the PU.1-induced upregulation of CSF1R. Notably, 5 d after exposure to 4-HT, we detected $CSF1R^{high}$ and $CSF1R^{low}$ cells in the population of PUER cells expressing MOZ-TIF2, but only $CSF1R^{low}$ cells were in the control PUER cell population (**Fig. 4d**). We did not detect CSF1R expression before addition of 4-HT, even in PUER cells expressing MOZ-TIF2 (**Fig. 4d**), indicating that functional PU.1 is required for MOZ-TIF2–induced CSF1R expression. Chromatin immunoprecipitation (ChIP) analysis indicated that PU.1, MOZ-TIF2 and possibly endogenous MOZ were recruited to the *Csf1r* promoter in the bone marrow cells of mice with MOZ-TIF2–induced AML (**Supplementary Fig. 11a**). In PUER cells expressing MOZ-TIF2, recruitment of MOZ-TIF2 and MOZ to the *Csf1r* promoter was detected after 4-HT treatment, but not before the treatment (**Supplementary Fig. 11b**), suggesting that the recruitment of MOZ-TIF2 and MOZ is dependent upon functional PU.1.

To determine whether PU.1 is essential for the development of MOZ-TIF2–induced AML, we infected wild-type and $Sfpi1^{-/-}$ fetal liver cells of E12.5 littermates with retroviruses encoding MOZ-TIF2 or N-Myc and transplanted them into irradiated mice. Although mice transplanted with $Sfpi1^{-/+}$ cells expressing MOZ-TIF2 developed AML 8–14 weeks after transplantation, mice transplanted with $Sfpi1^{-/-}$ cells were healthy for at least 6 months (**Fig. 4e**). In contrast, all mice transplanted with either wild-type or $Sfpi1^{-/-}$ cells expressing N-Myc developed AML 6–10 weeks after transplantation (**Fig. 4f**). When both PU.1 and MOZ-TIF2 were introduced into PU.1-deficient fetal liver cells, the transplanted mice developed leukemia (**Fig. 4g**). However, introduction of either PU.1 or MOZ-TIF2 alone was not sufficient for AML induction. Thus, we conclude that PU.1 is required for the initiation of MOZ-TIF2–induced AML.

To determine whether PU.1 is also required for the maintenance of MOZ-TIF2–induced AML, we infected fetal liver cells of PU.1 conditional knockout mice ($Sfpi1^{flox/flox}$ and expressing estrogen receptor (ER)-Cre) with MOZ-TIF2 and transplanted them into irradiated recipient mice, which developed AML. We next transplanted bone marrow cells of these mice into irradiated secondary recipients and then treated half of the mice with tamoxifen to induce PU.1 deletion. All of the control mice died of AML within 6 weeks, but none of the tamoxifen-treated mice developed AML for at least for 6 months (**Fig. 4h**). These results indicate that PU.1 is also required for the maintenance of MOZ-TIF2–induced AML stem cells.

Taken together, our results indicate that MOZ and its leukemia-associated fusion proteins activate PU.1-mediated transcription of the monocyte-specific gene *Csf1r*. MOZ fusion proteins might constitutively stimulate high *Csf1r* expression to induce AML (**Fig. 4i**). In contrast, we previously found that MOZ fusion proteins inhibit AML1-mediated activation of granulocyte-specific *Mpo* gene transcription[18]. Because MOZ fusion proteins are associated with monocytic leukemia, commitment to the monocytic lineage may be determined by differential regulation of target genes by MOZ fusion proteins (that is, upregulation of monocyte-specific genes such as *Csf1r* and downregulation of granulocyte-specific genes such as that encoding myeloperoxidase). It is also likely that the normal MOZ protein modulates *Csf1r* expression to an appropriate level to regulate normal hematopoiesis (**Fig. 4i**), as *Csf1r* expression was impaired in $MOZ^{-/-}$ fetal liver cells (**Supplementary Fig. 12**).

Although AML induction was suppressed in mice transplanted with $Csf1r^{-/-}$ cells, half of these mice developed AML, albeit at a longer latency. Thus, MOZ-TIF2 can provoke either a rapid induction of AML in a CSF1R-dependent manner or a slower induction in a CSF1R-independent manner. There are several possibilities to explain this CSF1R independence. First, we observed increased HoxA9 expression in both $CSF1R^{high}$ and $CSF1R^{low}$ cells. HoxA9 overexpression is reportedly not sufficient to induce AML and additional mutations or oncogene activation is required for AML induction in this context[21,22]. Thus, MOZ-TIF2–transfected $Csf1r^{-/-}$ cells might require additional mutations to induce leukemia. Second, because we used a retrovirus vector to introduce MOZ-TIF2, it is possible that oncogene activation by retroviral integration might mediate AML pathogenesis.

In conclusion, our results indicate that PU.1-mediated upregulation of *Csf1r* is crucial for leukemia stem cell potential induced by MOZ-TIF2. Our findings add to previous work associating CSF1R with AML. CSF1R upregulation has been reported in human[23–25] and mouse[26] AML. CSF1R is also known as the oncoprotein c-Fms, and transplantation of bone marrow cells expressing the v-fms oncoprotein induces multilineage hematopoietic disorders[27]. A chromosomal translocation resulting in expression of a fusion protein in which RNA-binding motif protein-6 (RBM6) is fused to CSF1R has recently been reported to be associated with AML[28]. CSF1R may thus be crucial for not only leukemia induced by MOZ fusions but also a wider subset of AML.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We would like to thank D.E. Zhang for the CSF1R promoter mutant lacking PU.1-binding sites, Y. Kamei and A. Iwama for MOZ-TIF2 cDNA, H. Ichikawa for N-MYC cDNA, T. Taya for SaOS2 cells (National Cancer Center Research Institute) and A. Kuchiba for help with statistical analyses. This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor and Welfare and from the Japanese Ministry of Education, Culture, Sports, Science and Technology (I.K.), by the Program for Promotion of Fundamental Studies from the National Institute of Biomedical Innovation of Japan (I.K.), and by US National Institutes of Health grants R01-CA41456 (D.G.T.), CA32551 and 5P30-CA13330 (E.R.S.).

AUTHOR CONTRIBUTIONS

Y.A., I.K., T.K. and M.S. conducted experiments in AML mice. Y.A., H. Shima and I.K. performed western blotting, immunoprecipitation, GST pull down, ChIP and reporter assays. P.Z. and D.G.T. conducted experiments in PU.1-deficient mice. E.R.S. designed and performed experiments in CSF1R-deficient mice. K.T. and E.J. analyzed expression of CSF1R in human AML cells. H. Singh designed and performed experiments in PUER cells. H.O. prepared Ki20227. I.K. and Y.A. analyzed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Human subjects, mice and cells. The study involving human samples was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent. C57BL/6 mice were purchased from CREA Japan. NGF-FKBP-Fas transgenic mice[16] (Jackson Laboratories), *Csf1r*-deficient mice[22] (provided by E.R.S.), PU.1-null (*Sfpi1*$^{-/-}$) and PU.1 conditionally deficient (*Sfpi1* floxed) mice[23] (provided by D.G.T.), CreERT2 knock-in mice (TaconicArtemis GmbH)[24] and MOZ-deficient mice[5] were backcrossed to C57BL/6 mice at least five times. Mouse experiments were performed in a specific pathogen–free environment at the Japan National Cancer Center animal facility according to institutional guidelines and with approval of the Japan National Cancer Center Animal Ethics Committee. PUER cells[20] were provided by H. Singh.

Generation of acute myeloid leukemia mouse models. MSCV-MOZ-TIF2-IRES-EGFP, MSCV-N-Myc-IRES-EGFP, MSCV-CSF1R-pgk-pac and MSCV-PU.1-pgk-pac constructs were generated by inserting cDNAs encoding MOZ-TIF2, N-Myc, CSF1R or PU.1 into the appropriate vector. The constructs were transfected into Plat-E cells[31] cells using the FuGENE 6 reagent (Roche Diagnostics) and supernatants containing retrovirus were collected 48 h after transfection. c-Kit$^+$ cells (1×10^5 cells) were selected from bone marrow or fetal liver cells using CD117-specific MicroBeads (Miltenyi Biotec); the cells were then incubated with retroviruses using RetroNectin (Takara Bio) for 24 h in StemPro-34 serum-free medium (Invitrogen) containing cytokines (20 ng ml^{-1} stem cell factor (PeproTech), 10 ng ml^{-1} interleukin-6 (PeproTech), 10 ng ml^{-1} interleukin-3 (a gift from Kirin Pharmaceuticals)). The infected cells were then transplanted together with bone marrow cells (2×10^5) into lethally irradiated (9 Gy) 6- to 8-week-old C57BL/6 mice by intravenous injection. Secondary transplants were performed by intravenous injection of bone marrow cells from primary AML mice into sublethally irradiated (6 Gy) C57BL/6 mice.

Administration of AP20187, imatinib or Ki20227. AP20187 (a gift from Ariad Pharmaceuticals; 10 mg per kg body weight) was administered daily by intravenous injection for 5 d, and then 1 mg per kg body weight AP20187 was administered every 3 d thereafter as described previously[10]. Mice were orally administered imatinib mesylate (Novartis Pharmaceuticals; 100 mg per kg body weight), Ki20227 (ref. 13) (a gift from Kirin Pharmaceuticals; 20 mg per kg body weight) or solvent twice daily from 7 d after transplantation.

Immunofluorescent staining, detection of side population cells, flow cytometric analysis and cell sorting. Bone marrow cells from mice with AML were preincubated with rat IgG and then incubated on ice with the following staining reagents: antibody to CD115 (AFS98) conjugated to phycoerythrin (PE) (eBioscience), antibody to Mac-1 (M1/70) conjugated to PE-Cy7 (eBioscience), antibody to Gr-1(RB6-8C5) conjugated to allophycocyanin (APC) (BD Pharmingen) and antibody to c-Kit (2B8) conjugated to APC (BD Pharmingen). For the detection of side population cells, bone marrow cells were stained with 5 μg ml^{-1} Hoechst 33342 in the presence or absence of 50 μM verapamil at 37 °C for 60 min. Flow cytometric analysis and cell sorting were performed using the JSAN cell sorter (Baybioscience) and the results were analyzed with FlowJo software (Tree Star).

Reporter analysis. *CSF1R*-luciferase constructs were generated by insertion of *CSF1R* promoter constructs, either wild type or lacking the PU.1-binding site[32], into pGL4.10 (luc2) (Promega). SaOS2 cells (a gift from T. Taya) were transfected with *CSF1R*-luciferase constructs and pGL4.75 (hRL-CMV) (Promega) together with various expression constructs (pLNCX-AML1 (ref. 18), pLNCX-PU.1 (ref. 33), pLNCX-MOZ[18], pLNCX-MOZ-TIF2 (ref. 18) and pLNCX-MOZ-CBP[18]) in 24-well plates, and luciferase activity was assayed 24 h after transfection using the microplate luminometer GLOMAX (Promega). The results shown for the reporter assays represent average values for relative luciferase activity generated from at least three independent experiments; relative values were obtained by normalizing to the luciferase activity of phRL-CMV, which served as an internal control.

