The same site on LEDGF IBD domain represents therapeutic target for MLL leukemia and HIV

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Abstract

Lens epithelium-derived growth factor (LEDGF) is a chromatin associated protein implicated in leukemia and HIV-1 infection. LEDGF associates with MLL fusion proteins and menin and is required for leukemic transformation. To better understand the molecular mechanism underlying the LEDGF IBD domain interaction with MLL fusion proteins in leukemia we determined the solution structure of the MLL-IBD complex. We found a novel MLL motif, IBM2 (Integrase Binding Motif 2), which binds to a well-defined site on IBD. Point mutations within IBM2 abolished leukemogenic transformation by MLL-AF9, validating that this newly identified motif is essential for the oncogenic activity of MLL fusion proteins. Interestingly, the IBM2 binding site on IBD overlaps with the binding site for the HIV integrase (IN) and IN was capable to efficiently sequester IBD from the menin-MLL complex. A short IBM2 peptide binds to the IBD domain directly and inhibits both the IBD-MLL/menin and IBD-IN interactions. Our findings show that the same site on IBD is involved in binding to MLL and HIV integrase, revealing an attractive approach to simultaneously target LEDGF in leukemia and HIV.

Key points

Inhibiting LEDGF interaction with a novel fragment of MLL represents attractive approach to develop new drugs for MLL leukemias.

Structural studies reveal a new pocket on LEDGD IBD domain suitable for targeting by small molecule inhibitors.
Introduction

Lens epithelium-derived growth factor (LEDGF) is a chromatin associated protein belonging to the hepatoma-derived growth factor (HDGF) family (1). It functions as a transcriptional coactivator activating stress-related genes (2, 3) and promotes growth and survival of lens epithelial cells, keratinocytes and fibroblasts (4). Accumulating data involve LEDGF in acute myeloid leukemia (AML). Recurring chromosomal translocations of LEDGF with nucleoporin NUP98 gene have been identified in pediatric AML (5, 6). Furthermore, the LEDGF/p75 isoform is consistently upregulated in a subset of AMLs resistant to chemotherapy (7). LEDGF is a critical co-factor of MLL fusion proteins required for leukemic transformation (8). Importantly, disruption of the LEDGF interaction with MLL fusion proteins abolishes development of acute leukemia in vivo, suggesting that the LEDGF-MLL interaction represents a valuable target for development of novel anti-leukemic drugs (8).

LEDGF plays an essential role in human immunodeficiency virus type 1 (HIV-1) pathogenesis (9). LEDGF is a key host protein required for chromatin binding activity of HIV-1 integrase (10). The catalytic domain of HIV integrase binds to the C-terminal Integrase Binding Domain (IBD) of LEDGF (11), and multiple studies validated this protein-protein interaction as necessary for HIV-1 replication (9, 11, 12). Targeting the IBD-IN interaction attracted a strong attention as a valuable approach to develop novel anti-retroviral drugs for HIV infection (13) and a number of studies reported small molecules and peptidomimetics binding to the HIV integrase and blocking its interaction with the LEDGF IBD. Such compounds interfere with HIV integrase activity and inhibit replication of HIV-1 in cell culture (14, 15).
Biochemical and structural studies revealed that the LEDGF IBD domain also interacts with menin and MLL (8, 16). Therefore, the IBD plays a dual role as a co-factor of MLL fusion proteins in leukemia and as a cellular co-factor of viral integrase in HIV infection. Consequently, protein-protein interactions of LEDGF IBD domain might constitute an attractive target for development of novel therapeutics. However, molecular details of IBD interaction with MLL and menin are not entirely understood. A recently reported crystal structure of the menin-MLL-LEDGF complex revealed that a helical fragment of MLL spanning residues 113-134 is involved in the interactions with LEDGF IBD domain (16). On the other hand, functional studies have shown that deletion of MLL residues 123–153 abolished MLL binding to LEDGF(8), revealing that longer fragment might be required for formation of the MLL-LEDGF complex. In order to understand the molecular mechanism underlying the IBD domain interaction with MLL fusion proteins we determined the solution structure of the MLL-IBD domain complex. Importantly, we identified a novel motif within MLL, which directly interacts with IBD domain and is critical for leukemogenic activity of MLL-AF9. Interestingly, we also found that the same site on IBD is involved in binding to this newly identified MLL fragment and to the HIV integrase. Our work provides an important structural basis to simultaneously target the protein-protein interactions of LEDGF in leukemia and HIV.

