Proteolytically cleaved MLL subunits are susceptible to distinct degradation pathways

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Summary

The mixed lineage leukemia (MLL) proto-oncogenic protein is a histone-lysine N-methyltransferase that is produced by proteolytic cleavage and self-association of the respective functionally distinct subunits (MLLN and MLLC) to form a holocomplex involved in epigenetic transcriptional regulation. On the basis of studies in Drosophila it has been suggested that the separated subunits might also have distinct functions. In this study, we used a genetically engineered mouse line that lacked MLLC to show that the MLLN–MLLC holocomplex is responsible for MLL functions in various developmental processes. The stability of MLLN is dependent on its intramolecular interaction with MLLC, which is mediated through the first and fourth plant homeodomain (PHD) fingers (PHD1 and PHD4) and the phenylalanine/tyrosine-rich (FYRN) domain of MLLN. Free MLLN is destroyed by a mechanism that targets the FYRN domain, whereas free MLLC is exported to the cytoplasm and degraded by the proteasome. PHD1 is encoded by an alternatively spliced exon that is occasionally deleted in T-cell leukemia, and its absence produces an MLL mutant protein that is deficient for holocomplex formation. Therefore, this should be a loss-of-function mutant allele, suggesting that the known tumor suppression role of MLL may also apply to the T-cell lineage. Our data demonstrate that the dissociated MLL subunits are subjected to distinct degradation pathways and thus not likely to have separate functions unless the degradation mechanisms are inhibited.

Key words: MLL, Degradation, Proteolysis

Introduction

The mixed lineage leukemia (MLL) protein is an epigenetic transcriptional regulator that is crucial in many developmental and homeostatic processes. It maintains proper Hox gene expression during embryogenesis and hematopoiesis (Jude et al., 2007; McMahon et al., 2007; Yu et al., 1998; Yu et al., 1995) and regulates expression of cyclin-dependent kinase inhibitors (CDKIs) in fibroblasts (Milne et al., 2005). Misregulation of MLL-dependent transcriptional pathways is associated with various pathologies. Gain-of-function mutations of MLL in the hematopoietic lineage result in constitutive expression of Hox genes leading to acute leukemia (Ayton and Cleary, 2001; Hess, 2004; Krivtsov and Armstrong, 2007), whereas loss of the MLL- and MLL2-complexes through mutations of menin, an essential MLL-associated cofactor (Hughes et al., 2004; Yokoyama et al., 2004), leads to decreased expression of CDKIs in the endocrine tissues, hyper proliferation of endocrine cells, and development of multiple endocrine neoplasias (Bertolino et al., 2003; Crabtree et al., 2001; Karnik et al., 2005; Milne et al., 2005). Thus, MLL regulates growth-regulatory transcriptional circuits that are subject to perturbations in various malignancies.

MLL is translated as a large precursor protein that subsequently undergoes proteolytic processing into two fragments (MLLN and MLLC) that self-associate through non-covalent interaction to form an intramolecular complex (Hsieh et al., 2003b; Yokoyama et al., 2002). MLL is processed by the Taspase 1 endopeptidase, which specifically cleaves at sites that are evolutionarily conserved with MLL2 and Drosophila TRX (Hsieh et al., 2003a; Hsieh et al., 2003b; Yokoyama et al., 2002); however, the biological significance of processing remains unclear. MLLN appears to comprise a targeting subunit that contains several motifs involved in DNA binding (AT hooks, CXCC domain) (Ayton et al., 2004; Birke et al., 2002; Zeleznik-Le et al., 1994) and chromatin recognition [plant homeodomain (PHD) fingers, bromo domain]. In particular, the third PHD finger (PHD3) was shown to associate with di- or tri-methylated histone H3 lysine 4, which might be regulated by Cyp33 binding (Fair et al., 2001; Chang et al., 2010; Milne et al., 2010; Wang et al., 2010b). PHD3 is not present in the leukemic MLL fusion proteins and diminishes oncogenic ability if artificially included in an MLL fusion protein (Muntean et al., 2008; Chen et al., 2008). MLLN associates with menin and LEDGF, which are also crucial for linking MLL proteins with target chromatin (Yokoyama and Cleary, 2008). Lastly, binding to MYB and the PAF1 complex is also implicated in the target recognition (Jin et al., 2010; Milne et al., 2010; Muntean et al., 2010). By contrast, MLLC has features of a transcriptional effector subunit that possesses a potent transactivation domain (Yokoyama et al., 2002; Zeleznik-Le et al., 1994) and a methyltransferase (SET) domain specific for lysine 4 of histone H3, an epigenetic mark associated with transcriptionally active states (Milne et al., 2002; Nakamura et al., 2002). The SET domain also associates with accessory factors (WDR5, RBBP5 and ASH2L) that promote optimal substrate recognition and enzymatic activity (Dou et al., 2006; Steward et al., 2006; Southall et al., 2009; Yokoyama et al., 2004). Intramolecular interaction is mediated in part by the FYRN (also called ATA1) and FYRC (also called ATA2) domains (Caldas et
al., 1998; Hsieh et al., 2003b; Yokoyama et al., 2002), which directly associate with each other (Garcia-Alai et al., 2010; Hsieh et al., 2003b; Pless et al., 2011). Thus, the MLL complex is thought to consist of an MLLC effector subunit tethered to the MLLN targeting subunit by non-covalent association. This model has prompted the hypothesis that conditional association or disassociation of the MLLC subunit might serve important roles in MLL-dependent transcriptional regulation. Supporting this hypothesis, a genome-wide association analysis in Drosophila showed that TRXN and TRXC could differently localize at some loci (Schuettengruber et al., 2009).