Immunoprecipitation and immunoblotting. For Flag tag immunoprecipitation experiments, cells were lysed in a lysis buffer containing 250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1% NP-40, 5 mM dithiothreitol, 1 mM phenylmethanesulfonylfluoride and Complete protease inhibitor (Roche). Cell lysates were incubated with Flag-specific antibody–conjugated agarose beads (Sigma) and rotated at 10 r.p.m. (TAITEC RT-50) at 4 °C overnight. The adsorbed beads were washed three times with lysis buffer. Precipitated proteins were eluted from the beads by Flag peptide and dissolved with the same volume of 2× SDS sample buffer. When immunoprecipitation was not performed, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence with ECL plus Detection Reagents (Amersham Biosciences). The primary antibodies used in this study were Flag-specific antibody (M2) (Sigma), hemagglutinin-specific antibody (3F10) (Roche) and MOZ-specific antibody[18], which was generated by immunizing rabbit with peptides corresponding residue 441–460 of human MOZ.

GST pull-down assay. The HindIII-ClaI fragment corresponding to the N-terminal region (1–664) of MOZ was cloned into the pSP64polyA vector. [^{35}S]-MOZ (1–664) was produced by incubating pSP64polyA-MOZ with [^{35}S]-methionine using the TNT Coupled Rabbit Reticulocyte Lysate System (Promega). pGEX-6P-PU.1 and pGEX-6P-AML1 were generated by subcloning full-length human PU.1 and AML1 cDNAs into pGEX-6P (GE Healthcare). GST, GST-PU.1 and GST-AML1 were produced in *Escherichia coli* BL21 containing pGEX-6P, pGEX-6P-PU.1 and pGEX-6P-AML1, respectively. The [^{35}S]-MOZ (1-664) protein was incubated with GST-, GST-PU.1– or GST-AML1–conjugated glutathione-agarose at 4 °C for 60 min in lysis buffer, washed three times with lysis buffer, analyzed by SDS-PAGE and detected by autoradiography.

Statistical analyses. We performed unpaired two-tailed Student's *t* tests for comparisons and a log-rank test for survival data with JMP8 software (SAS Institute).

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doi:10.1038/nm.2122

Cancer Cell

A Higher-Order Complex Containing AF4 and ENL Family Proteins with P-TEFb Facilitates Oncogenic and Physiologic MLL-Dependent Transcription

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DOI 10.1016/j.ccr.2009.12.040

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 Rabbitts, 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 effecter 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 immunoaffinity 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

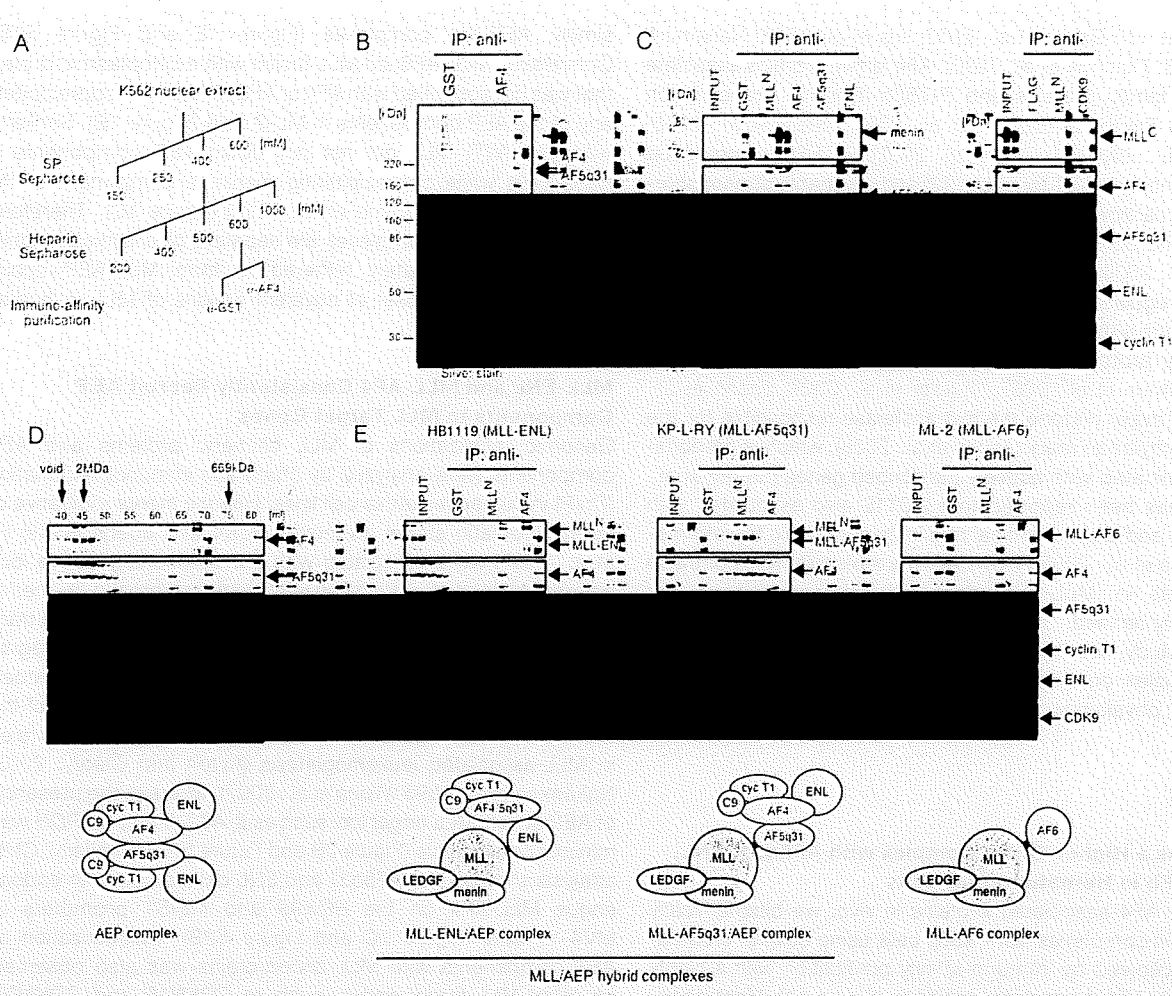


Figure 1. Heterologous Associations of Wild-Type and Oncogenic AF4 and ENL Family Proteins

(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 immunoblotted 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 transformation, 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 evolutionally conserved with AF9 and *Saccharomyces cerevisiae*

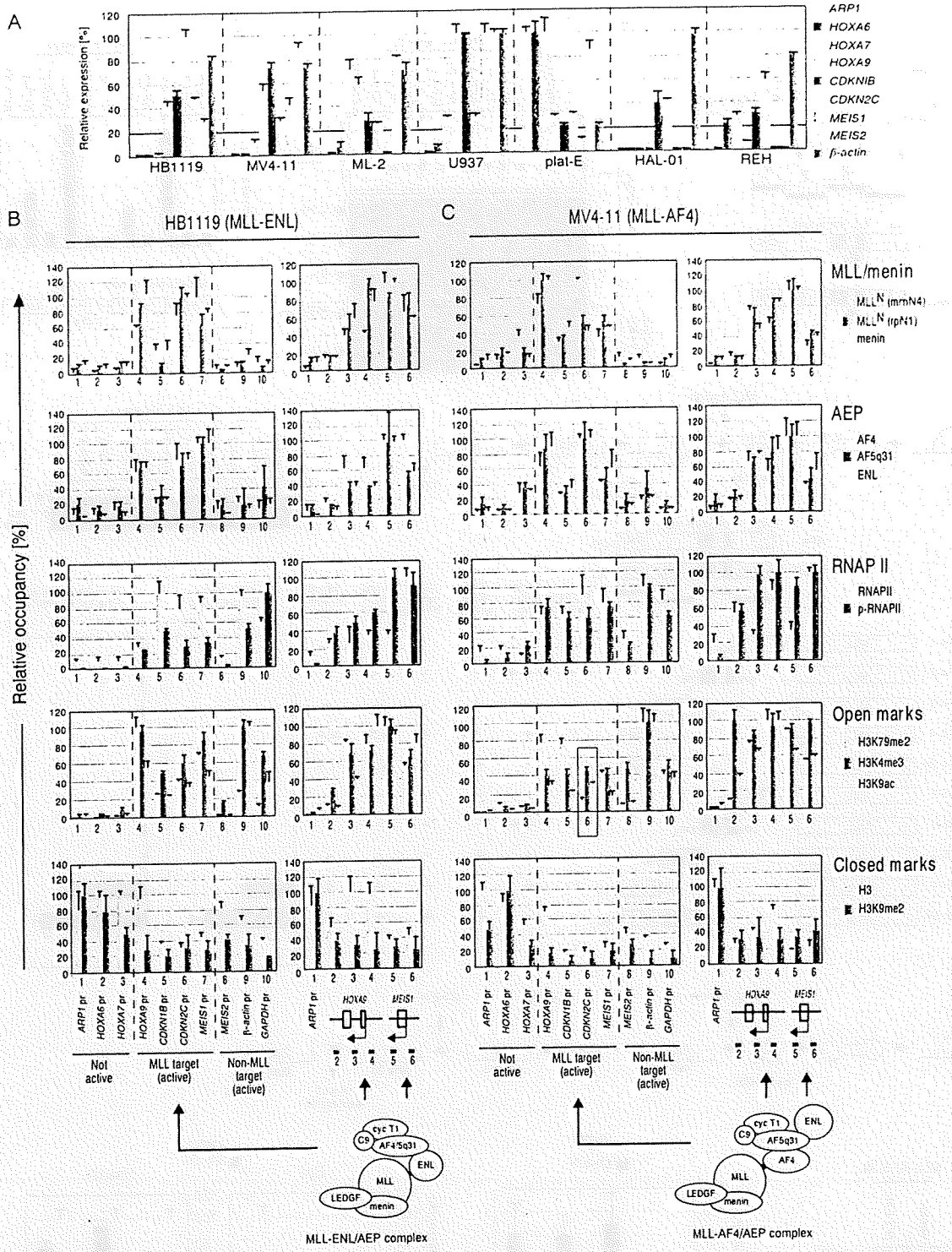
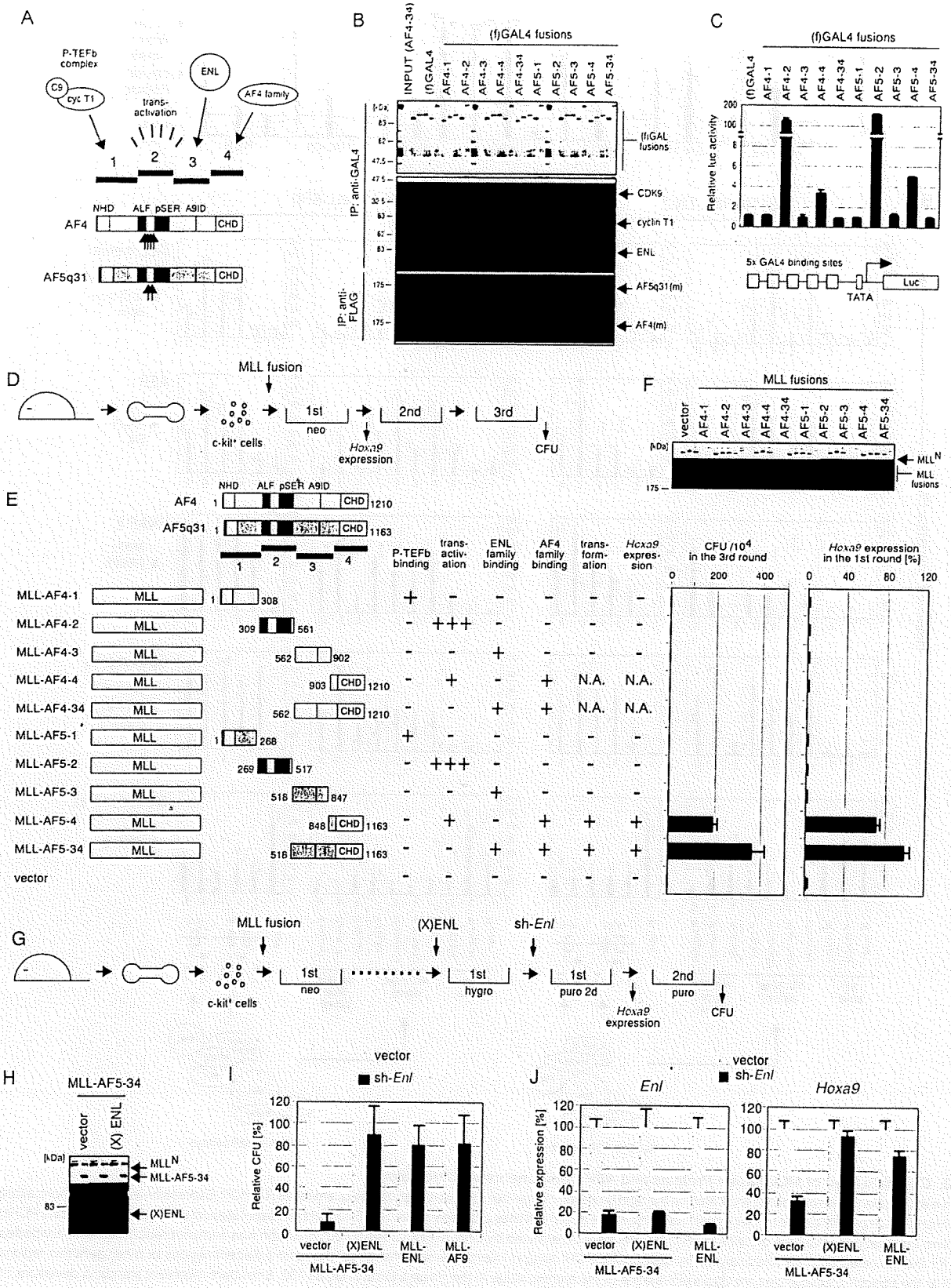


