Interplay of RUNX1/MTG8 and DNA Methyltransferase 1 in Acute Myeloid Leukemia

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Abstract

The translocation t(8;21)(q22;q22) in acute myeloid leukemia (AML) results in the expression of the fusion protein RUNX1/MTG8, which in turn recruits histone deacetylases (HDAC) to silence RUNX1 target genes [e.g., interleukin-3 (IL-3)]. We previously reported that expression of the RUNX1/MTG8 target gene IL-3 is synergistically restored by the combination of inhibitors of HDACs (i.e., depsipeptide) and DNA methyltransferases (DNMT; i.e., decitabine) in RUNX1/MTG8-positive Kasumi-1 cells. Thus, we hypothesized that DNMT1 is also part of the transcriptional repressor complex recruited by RUNX1/MTG8 by RUNX1/MTG8 and DNMT1 were concurrently released from the IL-3 promoter by exposure to depsipeptide or stabilized on the promoter by decitabine treatment. Finally, we proved that RUNX1/MTG8 and DNMT1 were functionally interrelated by showing an enhanced repression of IL-3 after coexpression in 293T cells. These results suggest a novel mechanism for gene silencing mediated by RUNX1/MTG8 and support the combination of HDAC and DNMT inhibitors as a novel therapeutic approach for t(8;21) AML. (Cancer Res 2005; 65(4): 1277-84)

Introduction

The translocation t(8;21)(q22;q22) is one of the most common nonrandom cytogenetic aberrations occurring in acute myeloid leukemia (AML) and is associated with a relatively favorable prognosis (1). At the molecular level the translocation results in the fusion of the amino-terminal portion of RUNX1 (also known as AML1 or CBFA2) on chromosome 21q22 with nearly full length MTG8 (ETO or CBFA2T1) on chromosome 8q22, thereby creating a novel chimeric gene, RUNX1/MTG8 (2, 3).

Of the two fusion partners, RUNX1 is a member of the runt domain family and encodes the subunit of core binding factor (CBF; ref. 4). Through its runt homology domain (RHD), RUNX1 binds to both the subunit of CBF (CBF) and the enhancer core sequence 5′-TGT/GAG-3′ (2, 5) and plays a critical role as a master regulator of the expression of several hematopoietic-specific target genes, including interleukin-3 (IL-3; ref. 6), GM-CSF (7), CSF-1R (8) and p14ARF (9), among others. As a transcription factor, RUNX1 requires additional cofactors including histone acetyltransferases that mediate transcription-permissive chromatin changes and, thereby, expression of the target genes (10, 11).

The other fusion partner, MTG8, is a putative gene repressor that makes distinct contacts with multiple histone deacetylases (HDAC) while interacting with mSin3A and N-CoR through its oligomerization domains (9, 12–15). Incapable of binding DNA directly, MTG8 and its associated corepressors are recruited to the target gene promoters by sequence-specific interactions with DNA-binding proteins, such as promyelocytic leukemia zinc finger (PLZF; ref. 16), growth factor independence-1 (GFI-1; ref. 17) and BCL-6 (18). In the RUNX1/MTG8 chimeric protein, the MTG8 fusion partner retains the ability to form stable complexes with N-CoR/mSin3A and HDACs while being recruited to the target gene promoter by the RUNX1 moiety (13, 19).

Previous studies have showed that both deacetylation of chromatin histones and methylation of gene promoter CpG islands play an important role in gene silencing in normal and malignant hematopoiesis (20). Emerging data support the interplay between these two mechanisms mediated through MeCP2 and other methyl-binding proteins that functionally link the DNA methyltransferases (DNMT) to HDACs (21, 22). This model has gained further support by studies showing that both DNA hypomethylating agents and HDAC inhibitors synergistically reactivate epigenetically silenced genes (23). We previously reported that the HDAC inhibitor depsipeptide (FR228) and the DNMT inhibitor decitabine (5-aza-2′-deoxycytidine) synergistically increase the transcription of the RUNX1 target gene IL-3 in RUNX1/MTG8-positive cells (24), suggesting that both DNA demethylation and histone deacetylation contribute to the transcriptional repression activity of the fusion protein. Indeed, this might represent a common mechanism for chimeric oncogenic transcription factors in AML because in a recent report the fusion protein PML/RAR expressed in t(15;17) acute promyelocytic leukemia (25) was shown to repress RAR target genes by interacting with both HDACs and DNMTs (26–29).

In the current study, we show that RUNX1/MTG8 acts as a potent negative regulator of gene transcription by associating to DNMT1, in addition to HDAC1. We showed that DNMT1 was part of the RUNX1/MTG8 repressive complex and cooperated with this oncogenic factor to block gene transcription. The relevance of these findings is substantial because DNMT and HDAC inhibitors are making their way into the clinic, and combinations of these...
agents might target DNMT1/HDAC-dependent transcriptional repression in patients with RUNXI/MTG8-positive AML.

