hDOT1L Links Histone Methylation to Leukemogenesis

Yuki Okada,1,2 Qin Feng,1,2 Yihui Lin,4 Qi Jiang,2,3 Yaqiang Li,4 Vernon M. Coffield,2,3 Lishan Su,2,3 Guoliang Xu,2 and Yi Zhang1,3,*

1Department of Biochemistry and Biophysics
2Department of Microbiology and Immunology
3Lineberger Comprehensive Cancer Center
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599
4State Key Laboratory of Molecular Biology
Institute of Biochemistry and Cell Biology
Chinese Academy of Sciences
Shanghai 200031
China

Summary

Epigenetic modifications play an important role in human cancer. One such modification, histone methylation, contributes to human cancer through deregulation of cancer-relevant genes. The yeast Dot1 and its human counterpart, hDOT1L, methylate lysine 79 located within the globular domain of histone H3. Here we report that hDOT1L interacts with AF10, an MLL (mixed lineage leukemia) fusion partner involved in acute myeloid leukemia, through the OM-LZ region of AF10 required for MLL-AF10-mediated leukemogenesis. We demonstrate that direct fusion of hDOT1L to MLL results in leukemic transformation in an hDOT1L methyltransferase activity-dependent manner. Transformation by MLL-hDOT1L and MLL-AF10 results in upregulation of a number of leukemia-relevant genes, such as Hoxa9, concomitant with hypermethylation of H3-K27. Our studies thus establish that mistargeting of hDOT1L to Hoxa9 plays an important role in MLL-AF10-mediated leukemogenesis and suggests that the enzymatic activity of hDOT1L may provide a potential target for therapeutic intervention.

Introduction

Covalent modifications of histone tails play important roles in regulating chromatin dynamics and gene expression (Strahl and Allis, 2000). Among the various modifications, histone methylation has attracted great attention due to its diverse functions, which include transcriptional regulation (Lachner et al., 2003; Zhang and Reinberg, 2001). Histone methylation occurs on both lysine and arginine residues that are usually located on the N-terminal tails of histones H3 and H4. With the exception of yeast Dot1 (Ng et al., 2002; van Leeuwen et al., 2002) and its human homolog hDOT1L (Feng et al., 2002), all the histone lysine methyltransferases (HKMases) identified thus far contain an evolutionarily conserved motif, the SET (Suv3-9, E(z), Trx) domain. Many of the HKMases have been linked to cancer (Schneider et al., 2002). For example, the H3-lysine 4 (H3-K4) methyltransferase MLL is frequently translocated in leukemia (Ayton and Cleary, 2001; Milne et al., 2002; Nakamura et al., 2002) and the EZH2 component of the H3-lysine 27 (H3-K27) methyltransferase, the EED-EZH2 complex, is overexpressed in a variety of cancers and its protein level may be directly linked to the invasiveness of these cancers (Bracken et al., 2003; Kleer et al., 2003; Varambally et al., 2002).

Chromosomal translocation is one of the major causes of human cancer, particularly in acute leukemias. The most common chromosome rearrangements found in leukemia patients involve the mixed lineage leukemia gene MLL (also called ALL or HRX) located at 11q23 (Ayton and Cleary, 2001; Daser and Rabbitts, 2004). MLL is the human homolog of Drosophila Trithorax (Trx), a protein involved in maintaining the “on” state of homeotic box (Hox) gene expression during embryonic development. MLL contains a number of functional motifs including the N-terminal AT hook DNA binding motif and the C-terminal SET domain required for its H3-K4 methyltransferase activity (Milne et al., 2002; Nakamura et al., 2002). As a result of chromosome translocation, the MLL N terminus becomes fused in frame to one of more than 30 partner proteins (Ayton and Cleary, 2001; Daser and Rabbitts, 2004). Regardless of whether the fusion partner is normally localized to the cell nucleus or cytoplasm, the chimeras are always nuclear (Dimartino and Cleary, 1999). Since the DNA binding domain of MLL is retained in the fusion proteins, the MLL target genes are usually differentially regulated as a result of a gain of function that is associated with the fusion partner proteins. Hoxa9 has emerged as one of the most relevant MLL target genes in human acute myeloid leukemia (AML) since it is always upregulated in AML (Golub et al., 1999). Indeed, the leukemogenic potential of Hoxa9 was directly demonstrated by the development of AML in mice receiving transplantation of bone marrow cells overexpressing Hoxa9 (Kroon et al., 1998). Recent studies indicate that both Hoxa9 and Hoxa7 are required for MLL fusion proteins to transform myeloid progenitor cells (Ayton and Cleary, 2003). However, the mechanism by which different MLL fusion proteins upregulate Hoxa9 and how higher levels of Hoxa9 lead to leukemia are not known.

Using biochemical and genetic approaches, we and others have previously demonstrated that lysine 79 of histone H3, located in the globular domain, is methylated by a non-SET domain protein called yeast Dot1 and its human homolog hDOT1L (Feng et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002). Here we report the identification of AF10, an MLL fusion partner in human leukemia, as an hDOT1L-interacting protein. The interaction is mediated by an octapeptide motif and a leucine zipper (OM-LZ) region of AF10 required for leukemic transformation by MLL-AF10. Importantly, fusion of hDOT1L to MLL is capable of leukemic transformation in an hDOT1L HMTase activity-dependent manner. Moreover, an HMTase-defective hDOT1L mutant is capable of suppressing growth of MLL-AF10-transformed...
cells. Transformation of progenitor bone marrow cells by MLL-AF10 or MLL-hDOT1L results in upregulation of a set of leukemia-relevant Hox genes, including Hoxa9, concomitant with hypermethylation on H3-K79. Our study thus reveals a role for hDOT1L-mediated H3-K79 methylation of MLL target genes in leukemogenesis and raises the intriguing possibility that the HMTase activity of hDOT1L can serve as a target for therapeutic intervention of human leukemia.