Figure 2. Colocalization of MLL Fusion Proteins and AEP Components on Chromatin

(A) Relative expression of various genes (indicated on the right) in seven human cell lines was analyzed by quantitative RT-PCR. Expression levels were normalized to *GAPDH* and are depicted relative to the highest value among the seven cell lines arbitrarily set as 100. Error bars represent standard deviations of triplicate PCRs.

(B) Genomic localizations of various proteins in HB1119 cells were determined by ChIP assay. Cross-linked chromatin was immunoprecipitated with antibodies specific for the indicated proteins and analyzed by quantitative PCR using primer/probe sets that target promoter-adjacent regions or other genomic regions indicated at the bottom. Occupancies are displayed relative to the highest value in the group arbitrarily set as 100. Error bars represent standard deviations of triplicate PCRs. Genes expressed more than 20% of the highest levels in (A) are defined as active genes.

(C) A comparable analysis as in (B) was performed for MV4-11 cells, which harbor a t(4;11) translocation and express MLL-AF4 proteins. The purple rectangle highlights a locus on which dimethyl H3K79 marks were absent, but the MLL-AF4/AEP complex was present. See also Figure S2.


A
P-TEFb complex
C9
cyc T1
ENL
AF4 family
trans-activation
1
2
3
4
NHD
ALF
pSER
A9ID
CHD
AF4
AF5q31
B
(f)GAL4 fusions
INPUT (AF4-34)
(f)GAL4
AF4-1
AF4-2
AF4-3
AF4-4
AF4-34
AF5-1
AF5-2
AF5-3
AF5-4
AF5-34
IP: anti-GAL4
IP: anti-FLAG
(f)GAL fusions
CDK9
cyclin T1
ENL
AF5q31(m)
AF4(m)
C
(f)GAL4 fusions
Relative luc activity
5x GAL4 binding sites
Luc
TATA
D
MLL fusion
c-kit+ cells
1st
neo
2nd
3rd
Hoxa9 expression
CFU
E
AF4
AF5q31
MLL-AF4-1
MLL-AF4-2
MLL-AF4-3
MLL-AF4-4
MLL-AF4-34
MLL-AF5-1
MLL-AF5-2
MLL-AF5-3
MLL-AF5-4
MLL-AF5-34
vector
P-TEFb binding
trans-activ-ation
ENL family binding
AF4 family binding
trans-form-ation
Hoxa9 expres-sion
CFU /10^4 in the 3rd round
Hoxa9 expression in the 1st round [%]
F
MLL fusions
vector
MLL^N
MLL fusions
G
MLL fusion
(X)ENL
sh-Enl
c-kit+ cells
1st
neo
1st
hygro
1st
puro 2d
2nd
puro
Hoxa9 expression
CFU
H
MLL-AF5-34
vector
(X) ENL
MLL^N
MLL-AF5-34
(X)ENL
I
vector
sh-Enl
Relative CFU [%]
vector
(X)ENL
MLL-ENL
MLL-AF9
MLL-AF5-34
J
Enl
Hoxa9
vector
sh-Enl
Relative expression [%]
vector
(X)ENL
MLL-ENL
MLL-AF5-34

Anc1 (designated AHD: Anc1 homology domain), displayed transactivation potential that correlated with association with AF4 family proteins (Figure 4C). The AHD of ENL also mediated association with DOT1L (Figure 4D), consistent with results of previous studies (Mueller et al., 2007). Mutations of MLL-ENL that abolished AF5q31 and DOT1L interaction (including a single L550E point mutation) resulted in failure to up-regulate *Hoxa9* transcription and transform myeloid progenitors (Figures 4E–4G). Similarly, the portion of AF9 retained in MLL-AF9 oncoproteins, which includes AHD (residues 502–568) (Figure 4A), mediated AF5q31 and DOT1L association and conferred GAL4-dependent transactivation, MLL-dependent *Hoxa9* expression, and myeloid transformation (Figures 4B–4G). Unlike MLL-AF5q31-transformed cells, MLL-ENL- and MLL-AF9-transformed cells did not require WT Enl because their clonogenicities were unaffected by its knockdown (Figures 3I and 3J), consistent with the observation that MLL-ENL did not form a complex with WT ENL in HB1119 cells (Figure 1E). These results suggest that association with AF4 family proteins and/or DOT1L is required for the oncogenic properties of MLL-ENL and MLL-AF9.

Interactions of ENL with DOT1L or AF4 Family Proteins Are Mutually Exclusive

To determine whether ENL can simultaneously coassociate with AF4 family proteins and DOT1L, IP analysis was performed on cells transiently expressing ENL, AF5q31, and DOT1L. Although ENL coprecipitated both AF5q31 and DOT1L, the latter two did not pull down each other (Figure 5A), indicating that the three proteins do not form a trimeric complex. Similarly, GAL4-AF5-3 effectively coprecipitated ENL but not DOT1L under conditions where GAL4-ENL successfully pulled down DOT1L (Figure S4). These data demonstrate that the associations of ENL family proteins with AF4 family proteins or DOT1L are mutually exclusive. Therefore, the ENL/DOT1L complex is a separate entity from AEP (Figure 5B).

Recruitment of AEP, versus DOT1L, Plays a Predominant Role in MLL-Dependent Leukemogenesis

The ability of MLL-ENL to associate with AF4 family proteins or DOT1L raised the issue of which interaction (MLL-ENL/AEP vs. MLL-ENL/DOT1L) is essential for leukemic transformation (Figure 5B). To address this issue, an artificial MLL fusion with DOT1L (MLL-DOT1L) that does not associate with AF4 (Figure 5C) but retains the HMT catalytic domain (thus mimics the MLL-ENL/DOT1L complex) was assessed for its transformation potential. MLL-DOT1L failed to sufficiently activate *Hoxa9* expression to immortalize myeloid progenitors (Figures 5D and 5E), despite the comparable levels of protein expression in packaging cells (Figure 5F) and mRNA expression in first-round colonies (Figure 5E). In the same experimental condition, MLL-AF5q31 successfully transformed myeloid progenitors (Figure 5E) without being able to directly associate with DOT1L (Figure 5C). These results, which contrast with those of previous studies (Okada et al., 2005), indicate that simple recruitment of DOT1L HMT activity alone to MLL target genes is not sufficient for transformation and support a more predominant role for AEP recruitment.

Nevertheless, DOT1L-dependent H3K79 methylation colocalized with the presence of MLL-ENL at all target loci tested in HB1119 cells (Figure 2B), indicating that not only AEP components but also DOT1L is consistently recruited by MLL-ENL. In MV4-11 cells, H3K79 methylation marks also colocalized at most of the MLL-AF4-occupied loci, consistent with previous observations (Krivtsov et al., 2008; Guenther et al., 2008), despite the apparent inability to directly recruit DOT1L (Figures 2C and 5C). However, the signal intensities of H3K79 dimethylation were relatively low at MLL-AF4-target loci, compared with those at MLL-ENL-target loci (compare relative intensities to those of *β-ACTIN* and *GAPDH*, which served as internal standards) (Figures 2B and 2C; Figure S2) and were minimal at the *CDKN2C* promoter in spite of the localization of abundant AEP components (Figure 2C, purple rectangle). Thus, DOT1L-dependent

Figure 3. Formation of an AEP-Like Complex Is Required for MLL-AF5q31-Dependent Myeloid Transformation

(A) The structures of AF4 and AF5q31 are schematically illustrated. Subregions (1–4) of AF4 and AF5q31 are indicated with associated functions. Upward arrows indicate the sites of fusion with MLL in human leukemia oncoproteins (Jansen et al., 2005) (A9ID, AF9 interaction domain; Srinivasan et al., 2004).

(B) The four subregions fused to GAL4 DNA binding domain were expressed in 293T cells (upper four panels) or coexpressed with myc-tagged AF4 or AF5q31 [AF4(m) or AF5q31(m)] (lower two panels) and analyzed by IP western blotting. IP antibodies are indicated on the left and proteins detected by western blotting are indicated on the right. (f)GAL4 fusions and myc-tagged AF4 family proteins were visualized with anti-FLAG and anti-myc antibodies, respectively.