Methods

Molecular biology

LEDGF constructs for bacterial expression have been ordered from GenScript and cloned into pET32a. Full length LEDGF cDNA was amplified using rt-PCR from mRNA extracted from
HEK293 cells. The expression vector pCMV Flag-MLL-AF9 was prepared using pMSCV Flag-MLL-AF9 as template (17). cDNA encoding IN and MLL_{110-160} constructs were synthesized by GeneArt Strings (Invitrogen). Detailed description of constructs is provided in **Supplementary Methods**.

**Protein expression and purification**

IBD domain, MLL fragments and the MLL-IBD fusions for *in vitro* studies were expressed in *E. coli* as insoluble proteins. Inclusion bodies were solubilized in 6 M guanidine hydrochloride and refolded on-column bound to Ni-NTA resin (Qiagen). The thioredoxin-His\textsubscript{6} tag has been cleaved by 3C protease and final proteins were purified by ion exchange chromatography using SP sepharose (GE Healthcare). IBD was concentrated and dialyzed into 50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM DTT. All other proteins were concentrated and dialyzed into 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM β-mercaptoethanol. Full-length human menin was purified as described elsewhere (18). Proteins labeled with stable isotopes (\textsuperscript{13}C, \textsuperscript{15}N) were expressed in M9 minimal media. HIV integrase was captured by HisTrap HP (GE Healthcare) followed by ion exchange chromatography on a Q sepharose column (GE Healthcare).

**NMR binding experiments**

Mapping of IBD–MLL interaction was performed using \textsuperscript{15}N-labeled IBD and unlabeled MLL fragments. To determine binding affinity, 70 μM \textsuperscript{15}N-labeled IBD was titrated with MLL_{1-160} and the two mutants F129A and F148A,L149A (FLAA) and chemical shift perturbations were measured from HSQC spectra. The $K_d$ values were determined using previously established procedure (19).
The IBD2 peptide corresponding to MLL 146-153 fragment and has been ordered from GenScript.

**Structure determination of MLL-IBD**

NMR spectra were measured on Bruker Avance III 600MHz NMR spectrometers at 25°C. Protein samples contained 0.34 mM $^{13}$C/$^{15}$N-labeled MLL-IBD in 50mM phosphate buffer, pH 6.5, 50mM NaCl supplemented with 5% D$_2$O. Backbone assignment was completed based on triple-resonance experiments and structure has been calculated using Cyana 2.1 (20) based on cross-peaks derived from NOESY spectra and dihedral angle restraints from Talos+ (21). More detailed procedure is described in *Supplementary Data*. The coordinates for the MLL-IBD structure have been deposited in the PDB with the accession number 2MTN.

**Isothermal Titration Calorimetry**

Menin-MLL$_{1-160}$ complex was purified by size exclusion chromatography and titrated with IBD using a VP-ITC titration calorimeter. Menin-MLL$_{1-160}$ complex was used at 20μM concentration, 1000-fold above $K_d$ for menin-MLL$_{1-160}$ interaction (18). Further details are provided in *Supplementary Methods*.

**Co-immunoprecipitation of MLL-AF9 with menin and LEDGF**

HEK293 cells were co-transfected by pCMV-Flag-MLL-AF9 or pCMV-Flag-MLL-AF9-FLAA with pMSCV-Myc-menin and pMSCV-LEDGF-HA plasmids using Fugene 6 (Promega). Forty-eight hours after transfection, whole-cells lysates were immunoprecipitated with anti-FLAG M-2 Magnetic Beads (M8823) (Sigma-Aldrich) and analyzed by SDS-PAGE and Western blotting.
using anti-N-MLL antibody (05-764) (Millipore), anti-β-actin antibody(A00702)(GenScript), anti-menin antibody and anti-HA tag antibody(2367S) (Cell signaling).

**AlphaLISA assays**

To assess the inhibition of IBD-menin-MLL interaction we optimized AlphaLISA assay using 62.5 nM His-Trx-MLL160, 62.5 nM menin and 62.5 nM Flag-IBD. Proteins were incubated for 1h in buffer containing 50 mM MOPS (pH 7.25), 50 mM NaCl, 1 mM TCEP, 0.02% (w/v) bovine serum albumin 0.025% (v/v) Tween-20. Ni-chelate-coated donor beads and anti-Flag acceptor beads (10 μg/mL final concentration) were added and incubated for 1h. The luminescence signal was recorded using excitation at 680 nm and emissions at 615 nm using the PHERAstar microplate reader (BMG). Competition experiments with either IBM2 peptide or HIV-IN were performed with 2-fold serial dilutions. Data were analyzed using the Origin 7.0.