Results

Identification of AF10, an MLL Fusion Protein in Leukemia, as an hDOT1L-Interacting Protein

We have previously cloned the human homolog of yeast DOT1 and demonstrated its H3-K79-specific methyltransferase activity (Feng et al., 2002). To understand the function of hDOT1L, we searched for its functional partners by yeast two-hybrid screening using hDOT1L as bait. This screen resulted in repeated isolation of cDNAs encoding the C-terminal half of AF10 (data not shown), a frequent fusion partner of MLL and CALM in leukemia (Chaplin et al., 1995; Dreyling et al., 1996). To confirm the interaction, we coexpressed AF10 and hDOT1L that were tagged with Flag and HA, respectively. Following immunoprecipitation and Western blot analysis, we demonstrated that the two proteins can be coimmunoprecipitated (Figure 1A, compare lanes 3 and 6).

To demonstrate an association of the two proteins at endogenous protein levels, we raised a polyclonal antibody against hDOT1L. While this antibody can recognize as little as 2 ng of recombinant hDOT1L in Western blotting, it failed to detect endogenous hDOT1L in a number of cell sources (data not shown). In an effort to identify a cell source for detection of the endogenous hDOT1L protein, both Northern blotting and microarray analysis were performed. Results indicated that both human and mouse DOT1L genes are broadly expressed (Figure S1) (Zhang et al., 2004). Interestingly, hematopoietic stem cells and some leukemia/lymphoma cell lines exhibit relatively higher expression levels (Figure S1B). Due to its relatively higher level of hDOT1L expression, Raji cells were used for nuclear extract preparation and coimmunoprecipitation experiments. Results shown in Figure 1B demonstrate that the antibody against AF10 not only immunoprecipitated itself but also coimmunoprecipitated hDOT1L (lane 6). The immunoprecipitation is specific, as a parallel immunoprecipitation using equal amounts of mouse IgG failed to immunoprecipitate either protein (lane 3). In addition, we also attempted to coimmunoprecipitate AF10 using the hDOT1L antibody. However, hDOT1L protein can only be coimmunoprecipitated under denaturing conditions (Figure S2), indicating that either the hDOT1L epitopes or the entire protein is inaccessible by the hDOT1L antibody under native conditions. The above results, in combination with the mammalian two-hybrid results shown below, allow us to conclude that AF10 and hDOT1L associate in vivo.

The OM-LZ Region of AF10, Required for Leukemic Transformation, Is Both Necessary and Sufficient for AF10-hDOT1L Interaction

AF10 was initially discovered by virtue of its involvement in t(10;11)(p12;q23) chromosomal translocations found in acute myeloid leukemia patients (Chaplin et al., 1995). The genetic rearrangement results in fusion of the C-terminal half of AF10 to the N-terminal third of MLL. AF10 contains several motifs common to transcription factors, and the minimal portion of AF10 that is leukemogenic when fused to MLL contains a leucine zipper (LZ) motif and an octapeptide motif (OM) that is highly conserved between homologs of AF10 from C. elegans to human (Figure 1C). To characterize the nature of the AF10-hDOT1L interaction, we mapped the interaction region using a mammalian two-hybrid assay. AF10 and hDOT1L that were fused to the VP16 activation domain and the Gal4-DNA binding domain, respectively, were coexpressed in 293T cells in the presence of a luciferase reporter containing five Gal4 binding sites. Results shown in Figure 1D demonstrate that the 80 amino acid (aa 719–800) OM-LZ region is sufficient to mediate the interaction. Interestingly, this same region was previously reported to be required for leukemic transformation by the MLL-AF10 fusion protein (DiMartino et al., 2002). To evaluate whether the OM-LZ region is necessary for the interaction, we created an AF10 construct where this region was deleted. Flag-AF10 with or without this region was coexpressed with hDOT1L- HA and their ability to interact was analyzed by coimmunoprecipitation. Although both constructs expressed at a similar level (Figure 1E, left panel), deletion of the OM-LZ region abrogated the AF10-hDOT1L interaction (Figure 1E, right panel, compare lanes 6 and 9). Therefore, we conclude that the OM-LZ region on AF10 required for leukemogenesis is both necessary and sufficient in mediating the AF10-hDOT1L interaction.

To evaluate the relative contribution of OM and LZ to the AF10-hDOT1L interaction, we created constructs harboring mutations in the two regions individually or in combination and analyzed their effect on the AF10-hDOT1L interaction using the mammalian two-hybrid assay described above. Results shown in Figure 1F indicate that while mutation in each of the two regions greatly reduced their interaction, mutations in both regions together completely abrogated the interaction. Thus, we conclude that both OM and LZ contribute to the hDOT1L-AF10 interaction. This result, in combination with the previous demonstration that both OM and LZ contribute to the leukemic transformation capability of MLL-AF10 (DiMartino et al., 2002), raises the possibility that recruitment of hDOT1L by MLL-AF10 may directly contribute to the leukemic transformation capability of MLL-AF10.

Transformation of Murine Myeloid Progenitor Cells by MLL-hDOT1L Requires the HMTase Activity of hDOT1L

The above results suggest that hDOT1L may participate in MLL-AF10-mediated leukemic transformation. To determine whether MLL-AF10 is capable of recruit-
Figure 1. The OM-LZ Region of AF10, Required for Leukemogenesis of MLL-AF10, Is Both Necessary and Sufficient for hDOT1L and AF10 Interaction

(A) AF10 and hDOT1L coimmunoprecipitate when coexpressed in 293T cells. Flag and HA antibodies were used for immunoprecipitation and Western blotting, respectively.

