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Journal Article

Kyoto University
MLL Becomes Functional through Intra-Molecular Interaction Not by Proteolytic Processing

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Abstract

The mixed lineage leukemia (MLL) protein is an epigenetic transcriptional regulator that controls proliferative expansion of immature hematopoietic progenitors, whose aberrant activation triggers leukemogenesis. A mature MLL protein is produced by formation of an intra-molecular complex and proteolytic cleavage. However, the biological significance of these two post-transcriptional events remains unclear. To address their in vivo roles, mouse mutant alleles were created that exclusively express either a variant protein incapable of intra-molecular interaction (designated de) or an uncleavable mutant protein (designated uc). The de homozygous mice died during midgestation and manifested devastating failure in embryonic development and reduced numbers of hematopoietic progenitors, whereas uc homozygous mice displayed no apparent defects. Expression of MLL target genes was severely impaired in homozygous fibroblasts. These results unequivocally demonstrate that intra-molecular complex formation is a crucial maturation step whereas proteolytic cleavage is dispensable for MLL-dependent gene activation and proliferation in vivo.

Introduction

MLL (also known as MLL1, HRX and KMT2A) is an epigenetic transcriptional regulator that serves essential roles in embryonic and hematopoietic development. During embryogenesis, MLL maintains expression of Homeobox (HOX) genes to confer cellular identities along the anterior-posterior body axis [1], [2]. In the hematopoietic lineage, MLL regulates expression of a subset of HOX genes [3], [4] that promotes self-renewal of hematopoietic stem cells (HSCs) and expansion of immature progenitor pools [5], [6]. Hence, MLL deficiency in mice causes hematopoietic failure accompanied with insufficient expansion of immature hematopoietic progenitors [3], [7], [8]. Furthermore, MLL suppresses premature-senescence in both human and mouse fibroblasts in part by maintaining HOX gene expression [8], [9], [10]. Therefore loss of MLL function causes premature senescence. Conversely, MLL gain-of-function mutations caused by chromosomal translocations in hematopoietic cells result in constitutive expression of HOX genes that aberrantly enhance proliferation [11], [12] and suppress senescence to cause acute leukemia [8], [10].

MLL is translated as a large precursor protein (~430 kD) that subsequently undergoes proteolytic processing into two fragments (MLL N and MLL C) by the Taspase 1 endopeptidase [13], [14], [15], which specifically cleaves sites that are evolutionarily conserved with MLL2 (also known as MLL4, HRX2 and KMT2B) and Drosophila TRX. The respective MLL N and MLL C fragments form a holocomplex by non-covalent intra-molecular interaction [14], [15]. Although these MLL fragments are susceptible to distinct degradation pathways [9], the MLL N/MLL C holocomplex is stably expressed because intra-molecular complex formation masks structures that would otherwise lead to degradation. However, the biological significance of these maturation processes in vivo remains unclear.

In the current study, the in vivo roles of intra-molecular complex formation and proteolytic processing in MLL functions were examined using knock-in mouse lines with targeted mutations that selectively prevent self-association and proteolytic cleavage, respectively.

Materials and Methods

Ethics statement

All animal work has been conducted according to the institutional guidelines with the approval of Stanford University (8939) and National Cancer Center Research Institute (T08-030-N, T08-030-CCB02).
Maturation Mechanism of MLL

