

Figure 4. A, sequence alignment of *miR-22* and the 3' UTR of *p21* mRNA is indicated at the top. Mutant sequences used for the reporter gene assay are listed (Mut 1 and Mut 2). B, reporter gene assay. Error bar indicates SD ($n = 6$). C, expression level of p21 protein in the presence of *miR-22*. *Mir-34a* was used as positive control. D, expression levels of *p21* mRNA in the presence of *miR-22*. Cells were transfected with *miR-22*, and incubated for 3 days. The relative expression levels of *p21* mRNA were quantified by TaqMan assay. E, effect of *miR-22* on the activation of p21 expression after exposure to ADR. HCT 116 cells were transfected with 5 nmol/L of either *miR-22* or *miR-NC* and incubated for 48 hours, and further incubated in the presence of ADR for the indicated times. F, indirect immunocytochemistry. Cells were transfected as described above, and incubated in the presence of ADR for the indicated times. Cells were subjected to immunostaining. G, activation of *p21* expression in the presence or absence of *miR-22* after exposure to ADR. Cells were prepared as described in (E), and total RNAs were prepared from each time point. Relative expression levels of *p21* mRNA were quantified by TaqMan assay.

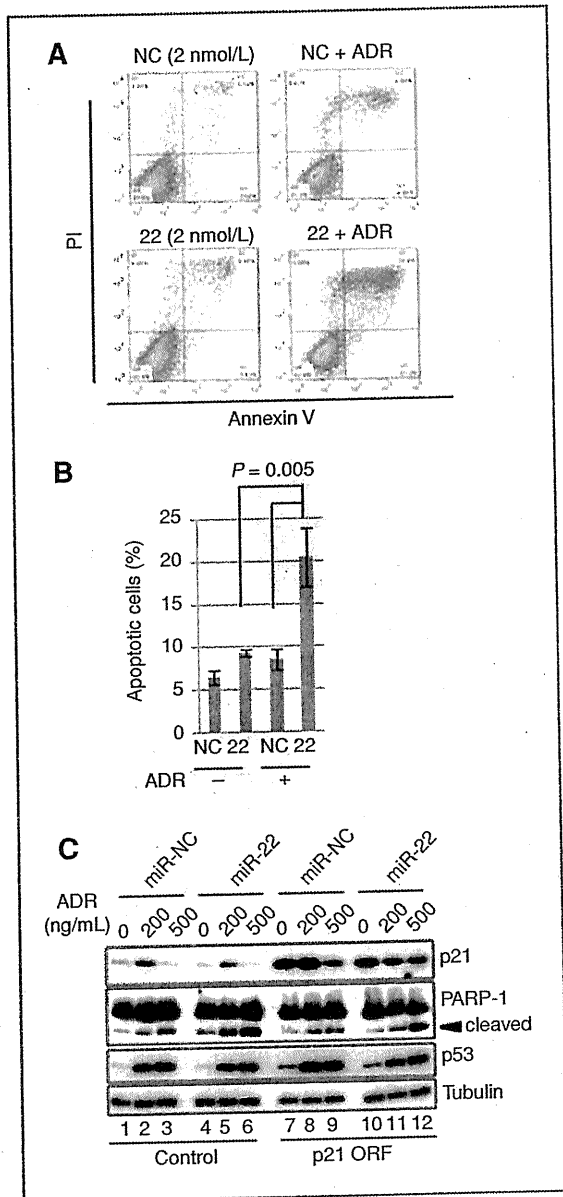


Figure 5. A, sensitization to p53-dependent apoptosis by *miR-22*. HCT 116 cells, transfected with either 2 nmol/L of *miR-22* or *miR-NC*, were incubated in the presence or absence of 100 ng/mL ADR for 12 hours. Apoptotic cells were determined by FACS. B, quantification of apoptotic cells using 3 independent FACS experiments as described in (A). Data show mean with SD. C, expression of p21 protein reduced *miR-22*-induced sensitization of apoptosis. HCT 116 cells were transfected with either *miR-NC* or *miR-22* lentivirus. After selection, cells transfected with either *miR-NC* or *miR-22* were treated with indicated concentration of ADR for 24 hours and cleaved PARP-1 was detected by immunoblotting.

and its role in the determination of p53-dependent cellular fate through the formation of the p53-*miR-22*-p21 axis was shown. This axis might be activated by specific stresses that require the elimination of damaged cells. The current

findings provide a novel insight into the regulatory mechanism of cell fate determination by a specific molecule, *miR-22*, in response to various oncogenic stresses and in a p53-dependent manner.

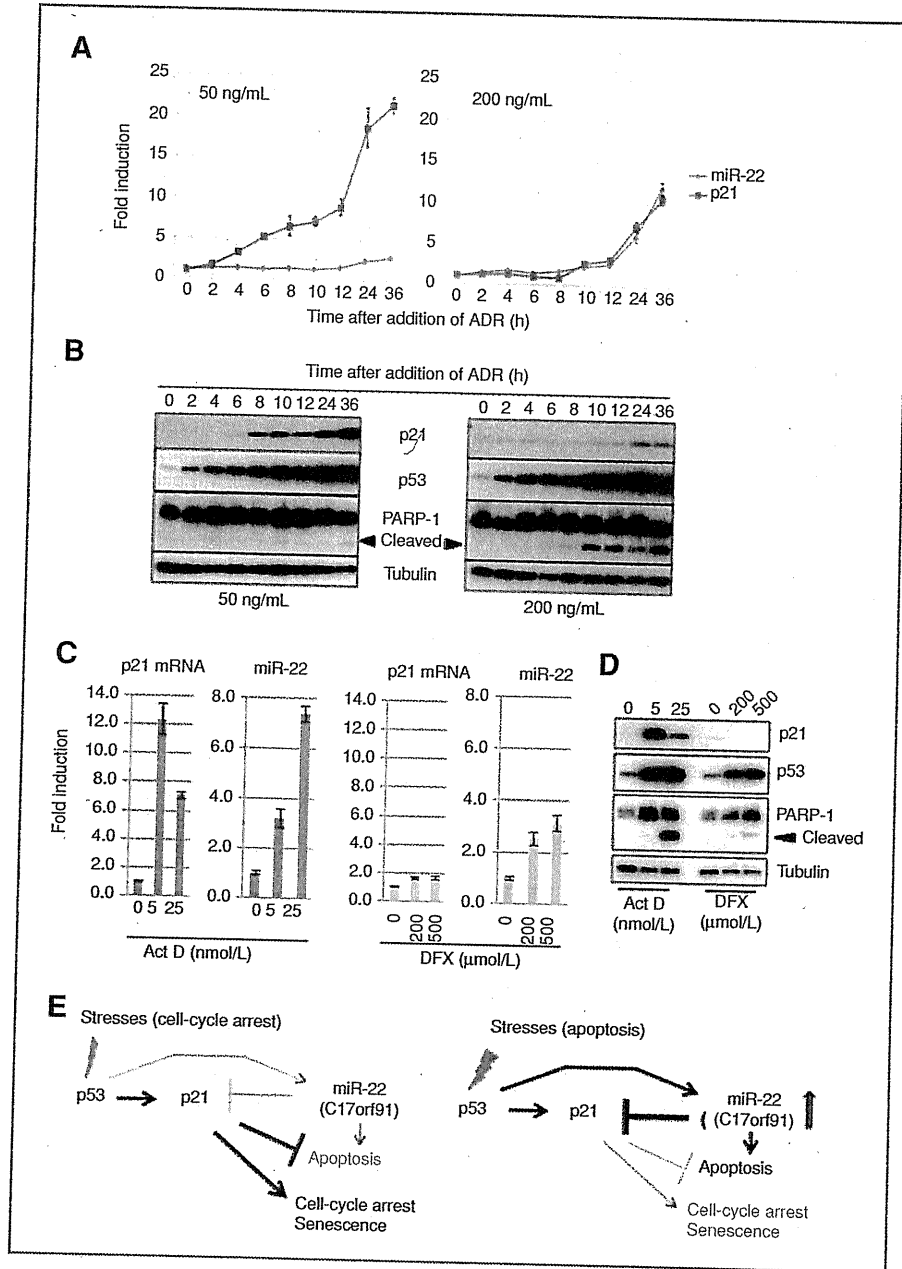
As depicted in Fig. 6E, 2 modes of action of the p53-*miR-22*-p21 axis were suggested in response to the different intensity of the stresses applied. In brief, p53 only activates p21 to induce cell-cycle arrest against weak stresses in the p53-p21 pathway. On the other hand, severe damage transcriptionally activates both p21 and *miR-22*, and *miR-22* represses p21 expression through the inhibition of protein synthesis and enhancement of p21 mRNA degradation. Under severe damage conditions, apoptosis may be induced by entry into the cell cycle via direct repression of p21 by *miR-22*.