(B) Coimmunoprecipitation of endogenous hDOT1L and AF10. To stabilize hDOT1L protein, Raji cells were treated with 10 μM MG-132 (Peptides International) for 10 hr before harvesting. An anti-AF10 monoclonal antibody was used for immunoprecipitation. The same amount of mouse IgG was used as a control. Antibodies used for Western blotting are indicated. In, FT, and IP represent input (3%), flow-through, and bound, respectively.

(C) Diagram of AF10 functional motifs and sequence alignment of the OM-LZ region. Arrow indicates the break point for MLL-AF10 fusion. Human AF10 (GenBank number AAT47519) and its homologs from mouse (GenBank number O54826), Drosophila (GenBank number AAF72595), and C. elegans (GenBank number 2122400A) were compared. Conserved amino acids are marked in red. Mutations on the amino acids marked by * affect the interaction between hDOT1L and AF10 (E).

(D) Mammalian two-hybrid analysis located the OM-LZ region of AF10 to be sufficient for mediating AF10 and hDOT1L interaction. The bars represent average fold of activation from four independent experiments.

(E) Coimmunoprecipitation experiments demonstrating that the OM-LZ region of AF10 is necessary for the AF10 and hDOT1L interaction. Western blotting shown on the left panel indicates that the wild-type and the deletion mutant proteins were expressed to a similar level.

(F) Mammalian two-hybrid assays demonstrating that both the evolutionarily conserved octapeptide (EQLLERQW) motif (OM) and the leucine zipper (LZ) of AF10 contribute to the interaction. The mutations are marked by * as in (B). The bars represent average fold of activation from four independent experiments.

To test this possibility, we analyzed the ability of MLL-hDOT1L to immortalize murine progenitor bone marrow cells using a myeloid colony-formation assay outlined in Figure 2B. Toward this end, a modified murine stem cell virus (MSCV) derived retroviral vector, designated as MSCN, was used to transduce freshly harvested bone marrow progenitor cells from mice pretreated with 5-fluorouracil. MSCN constructs encoding MLL sequences 5’ of the translocation breakpoint (MLL-N), MLL-AF10 that mimics the most frequent fusion involving the two proteins, and MLL fused to various length
of hDOT1L were made (Figure 2C, left panel and data not shown). To facilitate monitoring expression of these constructs, an N-terminal Flag-tag was added to each of them. Due to the size limitation for efficient packaging of retroviral vectors, MLL-hDOT1L fusion constructs that encode longer than the first 670 amino acids of hDOT1L have dramatically decreased retroviral transduction efficiency (data not shown). This prevented us from analyzing MLL-hDOT1L that contains the full-length hDOT1L. Nevertheless, retroviruses with similar titer as that of MLL-AF10 were successfully generated with MLL-hDOT1L(1–670) (data not shown).

We have shown previously that the N-terminal 416 amino acids of hDOT1L have robust H3-K79 HMTase activity in vitro (Min et al., 2003b). Importantly, HMTase assays demonstrate that MLL-hDOT1L(1–670) is enzymatically active, while the protein harboring the “GSG163-165RCR” mutations is enzymatically inactive (data not shown). Western blotting of extracts from transiently transfected retroviral packaging cells confirmed that MLL-N, MLL-AF10, MLL-hDOT1L, and hDOT1L constructs were efficiently expressed (data not shown). Plating of bone marrow progenitor cells transduced with the various MSCN constructs under selective conditions showed a variable number (50–200) of primary or first round colonies that are consistent with their respective virus titer. However, significant differences were observed in a second round plating of 10^4 cells pooled from colonies harvested from the first round of cultures. Compared to the first round, vector

Figure 2. The HMTag Activity of hDOT1L Is Required for Bone Marrow Transformation Capability of MLL-hDOT1L
(A) Immunofluorescence staining revealing colocalization of hDOT1L with MLL-AF10 to a few large foci when the two proteins are coexpressed.
(B) Schematic presentation of the retroviral transduction procedures.
(C) Diagram of retroviral constructs expressing MLL, MLL-AF10, and MLL-hDOT1L (left panel). The numbers refer to the amino acid number of corresponding proteins. The HMTag-defective mutant contains a GSG to RCR mutation in the SAM binding domain (Feng et al., 2002). Colony numbers generated in the first, second, and third round of plating of 1 × 10^4 transduced bone marrow cells are shown (right panel). The various controls and fusion proteins transduced are indicated. Data presented are an average of four independent experiments with error bars.
alone, MLL-N, hDOT1L, and the MLL-hDOT1L mutant transduced cultures produced a decreased number of colonies. In contrast, MLL-hDOT1L- and MLL-AF10-transduced cells gave rise to hundreds if not thousands of colonies, an amount significantly higher than that from the first round of plating (Figure 2C, right panel). When a second round of plating was assayed, only MLL-hDOT1L- and MLL-AF10-transduced cells gave rise to increased number of colonies compared with that from the second round of plating (Figure 2C, right panel). These results allow us to conclude that similar to MLL-AF10, MLL-hDOT1L has leukemic transformation capability and the ability depends on the HMTase activity of hDOT1L.

hDOT1L HMTase Activity Is Required for Maintenance of the Transformed Cell Status in Cells Transformed by MLL-AF10

Having established a role for the HMTase activity of hDOT1L in transforming murine primary bone marrow progenitor cells, we asked whether the HMTase activity is also required for maintaining the MLL-AF10-transformed cell status. To address this question, we examined the effect of the wild-type hDOT1L and the HMTase-defective mutant hDOT1L on the sustaining growth capability of MLL-AF10-transformed bone marrow cells. Full-length wild-type and HMTase-defective mutant hDOT1L were cloned into retroviral vectors expressing the Blastidicin resistance gene. Viruses generated were used to infect MLL-AF10-transduced/transformed bone marrow cells derived from second round plating (Figure 3A). Cells expressing hDOT1L were selected in the presence of Blastidicin. After 10 days of methylcellulose culture, we noticed a dramatic difference in the colony-formation assay (Figure 3B). While transduction of wild-type hDOT1L to MLL-AF10-expressing cells does not significantly alter the colony-formation capability when compared with transduction of an empty vector, transduction of HMTase-defective hDOT1L to MLL-AF10 significantly suppressed the ability of MLL-AF10 to support colony formation.