A

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**Genotypes**

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**Images**

B

- Wild type locus
- Targeting vector
- Mutated locus

C

- Genotypes

D

- Primer positions

E

- Genotypes

F

- E13.5

G

- Exon 10 and 12

H

- Graphs of MLL(N), MLL(M), and MLL(C) expression
Figure 1. Intra-molecular interaction is required for MLL-dependent gene activation. A. MLL proteins produced by dC and de mutations are shown schematically. The characteristics of each mutant are shown on the right. The N-terminal intra-molecular interaction domain (NIID) includes PHD1, PHD4 and FYRN and the C-terminal intra-molecular interaction domain (CIID) is FYRC. Positions of the three taqman probes for MLL (M), MLLM (M) and MLLC (C) used in Figure 1G are indicated by red bars. Genomic sequences around the exon 11 of the human MLL and its murine counterpart are shown at the bottom. B. Genomic structures of the wild type allele and the recombined allele are shown with the targeting vector. C. Diagnostic PCR identifies the recombined allele in genetically engineered mice. +/+: wild type. de/de: heterozygous D. Sequence of the exon junction of Mll de transcript. The PCR product generated by RT-PCR of de homozygous MEFs was sequenced. E. Genotypes at various developmental stages. Viability of embryos was confirmed by presence of heart contractions. de/de: de homozygous F. Morphologies of E13.5 embryos of de mutants.

Generation of knock-in mice

Targeting vectors containing the mutations and the cassettes of Neomycin resistance gene (neo) and Diphtheria toxin gene (DT) (kindly provided by Dr. Takeshi Yagi) were constructed by PCR-mediated mutagenesis and restriction enzyme digestion/ligation. The targeting vector for the ac mutation was constructed in the same manner as the previously published dC mutant allele (8). ES cells (CGR8.8) were transfected with the linearized targeting vectors and screened for positive clones by PCR. Homologous recombination was confirmed by LA-PCR (Takara Bio Inc., Otsu, Japan) using primer pairs specific for both ends of the targeting construct (primer sequences available upon request). Targeted ES clones were transiently transfected with a Cre recombinase expression vector (kindly provided by Dr. Takeshi Yagi) and subsequently screened for clones with appropriate excision of the neo cassette. Blastocyst injections were performed by the Transgenic Research Facility of Stanford University. Knock-in mouse lines were maintained by backcrossing onto a C57BL/6 genetic background. Genotyping of mice for the de allele was performed by PCR using a primer set (5'-tgaaagttgggtgaagttgtaagg-3' and 5'-agatggagtccacaggatca-3') that detected both the mutant allele (~500 bp) and the wild type allele (800 bp). Genotyping of mice for the ac allele was performed by PCR using a primer set (5'-gtctggaagacacactcctgta-3' and 5'-ctatcagagggaacggcatctcgg-3') that detected both the mutant allele (~300 bp) and the wild type allele (250 bp).

Cell culture

293T, plat-E, and mouse embryonic fibroblast (MEF) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal calf serum and non-essential amino acids.

Western blotting

Western blotting was performed as described previously [15]. The mouse monoclonal anti-MLL3 antibody (mAb4) and anti-MLL5 antibody (9-12) were previously described [8, 15]. Goat anti-membrane antiserum (C19) was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA) and mouse anti-actin antibody (MAB 1501R) was purchased from Millipore (Billerica, MA).

RT-PCR

Reverse transcription (RT) was performed as described previously [8]. RT-PCR of the mouse de variant transcript was performed using a primer set flanking the exon 11 counter part sequences (5’-agagtggtctgctaggtgctagaaggtggtaagttgtaagg-3’ and 5’-tttctctggtggggggtggggg-3’). Quantitative PCR (qPCR) was performed in triplicate and average expression levels (with standard deviations) normalized to that of Gapdh or Actb were calculated using a standard curve and the relative quantification method as described in ABI User Bulletin #2. Taqman probes for various genes [Gapdh: Mm00445296_g1, Mll(M):Mm01179266_g1, Mll(M):Mm01179218_m1, Mll(C):Mm01179293_m1, Hoxc8:Mm00439699_m1, Cdkn2a:Mm01179264_m1, Hoxc9: Mm00439372_m1, PAI-1:SeqPrimeF:Mm00435860_m1, Cdkn2c:Mm00439635_m1, Ledgf/Pot1:Fm01259222_m1, Hoxa10:Mm00439364_m1, Hoxa7:Mm00657963_m1] were purchased from Applied Biosystems (Foster City, CA).

