組織発現を検討したところ、腸管に特異的に発現し、さらに腸上皮細胞の微絨毛に局在することが明らかとなった。また、pp90の細胞内領域のチロシンリン酸化には、細胞質型チロシンキナーゼのSrcやFynが重要であり、SAP-1の強制発現により脱リン酸化されることが分かった。さらに、pp90の強制発現により、MAPキナーゼの活性化や複数のシグナル分子のチロシンリン酸化が誘導されることが明らかとなった。

(2) マウスB16メラノーマにおいても、SIRPαが高度に発現することを確認した。そこで、B16メラノーマ接種による肺転移モデルにおいて抗SIRPα抗体単独の効果を検討した結果、有意に肺転移を抑制することが分かった。また、ADCC活性を有する抗メラノーマ特異的抗体TA99との併用による腫瘍排除の効果を検討したところ、有意な相乗効果が観察された。現在、この機序につき細胞特異的SIRPα KOマウスなどを用いて検討中である。

D．考察

SAP-1はヒト胃がんや大腸がんに高度に発現するPTPであり、大腸がんモデルにおいてSAP-1ががんの発生を促進的に制御することを見出していたが、その分子基盤は不明であった。すでに、SAP-1の脱チロシンリン酸化基質として膜貫通型糖化分子であるpp90を同定しており、今年度はこのpp90の性状を詳細に解析した。pp90は腸上皮細胞に高度に発現し微絨毛に局在することから、この分子がin vivoにおけるSAP-1の脱チロシンリン酸化基質である可能性がより高まった。また、pp90のチロシンリン酸化の機序や下流の細胞内シグナルが一部明らかとなった。今後は、pp90の生理機能やSAP-1による発がん機序への関与につき検討する必要がある。すでに、SIRPα KO マウスにおいて抗メラノーマ特異的抗体による腫瘍排除が顕著に増強することを見出しており、抗SIRPα抗体を用いてCD47-SIRPα結合を阻害することで、ADCC活性を有する分子標的薬の効果増強の可能性が示唆された。今回、抗SIRPα抗体単独でも、メラノーマの肺転移を抑制したことから、抗SIRPα抗体が２つの異なる機序を介したがん治療薬として利用できる可能性が考えられた。今後、抗SIRPα抗体単独の効果が、この抗体自体が誘導するADCC活性を介したものであるか否か、あるいはがん細胞のmigrationを抑制し転移の過程を阻害しているかなどにつき、in vitro系における検討が必要である。

E．結論

本研究により、受容体型PTPであるSAP-1の作用機構に新規膜型分子であるpp90が関与する可能性が示唆された。また、Shp2の結合分子であるSIRPαの機能を人為的に操作することにより、がんの分子標的薬として利用できる可能性が示された。

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H．知的所有権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

別紙４

研究成果の刊行に関する一覧表

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Deregulated transcription factors in leukemia

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Abstract Specific chromosomal translocations and other mutations associated with acute myeloblastic leukemia (AML) often involve transcription factors and transcriptional coactivators. Such target genes include AML1, C/EBPα, RARα, MOZ, p300/CBP, and MLL, all of which are important in the regulation of hematopoiesis. The resultant fusion or mutant proteins deregulate the transcription of the affected genes and disrupt their essential role in hematopoiesis, causing differentiation block and abnormal proliferation and/or survival. This review focuses on such transcription factors and coactivators, and describes their roles in leukemogenesis and hematopoiesis.

Keywords Leukemia · Transcription factor · Transcriptional coactivator

1 Introduction

In human leukemias, specific chromosomal translocations are common and can result in the expression of specific fusion proteins. Chromosomal rearrangements often involve genes encoding transcriptional coactivators, such as MOZ/MORF, p300/CBP, PML and MLL, as well as transcription factors, such as AML1 and RARα are well known in acute myeloblastic leukemia (AML). Point mutations of transcription factors such as AML1, C/EBPα and GATA1 are also observed in leukemia. Such transcription factors and coactivators play important roles in the development, maintenance, and differentiation of hematopoietic cells. The deregulated transcription of these genes caused by translocations and mutations leads to leukemogenesis. In this review, the relationship between deregulated transcription factor expression and leukemogenesis is discussed in light of recent findings.

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2 AML1

The *AML1/RUNX1* gene is the most frequent target for chromosomal translocation in leukemia [1]. Situated on chromosome 21, it was originally identified at the breakpoint of the t(8;21) chromosomal translocation commonly found in the FAB M2 subtype of AML [2]. The t(8;21) rearrangement fuses the *AML1* gene to the *ETO/MTG8* gene, and results in the AML1-ETO fusion protein. The *AML1* gene has also been identified as the target in the following translocations: t(12;21) (TEL-AML1) [3, 4], t(16;21) (AML1-MTG16) [5], t(3;21) (AML1-EVI1) [6, 7], t(4;21) (AML1-EBP) [8], t(11;21)(q13;q22) (AML1-LRP16, LRP16-AML1) [9], t(2;21) (AML1-LAF4) [10], and t(11;21)(q12;q22) (AML1-LPXN, LPXN-AML1) [11]. While the TEL-AML1 fusion is restricted to childhood pre-B cell lymphoblastic leukemia, AML1-ETO is found exclusively in myeloid leukemia. In its physiological role as a regulator of hematopoiesis, AML1, also known as CBFα2, dimerizes with CBFβ to form a heterodimeric transcription factor [12]. AML1 is essential for the development of hematopoietic stem cells (HSCs), and disruption of the *Aml1* gene in mice causes impaired definitive hematopoiesis and embryonic lethality [13, 14]. AML1 also regulates the maturation of megakaryocytes and differentiation of T and B cells [15]. AML1 interacts with transcriptional coactivators, such as the histone

acetyltransferase p300, with monocytic leukemia zinc finger protein (MOZ), PML I, and homeodomain-interacting protein kinase 2 (HIPK2), and activates their transcription [16–18]. By contrast, the AML1-ETO fusion protein interacts with histone deacetylases (HDACs), with transcriptional corepressors, such as nuclear receptor corepressor (N-CoR) and mSin3A, and with silencing mediator for retinoid and thyroid hormone receptors (SMRT) to inhibit AML1-dependent transcriptional activation [19–23]. Furthermore, it has been reported that AML1-ETO recruits DNA methyltransferase 1 (DNMT1) to silence target genes [24]. These findings indicate that AML1-ETO represses AML1-dependent transcriptional activation by recruiting transcription inhibitors, leading to leukemogenesis (Fig. 1). Induced expression of AML1-ETO can immortalize mouse bone marrow cells, but it is not sufficient to induce leukemia in mice, suggesting that additional mutation(s) are needed. Indeed, activating mutations of C-KIT, FLT3 and NRAS are frequently detected in cases of AML bearing t(8;21) [25–27]. Recently, Wang and coworkers [28] showed that mutated C-KIT cooperates with full-length AML1-ETO to induce AML in mice. It has been reported that a truncated form of AML1-ETO is sufficient to induce erythroleukemia in mice [29]. The t(12;21) chromosomal translocation, which generates the TEL-AML1 fusion gene, is present in childhood acute lymphoblastic leukemia (ALL). Recently, it has been shown that, as is likely for AML-ETO induction, a multistep process is required to induce TEL-AML1 leukemia [30, 31]. By studying a monochorionic twin pair bearing TEL-AML1 and by transplanting TEL-AML1-transduced cord blood cells into NOD/SCID mice, Hong and coworkers [30] have identified preleukemic cells in which the fusion gene first appears. Schindler and coworkers [31] have used TEL-AML1 knock-in mice to show that TEL-AML1 does not directly transform HSCs but makes them susceptible to the accumulation of further genetic hits. These results suggest that, after acquiring the translocation, additional genetic/epigenetic events are necessary for the development of TEL-AML1-associated leukemia.