The above results have two possible explanations. The first is that the mutant hDOT1L may have a general role in suppression of cell growth. Alternatively, the mutant hDOT1L may function in a dominant-negative fashion by preventing recruitment of wild-type hDOT1L to MLL-AF10 target genes such as Hoxa9 whose expression is required for maintaining the transformed cell status (Aytton and Cleary, 2003). To differentiate between these two possibilities, we repeated the above experiments using MLL-AFX-transformed cells. MLL-AFX was chosen because of the low latency, reduced penetrance, and hematologic features of the leukemias induced by this fusion protein are in contrast with the more aggressive features of leukemias induced by MLL-AF10 (So and Cleary, 2003), and consequently the two fusion proteins are likely to employ different mechanisms of leukemogenesis. In contrast with the effect observed on MLL-AF10-transformed cells, no significant effect on MLL-AFX-transformed cells was observed when wild-type or mutant hDOT1L were expressed (Figure 3B). These results indicate that the dominant-negative effect observed on MLL-AF10-transformed cells is specific and consistent with a role for the HMTase activity of hDOT1L in maintaining the growth capability of murine bone marrow cells transformed by MLL-AF10.

siRNA-Mediated mDOT1L Knockdown Impairs Cell Proliferation

The above results indicate that hDOT1L is critical in MLL-AF10-mediated leukemic transformation. To gain further supporting evidence for this conclusion, we attempted to attenuate the mouse DOT1L (mDOT1L) function in progenitor bone marrow cells using siRNA-mediated knockdown and asked whether the cells can still be transformed by MLL-AF10. To this end, two siRNA retroviral constructs that target different regions of the mDOT1L mRNA were made and their ability in knocking down mDOT1L in 3T3 cells was confirmed by RT-PCR (Figure S3A). As outlined in Figure 3C, progenitor bone marrow cells were transduced with the knockdown viruses. After selection under Blastidicin, the recovered colonies were analyzed for mDOT1L expression and for transduction by MLL-AF10 as well as MLL-AFX. Although knockdown viruses that express RNAi2 are more efficient in knocking down mDOT1L in 3T3 cells (Figure S3A), transduction of progenitor bone marrow cells with viruses expressing RNAi2 generated no knockdown colonies. This is likely due to the requirement of a certain level of mDOT1L for cell survival (see below). However, we were able to recover colonies from transduction by the viruses expressing RNAi1, which resulted in about 50% knockdown (Figure 3D and Figure S3A). Transduction of these recovered mDOT1L knockdown cells by MLL-AF10, MLL-AFX, or vector control generated no colonies (Figure 3E). In contrast, large numbers of colonies were generated when the mock vector knockdown cells were transduced by the same MLL-AF10, MLL-AFX-expressing viruses in parallel experiments (Figure 3E). No colony was generated from mDOT1L knockdown cells transduced by MLL-AFX, even though the bone marrow transformation capability of MLL-AFX is not dependent on hDOT1L (Figure 3B). Given this fact, we believe that a critical level of mDOT1L is required for cell survival. This interpretation is consistent with our observation in 3T3 cells where both RNAi1 and 2 expression resulted in cell growth arrest and cell death, and the more efficient RNAi2 exhibited a more severe phenotype (Figures S3B and S3C).

MLL-hDOT1L Immortalizes Murine Myeloid Progenitors

The similar GM (granulocyte-macrophase)-like morphology of the colonies formed from MLL-hDOT1L- and MLL-AF10-transduced bone marrow cells (Figure 4A) suggested that MLL-hDOT1L-transformed cells, like those transformed by MLL-AF10 (DiMartino et al., 2002), were likely to be of myeloid lineage. To test this possibility, we first verified that both MLL-hDOT1L and MLL-AF10 were expressed in cells derived from the respective colonies (Figure 4B). Then, cell lineages were analyzed by Wright-Giemsa staining and FACS using antibodies against specific cell-surface markers (Figures 4C–4F), Wright-Giemsa-stained cytospin prepara-
Figure 3. Role of hDOT1L and Its HMTase Activity in Maintenance of Transformed State and Cell Survival

(A) Diagram of the transduction procedure.

(B) The HMTase-defective hDOT1L attenuates proliferation of MLL-AF10-transformed cells, but not that of MLL-AFX-transformed cells. To adjust for the difference in titers of the different viruses, relative colony numbers were used (vector control was calculated as 100%). Data presented are an average of four independent experiments with error bars.

(C) Diagram of the sequential transduction of bone marrow cells with mDOT1L RNAi followed by MLL-AF10/MLL-AFX.

(D) Quantification of the mDOT1L knockdown efficiency by RT-PCR. Numbers of PCR cycles were indicated. GAPDH was used as a control.

(E) Effect of mDOT1L knockdown on colony formation of MLL-AF10- or MLL-AFX-transduced bone marrow cells. Colony numbers are indicated as an average of two independent experiments.