Flow cytometry analysis and sorting

Flow cytometry was performed as previously described [8, 16]. Single cell suspensions harvested from the bone marrow and thymus were stained in deficient RPMI (Irvine Scientific, Santa Ana, CA) containing 3% fetal calf serum, 1 mM EDTA and 10 mM HEPES. Conjugated monoclonal antibodies (mAbs) were obtained from either BD (Franklin Lakes, NJ) or eBioscience (San Diego, CA). The lineage cocktail included antibodies for Gr1 (RB6-8C5), B220 (RA3-6B2), TER119 (TER-119), CD3 (145-2C12), CD4 (GK1.5), and CD8 (53-6-7). The following mAbs were also used: Mac1/CD11b (M1/70), cKir (2B8), Sca1 (D7), CD48 (HM48-1), CD34 (49E8), CD16/32 (93), Flk2 (A2F10), CD45.2 (104), and CD43 (S7). Stained cells were analyzed with LSR-1A or LSR-II flow cytometer (BD). J-SAN (Bay bioscience, Kobe, JAPAN) was used for cell sorting. Cell Quest Pro or Diva (BD) was used for data acquisition, and FlowJo (Tree Star Inc., Ashland, OR) was used for analysis.

In vivo reconstitution assay

Fetal liver cells of de homozygous mutant (5×10^5 cells) or the wild type/heterozygous controls (5×10^5 cells) harvested from E12.5 embryos or white blood cells harvested from adult ac homozygous mice (1×10^5 cells) or the wild type control (1×10^5 cells) in the littermates were injected intravenously into lethally irradiated (1200 rads in two days or 900 rads in one day) C57BL/6 mice. Recipient mice were maintained on water supplemented with antibiotics for a few weeks after transplantation.
digoxigenin antibody coupled with alkaline phosphatase. The plasmid for the Hoxc8 probe was kindly provided by Dr. Licia Selleri.

**MEF proliferation and 3T3 senescence assays**

MEFs were derived from E11.5 embryos and analyzed as described elsewhere [8], [18]. The MEFs were plated at the concentration of $10^4$ cells/ml on Day 0 and the cell count was
Maturation Mechanism of MLL

A

B

C

D

E

F

G

Hoxc8

MLL (wild type)

MLLuc (uc mutant)

MLLN (dC mutant)

D (GAT) to A (GCT)

1st processing site

2nd processing site

(D2686) (D2718)

GCT

GCT

62%

N

D to A

stop

unc

kDa

175

83

82

unc/+ uc/+ uc/uc dC/+ dC/dC

Genotypes at various developmental stages

n

+/+

uc/+ uc/uc

E 10.5

14

5

5

4

E 11.5

20

6

9

5

E 12.5

37

9

19

9

E 13.5

16

5

6

5

E 14.5

20

5

10

5

new born

23

5

10

8

4 weeks old

65

11

38

16

Hoxc8

+/+

dC/dC

uc/uc

Cdkn2a

Cdkn1b

Cdkn2c

PAI-1

Proliferation assay

3T3 senescence assay
Figure 3. MLL processing is not required for MLL-dependent gene activation. A. Schematic structures of MLL proteins produced by dC and uc mutations. The characteristics of each mutant are shown on the right. The mutated processing sites are highlighted in red. Anti-menin blot serves as a loading control. uc/+: uc heterozygous. uc/uc: uc homozygous. dC/+: dC heterozygous. dC/ dC: dC homozygous. D. Genotypes at various developmental stages. Viability of embryos was confirmed by presence of heart contractions. E. Expression of Hoxc8 transcripts in E10.5 embryos. Whole mount in situ hybridization was performed using the Hoxc8 probe (Hoxc8). Arrows indicate sites of target gene expression. Previously published data for the wild type and dC homozygous embryos [8] are shown here for comparison. F. Expression of various genes in mutant MEFs. Three independently established MEF lines of wild type, uc homozygous and dC homozygous genotypes were examined by RT-qPCR for genes indicated at the tops of respective panels. Relative expression levels (normalized to Gapdh) are expressed relative to those of wild type-1 arbitrarily set as 1. Previously published data for wild type and dC homozygous MEFs [8] obtained in the same experiment are included for comparison. Error bars represent the standard deviations of triplicate PCRs. G. Proliferative capacities of uc homozygous MEFs. Proliferation assay (top) and 3T3 senescence assay (bottom) were performed for three lines each of wild type, uc homozygous and dC homozygous genotypes at passage 3. Previously published data for wild type and dC homozygous MEFs [8] obtained in the same experiments are included for comparison. doi:10.1371/journal.pone.0073649.g003

