
<td>17±7 (5253)</td>
</tr>
<tr>
<td>∆446/∆446</td>
<td>n=11</td>
<td>9±3 (1609)</td>
<td>86±38 (13163)</td>
<td>23±10 (3503)</td>
</tr>
</tbody>
</table>

* Wild-type and homozygous littermates are compared.
** Numbers are given as mean ± standard deviation per 5000 nucleated cell cultured and mean per liver (in parentheses).
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A

WT

\[\text{50} \quad \text{177} \quad \text{291} \quad \text{371} \quad \text{411} \quad \text{451}\]

\[\Delta 446\]

\[\Delta 390\]

\[\Delta 320\]

\[\Delta 293\]

\[\text{YWRPY}\]

B

Wild-Type

Aml-1

\(\text{Coh}\)

\[\text{NH}_2\]

\[\text{COOH}\]

\[\text{Knock-out (eryg)}\]

\[\text{Knock-out (eco)}\]

\[\text{Knock-out Vector}\]

\[\text{Knock-out Allele}\]

\[\text{5'-Probe}\]

\[14\text{kb}\]

\[11\text{kb}\]

\[7\text{kb}\]

C

\[\text{AML1+/}\]

\[\text{AML1/-}\]

\[\text{-/KI(\Delta 293)}\]

\[\text{-/KI(\Delta 320)}\]

\[\text{-/KI(\Delta 390)}\]

\[\text{-/KI(\Delta 446)}\]

\[\text{-/KI(WT)}\]

Day 6 EBs

Day 10 EBs

Ery-P

Ery-D

Myeloid

E-Mix
VWRPY-motif-dependent and -independent roles of AML1/Runx1 transcription factor in murine hematopoietic development

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