(C) Transactivation activity of respective GAL4 fusions was analyzed using the reporter gene shown below. Error bars represent standard deviations from triplicate analyses.

(D) The experimental scheme of myeloid progenitor transformation assays to evaluate the oncogenic potentials of various MLL mutants shows the time points at which CFU (colony forming unit) activity or *Hoxa9* expression was examined.

(E) The structures of various MLL-AF4/AF5q31 mutants and their associated functions are summarized schematically. *Hoxa9* levels were normalized to *Gapdh* and displayed relative to *MLL-AF5-34*-transduced cells arbitrarily set at 100%. Error bars represent standard deviations of three independent analyses (left) or triplicate PCRs (right). N.A., not applicable because of unstable expression of MLL fusion proteins.

(F) Protein levels of respective MLL mutants in virus-packaging cells were examined by western blotting with anti-MLL^N antibody. MLL-AF4-4 and MLL-AF4-34 proteins were not stably expressed.

(G) The experimental scheme to evaluate the effect of *Enl* knockdown on MLL transformation is shown schematically. (X)ENL, Xpress-tagged human ENL.

(H) Transduced myeloid progenitors were analyzed by western blotting with anti-MLL^N (top) and anti-Xpress (bottom) antibodies to detect exogenous MLL-AF5q31 and human (X)ENL, respectively.

(I) The clonogenic potentials of MLL-AF5-34-transformed cells transduced with or without *(X)ENL* are shown at the second plating after sh-RNA transduction (vector or sh-*Enl*). MLL-ENL- or MLL-AF9-transformed cells were also subjected to sh-RNA transduction for comparison. CFUs are expressed relative to the vector control arbitrarily set as 100. Error bars represent standard deviations of three independent analyses.

(J) Cells from first-round colonies following sh-RNA transduction (vector or sh-*Enl*) were analyzed by RT-PCR for expression of endogenous *Enl* or *Hoxa9*. Expression levels were normalized to *Gapdh* and displayed relative to the vector/vector control cells arbitrarily set at 100. Error bars represent standard deviations of triplicate PCRs. See also Figure S3.

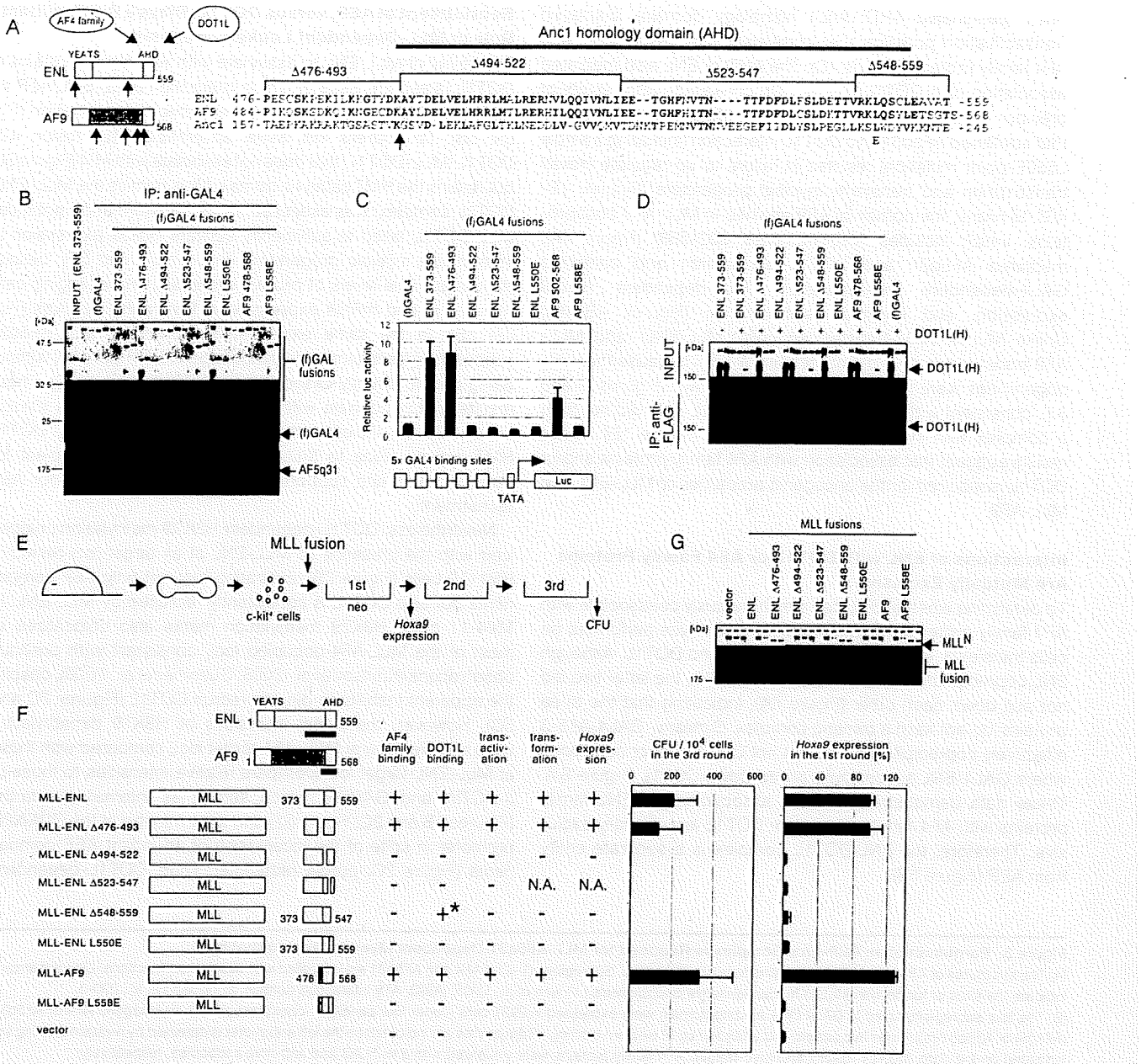


Figure 4. MLL-ENL and MLL-AF9 Transform Myeloid Progenitors via the AHD, which Is Responsible for Association with AF4 Family Proteins and DOT1L

(A) The structures of ENL and AF9 are schematically illustrated with associated functions (Zeisig et al., 2005). Aligned amino acid sequences for the minimum transformation domain are also shown with the positions of deletion or substitution mutations and AHD. Upward arrows indicate the sites of fusion with MLL in human leukemia oncoproteins (Jansen et al., 2005).

(B) Domain mapping of ENL family proteins for association with AF5q31 was performed with FLAG-tagged GAL4 fusion constructs of ENL (372–559 aa) and AF9 (478–568 aa). IP was performed with anti-GAL4 antibody, and the precipitates were immunoblotted with anti-FLAG antibody for (f)GAL4 fusions or anti-AF5q31 antibody for endogenous AF5q31.

(C) Transactivation activity of indicated GAL4 constructs was analyzed by luciferase assay as in Figure 3C.

(D) The same set of GAL4 fusion proteins used in (B) and HA-tagged DOT1L [DOT1L(H)] were coexpressed in 293T cells and analyzed by IP western blotting. IP was performed with anti-FLAG antibody and the precipitates were immunoblotted with anti-HA antibody.

(E) The experimental scheme is shown for myeloid progenitor transformation assays to evaluate the oncogenic potentials of MLL mutants.

(F) The structures of MLL-ENL and MLL-AF9 mutants and their associated functions are summarized with schematic representations. *Hoxa9* expression levels were normalized to *Gapdh* and displayed relative to the *MLL-ENL*-transduced cells arbitrarily set at 100%. Error bars represent standard deviations of three independent analyses (left) or triplicate PCRs (right). N.A., not applicable because of unstable expression of MLL fusion proteins. The asterisk indicates that association of ENL Δ548–559 mutant with DOT1L was detected but reduced substantially, compared with WT ENL.

(G) Protein levels of respective MLL mutants in virus packaging cells were examined by western blotting with anti-MLL^N antibody. MLL-ENL Δ523–547 was not stably expressed.

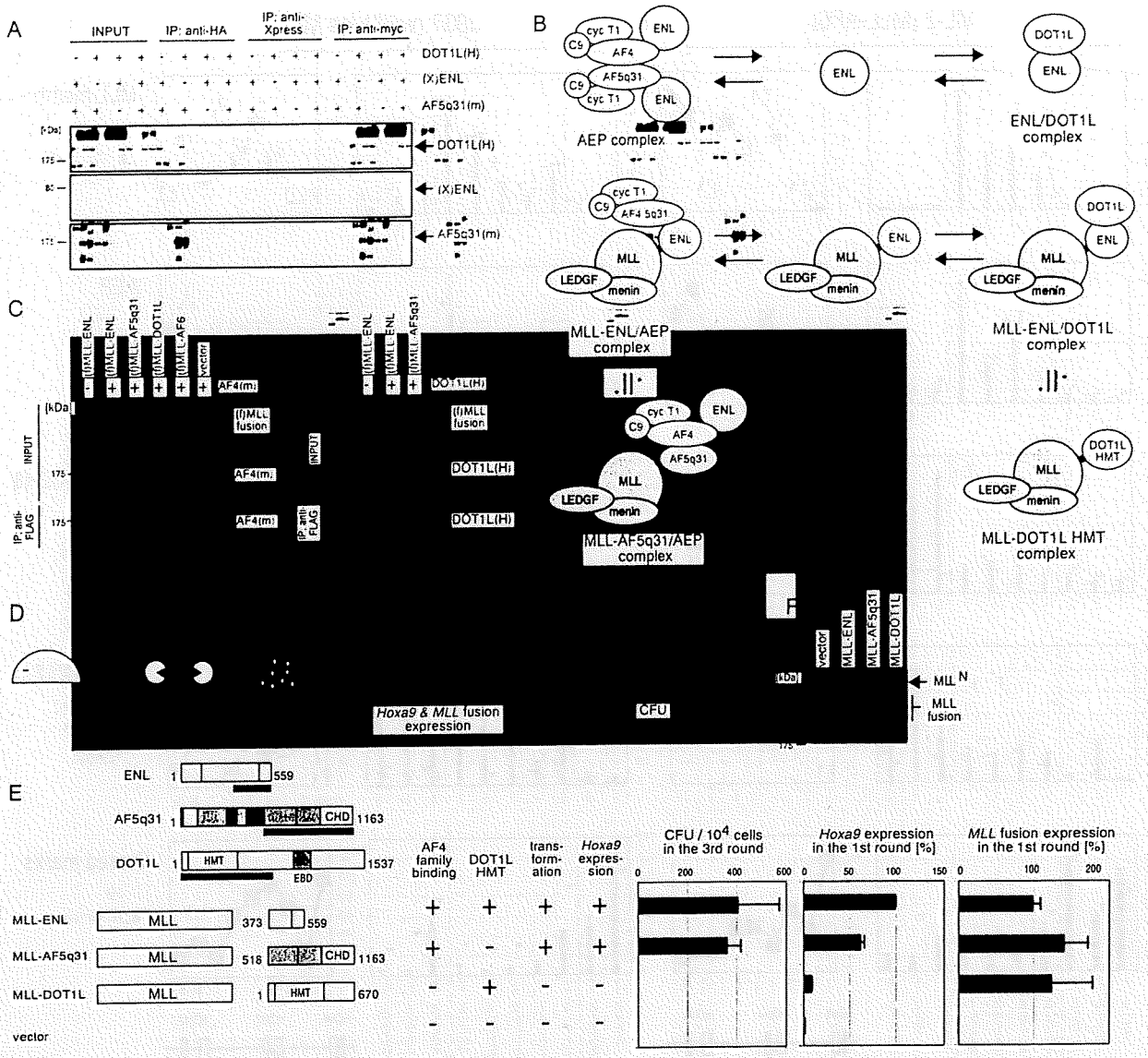


Figure 5. Associations of ENL Family Proteins with AF4 Family Proteins or DOT1L Are Mutually Exclusive

(A) AF5q31(m), (X)ENL, and DOT1L(H) were coexpressed in 293T cells and analyzed by IP western blotting. IP was performed with antibodies indicated on the top, and the precipitates were immunoblotted with anti-myc, anti-Xpress, or anti-HA antibody.