Figure 4. Processing of MLL is not required for adult hematopoiesis. A. Population analysis of Lin^− cKit^+ Sca1^− (MP) and Lin^− cKit^+ Sca1^+ (LKS) compartments and their subcompartments in the BM of adult uc mutant mice. Lineage cocktail (anti-CD3, CD4, CD8, B220, TER119, Gr-1, Mac1/CD11b) was used to define lineage negative fractions. B. Frequencies of hematopoietic progenitors (Flk2^− MPP, Flk2^+ MPP, CMP, GMP and MEP) and HSCs in uc mutant mice. Error bar represents standard deviations of three independent samples. doi:10.1371/journal.pone.0073649.g004
from the femurs of mice. C-kit positive cells were enriched by immuno-magnetic selection using an Auto MACS (Miltenyi Biotech), transduced with recombinant retrovirus by spinoculation, and plated in methylcellulose medium (M3231, Stemcell Technologies) containing SCF, IL-3, IL-6 and GM-CSF. The colony-forming units (CFUs) per 10^5 plated cells were enumerated after 5–7 d of culture and expressed as the average and standard deviation of at least triplicate determinations.

**Results**

**Intra-molecular complex formation of MLL fragments is essential for MLL-dependent gene activation**

The intra-molecular interaction domains of MLL have been defined as PHD fingers 1 and 4 (PHD1 and PHD4), FYRN, and FYRC domains [8], [15] (Figure 1A). Most of PHD1 is encoded by exon 11 of MLL. It has been reported that an MLL variant protein lacking exon 11 sequences is dominantly expressed in some cases of acute lymphoid leukemia [21]. We previously reported that this variant protein can be transiently expressed and efficiently processed but is incapable of forming an MLLN/MLLC holocomplex [8]. To investigate the in vivo roles of intra-molecular complex formation, a mutant allele (designated de) that lacks the exon 11 counterpart of mouse was generated in ES cells (Figure 1B) and its successful recombination was confirmed by diagnostic genomic PCR (Figure 1C) and RT-PCR followed by sequencing (Figure 1D). Another mutant allele (designated dC), which served as a control for null mutation, was previously engineered to contain a stop codon at the second processing site [8], thereby exclusively expressing MLLN due to the inability to translate downstream MLLC sequences within the Mll mRNA (Figure 1A).

Unlike wild type and de heterozygous mice, de homozygous mice died during midgestation (Figure 1E) with a similar phenotype displayed by dC homozygous (dC/dC) mice [8], including subcutaneous edema and hemorrhage (Figure 1F). Mouse embryonic fibroblasts (MEFs) derived from de homozygous embryos expressed MLL proteins at low or undetectable levels whereas their respective mRNAs were expressed at normal levels (Figure 1G), confirming previous findings that MLL protein fragments are subjected to degradation if unable to self-associate in an intra-molecular complex [8], [14]. Consequently, expression of MLL target genes including Hoxc8, Hoxc9, Cdkn1b and Cdkn2c mice was severely reduced in de homozygous MEFs [22], [23] (Figure 1H). These results clearly demonstrate that intra-molecular complex formation is required for stable expression of MLL proteins and thus for MLL-dependent gene activation in vivo.