Antiapoptotic function of p21 has recently attracted attention for its oncogenic action, which is opposed to a traditional tumor suppressor function. Lack of p21 induces apoptosis through the accumulation of DNA damage in leukemic stem cells (41). Disruption of the p21 gene sensitized cancer cells to apoptosis after treatment with chemotherapeutic agents (37, 38). Recently, the small molecule RITA, an activator of p53, was shown to efficiently induce apoptosis through inhibition of p21 (42). Furthermore, a single recombinant adenovirus containing p53 cDNA and synthetic p21 shRNA also efficiently induced apoptosis in colon cancer cell lines (43). These findings indicate that the downregulation or inhibition of p21 after activation of p53 in stressed cells is one of the key factors as an anticancer mechanism by inducing the change of cellular phenotype from cell-cycle arrest to apoptosis, which could be the mechanism triggered by *miR-22* as an intrinsic stress-response network.

MicroRNAs are known to repress multiple target mRNAs, leading to efficient shut down or activation of intracellular networks (44, 45). Indeed, introduction of *miR-22* broadly and significantly modulates cellular networks in p53 wild-type HCT 116 cells (Supplementary Tables S3, S4, and Fig. S3D). Furthermore, high levels of expression of *miR-22* alone clearly showed apoptosis without activation of p53 in HCT 116 cells (Fig. 2A), where p21 might not be a promising target of *miR-22*, suggesting that other *miR-22* target genes also contribute to the induction of p53-dependent apoptosis.

In addition to the *miR-22* function in p53 wild-type colon cancer cells, another interesting feature is that *miR-22* expression induces cell-cycle arrest in p53 knockout and mutant cell lines (Supplementary Fig. S3B and C). We searched for a TargetScan database to obtain a list of potential *miR-22* targets and conducted gene ontology analysis to identify genes whose repression theoretically induces cell-cycle arrest. These analyses indicated that several positive cell-cycle regulators, *CDK6*, *CDK3*, *SIRT1*, *CDC25B*, and *HDAC4*, are possible targets of *miR-22*. We analyzed the protein levels of CDK6 and SIRT1 by immunoblot analysis, and found no significant changes in their protein levels in the presence of *miR-22* in SW480 cells. Then, we re-evaluated the data of AGO2-IP on ChIP analysis using HCT 116 cells. Interestingly, *CDK3*, *CDC25B*, and *HDAC4* mRNAs were enriched in the AGO2 complex in a *miR-22*-dependent manner (data not shown). Although it is currently unclear that these mRNAs are directly

Figure 6. A and B, kinetics of *miR-22* and *p21* increments after exposure to ADR. Time-dependent increments of *p21* mRNA and *miR-22* after exposure to different doses of ADR were quantified by RT-PCR. Relative expression of *p21* and *miR-22* was calculated by $2^{-\Delta\Delta Ct}$ using *GAPDH* as an internal standard (A). Protein levels of *p21*, *p53*, and cleaved PARP-1 were analyzed by immunoblotting in cells treated with different doses of ADR (B). C and D, activation of *miR-22* expression by Act D. Cells were treated with specific concentration of either Act D or deferoxamine for 24 hours, and *p21* and *miR-22* levels were determined as described above. E, 2 modes of action of *miR-22* in the p53 network.



downregulated by *miR-22*, *miR-22* may induce p53 independent cell-cycle arrest through the repression of these genes.

The present data suggest a tumor-suppressive role of *miR-22* in colon cancer cells. *miR-22* was also reported to be downregulated in estrogen receptor (ER)-positive breast cancers, and repression of ER expression by *miR-22* suppressed cell proliferation (46, 47). On the other hand, *miR-22* was recently suggested to have an oncogenic role through the direct silencing of *PTEN* and its upregulation in prostate cancer cell lines (48). These authors identified the

transforming activity of *miR-22* in mouse embryonic fibroblast cooperatively with *c-Myc*, and showed that the overexpression of *miR-22* in the prostate cancer cell line DU145, harboring p53 mutations in both alleles, caused an enhancement of colony formation. The reported paradoxical function of *miR-22* implies that *miR-22* could act as a tissue-specific or context-dependent tumor suppressor gene.

Chromosome 17p13.3, where the *miR-22* gene resides, is well known to be a target for allelic loss, and loss of heterozygosity in 17p13.3 is often found independently of

the *TP53* mutation in human cancers, including lung and breast cancer (49, 50). Furthermore, an unknown tumor suppressor gene has been suggested to be present at this locus. The present data suggest that *miR-22* is a candidate haploinsufficient-type tumor suppressor gene within this region, and its hemizygous loss or downregulation reduced apoptosis induction in response to stresses, even in cells retaining an intact *TP53*.