To assess the inhibition of HIV-IN interaction with IBD we employed a very similar protocol as above using 62 nM His-HIV-IN and 1 μM flag-IBD. Ni-chelate-coated donor beads and anti-Flag acceptor beads were used at 2.5 μg/mL concentration. Competition experiments were performed with IBM2 using 3-fold serial dilutions.

**Virus Production and Bone Marrow Transformation Assays**

The pMSCV (for MLL-AF9 and the F129A and FLAA mutants) constructs were transfected using Fugene 6 reagent (Roche) into Plat-E cells selected in puromycin (1 μg/ml) and blasticidin (10 μg/ml). Media containing the recombinant retrovirus was collected for transduction at 48 and 72 hours post transfection. BMT assays were performed by transducing Lin- BM cells with retrovirus containing MLL-AF9 (or mutants) as described before (17). To assess the growth, the
transduced cells were cultured in IMDM media supplemented with 15% FBS, 1% penicillin/streptomycine, SCF and IL-3 and counted daily for 6 days. Colony formation was assessed by plating transformed cells in methylcellulose medium (STEMCELL Technologies), supplemented with IL-3, IL-6, SCF, GM-CSF and 1 mg/ml G418. After three rounds of plating, CFU (with > 50 cells) was scored under the microscope; colonies were stained using 0.1% INT and scanned. Cells harvested at the end of the experiment were cytospun and stained with the Hema 3 Stain Kit (Thermo Fisher Scientific). Images were acquired using a 100x lens under an Olympus BX-51 microscope (Olympus).

Quantitative RT-PCR
Reverse transcription and quantitative PCR were performed as previously described (17). TaqMan primer probes for HOXA9 and β-ACTIN were purchased from Applied Biosystems. Expression levels were analyzed using the comparative ΔΔCt method as described in ABI User Bulletin #2.

Chromatin immunoprecipitation (ChIP)
ChIP assays were performed as previously described using cells transfected with Hoxa9-LUC promoter, pMSCV-MLL-AF9 or the F129A or FLAA mutant plasmids (22). Antibodies against menin, LEDGF, AF9 and IgG were obtained from Bethyl Laboratories, Inc. qPCR was performed on the precipitated DNAs with TaqMan primers and probes from Applied Biosystems. TaqMan probes for Hoxa9 were the same as used previously (22).

Dual luciferase assay
HEK293 cells were co-transfected with pMSCV-MLL-AF9, pCMV-LEDGF, MSCV-menin, Renilla luciferase reporter (internal control), Hoxa9-LUC reporter and increasing concentrations of pMIGR1-IN with FuGene 6 according to the manufacturer’s instructions. Luciferase assays were performed using the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Emission was detected using a Monolight 3010 Luminometer (BD Biosciences).

**Bone marrow co-transduction assay**

Lin-c-Kit+ cells isolated from C57BL/6 mice were transduced with MSCV-neo-Flag-MLL-AF9. The following day the cells were transduced with retroviruses packaged with: MSCV-puro-IN or MSCV-puro-MLL110-160, MSCV-puro-MLL110-160 FLAA, MSCV-puro-MLL110-160 F129A. Cells were selected with G418 (1mg/ml, Gibco) and puromycin (2µg/ml, Sigma) for a week. Following the selection, cells were cultured for two weeks. Viable cells were counted using trypan blue staining. Following day 14, expression of Hoxa9 was analyzed using qRT-PCR, c-Kit level by FACS and morphology of cells by Wright-Giemsa staining. In addition, three rounds of serial replating assay were performed. More detailed procedure is described in Supplementary Data.

**Results**

*Two separate MLL motifs are involved in the interaction with LEDGF IBD domain*

To accurately map the MLL fragment necessary for the interaction with LEDGF we tested binding of various MLL fragments to IBD domain of LEDGF using NMR. First, we found that MLL1-160 binds to the IBD in the absence of menin with moderate affinity (Kd=14.7 ±
Further mapping revealed that MLL fragment comprising residues 100-160 is required for binding to the IBD domain (Figure 1C and Supplementary Figure S1). Interestingly, a recently published crystal structure of menin-MLL-LEDGF complex lacks any contacts involving MLL upstream of residue 135 (16). To understand the role of MLL residues in binding to the IBD we determined the solution structure of MLL-IBD complex using NMR spectroscopy. To overcome a modest affinity we designed a covalent complex by fusion of MLL to the IBD domain, which is a frequently adopted approach for structure determination of protein-protein complexes by NMR (23, 24).