Measurements revealed that colonies derived from MLL-hDOT1L-transduced cells have similar features as those derived from MLL-AF10. The majority of the cells appear to be immature myeloblasts. However, a few differentiated macrophage-like cells were also visible (Figure 4C). Immunophenotyping of the cells from the third round of plating revealed that almost all of the cells derived from MLL-AF10- or MLL-hDOT1L-transduced colonies expressed the early hematopoietic progenitor cell marker c-Kit and about half of them expressed the myeloid cell marker CD11b but not Gr-1 (Figures 4D and 4E). None of the cells expressed the B cell lineage marker B220.
or the T cell lineage marker CD3 (Figure 4F). In addition, markers for mast cells (IgE) or erythrocytes (Ter119) were also absent (data not shown). These results suggest that although the colony numbers formed by MLL-hDOT1L and MLL-AF10 transduction are not the same, both are capable of immortalizing bone marrow progenitor cells at a relatively early stage of myeloid differentiation. Nevertheless, MLL-hDOT1L cannot completely phenocopy MLL-AF10 (see below). Whether the difference is caused by the use of a partial, instead of the full-length, hDOT1L in the MLL fusion or additional factors are recruited by AF10 remains to be determined.

\textbf{Hoxa9 and Hoxa7 Upregulation Is Associated with Transformation}

In addition to participating in embryogenesis, Hox genes also play important roles in normal hematopoiesis and leukemogenesis (Buske and Humphries, 2000; Sauvageau et al., 1994). To understand how hDOT1L and its associated HMTase activity contribute to leukemogenesis, we determined the expression profile of Hoxa genes in vector-transduced and MLL-AF10- or MLL-hDOT1L-transduced murine progenitor bone marrow cells. Toward this end, we analyzed the expression levels of all 13 Hoxa genes by semiquantitative reverse transcriptase PCR (RT-PCR). Similar to a previous report (Ayton and Cleary, 2003), cells immortalized by MLL-AF10 resulted in upregulation of a number of genes in the Hoxa locus relative to that of vector-transduced cells (Figure 5A). Consistent with the fact that MLL-hDOT1L did not completely phenocopy MLL-AF10 in the transformation assay, cells derived from MLL-hDOT1L-transduced colonies had fewer numbers of Hoxa genes upregulated relative to that from MLL-AF10-transduced cells. However, Hoxa9, Hoxa7, and Meis1 are upregulated in cells transformed by either MLL-AF10 or MLL-hDOT1L, consistent with the fact that these genes are required for MLL fusion protein-mediated transformation (Ayton and Cleary, 2003). Similar to a previous report about the upregulation of Hoxa5 and Hoxa11 by MLL fusion proteins (Ayton and Cleary, 2003), we also detected upregulation of these two genes in MLL-hDOT1L-transduced cells. Whether upregulation of either one or both is a requirement for leukemic transformation remains to be determined.

\textbf{Hoxa9 Is Required for MLL-AF10- and MLL-hDOT1L-Mediated Bone Marrow Cell Immortalization}

Previous studies have demonstrated that both Hoxa9 and Hoxa7 are independently required for immortalization of bone marrow cells by MLL-ENL (Ayton and Cleary, 2003). Given that both Hoxa9 and Hoxa7 are upregulated in cells transformed by MLL-AF10 and MLL-hDOT1L, we evaluated the requirement of Hoxa9 in progenitor bone marrow cell transformation by MLL-AF10 or MLL-hDOT1L. Toward this end, progenitor bone marrow cells isolated from Hoxa9−/− mice were transduced by MLL-AF10 or MLL-hDOT1L and analyzed by the methylcellulose colony replating assay as described in Figure 2B. Results shown in Figure 5B indicate that the Hoxa9 gene is required for immortalization of progenitor bone marrow cells by both MLL-AF10 and MLL-hDOT1L because neither MLL-AF10 nor MLL-hDOT1L is capable of immortalizing Hoxa9-deficient progenitor bone marrow cells.
Figure 5. Expression of Hoxa9 Is Required for Transformation of Mouse Progenitor Bone Marrow Cells by MLL-AF10 and MLL-hDOT1L

(A) Upregulation of late Hoxa and Meis1 genes in cells transduced by MLL-AF10 and MLL-hDOT1L. Expression of Hoxa and Meis1 genes, analyzed by RT-PCR, in MLL-AF10- and MLL-hDOT1L-transformed cells (second round colonies) was compared with that in cells infected with an empty retrovirus vector (first round colonies). The expression of Meis1 was analyzed because Meis1 was reported to collaborate with late Hoxa genes in myeloid leukemogenesis (Nakamura et al., 1996). GAPDH serves as a control for equal input in RT-PCR of different samples. Template amounts and cycle numbers for PCR are described in Experimental Procedures.

(B) Hoxa9 knockout attenuates the transformation ability of MLL-AF10 and MLL-hDOT1L. Colony assays were performed as that in Figure 2. Colony numbers generated in the first, second, and third round from plating $1 \times 10^4$ transduced bone marrow cells are shown. Data represents average of three independent experiments with error bars.