In summary, the data presented suggest a role for *miR-22* as an intrinsic molecular switch in the p53 tumor suppressor network, functioning as a determinant of cell fate at a post-transcriptional level by inducing apoptosis via direct repression of *p21*. This system might function in the p53-dependent activation of a specific anticancer barrier in response to various oncogenic stresses, and dysfunction of *miR-22* might confer a chance of survival for damaged cells with tumorigenic potential.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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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 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.,

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 effector 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 effector 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/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

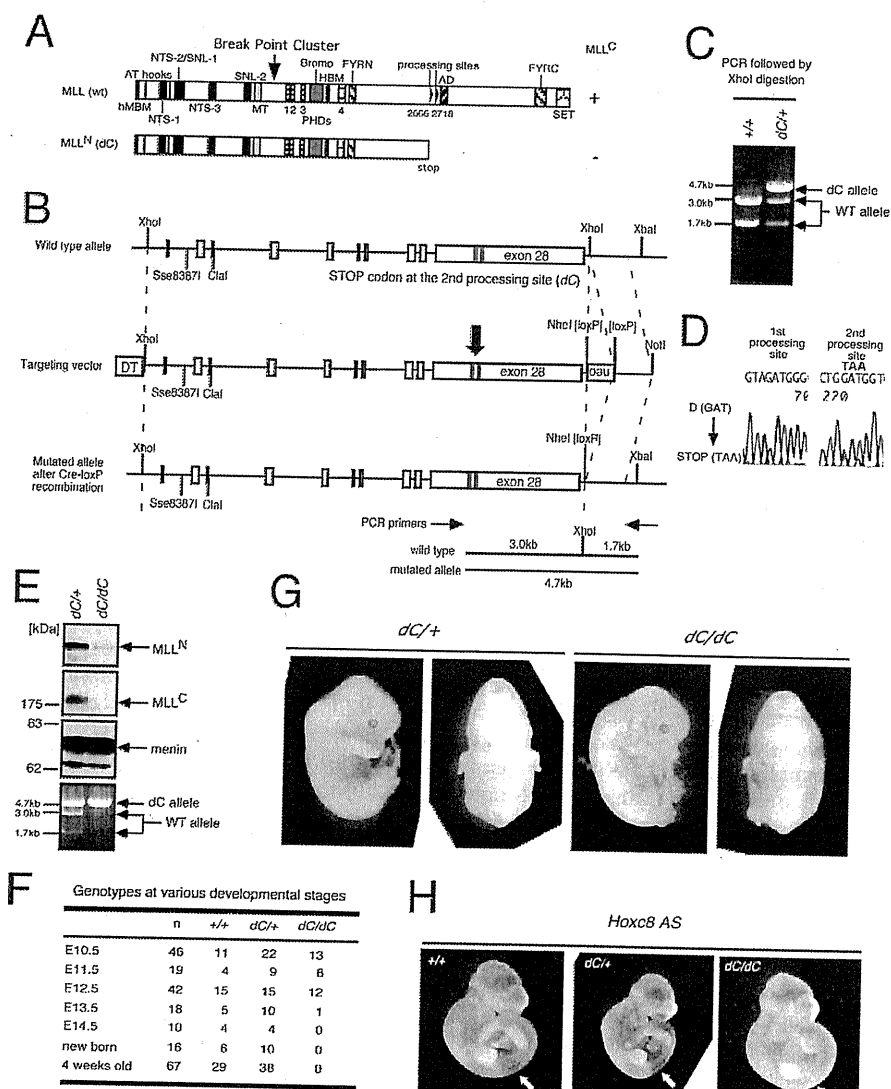


Fig. 1. MLL^C is required for MLL-dependent transcription during embryogenesis. (A) Schematic representations of dC-mutant MLL proteins. (B) The structure of the targeting vector. The positions of diagnostic primers are shown at the bottom. (C) Successful recombination of positive ES clones was confirmed 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 senescence inducer (Kortlever et al., 2006), was expressed at high levels in *dC/dC* MEFs, whereas *Pml*, another senescence inducer, was

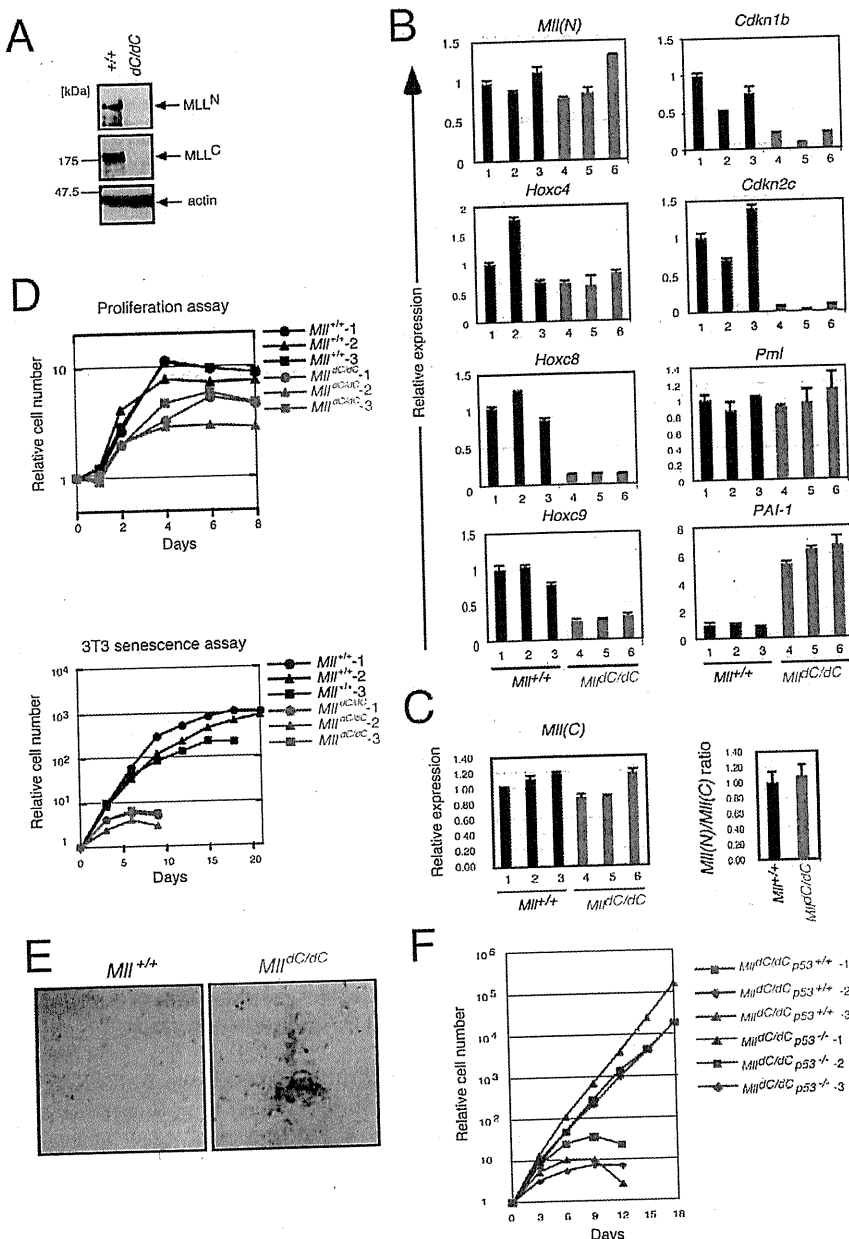


Fig. 2. The MLL holocomplex is required for MLL-dependent transcription in MEFs. (A) Protein expression of the MLL subunits in MEFs. Cell lysates prepared from wt or *dC/dC* MEFs were immunoblotted with anti-MLL^N (mmN4.4), anti-MLL^C(9–12) or anti-actin antibody. (B) Expression of various genes in *dC/dC* 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 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 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⁺, Scal⁺) 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 FYRN 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,