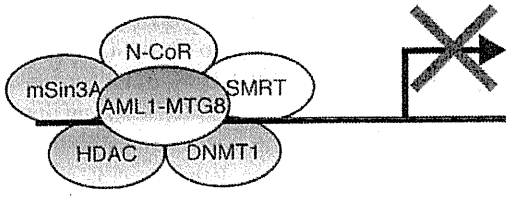

Fig. 1 AML1-MTG8 recruits transcription inhibitory factors. AML1-MTG8 interacts with HDAC, N-CoR, mSin3A, SMRT and DNMT1, and represses AML1-dependent transcriptional activation

3 Retinoic acid receptor α (RARα) and PML

RARα is a ligand-dependent transcription factor stimulated by retinoic acid. It forms a heterodimer with retinoid X receptor (RXR), which binds to specific DNA sequences to regulate transcription of target genes [32]. RARα interacts with transcriptional corepressors such as N-CoR, SMRT and HDAC [33, 34]. However, in the presence of retinoic acid, these transcriptional corepressors are released from RARα and transcriptional coactivators are recruited to stimulate RARα-mediated transcriptional activation. Acute promyelocytic leukemia (APL) is characterized by translocations involving the *RARα* gene on chromosome 17. The following fusions have been identified: t(15;17) (PML-RARα) [35–38], t(11;17) (PLZF-RARα) [39, 40], t(5;17) (NPM-RARα) [41, 42] and t(11;17) (NuMA-RARα) [43], but in more than 90% of APL cases, the *RARα* gene is fused to *PML*. The PML-RARα fusion protein interacts with transcriptional corepressors, as does RARα; however, at a physiological retinoic acid concentrations, these repressors are not released from PML-RARα, and in APL cells, RARα-regulated transcription is inhibited (Fig. 2a). The widely used APL therapeutic agent All-*trans* retinoic acid (ATRA) acts by releasing transcriptional corepressors from RARα, thereby inducing the differentiation of APL cells. The PML-RARα fusion also deregulates PML-dependent transcription, which plays an important role in myeloid differentiation. In *Pml*-knockout mice, granulopoiesis is impaired [44]. PML interacts with AML1 and enhances AML1-dependent differentiation of murine myeloid progenitor cells [45]. PML also interacts with PU.1 and C/EBPε, which are essential for granulopoiesis, and stimulates PU.1- and C/EBPε-induced myeloid differentiation. In contrast, PML-RARα inhibits the PML enhancement of PU.1-mediated differentiation [44] (Fig. 2b). While PML stabilizes transcriptional coactivators, such as p300 and HIPK2, to stimulate transcription by inhibiting SCF^{Fbx3}-mediated degradation of these transcription coactivators, PML-RARα destabilizes them, which inhibits transcription [46] (Fig. 2c). PML also stabilizes the transcriptional coactivators in PML nuclear bodies (NBs); conversely, PML-RARα disrupts the NBs and destabilizes the transcriptional coactivators. Thus, PML-RARα deregulates PML- and RARα-dependent transcription. It has recently been shown that, to repress transcription, PML-RARα binds to target gene promoter regions distinct from those recognized by RARα [47, 48]. Furthermore, the PML-RARα-binding regions often contain PU.1-binding motifs, and PML-RARα can thus repress PU.1-mediated transcription [48] (Fig. 2d). These results demonstrate that, in APL, PML-RARα deregulates transcription, particularly that mediated by PU.1, both by exerting a dominant negative effect and by gain-of-function

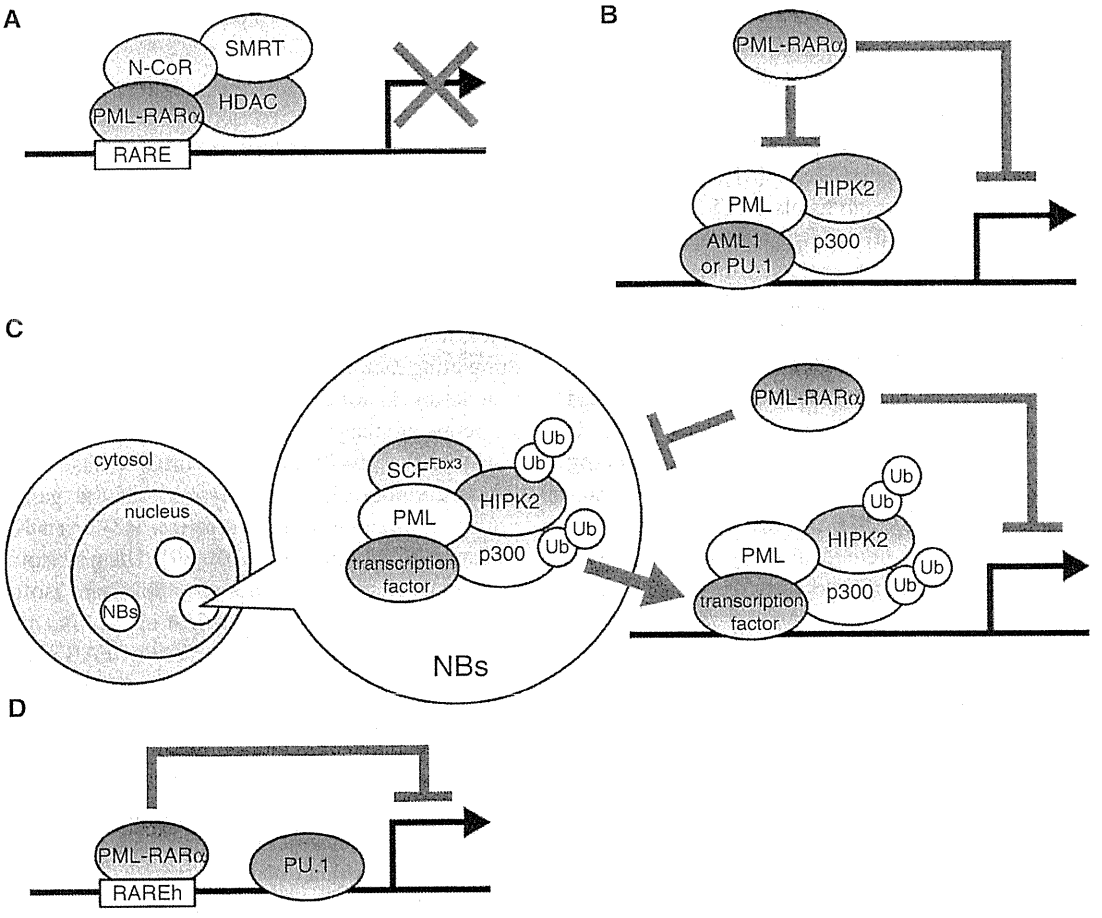

Fig. 2 PML-RARα deregulates RARα-, PML- and PU.1-dependent transcription. **a** PML-RARα interacts with N-CoR, SMRT and HDAC, and represses RARα-regulated transcription. *RARE* retinoic acid responsive element. **b** PML-RARα inhibits PML-regulated transcriptional activation by AML1 and PU.1, and granulopoiesis. **c** PML stabilizes transcription coactivators in NBs and activates transcription. *NBs* PML nuclear bodies. *Ub* ubiquitin. **d** PML-RARα binds at RAREh, at which site RARα cannot bind, and inhibits PU.1-dependent transcription. *RAREh* RARE half

for both PML and RARα. Compared to PML-RARα, the mechanism of leukemogenesis by other RARα fusion partner proteins is less well understood.