Hoxa9 Upregulation Correlates with H3-K79 Hypermethylation

Having demonstrated the requirement for the Hoxa9 gene in MLL-AF10- and MLL-hDOT1L-mediated leukemic transformation, we attempted to determine the molecular basis underlying Hoxa9 upregulation. Using myeloid progenitor cells immortalized by MLL-hDOT1L or MLL-AF10, we first determined the location of the fusion proteins on the Hoxa9 gene by ChIP analysis. Since the fusion proteins were tagged by an N-terminal Flag, a monoclonal antibody against Flag was used in the ChIP assay. As a negative control, a monoclonal antibody against HA was used in parallel experiments. DNA recovered from the ChIP experiments was amplified by PCR using 24 pairs of primers that cover the region from the last exon of Hoxa10 to the first exon of Hoxa7 (Figure 6A). Results revealed a similar distribution of the two fusion proteins with the exception of two regions (amplicons o-p, v-x). The presence of MLL-AF10, but not MLL-hDOT1L, in these two regions upstream of Hoxa7 is consistent with the fact that the Hoxa7 expression level is higher in cells transformed by MLL-AF10 relative to that transformed by MLL-hDOT1L (Figure 5A).

Having determined the localization of the MLL fusion proteins, we analyzed the histone modification patterns in the regions where both MLL-hDOT1L and MLL-AF10 are enriched (amplicons a, g, i, j, and s). Our analysis was focused on H3-K79 and H3-K4 methylation because these two sites are targeted by the hDOT1L and MLL HMTases, respectively (Feng et al., 2002; Milne et al., 2002; Nakamura et al., 2002). Consistent with binding of MLL to these sites in normal bone marrow cells transduced with an empty vector, H3-K4 methylation was detected (Figure 6B, compare lanes 5 and 6). In contrast, no significant H3-K79 methylation was detected at any of these sites (Figure 6B, lane 4), suggesting that hDOT1L is not recruited to the Hoxa9 gene in normal bone marrow cells. Interestingly, similar analysis in leukemic cells revealed robust H3-K79 methylation, regardless of whether the cells were transformed by MLL-hDOT1L or MLL-AF10 (Figure 5B, lanes 10 and 16). With the exception of amplicon “a,” all showed higher levels of H3-K79 methylation in MLL-AF10-trans-
formed cells when compared with that of cells trans- 
formed by MLL-hDOT1L (compare lanes 10 and 16). 
This could be explained in a number of ways. For ex- 
ample, fusion of MLL to hDOT1L could inhibit hDOT1L 
HMTase activity. Alternatively, the lack of an hDOT1L 
C-terminal in the fusion protein could prevent recruit- 
ment of cofactors required for optimal hDOT1L HMTase 
activity. Comparison of the H3-K4 methylation levels in 
normal and leukemic cells (Figure 6B, compare lanes 
11 and 17 with lane 5) revealed a general decrease, 
which is consistent with the fact that MLL-hDOT1L or 
MLL-AF10 may compete with endogenous MLL for 
binding to these sites. Collectively, the above results 
support the notion that leukemogenesis by MLL-AF10 
involves upregulation of Hox9 mediated through re- 
cruitment of hDOT1L and subsequent H3-K79 methyla- 
tion. How methylation on H3-K79 leads to transcription 
activation remains to be determined.

Discussion

MLL belongs to the Trithorax group of proteins and is 
involved in maintaining the “on” state of Hox gene ex- 
pression. Recent studies indicate that one way in which 
MLL maintains Hox gene expression might be through 
methylation of H3-K4 at MLL binding sites (Milne et al., 
2002; Nakamura et al., 2002). Chromosome transloca- 
tions involving MLL are associated with the majority of 
myeloid leukemia in human patients (Ayton and Cleary, 
2001; Daser and Rabbitts, 2004). As a result of chromo- 
some translocation, the N terminus of MLL becomes 
fused in-frame to one of a wide variety of proteins. The 
fusion proteins retain the DNA binding domain of MLL 
and lose the C-terminal SET domain, thus the H3-K4 
methyltransferase activity. At the same time, the fusion 
proteins gain function that is associated with the part- 
tner protein. Since the DNA binding domain of MLL is 
retained in the translocation, MLL fusion proteins are 
expected to target the same sets of genes as those 
targeted by MLL. The fact that H3-K4 methylation is 
linked to transcriptional activation (Santos-Rosa et al., 
2002), in combination with the fact that MLL fusion pro- 
teins do not possess H3-K4 methyltransferase activity, 
imply that MLL target genes may be downregulated in 
leukemia involving MLL fusion. However, contrary to 
this prediction, most of the leukemia-relevant MLL 
target genes, such as the late HoxA genes, are upregu- 
lated in MLL fusion protein-mediated leukemias (Ayton 
and Cleary, 2003). To explain this seemingly contradic- 
tory outcome, the effect on transcription caused by the 
loss of the H3-K4 methyltransferase activity must be 
overcome by the transcriptional activity gained from 
the fusion partner. In this study, we provide several lines 
of evidence suggesting that one way to compensate for 
the loss of H3-K4 methyltransferase activity is the recruit- 
mnt of hDOT1L, an H3-K79 HMTase previously 
linked to transcriptional activation (Feng et al., 2002; 
Ng et al., 2002; van Leeuwen et al., 2002).
Figure 7. Models Illustrate the Involvement of hDOT1L in Hoxa9 Regulation and Myeloid Transformation by MLL-AF10

(A) During normal myeloid cell differentiation, MLL complex induces methylation on H3-K4 and maintains the basal level of Hoxa9 expression.

(B) In myeloid cells with MLL-AF10 fusion, hDOT1L is recruited to MLL target genes, such as Hoxa9, through an interaction with the OM-LZ of AF10, leading to hypermethylation of H3-K79 and upregulation of Hoxa9.

(C) The HMTase-defective hDOT1L mutant can function in a dominant-negative fashion to prevent recruitment of the wild-type hDOT1L leading to inhibition of Hoxa9 expression and leukemogenesis. The thickness of the arrows represents relative expression levels.