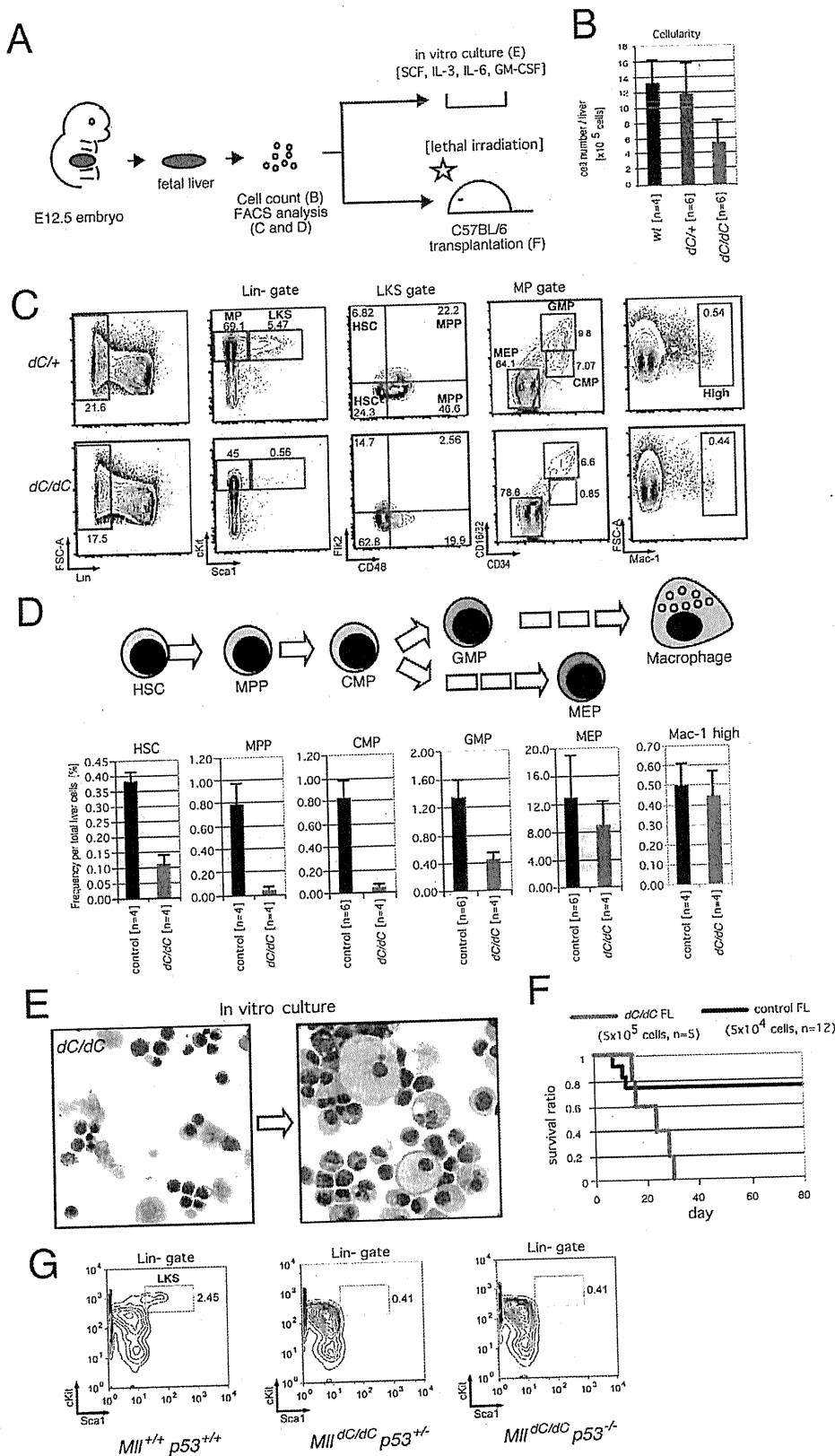


Fig. 3. The MLL holocomplex is required for HSC maintenance and expansion of hematopoietic progenitors, but not for differentiation. (A) The experimental scheme of B–F. (B) Cellularities of dC mutant fetal livers. Error bars represent the standard deviation of cell numbers of 4 or 6 livers. (C) Representative 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 × 10⁵) from dC/dC embryos (n=5) or control embryos (5 × 10⁴; 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.