(B) Putative conformations of various ENL complexes are shown schematically. ENL forms two distinct complexes: AEP and ENL/DOT1L. Similarly, MLL-ENL participates in two mutually exclusive associations to form the MLL-ENL/AEP and MLL-ENL/DOT1L complexes that are approximate to the MLL-AF5q31/AEP and MLL-DOT1L complexes, respectively.

(C) FLAG-tagged MLL fusion proteins [(f) MLL fusions] were coexpressed with AF4(m) or DOT1L(H) in 293T cells and were analyzed by IP western blotting. IP was performed with anti-FLAG antibody, and the precipitates were immunoblotted with anti-MLL^N, anti-myc, or anti-HA antibody.

(D) The experimental scheme is shown for myeloid progenitor transformation assays to evaluate the oncogenic potentials of MLL mutants.

(E) The structures of MLL-fusion proteins and their associated functions are summarized. Expression of *MLL* fusion genes or *Hoxa9* was examined by RT-PCR in first-round colonies. Expression levels were normalized to *Gapdh* levels and are displayed relative to the transcript levels in *MLL-ENL*-transduced cells arbitrarily set at 100. Error bars represent standard deviations of three independent analyses (left) or triplicate PCRs (middle and right). HMT, histone methyltransferase catalytic domain; EBD, ENL binding domain (Okada et al., 2005; Mueller et al., 2007).

(F) Protein levels of MLL fusions in virus packaging cells were analyzed by western blotting with anti-MLL^N antibody. See also Figure S4.

H3K79 methylation is associated with the presence of the MLL-AF4/AEP-hybrid complex, but the two distinct biochemical entities are not constitutively coupled. These results suggest that DOT1L is functionally linked to MLL-AF4 but normally recruited to target loci subsequent to AEP components.

AEP Is Indirectly Recruited to MLL-AF6-Occupied Loci to Sustain Transcription and Transformation

To investigate whether AEP involvement is restricted to MLL fusions with AF4 and ENL family proteins, ChIP analyses were performed on ML-2 cells. Surprisingly, MLL-AF6 colocalized

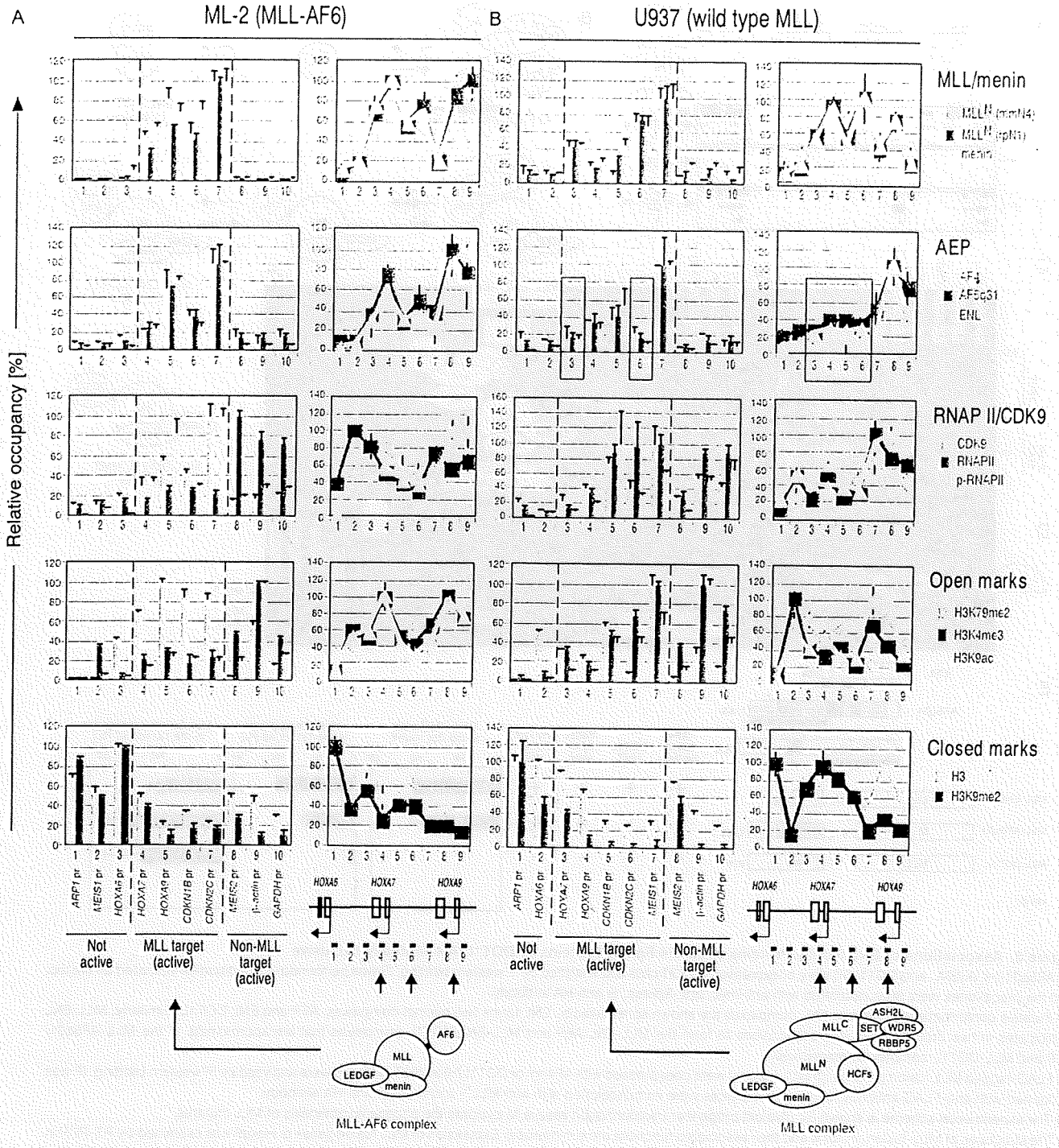


Figure 6. Indirect Recruitment of AEP to MLL-AF6- or WT MLL-Occupied Loci

(A and B) Genomic localizations of indicated proteins in ML-2 (A) and U937 (B) cells were determined by ChIP assay as in Figure 2B. The purple rectangle highlights regions where AEP is absent while the MLL complexes are present. ChIP data using anti-MLL^N (rpN1) and anti-menin antibodies are partially adapted from a previous report (Yokoyama and Cleary, 2008). See also Figure S5.

with AEP at the chromatin of MLL target genes (*HOXA7*, *HOXA9*, *CDKN1B*, and *CDKN2C*) (Figure 6A and Figure S5A) despite its inability to directly associate with AEP (Figures 1E and 5C). The occupancies of CDK9 and phosphorylated RNAPII coincided with the presence of AEP on MLL-AF6 target genes (Figure 6A). Characteristically, high levels of dimethyl H3K79 were associated with the presence of AEP, corroborating the functional link between AEP and DOT1L. These results suggest

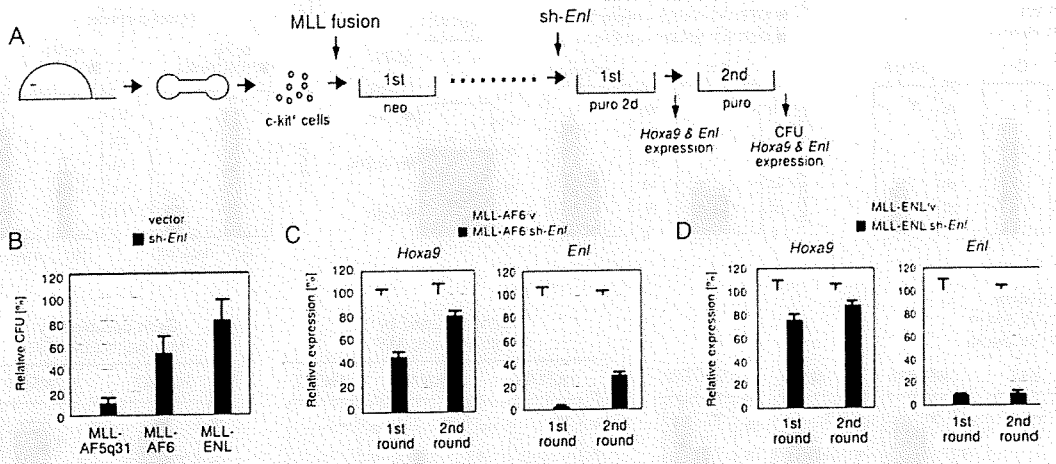


Figure 7. ENL Is Required for MLL-AF6-Dependent Transactivation and Transformation

(A) The experimental scheme to evaluate the effect of *Enl* knockdown on MLL transformation is shown.

(B) Clonogenic potentials are shown for myeloid cells transformed by MLL oncogenes (indicated below) at the second plating after sh-RNA transduction (vector or sh-*Enl*). CFU numbers are displayed relative to the vector control arbitrarily set as 100. Error bars represent standard deviations of three independent analyses.

(C) MLL-AF6-transformed cells from first- and second-round colonies following sh-RNA transduction (vector or sh-*Enl*) were analyzed by RT-PCR for expression of endogenous *Enl* or *Hoxa9*. Expression levels were normalized to *GAPDH* levels and are displayed relative to the transcript levels in vector control cells arbitrarily set as 100. Error bars represent standard deviations of triplicate PCRs.

(D) The same analysis as (C) was performed on MLL-ENL-transformed cells. Note that data in (B) and (D) are partially redundant with Figures 3I and 3J.

that the AEP complex can be recruited to MLL target loci via an indirect mechanism potentially serving a role in MLL-AF6-dependent leukemogenesis.

MLL-AF6-transformed cells were also dependent on Enl, because its knockdown reduced their clonogenicity and *Hoxa9* expression by 50% (Figures 7A and 7C). This was less severe, compared with MLL-AF5q31-transformed cells (Figure 7B), in part because of insufficient knockdown by the sh-RNA, since secondary colonies expressed *Hoxa9* at its normal levels accompanied with impaired knockdown of *Enl* (Figure 7C), indicating a selective proliferative advantage of cells in which *Enl* was incompletely knocked down (MLL-ENL served as a negative control in Figure 7D). Thus, transformation by MLL-AF6 is dependent on ENL, despite an inability to directly associate with AEP.