In searching for hDOT1L-interacting proteins, we identified AF10, a frequent fusion partner of MLL in leukemia patients. The fact that the hDOT1L-AF10 interaction is mediated through the OM-LZ domain of AF10 required for leukemogenesis raised the possibility that AF10 may function as a bridge for the recruitment of hDOT1L to the genes targeted by MLL-AF10 (Figure 1). Consistent with this possibility, direct fusion of hDOT1L to MLL resulted in transformation of bone marrow progenitor cells. Importantly, both the cell lineage (Figure 4) and Hoxa gene expression pattern (Figure 5A) of the transformed cells are similar to those transformed by MLL-AF10, supporting the possibility that one of the major function of AF10 in MLL-AF10 is to recruit hDOT1L. In addition, our study indicates that the transformation ability of MLL/hDOT1L is dependent on the H3-K79 methyltransferase activity of hDOT1L (Figure 2C). Furthermore, overexpression of an HMTase-defective hDOT1L mutant suppressed continued cell growth of MLL-AF10- but not MLL-AFX-transformed cells (Figure 3B). These results suggest that the enzymatic activity of hDOT1L is not only required for transformation but also required for maintaining the transformed status, raising the intriguing possibility that the hDOT1L HMTase activity may serve as a target for therapeutic intervention.

How does the enzymatic activity of hDOT1L contribute to leukemogenesis? Previous studies have demonstrated a critical role in the upregulation of late Hoxa genes, particularly Hoxa9 and Hoxa7, in leukemogenesis mediated by MLL-ENL (Ayton and Cleary, 2003; Kroon et al., 1998). Consistent with these observations, direct fusion of hDOT1L to MLL resulted in upregulation of a set of late Hoxa genes, including both Hoxa7 and Hoxa9, similar to those upregulated by MLL-AF10 (Figure 5A). Importantly, disruption of the Hoxa9 gene abrogated bone marrow transformation ability of MLL-AF10 or MLL-hDOT1L. ChIP assay demonstrated that binding of MLL-AF10/MLL-hDOT1L to the Hoxa9 gene in transformed cells correlates with hypermethylation of H3-K79 (Figure 6). Given that H3-K79 methylation has been linked to transcriptional activation (Feng et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002), hypermethylation on H3-K79 probably contributes to Hoxa9 upregulation and leukemic transformation.

Recent studies have demonstrated that MLL is cleaved by Taspase-1 into two subunits, MLLN (p300) and MLLC (p180), which form a heterodimer (Hsieh et al., 2003; Yokoyama et al., 2002). This cleavage is necessary for the expression of early Hoxa genes but is not required for the expression of late Hoxa genes, which are overexpressed in many MLL-fusion leukemias (Hsieh et al., 2003). Several protein complexes containing the MLL heterodimer have been recently purified (Hughes et al., 2004; Nakamura et al., 2002; Yokoyama et al., 2004). One complex contains MLL1/2, hAsh2, Rbp5, WDR5, HCFs, and Menin (Hughes et al., 2004; Yokoyama et al., 2004). The HCFs and Menin subunits in this complex associate with MLLN (Yokoyama et al., 2004). Based on our studies and in consideration of these recent developments, we propose the following models, presented in Figure 7, to explain the functions of MLL and its fusion proteins in normal and leukemia states. In normal myeloid differentiation (Figure 7A), the MLL complex uses its H3-K4 HMTase activity to establish H3-K4 methylation patterns on MLL target genes, resulting in appropriate levels of gene expression required for normal myeloid differentiation. In leukemia (Figure 7B), however, fusion of MLL to its partners, such as AF10, results in loss of the MLLC subcomplex as well as the MLLN-associated HCFs, leading to decreased levels of H3-K4 methylation. At the same time, protein factors or enzymatic activities that associate with the MLL fusion partners are recruited to the MLL target...
genes, leading to a different transcriptional outcome. In the case of MLL-AF10, the associated factor is hDOT1L, an H3-K79-specific HMTase. As a result, MLL target genes become hypermethylated on H3-K79, leading to their upregulation. This, in turn, may block myeloid differentiation and promote proliferation, leading to leukemia. The HMTase-defective hDOT1L apparently can function in a dominant-negative fashion by competing for AF10 binding (Figure 7C). In this case, methylation on both H3-K4 and H3-K79 of the MLL target genes will be kept at a very low level, resulting in very low or no expression of some MLL target genes. Expression of some of these genes may be required for cell proliferation or survival. As a result of the dominant-negative effect, cells that express an hDOT1L mutant may stop growing or undergo apoptosis.

In summary, our studies suggest that mistargeting of hDOT1L and a subsequent change in the histone methylation pattern from H3-K4 to H3-K79 of key MLL target genes, such as HoxA9, might be a crucial signal for oncogenic transformation of myeloid cells in at least some MLL-associated leukemias. The demonstration that the enzymatic activity of hDOT1L is crucial for leukemogenesis suggests that hDOT1L might be a potential target for therapeutic intervention. Thus, identification of small molecules that are capable of disrupting the interaction between hDOT1L and AF10 or inhibiting the HMTase activity of hDOT1L might lead to a novel treatment for leukemia. In this regard, our recent determination of the hDOT1L catalytic domain structure is of particular relevance (Min et al., 2003a).

Experimental Procedures

Yeast Two-Hybrid Screen

To identify hDOT1L-interacting proteins, a yeast two-hybrid screen was performed with the Matchmaker Gal4 two-hybrid system 3 (Clontech). The cDNA encoding the full-length hDOT1L was fused in-frame to the GAL4 DNA binding domain in the bait vector pGBKT7. This construct was transformed into Saccharomyces cerevisiae host strain AH109. A Pretransformed Mouse Testis MATCHMAKER cDNA library (Clontech) was screened following instructions of the manufacturer. Approximately 4.8 × 10^6 individual clones were screened and about 53 clones grew on the selected medium lacking His, Ade, Trp, and Ura. The clones were further selected by growth on SD-Ade−/His−/Leu−/Trp−/X-gal master plates. The prey plasmids were rescued and electroporated into E. coli strain Top10. Of the 23 clones recovered, 18 encode the C-terminal of AF10.