**Materials and Methods**

**Plasmids.** A 200-bp IL-3 promoter fragment (−200 to +1) containing two RUNXI/MTG8 binding sites and CpG dinucleotide sequences was subcloned into the NheI and HindIII sites of the luciferase reporter vector, pGL3-basic (Promega, Madison, WI). The 5′ and 3′ primers used for cloning the IL-3 promoter fragment were the following: NheI 5′-TTGCGGTACCTGTTTCACCTGATCTTGCATTAC-3′ and HindIII 5′-TGGAAACGCTTTGAGGTGCAAAGCCCTC-3′. The correct sequence of the IL-3 promoter fragment was confirmed by sequence analysis. PCs21 RUNXI/MTG8 expressing RUNXI/MTG8 (30) and pCMV-Myc-DNMT1 expressing the Myc-tagged full-length DNMT1 (provided by Dr. T. Kozurides, Wellcome Trust/Cancer Research UK Institute and Department of Pathology, Cambridge, United Kingdom) were also used.

**Cell Culture and Transfection.** 293T cells were grown in DMEM supplemented with 10% fetal bovine serum. Kasumi-1, Jurkat, and THP1 cells were incubated in RPMI 1640 supplemented with 10% fetal bovine serum. Transfection of the various constructs into 293T cells was done using LipofectAMINE 2000 with Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Pharmacologic treatment with depsipeptide and decitabine was done as previously published (24).

**Commmunoprecipitation and Western Blot.** Lysates of transfected 293T or leukemia cells were applied to immunoprecipitation using anti-MTG8 (New England Biolabs, Inc., Beverly, MA) and anti-DNMT1 (Oncogene Research, San Diego, CA) antibody. The immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Western blots were done using anti-myc tag (Cell Signaling Technology, Inc. Beverly, MA), anti-DNMT1, or anti-MTG8 antibody. SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) was used as a detection system.

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation (ChIP) was done using the ChIP Assay Kit (Upstate Biotechnology, Inc., Waltham, MA) according to the manufacturer’s protocol with antibodies against the Acetyl-Histone H4, Acetyl-Histone H3, HDAC1, McCP2 (Upstate Biotechnology, Inc.), DNMT1 (New England Biolabs), MTG8 (Oncogene Research), and RUNXI (provided by Dr. K-S. Chang) proteins. The immunoprecipitated chromatin was analyzed in triplicate by quantitative real-time PCR using the probe FAM5′-ATAATACGCTCGTTGTTCTTATGG-3′/TATGA-3′, forward primer 5′-TCCCTCTGCTGTTGTTCTTATGG-3′, reverse primer 5′-GGGGCGGATCCATCTGAGT- CATGGA-3′, reverse primer 5′-GGGAAGGATCCATCTGAGT- CATGGA-3′, forward primer 5′-ACGTGTTCTGTAAGTGACCATGGA-3′, and the IL-3 promoter, and the previously published conditions (24). The comparative ΔΔCt cycle threshold (Ct) method recommended by Applied Biosystems (Foster City, CA) was used to determine levels of IL-3 promoter enriched by each antibody used in the ChIP. The Ct for IL-3 promoter for each antibody and the DNA input were determined and the cycle number difference (ΔΔCt = DNA input − IL-3 promoter) was calculated for each replicate. Fold differences in enriched IL-3 promoter were expressed relatively to the non-antibody negative control, using the formula 2^−ΔΔCt. For qualitative PCR, we used IL-3 promoter forward primer 5′-CACAGGTAGTCCAGGTGATG-3′, IL-3 promoter reverse primer 5′-GGGGCGGATCCGGAAAGT3′, exon 5 forward primer 5′-TATGGCGATCCAACTTCTAAGT3′, and IL-3 gene exon 5 reverse primers 5′-GAGAAGGATCTGGACATGGA-3′. The cycle numbers were varied to ensure results within the linear range of the PCR.

**Reporter Assays.** 293T cells were seeded into 12-well plates at a concentration of 3 × 10^5 per well and grown overnight. Co-transfection was done by using 1 μg of the IL-3 reporter gene plasmid (pGL3-IL-3) and 0.1 to 5 μg of the indicated expression vectors (see Results section) or pGL3 empty vector used as control for basal promoter activity. Firefly luciferase activity was measured 48 hours after transfection by using the dual luciferase reporter assay system (Promega). Values were corrected to account for differences in transfection efficiency by cotransfecting pRL-TK Renilla luciferase plasmid and adjusting luciferase values accordingly.

All luciferase data represent the average of at least three independent experiments.