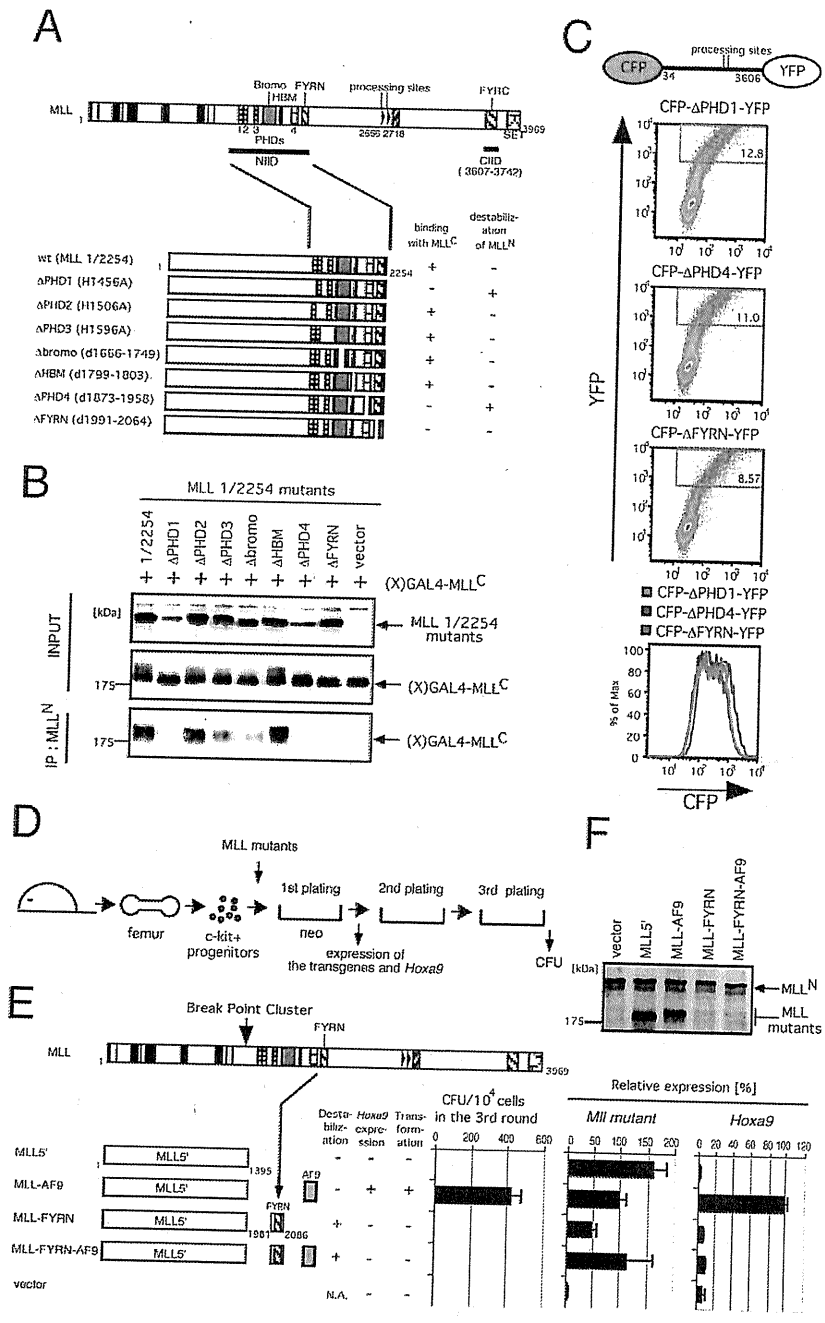


Fig. 4. PHD1 and PHD4 are required for intramolecular interaction in addition to FYRN, which also serves as a destabilization signal. (A) Schematic presentation of the various MLL 1/2254 substitution and deletion mutants analyzed. The binding property with MLL^C and susceptibility to destabilization 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 10⁴ 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

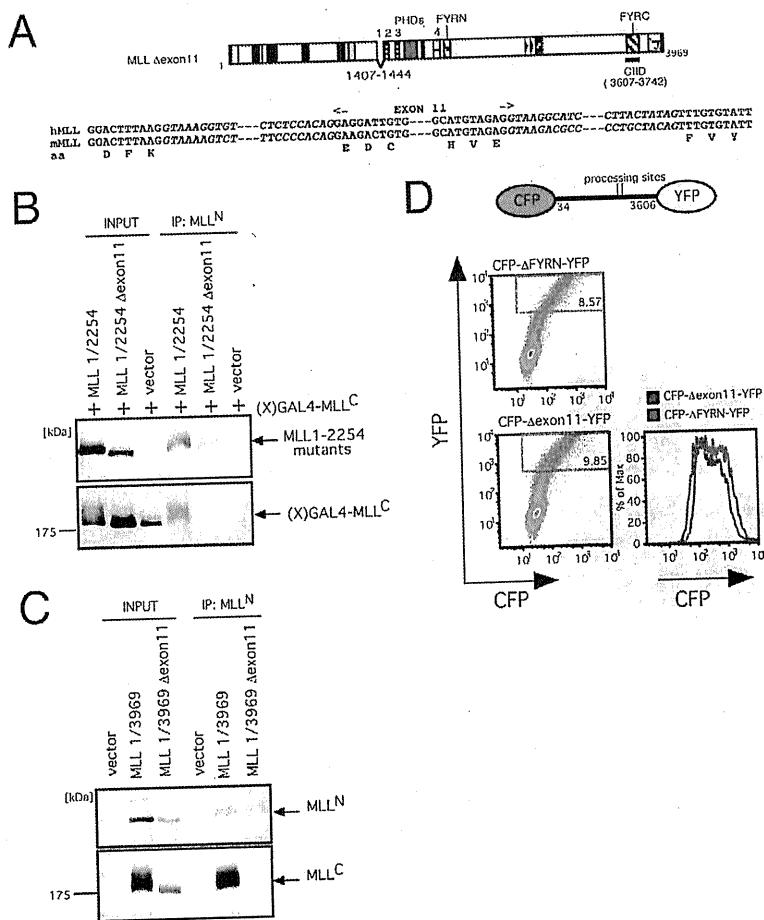


Fig. 5. The MLL Δexon11 mutant is incapable of forming an intramolecular MLL holocomplex and is susceptible to degradation. (A) Schematic representation of the Δexon11 mutant. The exon and intron structures around exon 11 are conserved between human (hMLL) and mouse (mMLL). (B) IP analysis was performed 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

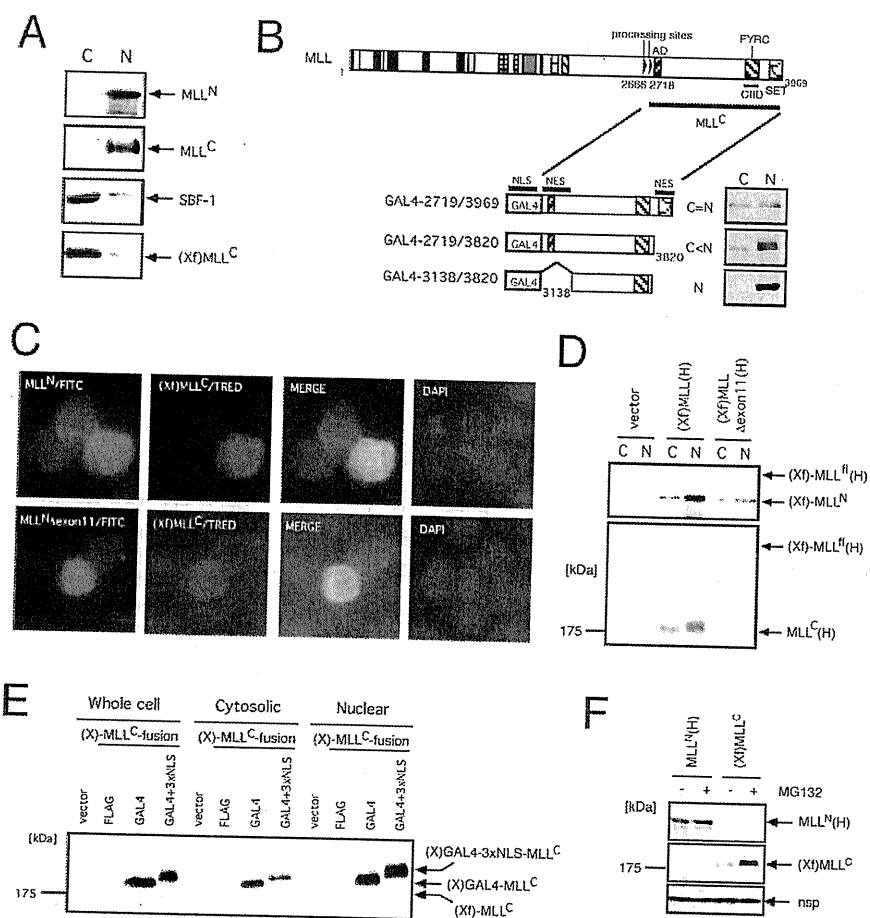


Fig. 6. MLL^C is exported to the cytosol after dissociation from MLL^N. (A) Subcellular localization of the endogenous MLL proteins and exogenous MLL^C. Xpress-FLAG-tagged (Xf) MLL^C (or vector) was transiently expressed in 293T cells that were lysed and separated into cytosolic (C) and nuclear (N) fractions 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 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 Dexon11 mutation. The MLL^N 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; Somerville 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