4 MOZ/MORF and p300/CBP

MOZ/MORF and p300/CBP are histone acetyltransferases, which function as transcriptional coactivators, and are also targets of chromosomal translocation in AML. The following fusions have been identified: inv(8) (MOZ-TIF2) [49, 50], t(8;16) (MOZ-CBP) [51], t(8;22) (MOZ-p300) [52, 53], and t(11;16) (MLL-CBP) [54–56]. MOZ interacts with transcription factors such as AML1 and PU.1 to stimulate transcription of their target genes [17]. *MOZ*-deficient mice die at E15 and have a pale appearance, a small liver (the major hematopoietic organ at this stage of fetal development) and defective erythrocyte maturation

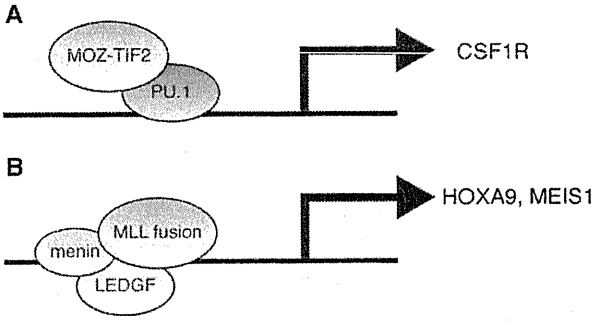

Fig. 3 MOZ fusion and MLL fusion abnormally activate transcription. **a** MOZ-TIF2 abnormally activates PU.1-dependent transcription and induces high expression of CSF1R. **b** MLL fusion abnormally upregulates *HOX* genes and maintains expression of HOXA9 and MEIS1

[57]. MOZ is required for the self-renewal of HSCs since it regulates the expression of c-Kit, c-Mpl and Hoxa9, which are important factors for maintaining stemness [57]. p300/

CBP acetylates histone and non-histone proteins, including p53 [58], GATA-1 [59] and c-Myb [60], and activates transcription factors essential for hematopoiesis, such as AML1, RARα and PU.1. MOZ-TIF2 fusion protein enables the transformation of non-self-renewing myeloid progenitors into leukemia stem cells [61]. These leukemia-initiating cells express high levels of CSF1R [62] and MOZ-TIF2 and MOZ-CBP fusion proteins abnormally activate PU.1-dependent transcription of this receptor (Fig. 3a). MOZ-TIF2 cannot initiate leukemia in mice with a PU.1-deficient background and leukemia cannot be maintained after conditional deletion of PU.1 [62]. These findings indicate that MOZ-TIF2-induced deregulated expression of PU.1-target genes such as CSF1R is essential for initiating and maintaining leukemia-initiating cells. MOZ-CBP also perturbs AML1-dependent transcription and inhibits the differentiation of murine myeloid cells [17]. Thus, the deregulation by fusion histone acetyltransferases of factors important for hematopoiesis is leukemogenic.

5 Mixed-lineage leukemia (MLL)

The *MLL* gene is the major target of chromosomal translocation in ALL and AML, and more than fifty fusion partner genes have been identified [63–65]. The major fusion partners are *ENL, AF4, AF9* and *AF10*. MLL-AF9 is mainly found in myeloid leukemia, and MLL-AF4 is restricted to infant or childhood pro-B cell lymphoid leukemia. Most of the other fusions, such as MLL-ENL and MLL-AF10, are found in both myeloid and lymphoid leukemia. Wild-type MLL is required for the maintenance of Hox gene expression [66]. MLL possesses histone methyltransferase activity specific for lysine 4 of histone H3, which is associated with the transcriptionally active state of chromatin [67, 68]. MLL associates with cofactors including menin, LEDGF, HCFs, ASH2L, WDR5 and RBBP5 to form a large complex [69, 70]. MLL fusion proteins in leukemia, however, lose the C-terminal region of wild-type MLL and cannot bind with the necessary complex subunits, except for menin and LEDGF, which are required for MLL and MLL fusions to interact with their target genes [69]. MLL fusion proteins constantly activate the *HOXA* genes, of which the *HOXA9* gene is thought to be the most important for leukemogenesis. The *HOXA* genes are homeobox genes that encode transcription factors (Fig. 3b). MLL-AF10, which is generated from the t(10;11) translocation, interacts with the H3K79 methyltransferase DOT1L via the AF10 portion and abnormally recruits DOT1L to HOX loci [71]. MLL-AF4 and MLL-ENL form a complex with P-TEFb transcription elongation factor [72]. Recruitment of this complex causes sustained target gene expression, which leads to the transformation of hematopoietic progenitors.

6 CCATT/enhancer binding protein alpha (C/EBPα)

The C/EBPα transcription factor plays a critical role in the proliferation and differentiation of myeloid cells [73]. *C/EBPα*-deficient mice have defective mature granulocytes and white adipose tissue [74, 75]. C/EBPα regulates the expression of myeloid genes, such as granulocyte colony-stimulating factor receptor (*G-CSFR*), macrophage colony-stimulating factor receptor (*M-CSFR/CSF1R*) and granulocyte–macrophage colony-stimulating factor receptor (*GM-CSFR*) [76–79]. C/EBPα mRNA has two different translation start sites, which result in the generation of either a full-length 42-kDa protein (p42) or an N-terminal truncated 30-kDa protein (p30) [80]. The p30 isoform lacks the transactivation domain and inhibits p42 isoform-mediated transcription. The *CEBPA* gene is mutated in approximately 10% of AML patients of FAB subtypes M1, M2 and M4 [81–83]. There are two major types of *CEBPA* mutation, one occurring at the N-terminal and one at the C-terminal. The N-terminal mutation is a frame-shift that eliminates p42 expression [82]. The C-terminal mutation is in-frame and disrupts the BR-LZ DNA-binding domain [81, 84]. Kirstetter and colleagues [85] showed that p42-deficient mice, which express only p30, develop AML with complete penetrance. The C-terminal mutation of C/EBPα increases the proliferation of long-term-HSCs, which leads to the expansion of premalignant HSCs [86]. The combination of N- and C-terminal C/EBPα mutations accelerates leukemia development. These results suggest that *CEBPA* mutation deregulates several differentiation steps in hematopoiesis, leading to the development of leukemia. Deregulated expression of C/EBPα in the absence of *CEBPA* mutations has been reported. In the presence of the t(8;21) translocation, C/EBPα mRNA expression is significantly reduced [87], and when inv(16) is present, C/EBPα expression is suppressed [88, 89]. Thus, in AML, mutations and the deregulated expression of C/EBPα are well documented, and these reports suggest that the loss of C/EBPα p42 isoform is critical for C/EBPα-related leukemogenesis.