In this study, we analyzed in vivo roles of the MLLN–MLLC holocomplex (hereafter referred to as MLL holocomplex) by engineering a knock-in mouse line with a stop codon introduced at the second processing site, thereby exclusively expressing MLLN (Fig. 1A,B). Diagnostic genomic PCR and sequencing of the PCR product confirmed that recombined embryonic stem (ES) cells harbored the targeted allele (Fig. 1C,D). Western blotting analysis confirmed the lack of MLLC expression in dC homozygous (MLLN/dC); hereafter referred to as dC/dC embryos (Fig. 1E). However, expression of the MLLN fragment was severely reduced, indicating that MLLN is unstable without MLLC, as previously suggested (Hsieh et al., 2003b). dC/dC embryos died during gestation at embryonic day 2209.

**Results**

**MLLC is required for MLL-dependent transcription and the stability of MLLN during embryogenesis**

To investigate the role of the MLLC subunit in vivo, we generated a knock-in mouse line with a stop codon introduced at the second processing site, thereby exclusively expressing MLLN (Fig. 1A,B). Diagnostic genomic PCR and sequencing of the PCR product confirmed that recombined embryonic stem (ES) cells harbored the targeted allele (Fig. 1C,D). Western blotting analysis confirmed the lack of MLLC expression in dC homozygous (MLLN/dC); hereafter referred to as dC/dC embryos (Fig. 1E). However, expression of the MLLN fragment was severely reduced, indicating that MLLN is unstable without MLLC, as previously suggested (Hsieh et al., 2003b). dC/dC embryos died during gestation at embryonic day 2209.

**Fig. 1. MLLC is required for MLL-dependent transcription during embryogenesis.** (A) Schematic representations of dC-mutant MLL proteins. (B) The structure of the targeting vector. The positions of diagnostic primers are shown at the bottom. (D) Successful recombination of positive ES clones was confirmed by PCR followed by digestion with XhoI. The XhoI site downstream of exon 28 was destroyed in the recombined allele as shown in B. (D) Sequences of PCR fragments amplified from genomic DNAs of the recombined ES clones. (E) Expression of MLL proteins in embryos at E11.5. Whole-embryo extracts were immunoblotted with anti-MLLN (mmN4.4), anti-MLLC (9–12) or anti-menin antibody. The diagnostic PCR data are shown in the bottom panel. (F) Genotypes at various developmental stages. The numbers of embryos or mice with the indicated genotypes are shown for each developmental stage. Viability was confirmed by presence of a beating heart. (G) Abnormal features of dC/dC embryo at E12.5. (H) Expression of Hoxc8 transcripts in E10.5 embryos. Whole-mount in situ hybridization was performed using antisense Hoxc8 probes (Hoxc8 AS). Arrows indicate positions of target gene expression.
13–14 manifesting subcutaneous edema, hemorrhage and hunched posture (Fig. 1F,G), similar to the phenotypes reported in mice with other \textit{Mll}-truncating mutations (Yagi et al., 1998; McMahon et al., 2007) and failed to maintain \textit{Hoxc8} expression at E10.5 (Fig. 1H). Thus, the MLL holocomplex is required for embryogenesis.

**Loss of MLL\textsuperscript{C} causes post-transcriptional degradation of MLL\textsuperscript{N} and p53-dependent premature senescence in fibroblasts**

To further analyze the effects of the loss of MLL\textsuperscript{C} on MLL-dependent transcription, we established wild type (wt) and \textit{dC/dC} mouse embryonic fibroblast (MEF) cell lines. Despite the comparable \textit{Mll} mRNA levels, MLL\textsuperscript{N} protein in \textit{dC/dC} MEFs was not detectable, indicating that MLL\textsuperscript{N} is degraded by a post-transcriptional mechanism (Fig. 2A,B). Expression of MLL target genes including \textit{Hoxc8}, \textit{Hoxc9}, \textit{Cdkn2c} and \textit{Cdkn1b} was severely impaired in \textit{dC/dC} MEFs, whereas \textit{Hoxc4} was unaffected (Fig. 2B). The mRNA sequence downstream of the artificially introduced stop codon was equally abundant as that of the upstream counterpart, and the ratios of the N-terminal and C-terminal portions of the \textit{Mll} mRNA were comparable between \textit{dC/dC} and the wild-type control MEFs (Fig. 2C). \textit{dC/dC} MEFs displayed a premature senescence phenotype both in a proliferation assay and in a 3T3 senescence assay (Fig. 2D,E) consistent with a previous human fibroblast study (Caslini et al., 2009). Moreover, \textit{PAI-1} (\textit{Serpine 1}), a well-known senescence inducer (Kortlever et al., 2006), was expressed at high levels in \textit{dC/dC} MEFs, whereas \textit{Pml}, another senescence inducer, was

![Fig. 2. The MLL holocomplex is required for MLL-dependent transcription in MEFs.](image-url)