The MLL-IBD fusion construct optimized for structural studies included a 110-160 fragment of MLL fused to a 337-442 fragment of LEDGF encompassing IBD domain. Comparison of the $^1$H-$^{15}$N HSQC spectra of the IBD saturated with MLL1-160 with the spectra of the MLL110-160-IBD fusion clearly shows that linking of the two protein fragments did not affect the structure of the complex (Supplementary Figure S2). We then determined the structure of the MLL-IBD complex in solution using a total of 1255 distance restraints, including 86 inter MLL-IBD distances (Supplementary Table 1). The overall structure of the complex is well defined with 2.0Å backbone r.m.s.d. for ordered IBD and MLL fragments in 20 lowest energy conformers. Based on the structure, we found that the IBD domain interacts with MLL110-160 via two distinct fragments, which we labeled, respectively, IBM1 (IBD binding motif 1, residues 123-134) and IBM2 (residues 147-152) (Figure 1C,D,E). While the IBM1 has been reported in the crystal structure (16), the IBM2 has not been observed before and represents a novel motif within MLL.

The IBM1 and IBM2 motifs form independent contacts with the IBD domain and are separated by a 12-amino acid long flexible linker. The IBM1 is an $\alpha$-helical motif, which packs
onto the site formed by two C-terminal helices of the IBD (Figure 1D,E). Interestingly, this helix is much longer in the ternary menin-MLL-IBD complex and contacts both, menin and the IBD (Figure 1H). A second motif, IBM2 is short and interacts with IBD domain in an extended conformation. The key contacts involve packing of MLL IBM2 side chains of F148 and F151 into a concave hydrophobic face of IBD domain lined by L363, I403 and F406 (Figure 1F).

*Both, IBM1 and IBM2 are necessary for high affinity association of LEDGF with menin-MLL complex*

It has been previously reported that menin is necessary to tether MLL to LEDGF (8). Here, we provide an evidence that MLL directly binds to the IBD domain in the absence of menin albeit with moderate affinity, $K_d = 14.7 \mu M$ (Fig 1A,B). To assess how menin affects the MLL-IBD interaction we employed Isothermal Titration Calorimetry (ITC) and quantified binding of the IBD domain to the menin-MLL$_{1-160}$ complex. As compared to the binary interaction, IBD binds to the menin-MLL complex with approximately 10-fold increased affinity ($K_d = 1.4\mu M$, Fig 1G).

In order to test whether both IBM1 and IBM2 motifs in MLL are required for high affinity binding of IBD to the menin-MLL complex we used mutagenesis. We introduced the mutation of F129A in IBM1, which has been shown previously to abolish MLL-ENL binding to LEDGF (8). To impair interaction of IBM2 with IBD we introduced single F148A and double F148A and L149A (FLAA) mutations (Figure 1F). We assessed how these mutations affect binary interactions between MLL$_{1-160}$ and IBD domain and using NMR we found that F129A has rather weak effect ([Supplementary Figure S3](#)), F148A decreased the binding ~10 fold while the FLAA mutation almost completely abolished this interaction ([Supplementary Figure S3](#)).
Subsequently, we employed ITC to test the impact of mutations within IBM1 and IBM2 on formation of the ternary complex with menin and IBD. Both, F129A and FLAA mutations result in a very severe decrease in binding affinity of the IBD domain to the menin-MLL1-160 complex (Figure 1G). Altogether, these data demonstrate that the intact fragment of MLL containing both, IBM1 and IBM2 is required to maintain high affinity menin-MLL-IBD complex.

**IBM2 is required for leukemogenic activity of MLL-AF9**

Interaction of LEDGF with the menin-MLL-fusion protein complex is essential for oncogenic activity of MLL fusion proteins (8). The F129A mutation has previously been shown to abolish binding of LEDGF to the menin-MLL-ENL complex as assessed by co-immunoprecipitation (co-IP) (8), which is consistent with our binding data (Figure 1G). To assess the role of the second motif, IBM2, we introduced FLAA mutation into MLL-AF9. The Co-IP clearly shows that MLL-AF9 interacts simultaneously with menin and LEDGF (Figure 2A). On the contrary, the MLL-AF9 FLAA mutant interacts only with menin but not with LEDGF (Figure 2A). These results demonstrate that binding of LEDGF to the menin-MLL-AF9 requires intact IBM2, recapitulating the *in vitro* binding data.