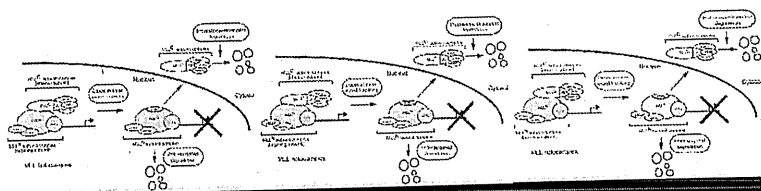


Fig. 7. A model of the two distinct degradation pathways for each MLL subunit.

progenitors and exhaustion of HSCs. Our analysis of the MLL-deficient hematopoietic system underscores a previously unappreciated role for MLL in LSK CD48⁺Fik2⁺ 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, *Mll*^C-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 mechanism appears to be 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 Δ exon11 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 endocrine 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 circuit (Franklin et al., 1998; Franklin et al., 2000; Karnik et al., 2005; Milne 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 Toriell 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 endocrine 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., 2007). 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-Sbf-1

(HAF3P.1) and polyclonal rabbit anti-MLL^N (rpN1) as described elsewhere (Yokoyama et al., 2002). Anti-HCF-1c (H12) antibody was provided by Winship Herr. 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-probe (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 (3F10; 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 [pLNCX (Xf)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 CFP 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 pLNCX vector (Clontech). The various MLL-AF9 mutants were generated by modification of the pMSCV MLL-AF9 vector (Somerville 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 cassettes (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'-CTGGCATTATGTTATTTGAACAGGCACCCC-3' and the reverse primer: 5'-TACACGTGGTAACCAGTCATCTGCAGCTCA-3' by LA-PCR followed by digestion with *XhoI*. 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 (Donehower 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 Licia Sella.

Quantitative RT-PCR

Reverse transcription (RT) and quantitative PCR (qPCR) were performed as described previously (Yokoyama et al., 2005). Taqman probes for *Actb* (Mm00607939_s1), *Gapdh* (Mm99999915_g1), *Hoxa9* (Mm00439364_m1), *Hoxc4* (Mm00442838_m1), *Hoxc8* (Mm00439369_m1), *Hoxc9* (Mm00433972_m1), *Cdkn1b* (Mm00439167_g1), *Cdkn2c* (Mm00483243_m1), *Pnl* (Mm00476969_m1), *Serpine1* (Mm00435860_m1), *MLL(N)* (Mm01179246_g1) and *MLL(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, 5×10^4 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 eBioscience (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), Scf (D7), CD48 (HM48-1), CD34 (49E8), CD16/32 (93), Fli2 (A2F10), CD45.2 (104) and CD43 (S7). Stained cells were analyzed with an LSR-IA or LSR-II flow cytometer. Cell Quest Pro or Diva (BD) were used for data acquisition, and FlowJo (Tree Star) was used for analysis.

Cytospin and in vitro differentiation to macrophages

Fetal liver cells were cultured for 1 week in methylcellulose medium (M3231; Stemcell Technologies; Vancouver, BC) containing SCF, IL-3, IL-6 and GM-CSF. Cytospin preparations were stained with May-Grunwald-Giemsa stain for assessment of cellular cytology as described elsewhere (Yokoyama et al., 2005).

In vivo reconstitution assay

Fetal liver cells from homozygous mutant (5×10^5 cells) or wt embryos (5×10^4 cells) were injected intravenously into lethally irradiated (900 rad) C57BL/6 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 RNeasy mini kit (Qiagen).

Indirect immunofluorescence

Indirect immunofluorescence was performed using 293T cells transfected with various *MLL* expression vectors as described elsewhere (Yokoyama and Cleary, 2008). Transfected cells were fixed and incubated with rabbit anti-*MLL*^N (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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A Higher-Order Complex Containing AF4 and ENL Family Proteins with P-TEFb Facilitates Oncogenic and Physiologic MLL-Dependent Transcription

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SUMMARY

AF4 and ENL family proteins are frequently fused with MLL, and they comprise a higher order complex (designated AEP) containing the P-TEFb transcription elongation factor. Here, we show that AEP is normally recruited to MLL-target chromatin to facilitate transcription. In contrast, MLL oncoproteins fused with AEP components constitutively form MLL/AEP hybrid complexes to cause sustained target gene expression, which leads to transformation of hematopoietic progenitors. Furthermore, MLL-AF6, an MLL fusion with a cytoplasmic protein, does not form such hybrid complexes, but nevertheless constitutively recruits AEP to target chromatin via unknown alternative mechanisms. Thus, AEP recruitment is an integral part of both physiological and pathological MLL-dependent transcriptional pathways. Bypass of its normal recruitment mechanisms is the strategy most frequently used by MLL oncoproteins.

INTRODUCTION

Leukemia is a heterogeneous disease with distinctive biological and clinical properties that are conferred by a variety of acquired genetic mutations (Gilliland, 2002). Chromosomal translocations of the *MLL* gene account for 5%–10% of acute leukemias and are generally associated with poor prognosis (Daser and Rabbits, 2004; Krivtsov and Armstrong, 2007; Pui et al., 2004). *MLL* gene rearrangements create fusion genes that contain the 5' portion of *MLL* and the 3' portion of its fusion partner, whose products cause sustained expression of MLL target genes and consequent enhanced proliferation of hematopoietic progenitors (Ayton and Cleary, 2003; Lavau et al., 1997; Cozzio et al., 2003). The amino-terminal portion of MLL serves as a targeting unit to direct MLL oncoprotein complexes to their target loci through DNA binding (Ayton et al., 2004; Slany et al., 1998) and association with menin and LEDGF (Yokoyama et al., 2005; Yokoyama and Cleary, 2008), whereas the fusion partner portion serves

as an effector unit that causes sustained transactivation (Cheung et al., 2007; Lavau et al., 2000; DiMartino et al., 2000; 2002; Slany et al., 1998; So and Cleary, 2002; 2003). To date, approximately 50 different fusion partners have been reported to form chimeric MLL oncoproteins (Huret et al., 2001). However, the mechanisms underlying this molecular diversity have not been revealed.