**Intra-molecular complex formation is required for expansion of hematopoietic progenitors**

The effect of intra-molecular complex formation on proliferative expansion of hematopoietic progenitors was examined by flow cytometry analysis of the fetal livers (FLs) derived from de homozygous E12.5 embryos, which contained fewer hematopoietic cells compared to the wild type/heterozygous control FLs (Figure 2A). The LKS compartment, which contains HSCs and multi-potent progenitors (MPPs), was markedly reduced in de homozygous FLs (Figure 2B, 2C). Further analysis using the CD48 marker [24] showed that de homozygous FLs produced HSCs 50% less efficiently than the wild type/heterozygous controls (Figure 2D, 2E). MPP frequency was more profoundly affected by loss of intra-molecular complex formation, phenocopying dC homozygous FLs [8]. Transplantation of FL cells into lethally irradiated mice showed that de homozygous FL cells were unable to reconstitute
the hematopoietic system, while a 10-fold lower cell number of the
wild type/heterozygous control FL cells successfully reconstituted
(Figure 2F). These results show that intra-molecular complex
formation is essential for the functions of MLL to generate
appropriate numbers of fetal HSCs and MPPs in vivo.

Proteolytic processing of MLL is not required for MLL-
dependent gene activation
To investigate the in vivo roles of proteolytic processing of
MLL, we generated a knock-in mouse line (designated uc)
with targeted germline mutations of the MLL processing sites. ES cells
were engineered to contain an alanine substitution mutation at the
critical aspartic acid residue in both processing sites (the murine
counterparts of human D2666 and D2718) [8], [10] thereby
expressing an uncleavable mutant of MLL (MLLuc) (Figure 3A).
Genomic PCR followed by sequencing confirmed that recombined
ES cells harbored the targeted allele (Figure 3B). Western blotting
analysis confirmed expression of MLLuc in uc mutant embryos
(Figure 3C).

uc homozygous mice were born at normal Mendelian
ratios (Figure 3D) with no apparent anatomic/functional defects.
Hoxc8, an MLL target gene [2], was properly expressed in uc homozygous
embryos when dC homozygous embryos failed to maintain its expression [8] (Figure 3E). Thus, an inability
to proteolytically process MLL does not compromise the
developmental roles of MLL.

To further investigate the possible effect of MLL processing on
MLL-dependent transcription, MEF cell lines of wild type, uc
homozygous and dC homozygous genotypes were analyzed by RT-
qPCR. In contrast to the comparable expression of Mll and Hoxc4
mRNAs among all the cell lines, expression of MLL target genes
including Hoxc8, Hoxc9, Cdkn2a and Cdkn1b [8], [22], [23] was
substantially reduced in dC homozygous MEFs but unaffected in uc
homozygous MEFs (Figure 3F). Although Taspase 1 knock-out
MEFs, which exclusively express the unprocessed form of MLL,
were reported to over-express Cdkn2a, uc homozygous MEFs
expressed Cdkn2a at comparable levels to the wild type control,
indicating that processing of MLL is not required for suppression
of Cdkn2a expression. Furthermore, in contrast to a report by
Takeda et al. [25], no severe growth retardation of uc homozygous
MEFs was observed in proliferation assays and in 3T3 senescence
assays, in the condition where dC homozygous MEFs displayed a
premature senescence phenotype [8] (Figure 3G). Consistent with
these results, PAI-1 (also known as Serpine-1), a well-known
senescence inducer [26], was highly expressed in dC homozygous
MEFs, but expressed at normal levels in uc homozygous MEFs.
Hence, processing of MLL is not required for MLL-dependent
transcription in contrast to the severe compromise of MLL
functions caused by the inability to self-associate.