**Reverse Transcription−PCR Assays.** Total RNA from untreated and drug-treated cells was extracted using the RNeasy Mini kit according to the manufacturer’s directions (Qiagen Inc., Valencia, CA). The reverse transcription (RT) step was carried out as previously described (31). Amplification of the IL-3 transcript was done by PCR as previously reported (24). To assess quantitatively endogenous IL-3 expression in 293T cells transfected with RUNXI/MTG8 or/and DNMT1, the IL-3 transcript was preamplified in a linear range (i.e., 10 cycles) before quantitative assessment by real-time PCR (24) using the comparative ΔΔCt method (Applied Biosystems). Following determination of the threshold cycles for IL-3- and 18S in samples run in triplicates, the cycle number difference (ΔΔCt = 18S − IL-3) was calculated and corrected to the cotransfected pRL-TK Renilla luciferase activity to account for transfection variations. IL-3 expression values were then expressed relatively to the samples transfected with the mock vector using the formula 2^−ΔΔCt (Applied Biosystems).

**Bisulfite Sequencing.** Genomic DNA was treated with sodium bisulfite according to published protocol (32) with the exception that the DNA purification steps were carried out with the Qiagen Gel Extraction kit (Qiagen; ref. 33). Reactions and sequencing were done as previously reported (33).

**In vitro Methylation of the IL-3 Promoter.** The IL-3 promoter insert was digested from the luciferase expression construct pGL3-IL-3 using the restriction enzymes KpnI and HindIII and extracted from the agarose gel. The insert DNA was incubated overnight with 60 units SsoI (CpG) methylase (methylated) or without SsoI methylase (mock methylated), as recommended by the manufacturer (New England Biolabs Inc.). Following DNA purification, the methylation status was confirmed by restriction digestion with the methylation-sensitive restriction enzyme HpaII. The methylated and mock-methylated DNA fragments were then ligated to the pGL3 luciferase vector, purified, and transfected into 293T cells. Luciferase activity was analyzed as described above.

**Results**

**RUNXI/MTG8 Binds the Endogenous IL-3 Promoter to Repress Gene Expression.** In this study, IL-3, one of the RUNXI target genes, was used as readout for RUNXI/MTG8-mediated transcriptional inhibition. Gene promoter requires an intact RUNXI binding site to be transactivated by the wild-type RUNXI (6) or repressed by the fusion protein RUNXI/MTG8 (34, 35). Therefore, to confirm that IL-3 is a target for RUNXI/MTG8, first we showed that IL-3 was not expressed in RUNXI/MTG8-positive Kasumi-1 cells, whereas it was readily detectable in RUNXI/MTG8-negative Jurkat cells, a T-lymphoblastic cell line that constitutively expresses IL-3 (Fig. 1A). Decitabine-treated Kasumi-1 cells were also used as a positive control based on our previous report showing reactivation of IL-3 expression in this cell line upon treatment with hypomethylating agents (24). Then, we did ChIP assays to show that RUNXI/MTG8 binds to the IL-3 promoter in Kasumi-1 cells, but not in the Jurkat cells (Fig. 1B). As shown, in Kasumi-1 cells, RUNXI and MTG8 were concurrently enriched on the IL-3 promoter, thereby indicating RUNXI/MTG8 binding. In contrast, in Jurkat cells, only RUNXI and not MTG8, which lacks DNA-binding activity, was enriched on the IL-3 promoter, indicating binding of the wild-type RUNXI protein. Expression of RUNXI/MTG8 and MTG8 protein in Kasumi-1 and Jurkat cells was verified by immunoblotting (Fig. 1C).

**Methylation of the IL-3 Promoter Functionally Represses IL-3 Transcription.** We recently showed that IL-3 is silenced and methylated in RUNXI/MTG8-positive cells (24). Consistent with our previous studies, here we showed by bisulfite sequencing 100% methylation of a region of the IL-3 promoter surrounding the
As both RUNX1/MTG8 binding and DNA methylation of the status, concurrently associate with the IL-3 DNMT1, which is important for maintaining gene methylation interdependent. Thus, we asked whether both RUNX1/MTG8 and for gene silencing, we investigated whether these events are cotransfected with RUNX1/MTG8 or/and DNMT1 expression was further showed by ChIP experiments done in 293T cells RUNX1/MTG8 and DNMT1 with the RUNX1/MTG8 and DNMT1. The simultaneous association of repressor complex that included these two proteins in addition to promoter, suggesting a promoter-specific binding of a transcription IL-3 the silenced gene in Kasumi-1 cells (Fig. 3A). Interestingly, despite being endogenously expressed (Fig. 4B), wild-type MTG8 did not seem to interact with DNMT1 because a coimmunoprecipitation band was detected only following cotransfection of DNMT1 and RUNX1/MTG8 (Fig. 4A). To further support the DNMT1-RUNX1/MTG8 interaction, immunoprecipitation using antibody specific to DNMT1 was done with RUNX1/MTG8-positive Kasumi-1 cells. RUNX1/MTG8-negative THP-1 myeloid cells were used as a negative control. The immunoblot was probed with an antibody to MTG8. We showed transcriptional start site and that includes two RUNX1/MTG8 binding sites in Kasumi-1 cells (Fig. 2A). Treatment with decitabine reduced CpG methylation of the IL-3 promoter by ~35% (Fig. 2A) while promoting gene IL-3 transcription in Kasumi-1 cells (Fig. 1A). To further support the notion that DNA methylation contributes to the IL-3 silencing, we showed a decrease in IL-3 promoter activity following in vitro SssI methylation of a region (~200 to +1) of the IL-3 promoter containing six CpG dinucleotides and two RUNX1 binding sites and subsequent transfection into 293T cells (Fig. 2B).