The AF4 and ENL protein families are the most frequent MLL fusion partners, accounting for two-thirds of *MLL*-associated leukemia incidence (Huret et al., 2001). The AF4 family comprises four paralogous proteins, including AF4, AF5q31, LAF4, and FMR2. The ENL family includes ENL and AF9 and has structural homology to the yeast Anc1 protein. The members of both protein families possess transactivation domains and therefore are thought to be involved in transcriptional regulation (Prasad et al., 1995; Ma and Staudt, 1996; Morrissey et al., 1997; Slany et al., 1998). All but *FMR2* have been reported to form fusion genes with *MLL* in leukemia (Domer et al., 1993; Taki

Significance

MLL is fused by chromosomal translocations in 5%–10% of acute leukemias to a variety of partner proteins (>50) of diverse molecular composition and function. Recent studies show that several of the more common *MLL* fusion partners (e.g., AF4, ENL, and AF9) associate in a higher-order complex containing transcription elongation factors. Here we show that this complex is biochemically distinct from the *MLL* histone methyltransferase complex, but nevertheless normally present at *MLL* target genes during physiologic gene expression. In acute leukemias, the complex is constitutively recruited to target chromatin by covalent fusion of *MLL* with one of several complex components or noncovalent mechanisms used by other *MLL* fusion proteins, thereby representing a unifying mechanism for *MLL*-mediated leukemogenesis that can be targeted by molecular therapy.

et al., 1999; von Bergh et al., 2002; Iida et al., 1993; Nakamura et al., 1993; Tkachuk et al., 1992). AF4 family proteins associate with ENL family proteins and P-TEFb (Positive Transcription Elongation Factor b) (Erfurth et al., 2004; Zeisig et al., 2005; Bitoun et al., 2007; Mueller et al., 2007). P-TEFb is composed of CDK9 and cyclin T1 (or cyclin T2) and is capable of phosphorylating the carboxy-terminal domain (CTD) of RNA polymerase II (RNAPII) and DSIF to facilitate transcriptional elongation (Saunders et al., 2006; Peterlin and Price, 2006). AF4 functions as a positive regulator of P-TEFb kinase (Bitoun et al., 2007), which, in turn, controls the transactivation activity or stability of AF4 and ENL family proteins. ENL family proteins also associate with DOT1L (Bitoun et al., 2007; Mueller et al., 2007; Zhang et al., 2006), the major histone methyltransferase responsible for the H3K79 methylation mark (Jones et al., 2008), which is predominantly associated with actively transcribed genes (Steger et al., 2008). It has been reported that DOT1L also associates with MLL-AF10 and plays a critical role in its oncogenic transformation (Okada et al., 2005). However, the molecular roles of these components in MLL-dependent leukemogenesis have not been clearly defined.

In this study, we investigated the contributions of a higher order complex containing AF4 and ENL family proteins with P-TEFb in physiologic and pathologic MLL-dependent transcription.

RESULTS

AF4 Forms a Higher Order Complex with AF5q31, ENL, and P-TEFb in Hematopoietic Cells

To identify AF4-associated proteins *in vivo*, we biochemically purified AF4 complexes from K562 cells using column chromatography followed by immuno-affinity purification with a highly specific anti-AF4 monoclonal antibody (Figure 1A). Mass spectrometry identified AF5q31, ENL, CDK9, and cyclin T1 in the purified materials (Figure 1B). Reciprocal immunoprecipitation (IP) further confirmed that all five proteins compose an endogenous bona fide complex (Figure 1C) consistent with previous observations (Erfurth et al., 2004; Zeisig et al., 2005; Bitoun et al., 2007; Mueller et al., 2007). In gel filtration analysis, the AF4 complex components codistributed in fractions that eluted at an average mass of ~0.8 MDa (Figure 1D). A similar complex was obtained using a monoclonal antibody specific for AF5q31 in the immuno-affinity step (see Figure S1A available with this article online). However, neither purification process yielded other proteins previously reported to interact with ENL (e.g., DOT1L and AF10) (Zeisig et al., 2005; Bitoun et al., 2007; Mueller et al., 2007). These data demonstrate that AF4, AF5q31, and ENL associate in an endogenous higher-order complex (hereafter referred to as "AEP" for the AF4 family/ENL family/P-TEFb complex) containing P-TEFb in hematopoietic lineage cells.

Leukemogenic Fusion Proteins Inappropriately Tether AEP Components with MLL

Co-IP analyses were performed to determine whether MLL chimeric oncoproteins participate in higher-order associations that recapitulate the composition of AEP. Reciprocal IP using human leukemia cell lines that express MLL-ENL, MLL-AF4, or MLL-AF5q31 showed that the respective fusion proteins form

similar AEP-like complexes (Figure 1E and Figure S1B). Conversely, MLL-AF6, an MLL fusion with a cytoplasmic protein that was not copurified with AF4 or AF5q31, did not coprecipitate any of the AEP components in ML-2 cells (Figure 1E). Similarly, wild-type (WT) MLL did not pull down AEP components in K562 cells while coprecipitating menin, a component of the MLL complex (Yokoyama et al., 2004) (Figure 1C). Therefore, the MLL and AEP complexes are separate biochemical entities that are inappropriately tethered to form MLL/AEP hybrid complexes by a subset of covalent fusions of MLL in human leukemia cells.

MLL-ENL and MLL-AF4 Consistently Recruit AEP Components to MLL Target Genes

Genomic localizations of MLL chimeric proteins and AEP components were analyzed by chromatin immunoprecipitation (ChIP) in human leukemia cell lines. Histone marks indicative of open chromatin states (tri-methyl H3K4 and acetyl H3K9) (Li et al., 2007) were associated with transcriptionally active loci, whereas histone marks indicative of closed chromatin (di-methyl H3K9 and high levels of histone H3) were associated with transcriptionally inactive loci (Figures 2A–2C), verifying the integrity of ChIP assays. In HB1119 cells, MLL-ENL specifically colocalized with AF4 and AF5q31 at promoter-adjacent regions of the *HOXA9* and *MEIS1* genes, which are known to serve critical roles in MLL-associated leukemogenesis (Ayton and Cleary, 2003; Nakamura et al., 1996; Wong et al., 2007), whereas the presence of AEP at non-MLL target loci such as *β-ACTIN* and *GAPDH* was minimal or negligible (Figure 2B and Figure S2A). Similarly, ChIP analysis showed that AF5q31 and ENL colocalized with endogenous MLL-AF4 on the *HOXA9* and *MEIS1* promoters in MV4-11 cells (Figure 2C and Figure S2B). Colocalization of AEP components with MLL oncoproteins was also observed on other MLL target genes, such as *CDKN1B* and *CDKN2C* (Milne et al., 2005), and the transcribed regions of *HOXA9* and *MEIS1* (Figures 2B and 2C), suggesting that MLL/AEP hybrid complexes may function in transcriptional elongation. Therefore, a subset of MLL oncoproteins results in consistent recruitment of AEP components at MLL target chromatin in leukemia cells.

Formation of a Higher Order MLL-AF5q31/AEP Hybrid Complex Is Required for Sustained Transcription of Target Genes and Transformation.