To test whether both the IBM1 and IBM2 motifs are required for the oncogenic activity of an MLL fusion protein we transduced murine bone marrow cells (BMCs) with MLL-AF9 and the two mutants F129A and FLAA. We found that only MLL-AF9 was capable of efficiently transforming BMCs and sustained proliferation (Figure 2B), while the BMCs transformed with either of the two mutants had severe defects in proliferation (Figure 2B). The BMCs transformed with MLL-AF9 form colonies in methylcellulose media and can be serially replated (Figure 2C). However, both F129A and FLAA mutations almost completely abolished colony
forming unit potential (Figure 2C,D). The oncogenic activity of MLL fusion proteins is also associated with high expression of Hoxa9, which is a downstream target of MLL-AF9 (25). To test whether disruption of the LEDGF interaction with menin-MLL-AF9 affects Hoxa9 expression we performed qRT-PCR. The BMCs expressing F129A and FLAA mutants show very low levels of Hoxa9 as compared to MLL-AF9 transformed BMCs (Figure 2E). Transformation with MLL-AF9 leads to a block in cellular differentiation of hematopoietic cells. Comparison of the morphology of BMCs transformed with MLL-AF9 and the two mutants revealed a more differentiated phenotype for cells harboring F129A and FLAA mutants compared to MLL-AF9 (Figure 2F), as assessed by increased cell size, higher cytoplasm to nuclear ratio and highly vacuolated cytoplasm. Furthermore, we tested whether F129A and FLAA mutations will impair development of leukemia in vivo. While nearly half of animals transplanted with MLL-AF9 developed leukemia, we have not observed any disease in mice transplanted with MLL-AF9 bearing either F129 or FLAA mutations (Figure 2G, Supplementary Figure S4). Altogether, these results demonstrate that mutations within the IBM1 and IBM2 that abolish the interaction of MLL with LEDGF result in loss of transforming properties by MLL-AF9.

Mutations in IBMs disrupt cooperative assembly of Menin-MLL-AF9-LEDGF complex on Hoxa9

To test whether disruption of the MLL-LEDGF interaction impairs the recruitment of MLL-AF9 to Hoxa9 loci we performed Chromatin Immunoprecipitation (ChIP) experiments. We found that the presence of F129A and FLAA mutations in MLL-AF9 significantly decreased the occupancy of MLL-AF9 at four distinct sites on the Hoxa9 promoter region (Figure 2H, I). Importantly, we also observed strong loss of menin binding and a very significant decrease in the
level of LEDGF at *Hoxa9* for both MLL-AF9 mutants (*Figure 2H, I*). These findings strongly suggest that ternary complex of menin, MLL-AF9 and LEDGF co-operatively assembles on *Hoxa9*.

**HIV integrase sequesters IBD domain from the menin-MLL-LEDGF complex**

LEDGF plays an intriguing dual role being a component of the menin-MLL and menin-MLL fusion complexes as well as a binding target of HIV-integrase (IN) required for the integration of the HIV genome into host chromatin (8, 9). Both activities of LEDGF require the IBD which is involved in the protein-protein interactions with menin-MLL and IN. Interestingly, the analysis of the crystal structure of IN-IBD complex (26) and our structure of MLL-IBD revealed that the integrase binding site on IBD overlaps with the binding site of IBM2 MLL motif (*Figure 3A*). For example, the W131 from IN occupies the same position as F148 from IBM2 (*Figure 3A*). Based on the structural analysis we hypothesized that IN should compete with MLL for binding to the IBD. Since the IN interacts with IBD with two orders of magnitude higher affinity (K\(_d\)=10nM) (27), than IBD binds to menin-MLL, we anticipated that IN should be capable of sequestering IBD from the ternary complex with menin-MLL. To test this, we developed an AlphaLisa assay (see Supplementary Methods) and, as predicted, IN proved to be a very potent competitor, disrupting the IBD-menin-MLL interaction with IC\(_{50}\)=301 nM (*Figure 3B*).