7 PU.1

PU.1 is an essential transcription factor in hematopoiesis. In *PU.1*-knockout mice, the differentiation of mature myeloid cells and B cells is impaired [90, 91]. PU.1 regulates the differentiation of progenitor cells, acting downstream of AML1 and upstream of C/EBPα [92]. In mice,

deletion of the distal enhancer of *Sfpi1*, the gene encoding PU.1, causes a reduction of PU.1 expression and leads to the development of AML [93]. As described above, PML-RARα inhibits PML enhancement of PU.1-regulated transcriptional activation. PML-RARα also binds the promoters of PU.1-regulated genes and represses expression. MOZ-TIF2 abnormally activates the PU.1-dependent transcription of CSF1R and does not induce leukemia in mice with a PU.1-deficient background. Furthermore, AML-MTG8 binds to PU.1 and represses PU.1-dependent transcription [94]. Thus, PU.1 is deregulated by several fusion proteins in leukemia. The PU.1 gene is mutated in 7% of patients with the FAB M0, M4 and M5 subtypes [95]. PU.1 mutations so far identified have been deletions and point mutations in the PEST domain, DNA-binding domain, or the transactivation domain. These mutants are impaired in their ability to coactivate target genes together with AML1 or c-Jun. However, another group analyzed sixty de novo AML samples and sixty myelodysplastic syndrome (MDS) samples, but was unable to identify PU.1 mutations, with the exception of intron mutations and a silent mutation [96]. This discrepancy may reflect ethnic differences in the patient groups, and the incidence and role of PU.1 mutations in AML remain controversial. In summary, PU.1 is an important transcription factor for granulopoiesis and several fusion proteins in AML deregulate PU.1, which leads to leukemogenesis. However, depending on the type of fusion protein generated, PU.1 can be hyper-activated (MOZ-TIF2) or hypo-activated (PML-RARα and AML1-MTG8).

8 GATA-1

GATA-1 is a transcription factor that regulates the differentiation of erythroid and megakaryocytic cell lineages, and is highly expressed in erythroid and megakaryocytic progenitors. Disruption of the *Gata-1* locus, situated on the X chromosome, is lethal at the yolk sac stage, as it causes anemia [97]. Suppression of GATA-1 leads to erythropoiesis arrest and the inhibition of the maturation of megakaryocytes [97, 98].

Children with Down syndrome (DS) have a high risk of developing acute megakaryoblastic leukemia (AMKL) [99]. Somatic mutations in *GATA-1* are found in DS-AMKL but rarely in non-DS AMKL. These mutations prevent full-length GATA-1 synthesis, and only a short-form, which lacks the N-terminal region, is generated. The activation domain for transcription lies in the N-terminal region of GATA-1, hence the short-form (GATA1s) has reduced transactivation potential [99]. Phenotypic analyses of DS patients with transient abnormal myelopoiesis who had GATA1 mutations suggested that levels of GATA1s were significantly associated with a risk of progression to DS-AMKL [100].

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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 (MLL^N and MLL^C) 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 MLL^C to show that the MLL^N–MLL^C holocomplex is responsible for MLL functions in various developmental processes. The stability of MLL^N is dependent on its intramolecular interaction with MLL^C, which is mediated through the first and fourth plant homeodomain (PHD) fingers (PHD1 and PHD4) and the phenylalanine/tyrosine-rich (FYRN) domain of MLL^N. Free MLL^N is destroyed by a mechanism that targets the FYRN domain, whereas free MLL^C 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 (MLL^N and MLL^C) 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. MLL^N appears to comprise a targeting subunit that contains several motifs involved in DNA binding (AT hooks, CXXC 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). MLL^N 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, MLL^C has features of a transcriptional effecter 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

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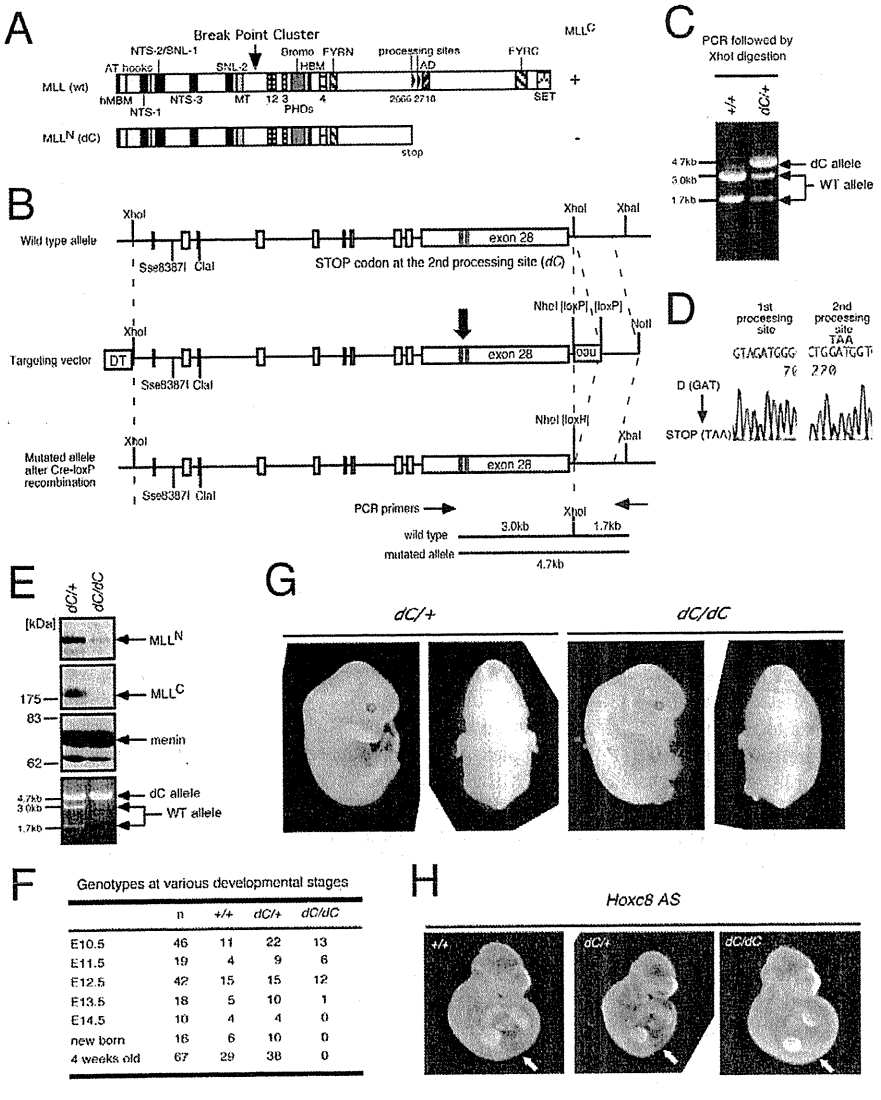
al., 1998; Hsieh et al., 2003b; Yokoyama et al., 2002), which directly associate with each other (Garcia-Alai et al., 2010; Hsieh et al., 2003b; Pless et al., 2011). Thus, the MLL complex is thought to consist of an MLL^C effecter subunit tethered to the MLL^N targeting subunit by non-covalent association. This model has prompted the hypothesis that conditional association or disassociation of the MLL^C subunit might serve important roles in MLL-dependent transcriptional regulation. Supporting this hypothesis, a genome-wide association analysis in *Drosophila* showed that TRX^N and TRX^C could differently localize at some loci (Schuettengruber et al., 2009).