(A) Protein expression of the MLL subunits in MEFs. Cell lysates prepared from wt or \textit{dC/dC} MEFs were immunoblotted with anti-MLLN (mmN4.4), anti-MLLC(9–12) or anti-actin antibody. (B) Expression of various genes in \textit{dC/dC} MEFs. Three independently established lines for the indicated genotypes were examined by quantitative RT-PCR for the genes indicated at the top of each panel. RNA was prepared from the third passage MEFs before they went into senescence. Relative expression levels (normalized to \textit{Gapdh}) of various transcripts are depicted relative to those of clone #1, which were arbitrarily set as 1. Error bars represent the standard deviations of triplicate PCRs. (C) Expression of \textit{Mll} was analyzed using a qPCR probe for a coding sequence downstream of the processing site as in B. The ratios of the \textit{Mll} mRNA signal detected by the probe upstream of the processing site (N) toward that by the probe downstream of the processing site (C) are shown in the right panel. (D) Proliferation and 3T3 senescence assays were performed on wt and \textit{dC/dC} MEFs. MEFs were analyzed after the third passage. A representative result, in which three clones each for the two genotypes were analyzed in a single experiment, is shown. Different clones were also analyzed and reproducibly showed a similar result. (E) Senescence-associated \beta-galactosidase assay of wt or \textit{dC/dC} MEFs. (F) Proliferation and 3T3 senescence assays were performed for three lines each of \textit{dC/dC} MEFs with or without homozygous p53 knockout alleles. MEFs were analyzed after the third passage.
unaffected (Fig. 2B). MEFs harboring homozygous dC and p53 null-mutations proliferated without going into senescence (Fig. 2F). Thus, the senescence triggered by loss of MLLC is p53 dependent. These results demonstrate that loss of MLLC leads to destruction of MLLN in vivo and abolishes MLL-dependent transcription to cause p53-dependent premature senescence in MEFs.

**MLLC is required for maintenance of hematopoietic stem cells and progenitors in fetal hematopoiesis**

The role of the MLL holocomplex in hematopoietic development was analyzed in E12.5 embryos because previous studies have shown that MLL affects fetal hematopoiesis (Ernst et al., 2004; McMahon et al., 2007; Yagi et al., 1998) (Fig. 3A). The livers of dC/dC embryos were hypocellular compared with wt and heterozygous counterparts (Fig. 3B). The relative frequency of LKS (Lin+, Kit+, Sca1+) cells, which include hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs), and common myeloid progenitors (CMPs) was severely reduced in dC/dC fetal livers but was partially restored at the granulocyte–monocyte progenitor (GMP) or megakaryocyte–erythroid progenitor (MEP) stages (Fig. 3C,D), indicating that MLL is particularly required for the maintenance of HSCs, MPPs and CMPs at E12.5. Interestingly, MPPs (CD48+ LKS), rather than HSCs (CD48 – LKS), were the most affected cells. In particular, the relative frequency of CD48+ Flk2+ LKS cells was severely decreased in dC/dC fetal livers compared with control livers (nearly to 1/100 of the control), whereas HSCs were reduced by ~70%. These results suggest that the MLL holocomplex is required for the expansion of not only HSCs but also MPPs.

Despite the severe defects in the early hematopoietic cell compartments, dC/dC fetal livers contained highly differentiated hematopoietic cells including Mac-1+ populations (Fig. 3C,D), which express the Mac-1 macrophage marker at high levels, and enucleated red blood cells (Fig. 3E). Moreover, dC/dC fetal liver cells differentiated into functional macrophages when cultured in the presence of granulocyte–macrophage colony stimulating factor (GM-CSF) in vitro (Fig. 3E), indicating that the potential for hematopoietic differentiation was preserved, which is consistent with previous studies of Mll-deficient mice (Yagi et al., 1998). Thus, the MLL holocomplex is not required for myeloid–erythroid differentiation. Nevertheless, transplantation of dC/dC fetal liver cells into lethally irradiated recipients failed to reconstitute the hematopoietic system, whereas one-tenth the dose of control fetal liver cells was sufficient to successfully reconstitute the system (Fig. 3F), revealing a profound functional deficiency similar to that observed in Mll knockout fetal liver and adult bone marrow (Jude et al., 2007; McMahon et al., 2007). Recipients of dC/dC fetal liver cells died 3–4 weeks after transplant, consistent with a defect in early progenitors, besides HSCs. This phenotype could not be rescued by the p53-null mutation, indicating that the hematopoietic defects caused by Mll mutations are not caused by p53-dependent senescence of hematopoietic progenitors (Fig. 3G). Taken together these results show that MLLC is required for the proper expansion of hematopoietic stem cells and/or progenitors but not for differentiation.

**MLLC associates with MLLN through PHD1, PHD4 and the FYRN domain to protect MLLN from the FYRN-targeted destruction pathway**

Next we investigated the molecular mechanism of MLL holocomplex formation and degradation of MLLN. Previously, the N-terminal intramolecular interaction domain (NIID) was tentatively located in a large region of MLLN containing the PHD fingers, bromo domain, HCF binding motif and FYRN domain (Yokoyama et al., 2002). It has been shown that the FYRN domain directly associates with MLLC in vitro (Hsieh et al., 2003b). To determine the mechanisms of intramolecular interaction, a series of MLLN deletion and substitution mutants (MLL 1/2254 mutants) was examined for their ability to interact with MLLC (Fig. 4A; supplementary material Fig. S1). Immunoprecipitation (IP) analysis revealed that PHD1 and PHD4 are required for intramolecular interaction, in addition to the FYRN domain (Fig. 4B; supplementary material Fig. S1). The three mutants deficient for MLLC binding were capable of binding to HCF-1, arguing against the possibilities of abnormal folding of these mutants (supplementary material Fig. S2A,B). Among the three mutants, the APHD1 and APHD4 mutants were unstable compared with those that associate with MLLC (Fig. 4A). However, the FYRN deletion mutant was as stable as the wt (MLL 1/2254) despite the inability to associate with MLLC, suggesting that the FYRN domain mediates not only MLLC interaction but also degradation of MLLN. Similar results were obtained using full-length MLL internal deletion and substitution mutants (supplementary material Fig. S3A,B) and serial C-terminal deletion mutants of MLLN (supplementary material Fig. S3C,D). Furthermore, expression levels of MLLN and MLLC within a single cell were analyzed by transiently expressing various MLL mutant proteins tagged with GFP and YFP at the N- and C-termini, respectively (all of the mutants lacked the C-terminal intramolecular interaction domain [CIID: 3607–3742aa] encompassing the FYRC domain, so that the processed fragments should dissociate from each other). Flow cytometry analysis showed that the MLLN fragment lacking the FYRN deletion was more stable than the ΔPHD1 or ΔPHD4 mutants (Fig. 4C; supplementary material Fig. S1).