To further assess whether IN disrupts the LEDGF-menin-MLL-AF9 interaction in cells we employed the luciferase reporter assay. We expected that overexpression of IN will reduce transactivation of the *Hoxa9* promoter induced by LEDGF-menin-MLL-AF9. Indeed, we observed a dose-dependent reduction of *Hoxa9* transactivation activity induced by HIV integrase.
expression (Figure 3C). Therefore, IN sequesters LEDGF from the menin-MLL fusion complex and reduces transactivation activity of the MLL-AF9 fusion protein. Intriguingly, these results suggest that overexpression of HIV integrase may reverse the leukemogenic activity of MLL fusion proteins in leukemia.

Expression of IN and MLL_{110-160} impairs leukemogenic transformation

To validate whether expression of HIV integrase or an MLL fragment impairs oncogenic transformation by MLL-AF9 we co-transduced BMCs with MLL-AF9 and IN or MLL_{110-160}. Expression of IN resulted in a significant growth inhibition of MLL-AF9 transformed cells, reduction in Hoxa9 expression, induction of differentiation as evident from cell morphology changes and a decrease in c-Kit expression quantified by flow cytometry (Figures 3D-I, Supplementary Figure S5). Expression of IN also resulted in strong reduction in colony formation of MLL-AF9 transformed BMCs. These data clearly demonstrate that IN impairs the MLL-AF9 fusion protein mediated oncogenic transformation. We have also tested whether expression of a short fragment of MLL encompassing the IBD binding motifs (residues 110-160) affects the activity of MLL-AF9. Indeed, the MLL_{110-160} fragment significantly reduced Hoxa9 expression, enhanced differentiation and reduced colony formation of MLL-AF9 transformed cells suggesting a dominant negative function (Figure 3D-I, Supplementary Figure S5). On the contrary, neither the F129A nor the FLAA mutant inhibited transformation mediated by MLL-AF9 (Figure 3D-I, Supplementary Figure S5), implying the dominant negative effect of MLL_{110-160} is specific.

IBM2 peptide disrupts menin-MLL-IBD and IN-IBD complexes
Targeting the interaction of LEDGF with the menin-MLL may constitute an attractive approach to reverse the oncogenic activity of MLL fusion proteins in leukemia (8). Our structural studies revealed that IBM2 of MLL interacts with a concave hydrophobic site on IBD domain, which may represent a “druggable” site to develop small molecule or peptidomimetic inhibitors. To validate this hypothesis, we assessed whether a short peptide corresponding to IBM2 can bind to the IBD domain. Indeed, an 8 amino acid long peptide derived from IBM2 directly binds to IBD and induces chemical shift perturbations on NMR spectra of IBD consistent with binding to the IBM2 site (Figure 4A,B). Based on the NMR titration experiment we estimated that IBM2-derived peptide binds to IBD with approximately 10-fold reduced affinity as compared to MLL1-160.

As shown above, the IBM2 site on IBD domain represents a binding site for both, MLL and IN. Therefore, IBM2-derived peptide should disrupt the IBD-menin-MLL as well as IBD-IN interactions (Figure 4C). Indeed, using the AlphaLisa assays we found that this peptide inhibits these two complexes with moderate affinity (IC50=318 μM and IC50=223 μM, respectively) (Figure 4D,E). Although, modest in vitro activity of the IBM2-derived peptide is insufficient to demonstrate cellular activity, it provides an important proof of concept that a short peptide can directly bind to the IBD domain and inhibit its interaction with both MLL-menin and IN. This finding paves the way towards development of more potent peptidomimetics or small molecules inhibitors of the protein-protein interactions between IBD and MLL as a future potential therapeutic for both, MLL leukemia and HIV infection.
Discussion

In this work we determined the structure of the MLL-IBD complex and found a new motif within MLL, labeled here as IBM2, that is involved in binding to the IBD domain of LEDGF. The newly identified IBM2 motif is necessary for the formation of a high affinity complex between LEDGF and menin-MLL-AF9. The mutation of IBM2 within MLL-AF9 disrupts its interaction with LEDGF and abolishes the leukemogenic activity of MLL-AF9. Interestingly, the IBM2 binding site (but not IBM1) on the IBD domain overlaps with the binding site for HIV integrase. We have further demonstrated that binding of IBD to integrase and menin-MLL is mutually exclusive. A short peptide corresponding to the MLL IBM2 fragment directly binds to the IBD domain and disrupts both, the IBD-menin-MLL and IBD-IN complexes (Figure 4). Altogether, these experiments validate the IBM2 site on IBD as an attractive site to develop therapeutic agents for MLL leukemias as well as to block the HIV integrase activity critical to virus replication.