AF4 and AF5q31 share extensive sequence similarity that resides in four subregions of the respective proteins (Figure 3A). A structure/function analysis (Figures 3B and 3C) revealed that: (1) P-TEFb interacts with AF4 and AF5q31 via subregion 1, which contains the N-terminal homology domain (NHD) (Nilson et al., 1997); (2) strong transactivation activity is conferred by subregion 2, consistent with previous observations (Prasad et al., 1995; Ma and Staudt, 1996; Morrissey et al., 1997); (3) ENL interacts with AF4 and AF5q31 through subregion 3 that encompasses the AF9 interaction domain (Srinivasan et al., 2004; Zeisig et al., 2005); and (4) the C-terminal homology domain (CHD) within subregion 4 mediates hetero-association of AF4 and AF5q31, which appears to be highly preferred over their respective homo-dimerization (Figure 3B). Preferential hetero-dimerization was also observed in co-IP experiments of endogenous or transfected MLL-AF5q31 (Figure 1E and

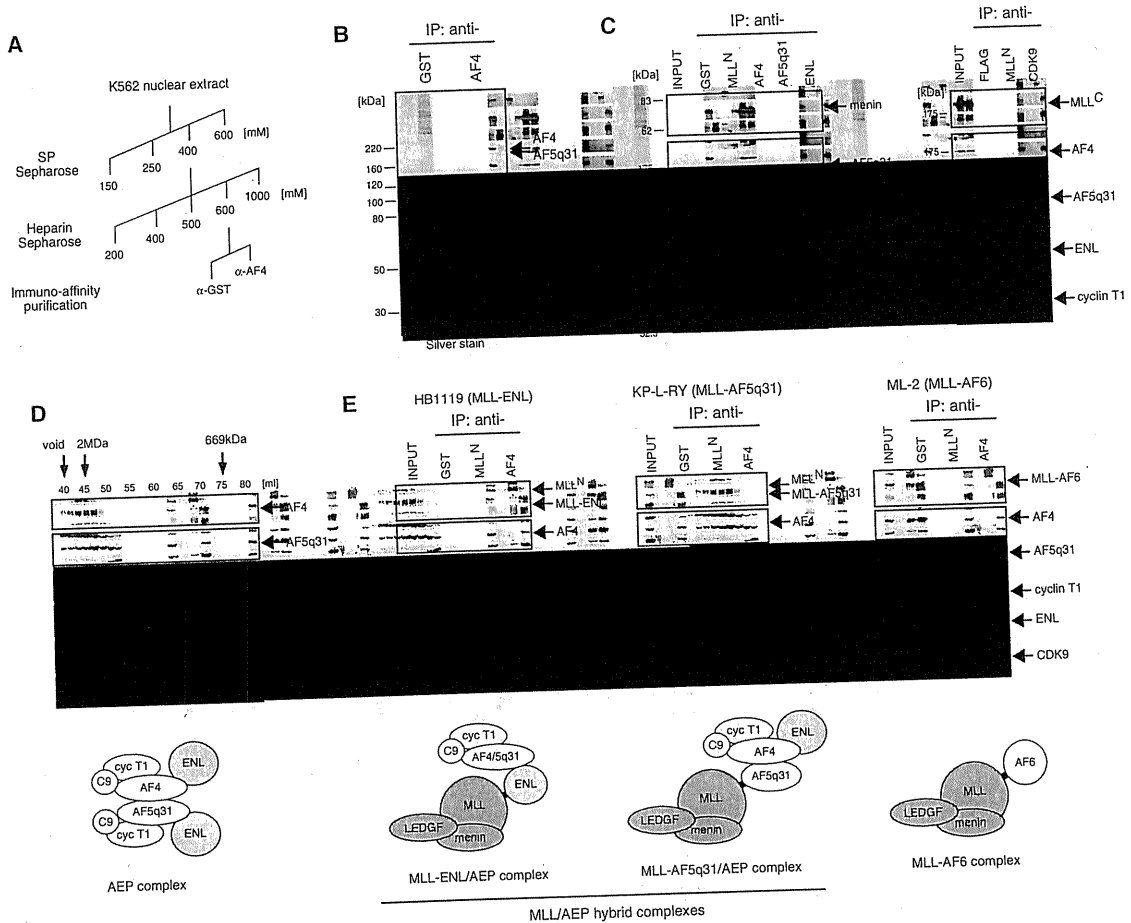


Figure 1. Heterologous Associations of Wild-Type and Oncogenic AF4 and ENL Family Proteins

(A) The scheme used for purification of the AF4 complex. (B) A silver-stained image shows the proteins immuno-purified using anti-AF4 antibody and subsequently identified by mass spectrometry, as indicated by arrows on the right. Anti-GST antibody served as a negative control. (C) K562 nuclear extracts were analyzed by IP western blotting. IP was performed with the antibodies indicated on the top, and the precipitates were immuno-blotted with the antibodies indicated on the right. Anti-GST and anti-FLAG antibodies served as negative controls. Asterisks indicate signals from IgG used for IP. (D) Selected fractions from gel filtration analysis of K562 nuclear extracts were analyzed by western blotting for AF4-associated factors (PARP served as a negative control). Molecular weight standards are shown on the top. A cartoon of a putative AEP complex is depicted. C9, CDK9; cyc T1, cyclin T1. (E) IP western blot analysis was performed as in (C) on human leukemia cell lines that harbor MLL chromosomal translocations and express MLL chimeric oncoproteins (indicated at tops). Cartoons of putative MLL fusion complexes are depicted below. See also Figure S1.

Figure S3A), as well as an interaction assay based on GAL4-dependent transactivation (Figure S3B).

MLL fusion proteins containing the respective subregions of AF4 or AF5q31 were assessed for their oncogenic potentials in a myeloid progenitor transformation assay (Figure 3D) (Lavau et al., 1997). Only MLL-AF5q31 constructs containing subregion 4 (MLL-AF5-4 and MLL-AF5-34) induced serial replating activity and up-regulation of *Hoxa9* transcription (Figures 3E and 3F). This result indicates that none of the single functions (i.e., P-TEFb recruitment, transactivation, or association with ENL) is sufficient for transformation but rather CHD-mediated association with endogenous AEP is required. The corresponding MLL-AF4-4 and MLL-AF4-34 proteins were not stably expressed and thus unable to be evaluated (Figure 3F). Although recruitment of Enl was not sufficient for MLL-AF5q31-dependent transforma-

tion, Enl was required because its knockdown by sh-RNA substantially decreased the clonogenicity and *Hoxa9* expression of MLL-AF5q31-transformed cells (Figures 3G–3J). This phenotype was rescued by exogenous expression of human ENL, thus verifying the target specificity of the sh-RNA. Hence, formation of a higher order MLL/AEP hybrid complex on target genes is necessary for MLL-AF5q31-dependent transformation.

Transforming Properties of MLL-ENL and MLL-AF9 Correlate with Association with AF4 Family Proteins and DOT1L

A similar structure/function analysis of MLL-ENL demonstrated that C-terminal ENL residues (494–559) are required for the interaction with AF5q31 (Figures 4A and 4B). This region, which is evolutionarily conserved with AF9 and *Saccharomyces cerevisiae*