AEP Facilitates the Physiologic MLL-Dependent Transcriptional Pathway

The foregoing results prompted studies of a potential relationship of AEP in physiologic transcriptional regulation by WT MLL. ChIP analyses of U937 cells, which lack an MLL chromosomal translocation (Dreyling et al., 1996; Guenther et al., 2005), showed that AEP colocalized with WT MLL at the *HOXA9*, *MEIS1*, and *CDKN1B* promoters (Figure 6B and Figure S5B). However, in contrast to MLL leukemia cell lines, colocalization was not observed at all of the MLL-occupied loci in U937 cells. For instance, the MLL complex occupied both the *HOXA7* and *HOXA9* loci, whereas AEP associated only with the latter (Figure 6B, purple rectangle). A similar disparity was observed at the *CDKN2C* promoter. These results suggest that AEP is recruited to WT MLL-occupied loci in a context-dependent manner, as opposed to its constitutive recruitment in MLL leukemia cells. The presence of AEP correlated more closely with active transcriptional marks such as phospho-RNAPII and acetyl-histone H3K9 (e.g., the *HOXA7-9* locus), suggesting that AEP recruitment to MLL-targeted chromatin facilitates transcription.

The role of ENL in physiologic MLL-dependent transcriptional maintenance was assessed by knocking down *Enl* in mouse embryonic fibroblasts (MEFs), in which *Hoxc8* is a target gene of the Mll/menin complex (Figure 8A) (Hughes et al., 2004; Milne et al., 2002). *Enl* knockdown caused reduction of *Hoxc8* expression, which could be prevented by antecedent expression of exogenous human ENL (Figure 8B). Thus, Enl is required for physiologic transcriptional regulation by the Mll/menin complex. Moreover, Dot1l-mediated histone methylation was decreased at the Hoxc8 promoter in *Men1* null MEFs (Figure 8C), indicating that the MLL/menin complex functions upstream of ENL/DOT1L functions.

Furthermore, *ENL* knockdown in U937 cells caused down-regulation of *HOXA9*, *CDKN1B*, and *MEIS1*, whose genomic loci were occupied by both MLL and AEP complexes, but did not affect expression of genes occupied by the MLL complex without AEP (*HOXA7* and *CDKN2C*) (*AF5q31*, *MLL*, or *β-ACTIN* served as negative controls) (Figures 6B and 8D). Thus, ENL is specifically required for the optimal transcription of genes occupied by both MLL and AEP complexes.

DISCUSSION

Our biochemical purification of AF4 family proteins demonstrates that they normally associate with ENL and the P-TEFb elongation factor in an endogenous complex (AEP) in hematopoietic cells. MLL oncoproteins fused with AEP components (AF4 or ENL family proteins) nucleate formation of MLL/AEP hybrid complexes that constitutively occupy MLL-target chromatin. This aberrant recruitment of AEP components causes sustained activation of MLL target gene transcription and transformation of hematopoietic progenitors. Although the AEP and

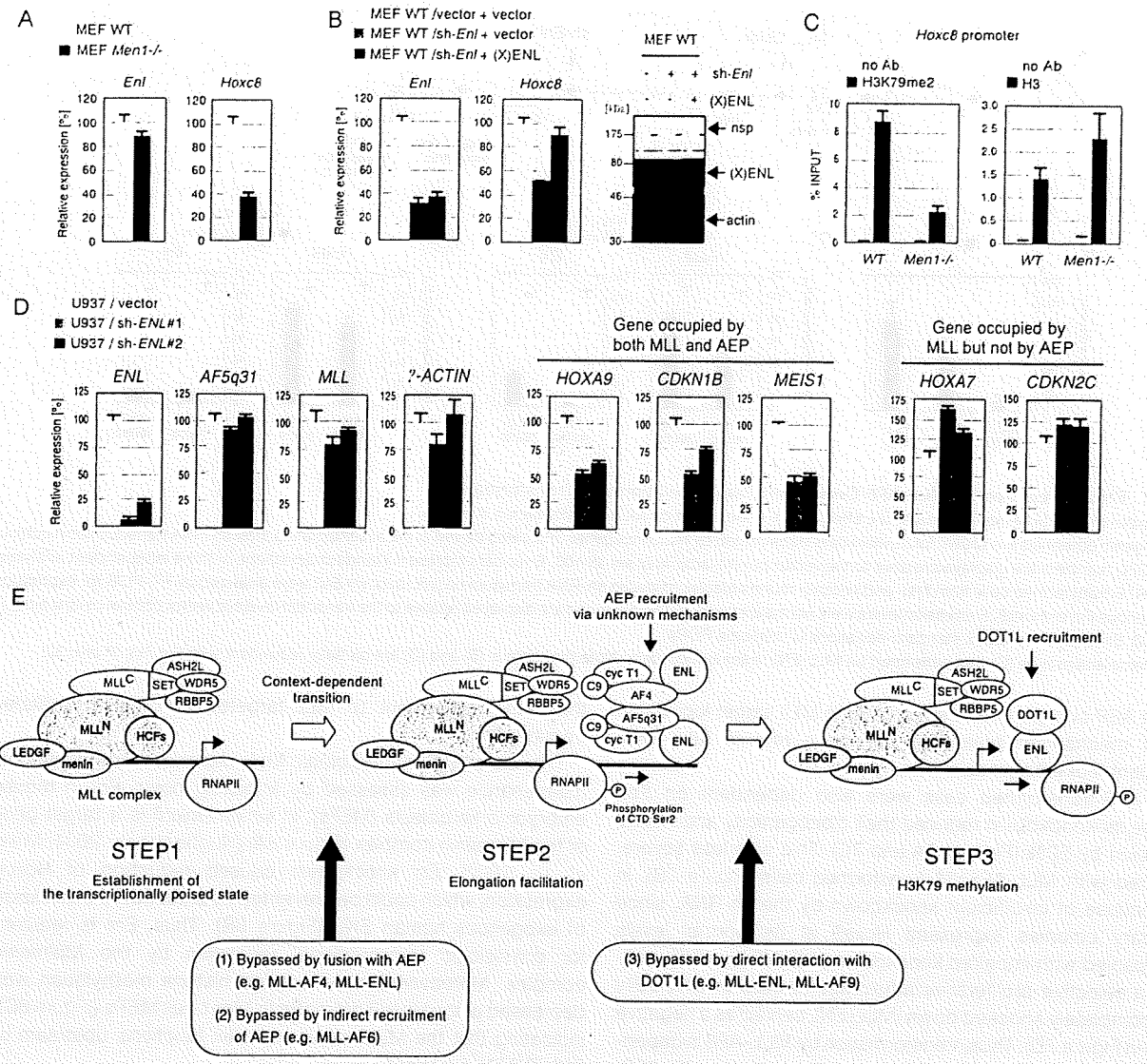


Figure 8. ENL Functions Downstream of Physiologic MLL-Dependent Transcriptional Pathways

(A) Expression levels of *Enl* and *Hoxc8* in WT or *Men1*$^{-/-}$ MEFs were determined by RT-PCR (normalized to *β-actin* levels and are displayed relative to the vector control arbitrarily set as 100). Error bars represent standard deviations of triplicate PCRs.

(B) Expression of *Enl* and *Hoxc8* with or without *Enl* knockdown/rescue was determined by RT-PCR. Expression levels were normalized to *β-actin* levels and are expressed relative to the vector/vector control arbitrarily set as 100. Error bars represent standard deviations of triplicate PCRs. Protein levels of the exogenously expressed (X)ENL (right panels) were assessed by western blotting with an anti-Xpress antibody (actin immunoblot served as a loading control). nsp, nonspecific band.

(C) ChIP assay was performed on WT or *Men1*$^{-/-}$ MEFs using anti-dimethyl H3K79 and histone H3 antibodies for the *Hoxc8* promoter-adjacent region and results displayed as relative ratio (%) to the input DNA. Error bars represent standard deviations of triplicate PCRs.

(D) The effects of *ENL*-knockdown are shown for two different sh-RNAs in U937 cells. Expression of various genes was analyzed by RT-PCR 4 days after transduction/puromycin selection. Expression values were normalized to *GAPDH* levels and displayed relative to the vector control arbitrarily set as 100. Error bars represent standard deviations of triplicate PCRs.

(E) A three-step model of MLL-dependent transcription.

MLL complexes are normally separate biochemical entities, our studies support a dependent role for the AEP complex in physiologic MLL target gene expression pathways, whose conditional recruitment mechanisms are often bypassed by leukemic MLL fusion proteins.

The AEP complex purified from leukemia cell lines under our experimental conditions contained ENL as an integral component but lacked a number of previously reported ENL-associated proteins, most notably the DOT1L histone methyltransferase (Mueller et al., 2007). Our domain-mapping analyses provide a molecular basis for its absence in that DOT1L and AF4 family proteins use the same binding surface within the AHD of ENL. Because of this physical constraint, DOT1L and AF4 family proteins are incapable of simultaneously associating with the

AHD to form an AF4/ENL/DOT1L trimeric complex. Therefore, retention of DOT1L in the AF4 complex previously identified in thymus homogenates (Bitoun et al., 2007) is likely mediated by other proteins (e.g., AF10 and RNAPII) but not by ENL/AF9. Our data suggest that an endogenous ENL/DOT1L complex and AEP normally exist as separate entities consistent with previous suggestions that ENL may participate in a mixture of different subcomplexes (Mueller et al., 2007).

A role for ENL in multiple subcomplexes raises the issue of which of its molecular interactions is essential for MLL leukemogenesis. This issue was addressed by assessing the oncogenic potential of MLL fused with the DOT1L catalytic domain, which effectively bypasses ENL. Contrary to a previous report (Okada et al., 2005), MLL-DOT1L was not sufficient for transactivation of MLL target genes and transformation of myeloid progenitors under our experimental conditions that read out the oncogenic properties of MLL-AF5q31 and MLL-ENL. This finding indicates that aberrant recruitment of AEP, not DOT1L, plays a primary rate-limiting role in transactivation and transformation by MLL fusion proteins, a conclusion further supported by structure/function analysis of MLL-AF5q31 showing that its CHD, which mediates hetero-interactions with AF4 family members, was necessary and sufficient for transformation.

Nevertheless, ChIP analyses by us and others show that H3K79 methylation marks are present at most MLL-AF4-target loci (Figure 2B) (Krivtsov et al., 2008; Guenther et al., 2008), indicating that there is a strong functional interconnection between AEP and DOT1L. DOT1L-dependent H3K79 methylation is associated with transcribed regions and stimulated by histone H2B K120 mono-ubiquitination (a histone mark accompanied with transcription), but is not required for transcription itself (Steger et al., 2008; McGinty et al., 2008). This finding suggests that DOT1L-dependent H3K79 methylation occurs after the traverse of RNAPII and may play roles in the maintenance of transcriptional memory rather than initiating transcription per se. In this context, our studies support dual roles for ENL, which is capable of interacting with AEP or DOT1L through its AHD to sequentially recruit them to the same target chromatin, possibly via its N-terminal YEATS domain that retains a chromatin binding property (Zeisig et al., 2005).