Targeting LEDGF-menin-MLL interaction represents an attractive approach for development of novel therapeutics for MLL leukemias (8, 28). We have recently developed small molecules that bind to menin and inhibit the menin-MLL interaction and demonstrate potent and selective activity in MLL leukemia cells (29, 30). Such approach, however, may have potentially undesired consequences as menin functions as a tumor suppressor in endocrine tissues (31). Therefore, inhibitors of the activity of MLL fusions that would not affect menin function are highly desired. In this work we found a novel MLL motif, IBM2, which interacts with IBD and is as essential for leukemogenic activity of MLL-AF9. We also demonstrated that a short peptide derived from MLL and corresponding to IBM2 motif is sufficient to bind to IBD
domain and disrupt the IBD-menin-MLL complex. Analysis of the MLL-IBD structure shows that IBM2 binds in an extended conformation and the side chains of F148 and F151 form contacts with the concave hydrophobic site on the IBD domain, strongly indicating that it represents a “druggable” site. Therefore, it is likely that more potent ligands, such as small molecules or peptidomimetics, can be developed as a potential therapeutic approach for MLL leukemia. The structure of the MLL-IBD complex reported here will significantly facilitate development of such inhibitors.

Interestingly, the structure of MLL-IBD revealed that the IBD domain utilizes the same site to bind MLL as well as IN. This finding has two key implications. First, due to high affinity binding, IN can sequester LEDGF from the menin-MLL complex. We demonstrated that expression of the IN significantly reduces transformation activity of MLL-AF9 (Figure 3) demonstrating that HIV integrase acts as a cellular inhibitor suppressing activity of MLL fusion proteins. Secondly, potent small molecule or peptidomimetic inhibitors that bind to IBM2 site will disrupt the interaction of LEDGF with IN, and therefore should inhibit the replication of HIV. Development of IN-LEDGF interaction inhibitors has been recognized as an attractive target for new anti-viral drugs and multiple studies identified small molecule inhibitors, all of which bind to IN (13). However, ligands binding to the LEDGF IBD domain are more desired to avoid resistance resulting from mutation of the viral integrase (32). Recently, peptides derived from LEDGF have been reported to disrupt MLL-LEDGF interaction and delay development of leukemia in animal model of MLL-AF9 (28). However, no potent small molecule inhibitors directly binding to the IBD domain have been identified to date and the structure of MLL-IBD reported here may significantly facilitate such efforts.
In summary, LEDGF plays a dual role as an oncogenic cofactor of MLL fusion proteins and also as a binding partner of HIV integrase required for viral infection. The finding that the same site on IBD domain is involved in binding of MLL and IN paves the way towards development of novel therapeutic agents with dual applications for both, MLL leukemias and HIV.

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Authorship

M.J.M. purified proteins, M.J.M and T.C. performed NMR experiments and determined the structure, T.P, S.H. and A.G.M. performed cellular assays, J.P. and A.Y. performed biochemical experiments. H.M. performed *in vivo* experiment. J.L.H, A.G.M, J.G. and T.C. planned the experiments and wrote the manuscript with an input from all authors.

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Figure legends

**Figure 1.** Characterization of MLL-IBD interaction and the solution structure of MLL-IBD complex.  
A. HSQC spectrum for 70μM IBD domain (green) titrated with 35 μM (cyan), 120 μM (blue) and 280μM MLL160 (black).  
B. Determination of IBD-MLL160 binding affinity from NMR titrations. Representative titration curves for three residues are shown and the average K_D is reported.  
C. Schematics of MLL constructs used for mapping the IBM1 and IBM2 binding motifs.  
D. 20 lowest energy structures of MLL-IBD. Well-ordered MLL fragments corresponding to residues 124-133 (IBM1) and 147-152 (IBM2) are shown in green and IBD domain (residues 349-428) is blue.  
E. Representative structure of MLL-IBD; coloring is the same as in D.  
F. Details of IBM2-IBD interaction. Key residues involved in the contacts are green (IBM2) and gray (IBD).  
G. Characterization of binding affinity using ITC. Equimolar complexes of menin with MLL160, MLL160 F129A and MLL160 FLAA were titrated with IBD domain. Experiments were performed twice and K_D and stoichiometry (N) are shown for representative experiments.  
H. Model of menin-MLL-LEDGF complex based on solution MLL-IBD structure and previously published crystal structure (PDB code 3U88).