Lack of MLL processing does not affect steady state
hematopoiesis
To investigate the role of MLL processing in hematopoiesis, we
analyzed the hematopoietic compartments of adult uc mutant mice
by flow cytometry (Figure 4A, 4B). HSCs, MPPs, and lineage-
restricted progenitors including common myeloid progenitors (CMPs), granulocyte/monocyte progenitors (GMPs) and mega-
karyocyte/erythroid progenitors (MEPs) in bone marrow (BM)
exhibited normal frequencies. Furthermore, T-cells in thymus, and
B- cells in BM also exhibited normal compositions (data not

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**Figure 6. MLL processing is not required for proliferation of oncogene-transformed myeloid progenitors.** A. Experimental scheme of
myeloid progenitor serial replating assay. Hoxa9 was transduced in Figure 6B and MLL-AF9 in Figure 6C and 6D. The time points at which colony
forming units and Hoxa9 expression were measured are shown. B. Clonogenic potentials of Hoxa9-transformed myeloid progenitors of wild type or uc
homozygous mutant origin. CFUs per 10^4 plated cells were enumerated at third and fourth round. Error bars represent standard deviations of three
independent samples. C. Clonogenic potentials of MLL-AF9-transformed myeloid progenitors of wild type or uc homozygous mutant origin. CFUs per
10^4 plated cells were enumerated at third and fourth round. Error bars represent standard deviations of three independent samples. D. Hoxa9
expression in the first round colonies of MLL-AF9-transformed cells. Relative expression levels (normalized to Gapdh) and expressed relative to those
of MLL-AF9-transformed wild type progenitors arbitrarily set as 100%. Error bars represent standard deviations of triplicate PCRs.
doi:10.1371/journal.pone.0073649.g006
shown). Thus, MLL processing is dispensable for steady-state hematopoiesis in adult mice.

The uncleavable mutant of MLL is not constitutively active

MLL maintains HOX gene expression to promote expansion of hematopoietic progenitors during myeloid differentiation [3], [4]. Because Hox gene expression progressively declines as cells differentiate [12], [27] (Figure 5A, upper panel), the transcriptional activity of MLL is presumed to decline in parallel. Transcripts for essential components of the MLL complex (MLL itself, menin and LEDGF) are also down-regulated as cells differentiate (Figure 5A, lower panel). However, their declining expression levels were not quantitatively in accord with those of Hox genes. Noticeably, their expression levels in differentiated cells (kit<sup>lo</sup>Mac<sup>hi</sup>) were similar to the levels present in MLL-ENL-transformed cells, whose growth is critically dependent on menin and LEDGF [28], [29], [30]. These data suggest that the decline of Hox gene expression during differentiation is not entirely due to the decrease of MLL complex components at the mRNA level; rather, a post-transcriptional regulatory mechanism might mediate down-regulation of MLL-dependent transcription. We hypothesized that dissociation of the MLL<sup>C</sup> subunit may occur during differentiation to extinguish MLL function, whereas uncleavable MLL might function as the constitutively active form. In this scenario, myeloid progenitors derived from uc homozygous mice should exhibit enhanced clonogenicity in vitro similar to that induced by MLL-ENL, which behaves as the constitutively active form that aberrantly maintains Hoxa9 expression and promotes proliferation (Figure 5B, 5C). However, uc homozygous myeloid progenitors did not display enhanced replating activity compared to the wild type control (Figure 5B, 5D). Thus, lack of processing does not render MLL constitutively active in hematopoietic progenitors.

MLL processing is not required for oncogene-dependent proliferation of myeloid progenitors

To examine whether MLL processing is required for the enhanced proliferation of hematopoietic progenitors induced by oncogenes, we transduced myeloid progenitors derived from uc homozygous mice with Hoxa9 expression vector and analyzed their serial replating activity (Figure 6A). Despite the lack of processing, Hoxa9-transformed uc homozygous myeloid progenitors exhibited clonogenicity comparable to the wild type control (Figure 6B). These results demonstrate that MLL processing is not required for proliferation of oncogene-transformed myeloid progenitors.
It has been reported that the wild type MLL protein is required for leukemic transformation by the MLL-AF9 oncogene [20]. This notion presumes that the function of MLL must be preserved for MLL-AF9 to activate Hoxa9 expression and transform myeloid progenitors. MLL-AF9 successfully transformed uc homozygous myeloid progenitors (Figure 6C) and activated Hoxa9 expression (Figure 6D) in a serial replating assay (Figure 6A). Hence, the uncleavable form of MLL is equivalent functional to the processed form of MLL in MLL-AF9-transformed myeloid progenitors.