In this study, we analyzed in vivo roles of the MLL^N–MLL^C holocomplex (hereafter referred to as MLL holocomplex) by engineering a knock-in mouse line in which the MLL^C effecter subunit cannot be produced. Our results show that the MLL holocomplex is responsible for MLL-dependent transcription in various developmental processes. We found that dissociated MLL subunits are subjected to distinct degradation pathways and therefore are not expected to have functions unless the degradation pathways are inhibited. A genetic lesion associated with human T-cell leukemias disables holocomplex formation and thus is expected to abolish MLL-dependent transcription, raising the possibility of a tumor suppressor role for MLL in the lymphoid lineage.

Results

MLL^C is required for MLL-dependent transcription and the stability of MLL^N during embryogenesis

To investigate the role of the MLL^C subunit in vivo, we generated a knock-in mouse line with a stop codon introduced at the second processing site, thereby exclusively expressing MLL^N (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 MLL^C expression in *dC* homozygous ($Mll^{dC/dC}$; hereafter referred to as *dC/dC*) embryos (Fig. 1E). However, expression of the MLL^N fragment was severely reduced, indicating that MLL^N is unstable without MLL^C, as previously suggested (Hsieh et al., 2003b). *dC/dC* embryos died during gestation at embryonic day



	n	+/+	dC/+	dC/dC
E10.5	46	11	22	13
E11.5	19	4	9	6
E12.5	42	15	15	12
E13.5	18	5	10	1
E14.5	10	4	4	0
new born	16	6	10	0
4 weeks old	67	29	38	0

Fig. 1. MLL^C 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. (**C**) Successful recombination of positive ES clones was confirmed by PCR followed by digestion with *Xho*I. The *Xho*I 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-MLL^N (mmN4.4), anti-MLL^C (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.

(E) 13–14 manifesting subcutaneous edema, hemorrhage and hunched posture (Fig. 1F,G), similar to the phenotypes reported in mice with other *Mll*-truncating mutations (Yagi et al., 1998; McMahon et al., 2007) and failed to maintain *Hoxc8* expression at E10.5 (Fig. 1H). Thus, the MLL holocomplex is required for embryogenesis.

Loss of MLL^C causes post-transcriptional degradation of MLL^N and p53-dependent premature senescence in fibroblasts

To further analyze the effects of the loss of MLL^C on MLL-dependent transcription, we established wild type (wt) and *dC/dC* mouse embryonic fibroblast (MEF) cell lines. Despite the comparable *Mll* mRNA levels, MLL^N protein in *dC/dC* MEFs was not detectable, indicating that MLL^N is degraded by a post-transcriptional mechanism (Fig. 2A,B). Expression of MLL target genes including *Hoxc8*, *Hoxc9*, *Cdkn2c* and *Cdkn1b* was severely impaired in *dC/dC* MEFs, whereas *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 *Mll* mRNA were comparable between *dC/dC* and the wild-type control MEFs (Fig. 2C). *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, *PAI-1* (*Serpine 1*), a well-known senescense inducer (Kortlever et al., 2006), was expressed at high levels in *dC/dC* MEFs, whereas *Pml*, another senescence inducer, was

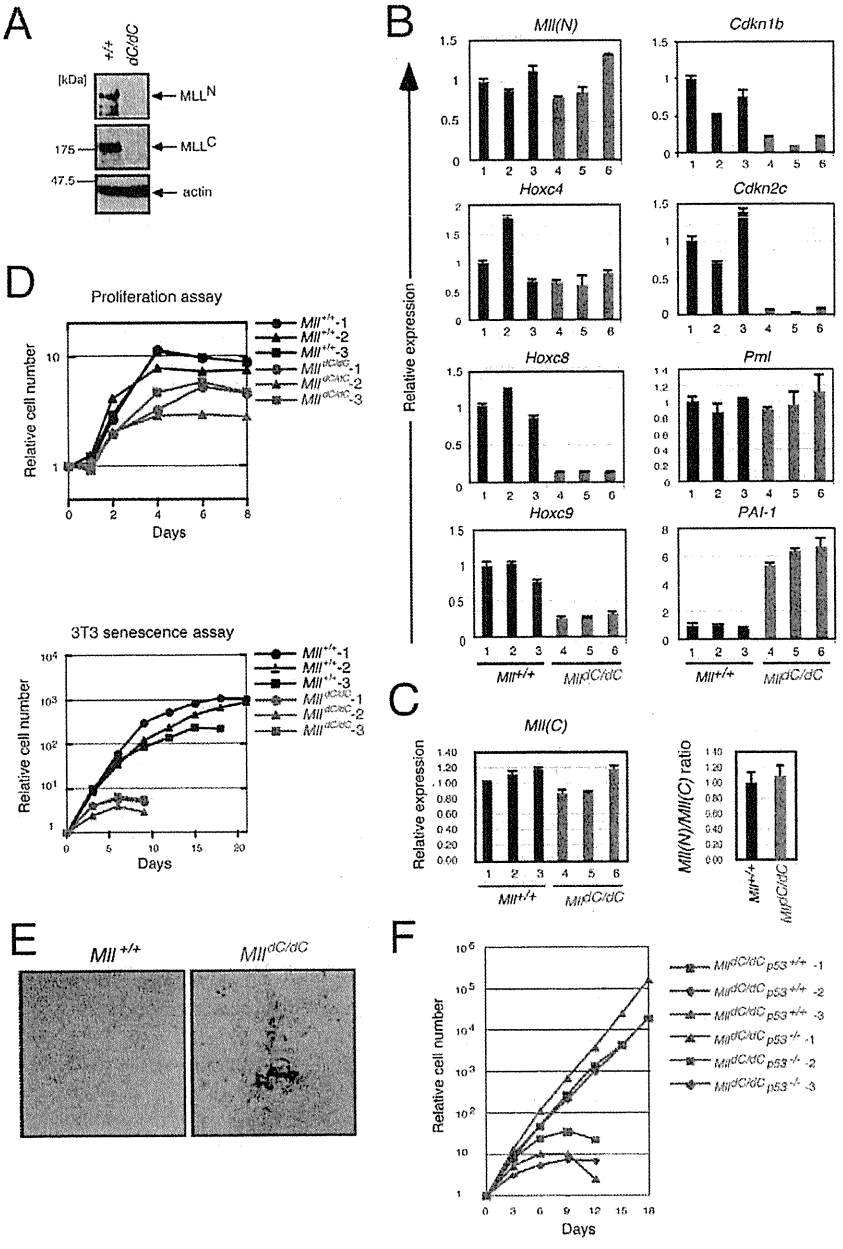


Fig. 2. The MLL holocomplex is required for MLL-dependent transcription in MEFs. (**A**) Protein expression of the MLL subunits in MEFs. Cell lysates prepared from wt or *dC/dC* MEFs were immunoblotted with anti-MLL^N (mmN4.4), anti-MLL^C(9–12) or anti-actin antibody. (**B**) Expression of various genes in *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 *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 *Mll* was analyzed using a qPCR probe for a coding sequence downstream of the processing site as in B. The ratios of the *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 *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 β-galactosidase assay of wt or *dC/dC* MEFs. (**F**) Proliferation and 3T3 senescence assays were performed for three lines each of *dC/dC* MEFs with or without homozygous p53 knockout alelles. 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 MLL^C is p53 dependent. These results demonstrate that loss of MLL^C leads to destruction of MLL^N in vivo and abolishes MLL-dependent transcription to cause p53-dependent premature senescence in MEFs.