To assess the destabilizing potential of the FYRN domain in the context of MLL oncoproteins, an artificial oncogenic protein, MLL–AF9, was engineered to contain the FYRN domain, and tested for its oncogenic and transcriptional activities in a myeloid progenitor transformation assay (Fig. 4D,E; supplementary material Fig. S1). The FYRN domain markedly destabilized MLL–AF9 and MLL5 (Fig. 4F). Furthermore, MLL–FYRN–AF9 was unable to sustain enhanced serial replating capacity unlike MLL–AF9, or maintain expression of MLL target genes such as Hoxa9 despite an intact AF9 portion and adequate transcription (Fig. 4E). Thus, the FYRN domain is sufficiently potent to destabilize and inactivate MLL oncoproteins. These results indicate that there is an intrinsic FYRN-targeted destruction pathway that destabilizes proteins with an exposed FYRN domain. Hence, intramolecular interaction of MLLN and MLLC is necessary not only for their holocomplex formation but also for the protection of MLLN from its specific destruction mechanism, by masking the FYRN domain.

**Deletion of PHD1 sequences encoded by exon 11 of MLL abolishes MLL holocomplex formation and leads to destabilization of MLLN**

Deletion of exon 11 of MLL [NM_005933 region 4242–4355; formerly described as ‘exon 8’ by Lockner et al. (Lockner et al., 1996)] causes in-frame fusion to produce a variant protein lacking 38 amino acids (Fig. 5A). Alternative splicing that deletes exon 11 occurs at low levels in normal cells and is aberrantly increased in some cases of acute lymphoblastic leukemia (Löchner et al., 1996; Nam et al., 1996). Because exon 11 spans part of PHD1, we tested,
Fig. 3. The MLL holocomplex is required for HSC maintenance and expansion of hematopoietic progenitors, but not for differentiation. (A) The experimental scheme of B–F. (B) Cellularities of dC mutant fetal livers. Error bars represent the standard deviation of cell numbers of 4 or 6 livers. (C) Representative FACS profiles of E12.5 fetal liver cells. Lineage cocktail (anti-CD3, -CD4, -CD8, -B220, -TER119 and -Gr-1) was used to define lineage negative fractions. A marked decrease in the LKS and multipotent progenitor populations was observed in dC/dC livers. HSCs are defined as CD48– within the LKS gate where MPPs are defined as CD48+ in this study (Christensen and Weissman, 2001; Kim et al., 2006). It should, however, be noted that an alternative model has also been proposed (Mansson et al., 2007). (D) Average frequencies of various hematopoietic cell sub-populations per total liver cells. Controls include wt and dC/+ . The number of embryos analyzed is indicated below. (E) The morphology of dC/dC fetal liver cells. Enucleated red blood cells were present in dC/dC fetal livers. Functional macrophages with engulfed materials emerged after 1 week in culture in methylcellulose medium containing GM-CSF, SCF, IL-3 and IL-6. (F) The ability of dC mutant fetal liver cells to reconstitute the hematopoietic system. Fetal liver cells (5×10^5) from dC/dC embryos (n=5) or control embryos (5×10^4; n=12) were injected into lethally irradiated recipients. The survival ratio during the monitoring period (80 days) is shown. (G) Hematopoietic defects of dC mutants are not caused by p53-dependent senescence. FACS plots of the fetal liver cells, with the various genotypes indicated below, are shown using the lineage cocktail, cKit and Sca1 antibodies.
using IP analysis, whether an exon 11 deletion mutant of MLL (designated \(\Delta\)exon11) forms an MLL\(^N\)–MLL\(^C\) holocomplex. Exon 11 deletion completely abolished MLL\(^N\)–MLL\(^C\) intramolecular interaction both when two fragments were separately expressed (Fig. 5B; supplementary material Fig. S1) and in the full-length context (Fig. 5C; supplementary material Fig. S1). Furthermore, the \(\Delta\)exon11 mutant was unstable compared with the FYRN deletion mutant (Fig. 5B–D; supplementary material Fig. S1), analogous to the \(\Delta\)PHD1 and \(\Delta\)PHD4 mutants (Fig. 4C). Thus, the MLL exon 11 deletion mutation associated with leukemia disrupts intramolecular interaction and thereby destabilizes MLL\(^N\).

**Free MLL\(^C\) is exported to the cytosol and degraded by the proteasome**

Next, we investigated the biological properties of the MLL \(\Delta\)exon11 mutant and its processed fragments. Following proteolytic processing, MLL\(^N\) and MLL\(^C\) normally colocalize in the nucleus as components of the MLL holocomplex (Fig. 6A; supplementary material Fig. S1). However, exogenously expressed MLL\(^C\) localized exclusively in the cytosol, whereas exogenous MLL\(^N\) resided predominantly in the nucleus (Fig. 6A). Covalent fusion of MLL with the GAL4 DNA binding domain, which contains potent nuclear localization signals (Silver et al., 1984), only partially
localized MLL\textsuperscript{C} in the nucleus, suggesting that it contains nuclear export sequences that antagonize GAL4-mediated nuclear localization (Fig. 6B; supplementary material Fig. S1). Deletion of the transactivation and SET domains yielded more complete nuclear localization of GAL4–MLLC proteins, implicating these domains in facilitating nuclear export (Fig. 6B). Coexpression of MLLN and MLLC resulted in nuclear colocalization of both subunits, whereas MLLN containing the \(\Delta\)exon11 mutation failed to relocate MLLC into the nucleus (Fig. 6C; supplementary material Fig. S1). Consistent with this notion, MLLC derived from the \(\Delta\)exon11 mutant resided exclusively in the cytosolic fraction (Fig. 6D; supplementary material Fig. S1). These results suggest that MLLC is stable when associated with MLLN in the nucleus, but is subjected to proteasome-dependent degradation in the cytosol when dissociated from MLLN (Fig. 7).