**MLL processing is not required for self-renewal of HSCs in vivo**

Previous studies have shown that MLL serves an important role in self-renewal of HSCs and therefore is necessary to reconstitute the hematopoietic system in vivo [3], [4], [7], [8] (Figure 2F). To examine the role of MLL processing in self-renewal, we performed serial in vivo reconstitution assays using wild type and uc homozygous donor cells derived from littermate mice (Figure 7A). uc homozygous BM cells successfully reconstituted the hematopoietic system as well as the wild type control (Figure 7B, 7C). Serial reconstitution assays showed that uc homozygous HSCs and the wild type control consecutively reconstituted three times on average before the eventual depletion of HSCs (Figure 7D), demonstrating that uc homozygous HSCs retain equivalent reconstituting potentials to the wild type control. These results indicate that MLL processing is not required for self-renewal of HSCs in vivo.

**Discussion**

In this study, we generated mouse mutant alleles that effectively inhibit intra-molecular complex formation and MLL processing, respectively, to address the in vivo roles of these two maturation steps. Loss of intra-molecular interaction had devastating effects and manifested a null phenotype, demonstrating that intra-molecular interaction of MLLN and MLLC is essential for MLL functions. These results indicate that loss of intra-molecular interaction exposes the FYRN domain, which triggers degradation and leads to loss-of-function of MLL as we previously reported [8]. On the other hand, in spite of the evolutionary conservation of the processing sites, loss of processing caused no measurable defects, indicating that processing is mostly dispensable for MLL-dependent functions. Furthermore, uc homozygous cells have normal proliferation capacity, demonstrated by proliferation assay of MEFs, serial replating assay of oncogene-transformed hematopoietic progenitors, and serial in vivo reconstitution assay. Therefore, our results do not support the previously suggested essential role of MLL processing in cell cycle progression. Taken together, MLL processing is not required for its function unlike many other bioactive peptides that become activated by proteolytic cleavage, whereas intra-molecular complex formation is an essential step in MLL maturation. Because cleavage occurs to the MLL mutant proteins that are incapable of intra-molecular interaction, the intra-molecular interaction itself is not a prerequisite for Taspase 1-dependent cleavage [8]. However, if cleavage occurs before the intra-molecular interaction, MLL fragments would dissociate from each other and be subjected to degradation. Hence, we propose a model in which intra-molecular interaction takes place first, followed by Taspase 1-dependent cleavage in the proper maturation process of MLL (Figure 7E).

Previous analysis of fetal and adult hematopoiesis showed that MLL is required for sufficient proliferative expansion of hematopoietic progenitors [3], [4], [7], [8]. During myeloid differentiation, MLL maintains expression of posterior Hoxa genes [3], [4], which are highly expressed in HSCs/MPPs and progressively down-regulated in more differentiated progenitors [12], [27] (Figure 4A). Posterior Hoxa genes are required to sufficiently promote proliferation of undifferentiated hematopoietic progenitors [5], [6], [12]. Hence, MLL-dependent Hox genes expression is necessary to maintain appropriate pool sizes of HSCs and immature progenitors. Our in vivo analyses of uc homozygous and de homozygous mutant mice show that intra-molecular complex formation but not MLL processing is required for proper expansion of hematopoietic progenitors. MLL fusion proteins generated by chromosomal translocations constitutively activate posterior HoxA genes to cause leukemia and therefore those mutations are defined as gain-of-function. Our analysis of de homozygous MEFs showed that loss of intra-molecular complex formation results in degradation of MLL proteins and impaired expression of MLL target genes. Thus, although deletion of exon 11 was originally found in ALL [21], this mutation is defined as loss-of-function, suggesting that this mutation likely contributes to leukemogenesis through different mechanisms from MLL fusion-dependent leukemogenesis.