MLL^C 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^{hi} 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 MLL^C is required for the proper expansion of hematopoietic stem cells and/or progenitors but not for differentiation.

MLL^C associates with MLL^N through PHD1, PHD4 and the FYRN domain to protect MLL^N from the FYRN-targeted destruction pathway

Next we investigated the molecular mechanism of MLL holocomplex formation and degradation of MLL^N. Previously, the N-terminal intramolecular interaction domain (NIID) was tentatively located in a large region of MLL^N 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 MLL^C in in vitro pull down assays (Hsieh et al., 2003b). To determine the mechanisms of intramolecular interaction, a series of MLL^N deletion and substitution mutants (MLL 1/2254 mutants) was examined for their ability to interact with MLL^C (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 MLL^C 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 ΔPHD1 and ΔPHD4 mutants were unstable compared with those that associate with MLL^C (Fig. 4A). However, the FYRN deletion mutant was as stable as the wt (MLL 1/2254) despite the inability to associate with MLL^C, suggesting that the FYRN domain mediates not only MLL^C interaction but also degradation of MLL^N. 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 MLL^N (supplementary material Fig. S3C,D). Furthermore, expression levels of MLL^N and MLL^C within a single cell were analyzed by transiently expressing various MLL mutant proteins tagged with CFP 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 MLL^N 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 MLL^N and MLL^C is necessary not only for their holocomplex formation but also for the protection of MLL^N 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 MLL^N

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,

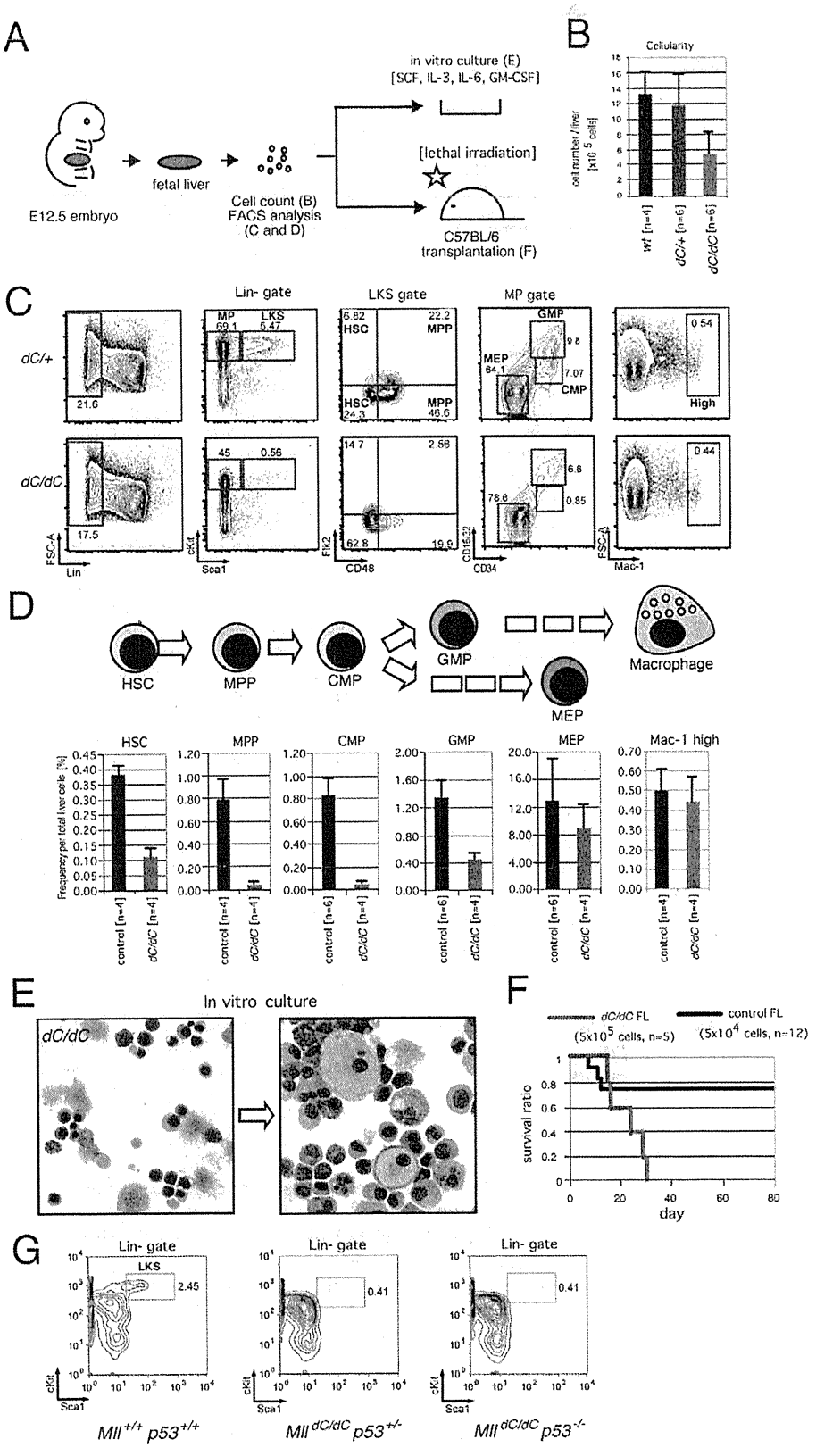


Fig. 3. The MLL holocomplex is required for HSC maintenance and expansion of hematopoietic progenitors, but not for differentiation. (**A**) The experimental scheme of B–F. (**B**) Cellularities of dC mutant fetal livers. Error bars represent the standard deviation of cell numbers of 4 or 6 livers. (**C**) 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.