**Discussion**

The MLL holocomplex is responsible for MLL-dependent transcription

Processing of MLL proteins is evolutionarily conserved; however, its consequences for MLL function are not well defined. Theoretically, processing enables production of free MLLN, dissociated from MLLC. To address the in vivo roles of MLL subunits, we created mutant mice that do not make MLLC. MllC\textsuperscript{-}deficient mice failed to maintain target gene expression and died during mid-gestation with an Mll\textsuperscript{-}null phenotype demonstrating that MLLC is required for the crucial transcriptional maintenance during embryogenesis.

MLL-dependent transcriptional maintenance was abolished in the \(dc/dc\) MEFs as expression of various MLL target genes was impaired and premature senescence was triggered. In human fibroblasts, MLL plays important roles in the maintenance of telomere integrity, and, therefore, knockdown of MLL induces the telomere-damage-response and p53-dependent senescence (Caslini et al., 2009). p53 activates expression of PAI-1 in the induction of replicative senescence (Kortlever et al., 2006). Consistent with these notions, loss of MLLC activates the p53–PAI-1 pathway.
because PAl-1 expression is induced in dC/dC MEFs, whereas the senescence phenotype can be rescued by a p53-null allele. Thus, MLLC is required for MLL function in the maintenance of the cellular homeostasis of fibroblasts.

Analysis of fetal hematopoiesis shows that the MLL holocomplex is also required for hematopoietic development. It has been reported that MLL is required for reconstitution of the adult hematopoietic system by maintaining the propagation of myeloid progenitors and quiescence of HSCs, but not for differentiation (Ernst et al., 2004; Jude et al., 2007; McMahon et al., 2007; Yagi et al., 1998). Consistent with previous reports, fetal liver cells deficient for MLLC had similar phenotypes. During hematopoietic development, MLL maintains expression of Hoxa9 (Jude et al., 2007; Yagi et al., 1998), which is highly expressed in HSCs and MPPs and progressively downregulated in more differentiated progenitors (Forsberg et al., 2005; Krivtsov et al., 2006; Somervaille and Cleary, 2006). Hoxa9 expression influences the proliferation status of undifferentiated hematopoietic cells because its forced expression expands the HSC or progenitor pools, including GMPs, whereas its loss produces the opposite effects (Kroon et al., 1998; Lawrence et al., 2005; Schnabel et al., 2000; Thorsteinsdottir et al., 2002; Wang et al., 2010a). MLL appears to maintain appropriate HSC pool sizes by sustaining Hox gene expression, the failure of which results in shortages of downstream
progenitors and exhaustion of HSCs. Our analysis of the MLL-deficient hematopoietic system underscores a previously unappreciated role for MLL in LSK CD48^+Flk2^+ cells, which is consistent with Hoxa9 being most highly expressed in MPPs (Forsberg et al., 2005) and could account for the inability of dC/dC fetal liver cells to reconstitute the hematopoietic system, even short-term, despite seemingly intact differentiation capacities. Hence, MLL^C is crucial to early stages of hematopoietic development.

From these results we conclude that the MLL holocomplex is responsible for MLL-dependent transcription because MLL^C is required in three different biological processes including embryogenesis, maintenance of cellular homeostasis in fibroblasts and hematopoietic development.

**MLL subunits are subjected to distinct degradation mechanisms upon dissociation**

We observed that free Mll^N was undetectable in dC homozygous MEFs despite comparable expression of Mll mRNAs. Furthermore, Mlf^−/− deficient embryos expressed only minimal amounts of Mll^N. These results indicate that free MLL^N is degraded by a post-transcriptional mechanism in vivo. In this study, we discovered that free MLL^C is exported to the cytosol and degraded via a proteasome-dependent pathway. Thus, both of the MLL subunits generated by processing are unstable if not forming an MLL holocomplex. Free MLL^N is destroyed by a unique mechanism that targets the FYRN domain. This degradation pathway is independent of the proteasome degradation pathway and therefore should be different from the previously reported SCF- or APC-proteasome-dependent mechanisms that dynamically regulate MLL protein levels during the cell cycle, which targets a different portion of MLL (amino acids 1–1400) (Liu et al., 2007). It is unclear at this point how free MLL^N fragments are degraded. It might involve autophagic degradation similar to the piecemeal microautophagy of the nucleus observed in yeast (Krick et al., 2009) or ‘nucleophagy’ observed in mammalian cells (Park et al., 2009).

FYRN domains are often found adjacent to FYRC in SET domain-containing proteins and together constitute a DAST domain, which is evolutionarily conserved between humans and plants (Alvarez-Venegas and Avramova, 2001). The FYRN domain directly associates with the FYRC domain through the hydrophobic residues of each domain, thereby its interaction surface is kept unexposed (Garcia-Alai et al., 2010; Pless et al., 2011; Hsieh et al., 2003b). Our data suggest that the FYRN domain harbors a destabilization signal that is normally masked by MLL^C within the MLL holocomplex. Supporting this notion, deletion of FYRN did not render MLL^N unstable, despite an inability to associate with MLL^C. The ability of FYRN to destabilize the MLL-AF9 protein and inactivate its transcriptional and oncogenic properties is consistent with this proposal and probably accounts for the absence of chromosomal translocations downstream of the FYRN domain in human leukemias (Meyer et al., 2009). Hence, FYRN might serve not only as a platform for interaction with FYRC but also as a destabilization signal that activates the targeted destruction process when exposed. The exposed hydrophobic surface of FYRN might trigger aggregation of the free MLL^N fragment, which is then subjected to autophagic degradation (Knaevelsrud and Simonsen, 2010).