MLL fragments generated by proteolytic processing associate with each other by non-covalent interaction, which potentially allows conditional dissociation of the MLL holocomplex. In accord with this hypothesis, genome wide ChIP analysis of Drosophila embryos showed that trithorax (TRX) protein fragments generated by processing differentially associate with the Drosophila genome [31]. Therefore, it was thought that MLLC might dissociate from MLLN in a context-dependent manner to become functionally inactive. In this scenario, the uncleavable mutant protein might function as the constitutively active form. However, unlike MLL fusion proteins, the uncleavable mutant did not enhance serial replating activity of hematopoietic progenitors (Figure 5D), indicating that conditional dissociation of MLLC is not the major mechanism for extinguishing MLL activity during myeloid differentiation.

It has been proposed that MLL processing might be important for maintaining expression of the Antenapedia complex (ANT-C) genes. This concept was originated by the study of a Drosophila TRX mutant (trx<sup>E3</sup>) that contains an internal deletion of amino acids encompassing the processing site and therefore exclusively expresses an uncleavable mutant protein [32], [33]. trx<sup>E3</sup> exhibits mildly reduced ANT-C expression in late stages and normal bithorax complex (BX-C) expression, whereas the TRX null mutant (trx<sup>111</sup>) displays mildly decreased expression of ANT-C genes in late embryonic development and severely decreased expression of BX-C genes in early embryonic development. Thus, it was hypothesized that TRX processing might be required specifically for late ANT-C expression in fly embryonic development. However, our current results show that uc homozygous mice have no developmental defects. Furthermore, expression of Hoxa4, which is a member of the ANT-C genes, in uc homozygous MEFs was comparable to the wild type control (Figure 3F). Expression of Hoxc8, which is a member of the BX-C genes, was severely decreased in de homozygous MEFs, but unaffected in uc homozygous MEFs. Thus, our results indicate that the role of MLL processing on Hox gene expression is minor, if any, in mammalian development.

Taspase 1 knock out mice demonstrate various defects including smaller body size, reduced MEF proliferation, and skeletal structural anomalies [25]. More specifically, Taspase 1 deficiency causes upregulation of CDK inhibitors such as p16 and ARF (the products of the Cldn2 gene) to inhibit proliferation of MEFs. However, uc homozygous mice were born with normal body size,
and uc homozygous MEFs did not display severely altered expression of Cdk5a2 (Figure 3E) nor manifested severe proliferation defects (Figure 3G) in contrast to a previous study that also analyzed mice engineered to express an unclavable MLL protein [25]. The basis for these differences between the two independently generated mouse lines is unclear since detailed description of the mice generated by Takeda et al. was not reported [25], but may be due to differences in genetic backgrounds of the ES cells or the targeting vector. Nevertheless, our results indicate that MLL processing does not serve rate-limiting roles for proliferation and suggest that the growth defects caused by Taspase 1-deficiency may be attributed to other substrates such as MLL2 [25] or TFIIA [34].

Taken together, our results indicate that MLL-processing has no biological roles in development, hematopoiesis and proliferation, whereas intra-molecular interaction of MLL is essential in all of those circumstances. However, evolutionary conservation of the processing sites suggests that MLL processing has some biological roles. It may be important under other circumstances not tested in those circumstances. However, evolutionary conservation of the processing sites suggests that MLL processing has some biological roles. It may be important under other circumstances not tested in this study such as stress conditions and immunity.

Conclusions

In the current study, we examined the in vivo roles of intra-molecular interaction and proteolytic processing of MLL in various assays. Loss of intra-molecular interaction caused loss-of-MLL function, whereas loss of processing caused no detectable functional alterations. These results unequivocally demonstrate that formation of an intra-molecular complex is, but the processing is not, required for MLL-dependent gene activation and cell proliferation.

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Author Contributions

Conceived and designed the experiments: AY. Performed the experiments: AY FF MJM CM. Analyzed the data: AY FF MJM CM. Contributed reagents/materials/analysis tools: IK MLC. Wrote the paper: AY MLC.

References