Constructs and Antibodies

For the mammalian two-hybrid assay (Clontech), various regions of hDOT1L were cloned into the pM vector (Clontech) to generate fusions with the Gal4 DNA binding domain. Various regions of AF10 were cloned into the pVP16 vector (Clontech) to generate fusions with the VP16 transcriptional activation domain. pG5LUC is a reporter vector which contains the luciferase coding region downstream of the adenovirus E1b gene minimal promoter and five GAL4 binding sites. For retroviral vector construction, the 5’LTR region of murine stem cell virus vector (MSCVneo, Clontech) was replaced by cytomegalovirus immediate early promoter sequences (MSCN). MSCN-MLL(N) and MSCN-MLL-AF10 were constructed from MSCV5-MLL and MSCV5-MLL-AF10 with a Flag tag upstream of the MLL gene. MSCN-hDOT1L was constructed by inserting hDOT1L cDNA (encoding amino acids 1–670) downstream of the MLL gene. MSCB vector was constructed by replacement of the neomycin-resistant gene of MSCN to the Blasticidin-resistant gene, and the full-length hDOT1L with or without mutations in the SAM binding site was inserted downstream of a Flag tag. RNAi constructs were made by synthesizing oligonucleotides encoding 21 bp short-hairpin RNA that target mDOT1L (RNAi1: 5'-GCACCATGA TGCGTCTCATGA-3', RNAi2: 5'-GCTGGCCATGGAGAACTATGT-3') and cloned into pMSCV-derived Blasticidinr retrovirus vector under the regulation of H1 RNA promoter. Antibodies against hDOT1L were generated in rabbits using the first 391 amino acids as antigen. The AF10 antibody was a gift from Dr. Bryan Young. Affinity-purified antibodies against H3-2mK79 and H3-2mK4 have been previously described (Feng et al., 2002).

Retrovirus Preparation, Transduction, and Colony Assays

Retroviral production and transduction of bone marrow progenitor cells were essentially carried out as described (Coffield et al., 2003). Briefly, MSCN or MSCB vectors containing MLL-AF10, MLL-hDOT1L, etc. were cotransfected with pTag-pol and pSV2g to 293T cells by calcium-phosphate method. At 48 to 72 hr post-transfection, the supernatants were collected and were used for transduction of bone marrow progenitor cells prepared as follows: 4- to 12-week-old C57BL/6 mice or 6-month-old Hoxa9−/− mice (provided by Dr. Michael Cleary) were injected intravenously with 5–fluourouracil (150 mg/kg), and bone marrow (BM) progenitor cells were harvested from both femurs at 5 days postinjection. Retroviral supernatants were used to transduce BM cells by spinoculation. Two days after infection, infected cells were plated into methylcellulose cultures.

For methylcellulose colony assays, 1 × 10^5 of retrovirally infected BM progenitor cells were plated in 0.9% methylcellulose (Cell Technologies) supplemented with 10 ng/ml of murine IL-3, IL-6, GM-CSF, 50 ng/ml SCF, and 3 μg/ml hEpo in the presence of 1 mg/ml G418 (GIBCO). After 7–10 days of culture, the number of colonies was counted and the single-cell suspensions prepared from G418-resistant colonies were replated into methylcellulose supplemented with the same growth factors without G418. Further plating was repeated every 7–10 days. For the experiments in Figures 3A and 3B, BM cells derived from a second round of plating were plated on plates containing 5 μg/ml of Blasticidin in the next day of the infection of MSCB vectors. For the experiments in Figures 3C–3E, 5 μg/ml of Blasticidin was used after the first infection, and 1 mg/ml of G418 was used after the second infection. For selection of ST3 cells (Figure S3), Blasticidin was applied with a final concentration of 2 μg/ml.

FACS Analysis

Cells harvested from the third round colonies of the methylcellulose plating were incubated for 20 min on ice with CD16/CD32 Fc block (BD Pharmingen, San Diego, California) and subsequently stained for 30 min on ice with phycoerythrin-, fluorescein isothiocyanate-, and allophycocyanin-conjugated isotype controls and monoclonal antibodies against CD11b, Gr-1, c-kit, B220, CD3, IgE, and Ter119 (BD Pharmingen). Cells were then washed with 2% FBS-containing PBS and analyzed using a FACSCalibur (BD Pharmingen), and collected data were analyzed with Summit V3.1 (Cytometry Inc., Fort Collins, Colorado).

Cell Culture, Transfections, Immunoprecipitation, Immunofluorescent Staining, as well as Hox Gene Expression and ChIP Assays

Please see Supplemental Data available with this article (see below).

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.cell.com/cgi/content/full/121/2/167/DC1/.

Acknowledgments

We thank Dr. Michael Cleary for MLL-AF10 and MLL-AFX constructs and Hoxa9 knockout mice and Dr. Bryan Young for AF10 antibody. We are grateful to Eric Kallin for critical reading of the manuscript. Y.O. is a research fellow of the Japan Society for the...
Promotion of Science. This work was supported by grants from Max-Planck Society, CAS, and China NSF to G.X. and NIH to L.S. (AI0330402, AI04840704) and Y.Z. (GM63076, GM68904).

Received: September 23, 2004
Revised: February 13, 2005
Accepted: February 17, 2005
Published: April 21, 2005

References