Because free MLL^N is degraded if it is dissociated from MLL^C, it is unlikely to possess any biological functions. However, free MLL^N might have functions in circumstances where the degradation pathway is inhibited. In Drosophila, it has been suggested that TRX^N associates with the genome without TRX^C at some loci. Whether MLL^N and MLL^C also differently associate with the human genome is currently unknown. Our data show that there is an intrinsic regulatory mechanism that effectively extinguishes MLL^N upon loss of intramolecular interaction. Therefore MLL^N must be protected from the degradation mechanism in order to function without MLL^C if it has a biological function (Fig. 7).

**PHD1 is necessary for holocomplex formation and implicated in tumor suppression**

Our structure–function analysis revealed that PHD1 and PHD4 are crucial for intramolecular interaction in the context of the full-length protein. PHD fingers serve as protein–protein interaction motifs (Fair et al., 2001). Recently, it has been shown that PHD3 specifically associates with di- and tri-methylated lysine 4 of histone H3 (Milne et al., 2010; Wang et al., 2010b; Chang et al., 2010). It is possible that PHD1 and/or PHD4 bind to specific motifs within MLL to enable or stabilize physical interaction between FYRN and FYRC. Such interactions could be modulated...
through post-translational modifications such as lysine methylation, thereby providing opportunities for a context-dependent regulation of holocomplex formation.

We demonstrate here that exclusion of exon 11 sequences from the MLL mRNA produces a variant protein lacking PHD1 that is unable to associate with MLL C, and thus subjected to degradation. Because the Δexon11 transcript is present in both normal and leukemia cells (Löchner et al., 1996; Nam et al., 1996; Takeuchi et al., 2008), alternative splicing of exon 11 might provide another context-dependent mechanism to conditionally extinguish MLL activity in vivo.

Genomic deletion and enhanced alternative splicing of MLL exon 11 have been reported in a subset of acute lymphoid leukemias (Löchner et al., 1996). Because exon 11 deletion abolishes holocomplex formation and renders MLL nonfunctional, its oncogenic mechanism should differ from MLL-fusion-associated leukemias, in which a gain-of-function mechanism plays the predominant role (Ayton and Cleary, 2001; Hess, 2004; Krivtsov and Armstrong, 2007). This raises the possibility that MLL serves as a tumor suppressor in the lymphoid lineage. In endothelial tissues, inactivating mutations of menin prevent the MLL–menin complex maintaining appropriate expression of Cdkn1b and Cdkn2c as part of a negative growth regulatory and tumor suppressor transcriptional factor (Franklin et al., 1998; Franklin et al., 2000; Karnik et al., 2005; Mlne et al., 2005). Indeed, these CDKIs are expressed at very low levels in dC/dC MEFs. These CDKIs have also been implicated in lymphoid growth control and tumorigenesis. Lymphoid cells from Cdkn2c knockout mice are hyper-proliferative to mitogens (Franklin et al., 1998), and inactivating mutations of Cdkn1b have been reported in T-ALL and other types of leukemia (Le Torriellec et al., 2008; Markaki et al., 2006). The Notch1 pathway, which is activated frequently in T-ALL, effectively reduces CDKN1B levels through upregulation of SKP2 expression (Dohda et al., 2007). Collectively, these data suggest that deletion of exon 11 might contribute to oncogenesis by downregulating CDKIs in lymphoid cells. Thus MLL might function as a tumor suppressor in the lymphoid lineage with a mechanism similar to that in endothelial tissues. However, conditional knockout studies show that MLL deficiency does not cause immediate expansion of lymphoid lineage populations (Jude et al., 2007; McMahon et al., 2008). These results do not support a rate-limiting role of MLL for proliferation of lymphoid cells, and suggest that additional mutations might be required for full transformation. Further investigation is required to determine whether MLL truly serves a tumor suppressor role in lymphoid lineages.

In summary, our studies define a crucial role for holocomplex formation of MLL subunits in various developmental processes, which is necessary to protect MLL N from the FYRN-targeted degradation mechanism. Our data suggest that if MLL N were to function without MLL C, the FYRN domain must be masked or modified to ensure MLL N stability; however, our studies provide no evidence for separate MLL N function in various MLL-dependent processes.

Materials and Methods

Cell culture

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

Subfractionation, immunoprecipitation and western blotting

Preparation of nuclear extracts, immunoprecipitation (IP) and western blotting were performed as described previously (Yokoyama et al., 2004). Primary antibodies included mouse monoclonal anti-MLL N (mmN4.4), anti-MLL C (mmC2.1), anti-Sbf1 (HAF3P.1) and polyclonal rabbit anti-MLL C (rpN1) as described elsewhere (Yokoyama et al., 2002). Anti-HCF-1 (H12) antibody was provided by Winship Harr. The mouse monoclonal anti-MLL C (9–12) antibody was generated using maltose-binding protein (MBP) fusion protein corresponding to residues 3084–3959 of human MLL. Goat anti-menin (C19), mouse anti-GAL4 (RK5C1) and omni-pan (D-8) recognizes the nearby sequence of the Xpress epitope antibodies were purchased from Santa Cruz Biotechnology, Inc. Additional primary antibodies included mouse anti-actin (MAB 1501R; Chemicon) and rat anti-HA (5F10; Roche). Agarose affinity beads coupled to mouse anti-FLAG (M2) monoclonal antibody were purchased from Sigma.