Our data demonstrate that AEP colocalizes with WT MLL on target promoters indicative of a role in physiologic as well as oncogenic MLL-dependent transcriptional pathways. Supporting this notion, knockdown of *ENL* impaired expression of MLL target genes in MEFs and U937 cells (Figures 8B and 8D), and *Af9*-deficient mice display homeotic transformations similar to those of *Mll*-deficient mice (Collins et al., 2002). The recruitment of AEP to MLL-target loci appears to be nonconstitutive because some MLL-occupied loci do not contain AEP (Figure 6B). Because the presence of the MLL complex does not invariably correlate with occupancy by AEP, other factors or signals yet to be identified are likely required for AEP recruitment. On the basis of these observations and speculations, we propose a three-step model in which WT MLL first establishes/maintains the transcriptionally poised state (Step 1), AEP is then recruited to facilitate onset of transcriptional initiation and/or elongation (Step 2), which is followed by DOT1L-dependent H3K79 methylation post-transcription (Step 3) (Figure 8E). In this model, ENL serves a key role in sequential recruitment of AEP and DOT1L, respectively.

To date, up to 50 different proteins have been reported to fuse with *MLL* in human leukemias. This promiscuity poses a question as to whether any common trait is shared among the fusion partners. We demonstrate here that AEP recruitment is a downstream event in physiologic MLL-dependent transcriptional pathways and is regulated in a context-dependent manner. MLL-AF4 and MLL-ENL family fusions transform myeloid progenitors by constitutively recruiting AEP to MLL-target loci through direct association. Thus, one of the major mechanisms of MLL-dependent transformation is constitutive activation of MLL-dependent transcription by direct recruitment of AEP, which circumvents the regulatory mechanisms that normally control AEP recruitment (Figure 8E).

AEP does not physically interact with MLL-AF6, but nevertheless consistently colocalizes with MLL-AF6 at target chromatin to activate transcription (Figures 6A and 8E). Although the mechanism of this aberrant AEP recruitment is unknown, it indicates that AEP serves an even broader role in MLL leukemogenesis beyond the subset of fusions with AEP components. Determination of whether this role may extend to other MLL fusion proteins requires further investigation. Nevertheless, our studies show that most of the frequently occurring MLL fusions (e.g., MLL-AF4, MLL-AF9, MLL-ENL, and MLL-AF6) use a similar strategy for leukemic transformation, in which AEP is constitutively recruited to MLL target genes either directly or indirectly.

A critical role for AEP in MLL-mediated leukemic transformation suggests that it may be an ideal target for molecular therapy of MLL-associated leukemias. In this regard, our results tentatively support the rationale for CDK9 inhibition as a potential therapeutic strategy, or inhibition of DOT1L whose activity appears to be functionally linked to AEP and possibly plays important roles in the maintenance of the epigenetic status of target genes. However, these molecules are likely to have more generalized roles other than AEP-dependent transcription (Jones et al., 2008; Peterlin and Price, 2006); therefore, serious side effects might occur if they are effectively inhibited. Thus, compounds that specifically target the function of AF4- and ENL family proteins but not P-TEFb or DOT1L may selectively inhibit MLL-dependent transcription and benefit the treatment of MLL-associated leukemias.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies

Highly specific monoclonal antibodies were generated against MBP fusion proteins containing portions of human AF4 (aa 782–979) (clone 2C.1), human AF5q31 (aa 489–680) (clone 1.3), and human ENL (414–472) (clone 3.1), respectively.

Cell Culture

Human leukemia cell lines K562, HB1119, SEM-K2, KP-L-RY, ML-2, MV4-11, and U937 were cultured in RPMI 1640 medium supplemented with 15% fetal calf serum and nonessential amino acids. MEFs were prepared from E11.5 p53 null embryos. The 293T and plat-E cell lines and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum and nonessential amino acids.

Purification of the AEP Complex, Immunoprecipitation, and Western Blotting

The purification procedure for AEP is described in the Supplemental Experimental Procedures. Immunoprecipitation and western blotting methods

are described elsewhere (Yokoyama et al., 2004; 2005). Primary antibodies used in this study are summarized in the Supplemental Experimental Procedures.

Quantitative RT-PCR

Reverse transcription and quantitative PCR were performed as described elsewhere (Yokoyama et al., 2005; Yokoyama and Cleary, 2008) using Taqman probes purchased from Applied Biosystems. The details of the probe set are summarized in the Supplemental Experimental Procedures. Expression levels (average values and standard deviations of triplicate determinations) normalized to housekeeping genes such as *GAPDH* and *β-ACTIN* were calculated using a standard curve and the relative quantification method as described in ABI User Bulletin #2.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitations were performed as described elsewhere (Weinmann and Farnham, 2002; Yokoyama and Cleary, 2008). Primary antibodies used in ChIP assays are summarized in Supplemental Experimental Procedures. Quantitative PCR was performed on the precipitated DNAs in triplicate using primers and probes described in Supplemental Experimental Procedures. The values relative to input were determined using a standard curve and the relative quantification method as described in ABI User Bulletin #2.

Vector Construction

cDNA fragments of AF4 and AF5q31 were cloned into pcDNA3.1/*myc*-His A (Invitrogen) for expressing c-*myc* tagged proteins, or pBICEP-CMV-2 (Sigma) for expressing FLAG-tagged proteins. pMSCV-neo constructs encoding MLL-ENL, MLL-AF9, and MLL-AF6 were described elsewhere (Ayton and Cleary, 2003; Somervaille and Cleary, 2006). pMSCV-hygro-Xpress tagged ENL and pMSCV-neo-Xpress tagged MLL-AF5q31-34 were generated by fusing the Xpress-tag sequence from pcDNA4 HisMax vector with the cDNAs of ENL or MLL-AF5q31, respectively. Other expression vectors for various MLL mutants were generated by restriction enzyme digestion or PCR-based mutagenesis. Various FLAG-tagged MLL fusions were also cloned into pCMV5 vector and used for IP analysis. The expression vectors for FLAG-tagged GAL4 fusion proteins were constructed by PCR using pM (Clontech) as template and cloned into pCMV5 vector. The sh-RNA expression vectors targeting murine *Enl* (TRCN0000084405) and human *ENL* (TRCN000019291[#1], TRCN000019293[#2]) were purchased from Open Biosystems.

Virus Production

Ecotropic retrovirus was produced using plat-E packaging cells (Morita et al., 2000). Lentivirus was produced by cotransfection of 293T cells with viral vectors and pCMV dR8.74 and pMD.G packaging constructs (Dull et al., 1998). Supernatant medium containing virus was harvested 48 hr after transfection and was used for transductions.

Myeloid Progenitor Transformation Assay

Myeloid progenitor transformation was assessed as described elsewhere (Lavau et al., 1997; Yokoyama and Cleary, 2008) using cells harvested from the femurs of CD45.1 inbred C57BL/6 mice. C-kit-positive cells were enriched by immunomagnetic selection using an Auto MACS (Miltenyi Biotech), were transduced with recombinant retrovirus by spinoculation, and were plated in methylcellulose medium (M3231, StemCell Technologies) containing SCF, IL-3, IL-6, and GM-CSF. The colony-forming units (CFUs) per 10^4 plated cells were quantified after 5–7 days of culture and were expressed as the average and standard deviation of at least triplicate determinations. For secondary transductions, 10^5 cells were transduced with retrovirus by spinoculation, were cultured in methylcellulose medium overnight, and were selected for drug resistance (hygromycin 750 μg/ml, puromycin 4 μg/ml) for at least 2 days prior to CFC enumeration.

Transactivation Assay

Transactivation assays were performed using 293T cells as described elsewhere (Yokoyama et al., 2002). Cells cultured in 24-well dishes were transfected with 25 ng of pRL-tk, 250 ng of pFR-luc, and 500 ng of pCMV5 FLAG-GAL4 fusion protein vector per well. Cells were lysed 24 hr later and analyzed for luciferase activity using a dual luciferase assay kit according to the manufacturer's instructions (Promega). Relative luciferase activities were normalized to renilla luciferase activities and expressed with the average values and standard deviations of triplicate determinations relative to the GAL4 DNA binding domain controls.

SUPPLEMENTAL INFORMATION

Supplemental information includes five figures and Supplemental Experimental Procedures and may be found with this article online at doi:10.1016/j.ccr.2009.12.040.

ACKNOWLEDGMENTS

We thank Dr. M. Meyerson for providing *Men1* conditional knockout mice, Dr. T. Kitamura for the plat-E cell line, Dr. K. Yamagata for the DOT1L expression vector, and Dr. Y. Zhang for the MLL-DOT1L expression vector. We thank C. Nicolas, M. Ambrus, B. Rouse, C. Hatanaka and M. Kawaguchi, for technical assistance. A.Y. was supported by a Special Fellow Award from the Leukemia and Lymphoma Society. These studies were supported by the Children's Health Initiative of the Packard Foundation and grants from the National Institutes of Health (CA55029 and CA116606) and in part by Grants-in-Aid for Cancer Research (21-6-1) and for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor of Japan.

Received: September 1, 2009
Revised: November 9, 2009
Accepted: December 4, 2009
Published online: February 11, 2010

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Biochemical and Biophysical Research Communications 390 (2009) 1355–1360

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Sirt1 physically interacts with Tip60 and negatively regulates Tip60-mediated acetylation of H2AX

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ARTICLE INFO

Article history:
Received 27 October 2009
Available online 4 November 2009

Keywords:
Sirt1
Tip60
H2AX
IR-induced foci (IRIF)

ABSTRACT

Sirt1 appear to be NAD(+)-dependent deacetylase that deacetylates histones and several non-histone proteins. In this study, we identified Sirt1 as a physical interaction partner of Tip60, which is a mammalian MYST-type histone acetyl-transferase that specifically acetylates histones H2A and H4. Although Tip60 also acetylates DNA damage-specific histone H2A variant H2AX in response to DNA damage, which is a process required for appropriate DNA damage response, overexpression of Sirt1 represses Tip60-mediated acetylation of H2AX. Furthermore, Sirt1 depletion by RNAi causes excessive acetylation of H2AX, and enhances accumulation of γ-ray irradiation-induced MDC1, BRCA1, and Rad51 foci in nuclei. These findings suggest that Sirt1 functions as negative regulator of Tip60-mediated acetylation of H2AX. Moreover, Sirt1 deacetylates an acetylated Tip60 in response to DNA damage and stimulates proteasome-dependent Tip60 degradation *in vivo*, suggesting that Sirt1 negatively regulates the protein level of Tip60 *in vivo*. Sirt1 may thus repress excessive activation of the DNA damage response and Rad51-homologous recombination repair by suppressing the function of Tip60.