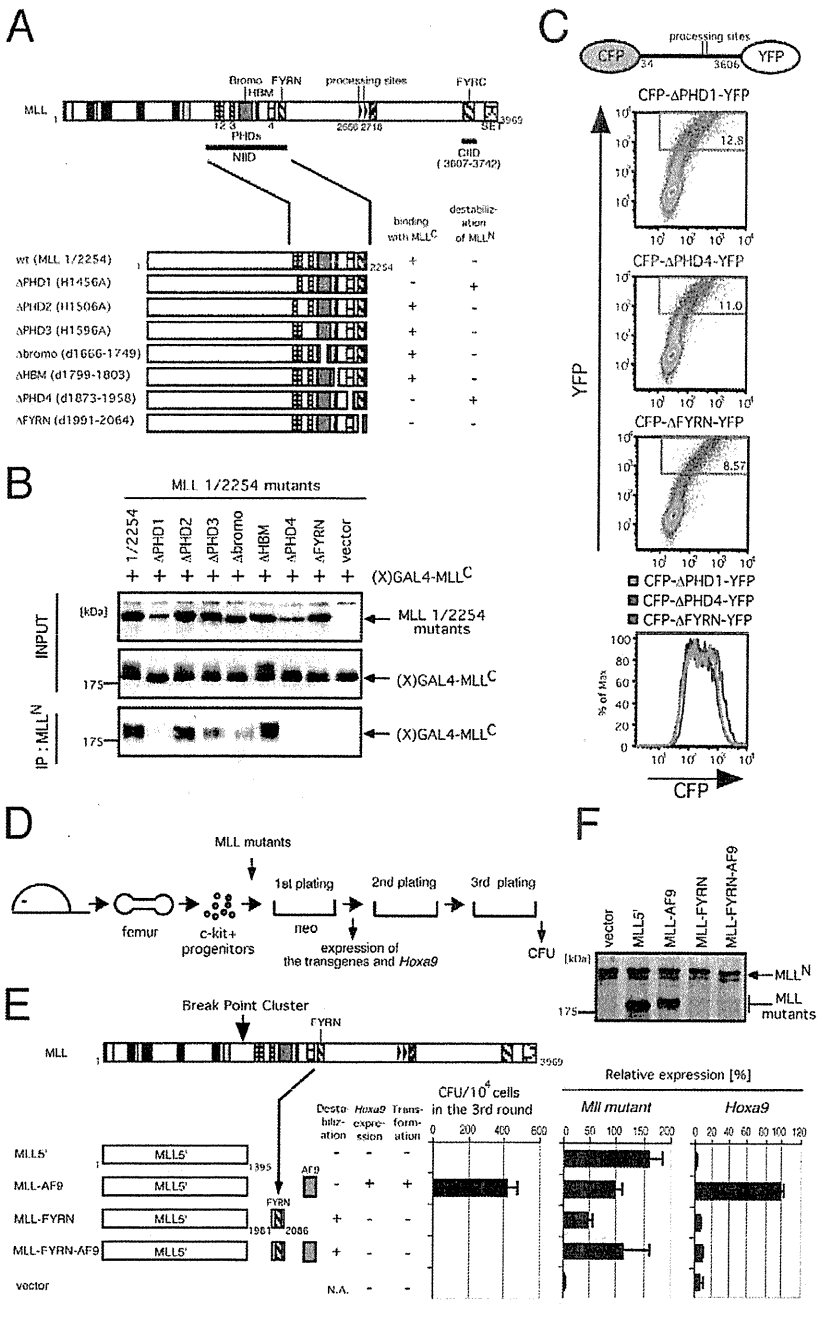


Fig. 4. PHD1 and PHD4 are required for intramolecular interaction in addition to FYRN, which also serves as a destabilization signal. (**A**) Schematic presentation of the various MLL 1/2254 substitution and deletion mutants analyzed. The binding property with MLL^C and susceptibility to destabilization are shown on the right. (**B**) Immunoprecipitation (IP) and western blotting analysis was performed for various MLL 1/2254 mutants that express residues 1–2254 with the indicated substitution and deletion mutations. MLL 1/2254 mutants were coexpressed with Xpress-tagged GAL4-MLL^C [(X)GAL4-MLL^C] in 293T cells. The cell extracts were subjected to IP with anti-MLL^N (mmN4.4) antibody followed by immunoblotting. The precipitates and input samples indicated on the left were immunoblotted with anti-MLL^N (mmN4.4; top panel) and/or anti-Xpress (middle and bottom panels) antibodies. (**C**) Various CFP–MLL 34/3606–YFP mutants were transiently expressed in 293T cells and analyzed by flow cytometry. A population that highly expressed YFP was gated and shown in overlay histograms for its CFP expression level. (**D**) The experimental scheme for the myeloid progenitor transformation assay. Expression of *Hoxa9* was analyzed at the end of the first round of plating. Colony forming units (CFUs) were measured at the end of the third round plating. (**E**) Schematic representation of the MLL–AF9 mutants with or without an FYRN domain. The destabilization property, *Hoxa9* expression, and transformation ability are summarized. CFUs per 10^4 cells at the third round are shown, with error bars representing the standard deviations from three independent analyses. Relative expression levels (normalized to the β-actin gene) of *Mll* mutant and *Hoxa9* transcripts in the first round colonies are depicted relative to MLL–AF9-transduced cells arbitrarily set as 100 (%). Quantitative PCR was performed with specific primers and probes for human *MLL* (which detects various *MLL* mutants but not endogenous mouse *Mll*) or mouse *Hoxa9* and standardized to the β-actin gene. (**F**) Protein expression of various FYRN mutants. MLL–AF9 mutants fused with or without an FYRN domain were expressed in plat-E cells and immunoblotted with anti-MLL^N antibody (mmN4.4).

using IP analysis, whether an exon 11 deletion mutant of MLL (designated Δexon11) 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 Δexon11 mutant was unstable compared with the FYRN deletion mutant (Fig. 5B–D; supplementary material Fig. S1), analogous to the ΔPHD1 and ΔPHD4 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 Δexon11 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

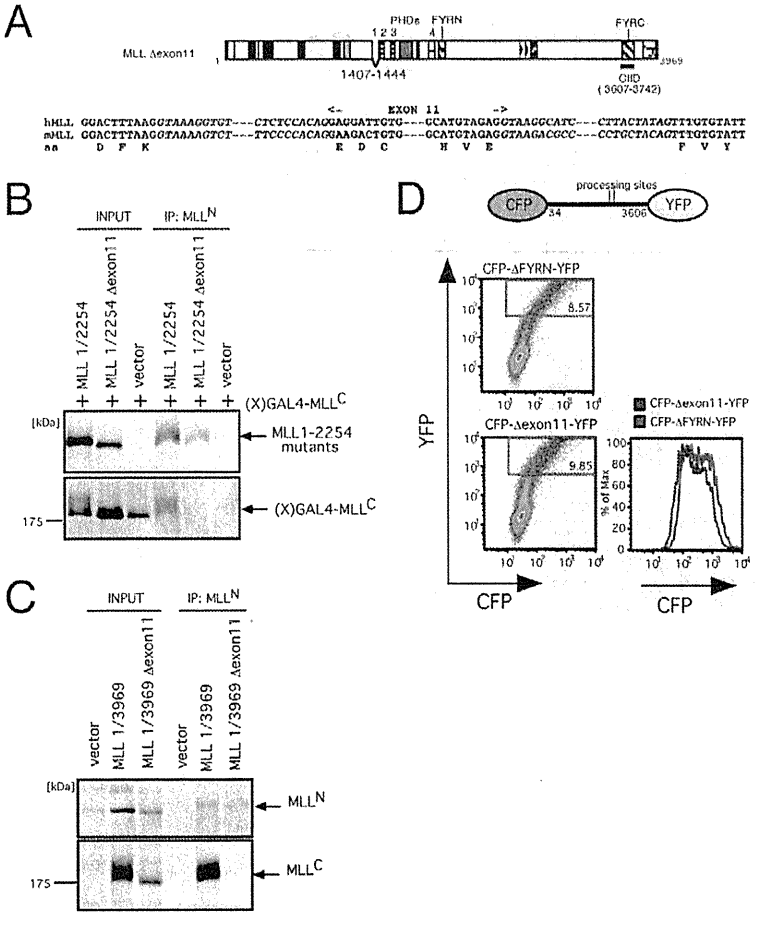


Fig. 5. The MLL Δexon11 mutant is incapable of forming an intramolecular MLL holocomplex and is susceptible to degradation. (**A**) Schematic representation of the Δexon11 mutant. The exon and intron structures around exon 11 are conserved between human (hMLL) and mouse (mMLL). (**B**) IP analysis was performed for the MLL 1/2254 Δexon11 mutant as in Fig. 4B. (**C**) IP analysis was performed for the MLL full-length Δexon11 mutant. IP analysis was performed for various MLL full-length Δexon11 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^N (mmN4) antibody followed by immunoblotting. The precipitates and input samples, indicated on the top, were immunoblotted with anti-MLL^N (mmN4; top panel) and anti-HA(3F10) antibodies (bottom panel). (**D**) The stability of MLL^N Δexon11 was analyzed as in Fig. 4C.