Construction of the expression vectors

Xpress–FLAG–MLL–HA expression vectors (plNXC (X(N)MLL(H)) and its derivatives) are described elsewhere (Yokoyama et al., 2002). Various MLL mutants were generated by polymerase chain reaction (PCR)-mediated mutagenesis and restriction enzyme digestion and/or ligation. The GAL4 DNA binding domain, Xpress epitope, 3xNLS and GFP and YFP portion were transferred from pM (Clontech), pcDNA4 His/Max (Invitrogen), pEF/myc/nuc/GFP (Invitrogen) and pECFP-C1/pEYFP-C1 (BD Biosciences) vectors, respectively. Assembled CDNAs were cloned into the plNXC vector (Clontech). The various MLL-AF9 mutants were generated by modification of the pMSCV MLL-AF9 vector (Sonnewalve and Cleary, 2006).

Generation of knockin mice

A mouse ES BAC DNA pool (Down-to-the-well™) was screened by PCR to isolate a BAC clone containing the Mll locus. Targeting vectors containing neo and DT cassette (kindly provided by Takeshi Yagi) were constructed by PCR-mediated mutagenesis, restriction enzyme digestion and ligation. ES cells (CGR8.8) were transfected with the linearized targeting vectors and screened for positive clones by PCR. Homologous recombination was confirmed by using the LA-PCR kit (Takara Bio Inc.) specific for both ends of the targeting construct (primer sequences available upon request). Targeted clones were transiently transfected with a Cre recombinase expression vector (kindly provided by Takeshi Yagi) and subsequently screened for clones with appropriate excision of the neo cassette. Diagnostic PCR for genotyping was performed using a primer pair of the forward primer: 5’-CTGGCATCATGTATTTTGAACAGGCACCCC-3’ and the reverse primer: 5’-TACACGTGGTAACCAGTCATCTGCAGCTCA-3’ by LA-PCR followed by digestion with Xhol. Blastocyst injections were performed by the Transgenic Research Facility of Stanford University. Germline transmission of the targeted Mll allele was confirmed by PCR genotype analysis. Knockin mouse lines were maintained by backcrossing onto a C57BL6 genetic background. p53 knockout mice were reported previously (Donoher et al., 1992).

Whole-mount in situ hybridization

In situ hybridization was performed on E10.5 embryos as described elsewhere (Capellini et al., 2006). Plasmids for probes were kindly provided by Lucia Selleri.

Quantitative RT-PCR

Reverse transcription (RT) and quantitative PCR (qPCR) were performed as described previously (Yokoyama et al., 2005). Tagman probes for chop (Mm00476969_m1), Gapdh (Mm99999915_g1), Homo (Mm00433964_m1), Hoxc4 (Mm00442839_m1), Hoxc8 (Mm00433969_m1), Hoxc11 (Mm00433972_m1), Cdkn1b (Mm00439167_g1), Cdkn2c (Mm00432343_m1), Pml (Mm00476969_m1), Serpine1 (Mm00435860_m1), Mlll(N) (Mm1179246_g1) and Mlll(C) (Mm01179235_m1) were purchased from Applied Biosystems. qPCR was performed in triplicate and average expression levels (with standard deviations) normalized to that of Gapdh or β-actin gene were calculated using a standard curve and the relative quantification method as described in ABI User Bulletin #2.

MEF proliferation and 3T3 senescence assays

MEFs were derived from E11.5 embryos and handled as described elsewhere (Sage et al., 2000). In proliferation assay, 5 × 10^4 cells were plated in a 60 mm dish on day 0 and the cells were counted after trypsinization and resuspension in medium at each time point. In 3T3 senescence assays, 5 × 10^5 cells were replated in a 60 mm dish every 3 days.

Flow cytometry

Flow cytometry analysis was performed at the fluorescence-activated cell sorter (FACS) facility of Stanford University as previously described (Ficara et al., 2008). Fetal liver single-cell suspensions were stained in deficient Roswell Park Memorial Institute medium (RPMI; Irvine Scientific) containing 3% fetal calf serum, 1 mM EDTA and 0.01 M HEPES. Conjugated monoclonal antibodies were obtained from either BD Pharmingen (BD) or ElbioScience (San Diego, CA). The lineage cocktail included Gr1 (RB6-8C5), B220 (RA3-6B2), TER119 (TER-119), CD3 (145-2C12), CD4 (GK1.5) and CD8. The following monoclonal antibodies were also used: Mac1/CD11b (M1/70), cKit (2B8), Sca1 (D7), CD48 (HM48-1), CD34 (49E8), CD4 (GK1.5) and CD8. The following monoclonal antibodies were also used: Mac1/CD11b (M1/70), cKit (2B8), Sca1 (D7), CD48 (HM48-1), CD34 (49E8), CD16/32 (93), Fik2 (AF210), CD45.2 (104) and CD43 (57). Stained cells were analyzed with an LSR-1A or LSR-II flow cytometer. Cell Quest Pro or Diva (BD) were used for data acquisition, and FlowJo (Tree Star) was used for analysis.
Cytosin and in vitro differentiation to macrophages

Fetal liver cells were cultured for 1 week in methylcellulose medium (M2321; Stemcell Technologies; Vancouver, BC) containing SCF, IL-3, IL-6 and GM-CSF. Cytosin preparations were stained with May–Grünwald–Giemsa stain for assessment of cellular cytology as described elsewhere (Yokoyama et al., 2005).

In vivo reconstitution assay

Fetal liver cells from homozygous mutant (5 × 10^6 cells) or wt embryos (5 × 10^6 cells) were injected intravenously into lethally irradiated (900 rad) C57BL6 mice. Recipient mice were maintained on water supplemented with neomycin.