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Introduction

The DNA damage response (DDR) involves cell signaling-dependent responses to DNA damage leading to activation of cell cycle checkpoints, DNA repair, and apoptosis [1]. DNA double-strand break (DSB) is particularly toxic lesion, and mammalian cells respond to DSB by rapidly recruiting DDR factors to sites of DSB. These factors include MDC1, 53BP1, the MRN complex (Mre11, Rad50, and NBS1), and BRCA1, which form large nuclear aggregates, namely irradiation-induced foci (IRIF) [2,3].

Histone H2AX is a key regulator of IRIF formation [4,5]. Although H2AX is phosphorylated on Ser139 by ATM in response to DSB, recent studies have shown that, in addition to the phosphorylation of H2AX, other modifications of histones surrounding DSB sites occur and play an important role in the DDR pathway. Tip60 is one of the critical factors regulating acetylation of chromatin surrounding DSB [6–10]. It acetylates H2AX at Lys5 in response to DNA damage, and regulates DDR and apoptosis [6,9,10]. Furthermore, histone H4 around DSB is transiently hyper-acetylated by Tip60, which is a process required for DSB repair [9]. During DDR in yeast, the NuA4 complex, which is equivalent to the mammalian Tip60 complex, is recruited to DSB, where it acetylates the lysines in the tail of histone H4, a process required for DSB repair [11]. Thus, the Tip60 complex (or its equivalent) appears to be involved in the DDR pathway in many eukaryotes.

We have identified Sirt1 as a physical interaction partner of Tip60. Sirt1 is an NAD(+)-dependent deacetylase and mammalian homologue of the yeast protein Sir2 [12]. A well-known substrate for Sirt1 is p53 [13,14]. Sirt1 deacetylates acetylated Lys382 on p53, and attenuates p53-dependent apoptosis. In addition, Sirt1 was found to control apoptosis by deacetylating Foxo3a, Ku70, the RelA/p65 subunit of NF-κB and NBS1 [15–18].

In the current study, we show that Sirt1 and Tip60 cooperatively regulate DDR signaling and DNA repair by controlling the state of H2AX acetylation.

Materials and methods

Detailed descriptions of the experimental data, methods and materials can be found in the Supplemental data.

Plasmid constructions. For transient expression of HA-Tip60, Flag-Tip60, and H2AX-Flag (pcDNA3.1-HA-Tip60, -Flag-Tip60, and -H2AX-Flag, respectively), PCR-amplified HA-Tip60, Flag-Tip60, and H2AX-Flag cDNA were each cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). The wild-type Sirt1-myc and H363Y Sirt1-myc expression vectors (pcDNA3.1 Sirt1-myc and pcDNA3.1 H363YSirt1-myc, respectively) were gifts from Dr. Tony Kouzarides.

Abbreviations: DDR, DNA damage response; DSB, DNA double-strand break; IRIF, irradiation-induced foci; γIR, γ-ray irradiation.

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.10.156

To produce retroviruses for establishing cell lines stably expressing HA-Tip60, HA-Tip60-ad, Sirt1-myc, and/or H363Y Sirt1-myc, Phoenix™ cells were transfected with LNCX-HA-Tip60, LNCX-HA-Tip60-ad, LPSX-Sirt1-myc, or LPSX-H363YSirt1-myc. The LNCX plasmid was purchased from Clontech, and the LPSX plasmid was a gift from Dr. Hitoshi Ichikawa.

Immunoprecipitation and transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen). For immunoprecipitations, cells were lysed in NP-40 lysis buffer (50 mM Tris, pH 8.0, 150 or 200 mM NaCl, 0.1% NP-40, 10% glycerol, and 1 mM DTT) or RIPA buffer (1× PBS, 1% NP-40, 0.5% DOC, 0.1% SDS, and 1 mM DTT) supplemented with protease inhibitors (Complete EDTA-free; Roche Applied Science, Indianapolis, IN, USA). After centrifugation (20,000*g* for 30 min at 4 °C), the supernatant was incubated with 1–2.5 μg of antibody and 10 μl protein G Sepharose beads (Amersham) or 10 μl anti-Flag M2 beads (Sigma–Aldrich) at 4 °C overnight and then washed extensively with NP-40 lysis buffer or RIPA buffer.

For the interaction between endogenous Tip60 and Sirt1, HEK293 nuclear extract was prepared from 2×10^8 cells and dialyzed to NP-40 lysis buffer. Anti-Tip60 (K-17 and N-17) or normal goat IgG was added to the lysate, and incubated overnight. Antibody-Tip60 immuno-complex was harvested with Dynabeads Protein G (Invitrogen) according to the manufacturer's protocol. To detect Tip60 and Sirt1 for Western blotting, rabbit anti-HTATIP (AP1081a, ABGENT) and rabbit anti-Sirt1 (Abcam), respectively, were used.

Results

Sirt1 physically interacts with Tip60

To investigate how Tip60 contributes to the DDR pathway, we searched for a physical interacting partner of Tip60. In budding yeast, Esa1, Sir2, Gcn5, Rpd3, and Hst1 are recruited to sites of DSB during homologous recombination repair [19]. Tip60 and Sirt1 are mammalian homologues of the yeast proteins Esa1 and Sir2, respectively, and they play opposing roles in several pathways in vertebrates, including the p53 and HIV Tat pathways [8,13,14,20,21]. Thus, we focused on Sirt1 as a potential regulator of Tip60. We first examined whether Sirt1 physically interacts with Tip60.

Sirt1-myc was co-immunoprecipitated with Flag-Tip60 (Fig. 1A, left panel). Likewise, Flag-Tip60 was co-immunoprecipitated with Sirt1-myc (Fig. 1A, right). The region of F-Tip60 253–513 a.a. (containing the MYST domain) was required for the interaction (Fig. S1). Additionally, endogenous Tip60 interacted with endogenous Sirt1 (Fig. 1B) before and after γIR-treatment. These results indicate that Sirt1 physiologically interacts with Tip60.

Sirt1 can repress the level of acetylated H2AX

Tip60 acetylates H2AX at Lys5 in response to DNA damage [10]. Therefore, we examined whether Sirt1 influences Tip60-mediated acetylation of H2AX. We first transiently expressed a C-terminally Flag-tagged H2AX protein (H2AX-Flag) in HEK293 cells and then treated the cells with 10 Gy γ-ray (γIR). Although exogenous H2AX-Flag was relatively overexpressed compared with endogenous H2AX, the majority of overexpressed H2AX-Flag was located in the mitotic chromatin (Fig. S2A, B). Moreover, γIR induced the phosphorylation of H2AX-Flag with similar kinetics as endogenous H2AX, indicating that the H2AX-Flag construct is functional *in vivo* (Fig. S2B).

To confirm that Tip60 acetylates H2AX at Lys5 as previously described [10], we co-expressed wild-type HA-Tip60 or HA-Tip60-ad with H2AX-Flag. For these experiments, we used a commercially available anti-acetylated Lys5 H2A antibody that can also detect the Lys5-acetylated form of H2AX (Fig. S3A). When co-expressed with a control vector, a small amount of H2AX-Flag was acetylated at Lys5 prior to γIR. Two hours after γIR, the level of Lys5-acetylated H2AX-Flag was dramatically increased (Fig. 1C). When co-ex-

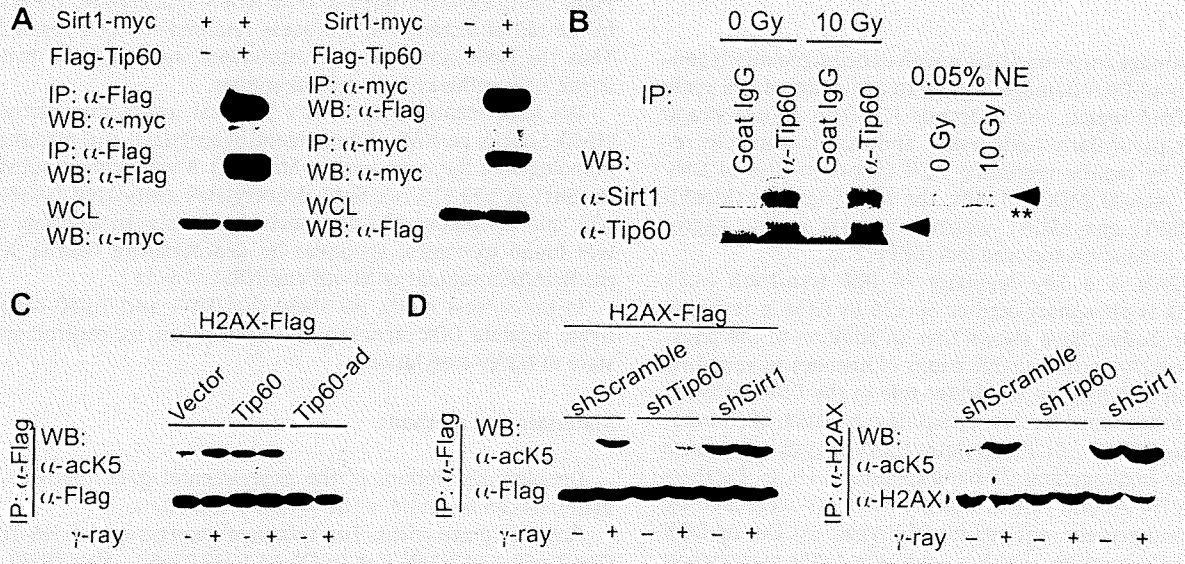

Fig. 1. The NAD(+)-dependent deacetylase Sirt1 interacts with Tip60 and represses Tip60-mediated acetylation of H2AX. (A) Flag-Tip60 or empty (mock) vector was co-introduced with Sirt1-myc or empty (mock) vector into HEK293 cells, and after 48 h, cells were lysed in NP-40 lysis buffer. Left panel: co-immunoprecipitation (co-IP) with anti-Flag (α-Flag) and Western blotting (WB) with anti-myc (α-myc). Right panel: co-IP with anti-myc and WB with anti-Flag. (B) Endogenous interaction of Sirt1 and Tip60. (C) HEK293 cells were co-transfected with pcDNA3.1-H2AX-Flag and pcDNA3.1-Flag-Tip60, or pcDNA3.1-Flag-Tip60-ad encoding wild-type Flag-Tip60 or Flag-Tip60-ad protein, respectively. Cells were irradiated with 10 Gy of γIR, harvested after 2 h and were lysed in RIPA buffer for IP of H2AX-Flag with anti-Flag. Anti-acetylated Lys5 H2A (α-acK5) and anti-Flag-HRP antibodies were used for WB. (D) In left panel, HEK293 cells were co-transfected with H2AX-Flag and pSuper-shScramble (control), pSuper-shTip60, or pSuper-shSirt1twice. 48 h after the last transfection, cells were treated with 10 Gy γIR and after 0 or 0.5 h incubation, cells were lysed in RIPA buffer for IP of H2AX-Flag. In right panel, HEK293 cells were transfected with pSuper-shScramble (control), pSuper-shTip60, or pSuper-shSirt1 three times at 24 h intervals. After 48 h, cells were treated with 10 Gy γIR and after 0 or 0.5 h incubation, and the state of acetylated endogenous H2AX was examined (see Supplemental materials and methods). WCL, whole cell lysate; **degradation product; NE, nuclear extract.