localized MLL^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–MLL^C proteins, implicating these domains in facilitating nuclear export (Fig. 6B). Coexpression of MLL^N and MLL^C resulted in nuclear colocalization of both subunits, whereas MLL^N containing the Δexon11 mutation failed to relocate MLL^C into the nucleus (Fig. 6C; supplementary material Fig. S1). Consistent with this notion, MLL^C derived from the Δexon11 mutant resided exclusively in the cytosolic fraction (Fig. 6D; supplementary material Fig. S1). These results suggest that MLL^N transports MLL^C into the nucleus through physical interaction, whereas MLL^C is actively exported to the cytosol if not anchored in the nucleus by MLL^N. Hence, the Δexon11 mutant is deficient for the MLL^C subunit.

The abundance of MLL^C proteins was markedly increased in the nuclear fraction when fused with GAL4 and additional nuclear localization signals (NLSs), indicating that MLL^C is relatively stable in the nucleus (Fig. 6E; supplementary material Fig. S1). Exogenously expressed MLL^C was markedly stabilized in the presence of MG132 proteasome inhibitor (Fig. 6F; supplementary material Fig. S1), whereas exogenously expressed MLL^N was unaffected, suggesting that the degradation mechanism of MLL^C is proteasome dependent but that of MLL^N is not. These results suggest that MLL^C is stable when associated with MLL^N in the nucleus, but is subjected to proteasome-dependent degradation in the cytosol when dissociated from MLL^N (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 MLL^N, dissociated from MLL^C. To address the in vivo roles of MLL subunits, we created mutant mice that do not make MLL^C. *Mll*^{C}-deficient mice failed to maintain target gene expression and died during mid-gestation with an *Mll*-null phenotype demonstrating that MLL^C 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 MLL^C activates the p53–PAI-1 pathway

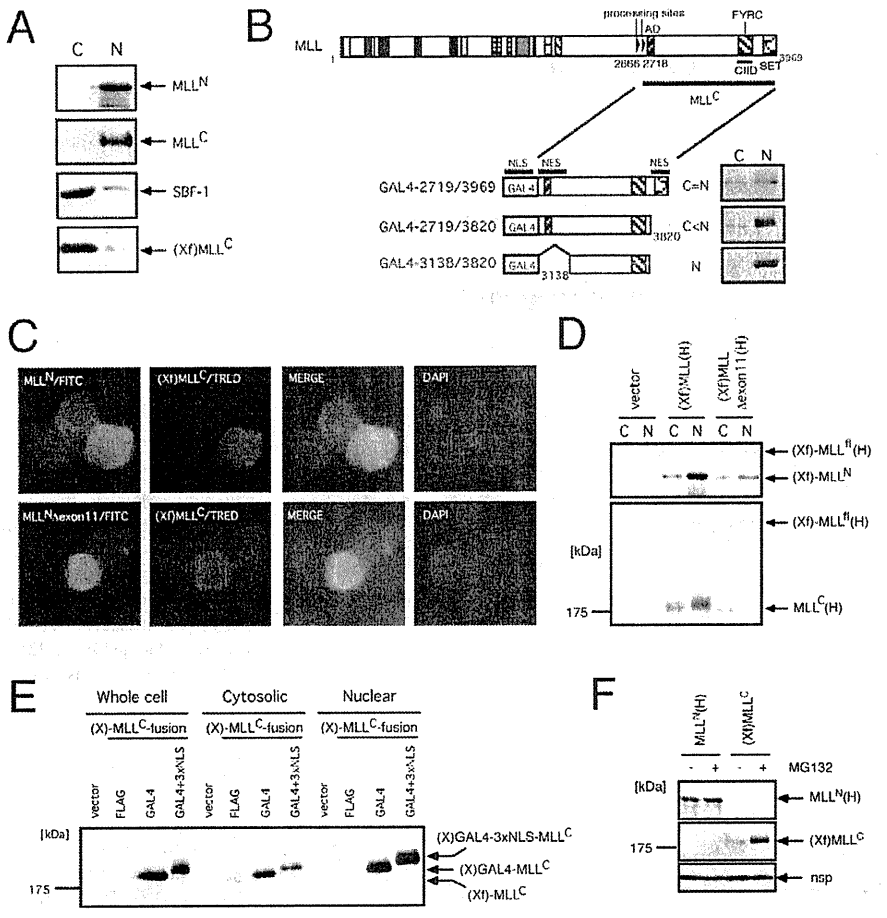


Fig. 6. MLL^C is exported to the cytosol after dissociation from MLL^N. (**A**) Subcellular localization of the endogenous MLL proteins and exogenous MLL^C. Xpress–FLAG-tagged (Xf) MLL^C (or vector) was transiently expressed in 293T cells that were lysed and separated into cytosolic (C) and nuclear (N) fractions and then immunoblotted for the indicated proteins with anti-MLL^N (mmN4.4), anti-MLL^C (mmC2.1), anti-SBF-1 (vector-transfected cells: upper three panels) or anti-Xpress antibodies [(Xf)MLL^C construct-transfected cells: bottom panel]. Sbf-1 served as a control for cytosolic localization. (**B**) Subcellular localization of various GAL4-MLL^C mutants. Various GAL4–MLL mutants, which are schematically illustrated, were analyzed for their subcellular localization as in A. GAL4 fusion proteins were visualized by anti-GAL4 antibody. (**C**) Colocalization of exogenous MLL^N or MLL^N Δexon11 with MLL^C. The indicated MLL^N mutants and (Xf)MLL^C fragments were coexpressed in 293T cells and analyzed by indirect immunofluorescence with anti-MLL^N (rpN1) and anti-Xpress antibodies. The MLL^N Δexon11 mutant served as a negative control. (**D**) Subcellular localization of exogenous full-length MLL proteins with or without the Δexon11 mutation. Full-length MLL tagged with Xpress and FLAG at its N-terminus and HA at its C-terminus [(Xf)MLL(H)] with or without deletion of exon 11 was analyzed as in A. Each fraction was immunoblotted with anti-Xpress or anti-HA antibody. (**E**) Expression of various MLL^C mutants driven by the same promoter and translation initiation sites with or without additional nuclear localization signals. (Xf)MLL^C, (X)GAL4–MLL^C, (X)GAL4–x3NLS–MLL^C were expressed in 293T cells, fractionated and immunoblotted with anti-Xpress antibody. (**F**) Sensitivities of MLL^N and MLL^C to the MG132 proteasome inhibitor. 293T cells were transfected with the corresponding expression vectors and cultured with and without 10 μM MG132 for 8 hours and subjected to western blotting with anti-Xpress antibody. A nonspecific band (nsp) serves as a loading control.

because *PAI-1* expression is induced in *dC/dC* MEFs, whereas the senescence phenotype can be rescued by a p53-null allele. Thus, MLL^C 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 MLL^C 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