Myeloid progenitor transformation assay

The myeloid progenitor transformation assay was described elsewhere (Lavau et al., 1997; Yokoyama and Cleary, 2008). A portion of the cells was lysed at the end of the first round of plating to prepare RNA using an RNAeasy mini kit (Qiagen).

Indirect immunofluorescence

Indirect immunofluorescence was performed using 293T cells transfected with various ML1 expression vectors as described elsewhere (Yokoyama and Cleary, 2008). Transfected cells were fixed and incubated with rabbit anti-ML1 (rpN1) or mouse anti-Xpress (omni probe D-8) antibodies, and then probed with FITC-conjugated goat anti-rabbit IgG or TRED-conjugated anti-mouse IgG (Santa Cruz Biotechnology). Cells were stained with DAPI (Vector Laboratories) and analyzed by immunofluorescence microscopy.

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Figure S1

Graphical representations of various constructs used in the manuscript.

MLL mutant proteins are shown schematically and grouped according to the experiments in which they were employed and the relevant figure numbers in the main text: (A) proteins shown in Fig. 4A and B, (B) Fig. 4C, (C) Fig. 4E and F, (D) Fig. 5B, (E) Fig. 5C, (F) Fig. 5D, (G) Fig. 6A, (H) Fig. 6B, (I) Fig. 6C, (J) Fig. 6D, (K) Fig. 6E, and (L) Fig. 6F. Predicted molecular mass (kDa) of each mutant is shown on the right.
Figure S1 continued

D

E

F

NIID  
pLNCX wt (MLL 1/2254)  
pLNCX MLL 1/2254 Δ exon11 (d1407-1444)  
pLNCX CFP-MLL 34/3606-YFP Δ exon11 (d1407-1444)

Predicted molecular mass
[248kDa]  
[244kDa]  
[158kDa]

HA

[433kDa]

[429kDa]

CFP

[441kDa]

[437kDa]

YFP

pLNCX wt (MLL 1/2254)  
pLNCX MLL 1/2254 Δ FYRN (d1991-2064)  
pLNCX CFP-MLL 34/3606-YFP Δ FYRN (d1991-2064)
Figure S1 continued
Figure S2

**MLL mutants that do not associate with MLL**

A. Schematic representation of the mutants. Predicted molecular mass of each mutant is shown on the right.

B. IP-western blot analysis was performed for various MLL 1/2254 mutants that do not associate with MLL. MLL 1/2254 mutants were transiently expressed in 293T cells. The cell extracts were subjected to immunoprecipitation (IP) with anti-HA (3F10) antibody followed by immunoblotting. The precipitates and input samples indicated on the top were immunoblotted with anti-HA (top panel) or anti-HCF-1C (H12) (bottom panel) antibodies.

C. IP-western blot analysis was performed for MLL1/2254 Δexon11 mutant as in panel B. HCF-1 binding was observed for the Δexon11 mutant.
Figure S1

Graphical representations of various constructs used in the manuscript.

MLL mutant proteins are shown schematically and grouped according to the experiments in which they were employed and the relevant figure numbers in the main text: (A) proteins shown in Fig. 4A and B, (B) Fig. 4C, (C) Fig. 4E and F, (D) Fig. 5B, (E) Fig. 5C, (F) Fig. 5D, (G) Fig. 6A, (H) Fig. 6B, (I) Fig. 6C, (J) Fig. 6D, (K) Fig. 6E, and (L) Fig. 6F. Predicted molecular mass (kDa) of each mutant is shown on the right.
Figure S1 continued
**Figure S2**

**MLL mutants that do not associate with MLLc can interact with HCF-1.**

A. Schematic representation of the mutants. Predicted molecular mass of each mutant is shown on the right.

B. IP-western blot analysis was performed for various MLL 1/2254 mutants that do not associate with MLLc. MLL 1/2254 mutants were transiently expressed in 293T cells. The cell extracts were subjected to immunoprecipitation (IP) with anti-HA (3F10) antibody followed by immunoblotting. The precipitates and input samples indicated on the top were immunoblotted with anti-HA (top panel) or anti-HCF-1C (H12) (bottom panel) antibodies.

C. IP-western blot analysis was performed for MLL1/2254 Δexon11 mutant as in panel B. HCF-1 binding was observed for the Δexon11 mutant.
**Figure S3**

The FYRN domain is required for holocomplex formation and destabilization in various contexts.

A. Schematic representation of the full-length MLL mutants. Binding property with MLL\(^\text{C}\), susceptibility to destabilization, and predicted molecular mass of each mutant are shown on the right.

B. IP-western blot analysis was performed for various MLL full-length mutants tagged with an HA epitope at the C-terminus. MLL mutants were transiently expressed in 293T cells. The cell extracts were subjected to IP with anti-MLL\(^\text{N}\) antibody (mmN4) followed by immunoblotting. The precipitates and input samples indicated on the top were immunoblotted with anti-MLL\(^\text{N}\) (mmN4) (top panel) or anti-HA (3F10) (bottom panel) antibodies.

C. Schematic representation of the various MLL deletion mutants. Binding property with MLL\(^\text{C}\), susceptibility to destabilization, and predicted molecular mass of each mutant are shown on the right.

D. IP-western blot analysis was performed for various MLL deletion mutants as in Figure 4B. MLL deletion mutants were co-expressed with Xpress-tagged GAL4-MLL\(^\text{C}\) [(X) GAL4-MLL\(^\text{C}\)] in 293T cells. The cell extracts were subjected to IP with anti-MLL\(^\text{N}\) (mmN4) antibody, and the precipitates and input samples indicated at the top were immunoblotted with anti-MLL\(^\text{N}\) (mmN4) (top panel) or anti-Xpress (middle bottom two panels) antibodies.

The FYRN domain is required for holocomplex formation and destabilization in various contexts.