**Figure 2.** Intact IBM2 is necessary for leukemogenic transformation by MLL-AF9.  
A. co-IP demonstrates that mutation FLAA in MLL-AF9 abolished the interaction with LEDGF but not with menin. HEK293 cells were transduced with Myc-menin, LEDGF-HA and Flag-MLL-AF9 or Flag-MLL-AF9 FLAA and co-immuno precipitated using anti-Flag antibody.  
B. Growth curves for BMC transformed with MSCV vector, MLL-AF9 and the F129A and FLAA mutants.
C. Activity of MLL-AF9 and the mutants in colony formation assay in transformed BMC. Colony counts after three rounds of replating (C) and representative colonies (D) are shown. E. qRT-PCR in BMC transformed with MLL-AF9 and the two mutants for three re-plating rounds showing expression of *Hoxa9*. Expression of *Hoxa9* has been normalized to β-actin and is referenced to the MSCV vector. F. Wright-Giemsa stained cytospins demonstrating differentiation of BMC cells transformed with MSCV, MLL-AF9 and the two mutants. G. Survival of mice transplanted with MSCV-MLL-AF9 and F129 and FLAA mutants. NRAS has been included to accelerate development of leukemia; n represents number of animals in each group. H. Schematic diagram showing the endogenous *Hoxa9* locus and probes used in the ChIP assay. TSS: transcription start site. I. ChIP assays performed in HEK293 cells transduced with MLL-AF9 and mutants F129A and FLAA to determine the recruitment of MLL-AF9, menin and LEDGF to the *Hoxa9* locus with 4 different probes. Antibodies against AF9, menin and LEDGF were used.

**Figure 3.** The same site on IBD domain interacts with IBM2 and HIV integrase. A. Superposition of MLL-IBD structure (MLL IBM2 is shown in magenta and IBD is green) onto IBD-IN complex (IN dimer is blue; based on pdb structure 2B4J). W131 from the integrase binds to the same site on IBD domain as F148 from IBM2 motif. B. IN disrupts menin-MLL-IBD complex in AlphaLisa assay by sequestering IBD domain from menin-MLL. C. Dual luciferase assay performed in HEK293 cells with Hoxa9-LUC reporter. Cells were co-transfected with menin, MLL-AF9, LEDGF and increasing amounts of integrase. D-I. Expression of IN and MLL_{110-160} impairs transformation of BMC with MLL-AF9. BMC were co-transduced with MSCV-neo-MLL-AF9 and MSCV-puro containing IN, MLL_{110-160}, MLL_{110-160} F129A or
MLL<sub>110-160</sub> FLAA. Expression of IN and MLL fragments has been validated using qRT-PCR (Supplementary Figure S5). D. Growth curves normalized to MSCV-puro. E. Expression of *Hoxa9* in transformed cells (day 14). F. Expression of c-Kit (day 14). G. Wright-Giemsa stained cytospins (day 14). H. Colony counts after three rounds of replating. I. Representative colonies in round 3.

**Figure 4.** IBM2 peptide binds to IBD domain and disrupts menin-MLL-IBD and IN-IBD interactions. A. HSQC spectrum of 70 µM IBD (black) titrated with 280 µM (blue) and 1mM (red) IBM2 peptide. B. mapping of chemical shift perturbations onto the structure of MLL-IBD complex upon binding of IBM2 peptide. Color coding represents magnitude of chemical shift perturbations: Δσ < 0.025ppm (yellow), 0.025 < Δσ < 0.08 ppm (orange) and Δσ > 0.08 ppm (red). The MLL fragment is shown in cyan and the key residues in IBM2 motif are blue. C. Schematics rationalizing the use of IBM2 peptide as competitor of menin-MLL-IBD and IN-IBD. The IC<sub>50</sub> values for competition experiments using IBM2 with menin-MLL-IBD and IN-IBD are shown, respectively, in D and E.
Figure 2
Figure 3
Figure 4

(A) 

(B) 

(C) 

(D) 

(E) 

\[ \text{IC}_{50} = 318 \mu M \] 

\[ \text{IC}_{50} = 223 \mu M \]
The same site on LEDGF IBD domain represents therapeutic target for MLL leukemia and HIV

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