RUNX1/MTG8 and DNMT1 Are Enriched on the IL-3 Promoter in Kasumi-1 Cells. As both RUNX1/MTG8 binding and DNA methylation of the IL-3 promoter appear to be important for gene silencing, we investigated whether these events are interdependent. Thus, we asked whether both RUNX1/MTG8 and DNMT1, which is important for maintaining gene methylation status, concurrently associate with the IL-3 promoter. A ChIP analysis revealed that both proteins were bound to the promoter of the silenced IL-3 gene in Kasumi-1 cells (Fig. 3A). Interestingly, HDAC1 and MeCP2 were also found associated with the IL-3 promoter, suggesting a promoter-specific binding of a transcription repressor complex that included these two proteins in addition to RUNX1/MTG8 and DNMT1. The simultaneous association of RUNX1/MTG8 and DNMT1 with the IL-3 endogenous promoter was further showed by ChIP experiments done in 293T cells cotransfected with RUNX1/MTG8 or/and DNMT1 expression plasmids (Fig. 3B). Notably, DNMT1 largely bound to the IL-3 promoter only in the presence of RUNX1/MTG8, suggesting a physical interaction between the fusion protein and DNMT1. Finally, to validate the results obtained in cell lines and transfected models, we analyzed RUNX1/MTG8-positive and RUNX1/MTG8-negative bone marrow samples from patients with AML. By a ChIP assay, we showed that RUNX1/MTG8, DNMT1, MeCP2, and HDAC1 were detectable above the background levels only on the IL-3 promoter of the RUNX1/MTG8-positive blasts (Fig. 3C). The IL-3 gene-coding region (i.e., exon 5) was used as a ChIP negative control for the DNA-protein interaction.

Physical Association of RUNX1/MTG8 with DNMT1. To assess if a physical interaction occurs between RUNX1/MTG8 and DNMT1, 293T cells were then transfected with RUNX1/MTG8 or/ and Myc-tagged DNMT1 plasmid. Cell lysates were immunoprecipitated with antibody to MTG8 and immunoblotting was done with anti-Myc tag antibody. RUNX1/MTG8 protein was found to be coimmunoprecipitated with DNMT1 (Fig. 4A). Control 293T cells, in which either DNMT1 or RUNX1/MTG8 alone were expressed, showed no reactivity and confirmed the specificity of the RUNX1/MTG8-DNMT1 interaction in cotransfected 293T cells. The same cell lysates were also subjected to immunoblotting with antibody to MTG8 and to Myc tag to confirm expression of the ectopic proteins (Fig. 4B).
that DNMT1 coprecipitated with RUNX1/MTG8 in Kasumi-1, but not in THP-1 cells, thus verifying that endogenous RUNX1/MTG8 was bound to endogenous DNMT1 (Fig. 4C). Notably, these results were also verified by immunoprecipitation with anti-ETO and immunoblotting with anti-DNMT1 (not shown).

**RUNX1/MTG8 Interacts with DNMT1 to Inhibit Gene Transcription.** To determine if the RUNX1/MTG8-DNMT1 complex is functionally important in silencing IL-3, 293T cells were cotransfected with RUNX1/MTG8 or/and DNMT1 and a luciferase reporter construct containing a region (−200 to +1) of the IL-3 promoter that includes six CpG dinucleotides and two RUNX1 binding sites (6). The IL-3 promoter-driven luciferase activity was measured 48 hours after transfection. RUNX1/MTG8 alone repressed the IL-3 promoter in a dose-dependent manner (Fig. 5A). Notably, RUNX1/MTG8 also synergized with DNMT1 in blocking the IL-3 promoter transcriptional activity (Fig. 5B). Expression of ectopic DNMT1 and RUNX1/MTG8 proteins following cotransfection with the luciferase plasmid was shown by immunoblotting (Fig. 5C).

For further verification, 293T cells were transfected with RUNX1/MTG8 or/and DNMT1, and real-time RT-PCR was used to quantify endogenous IL-3 expression normalized to luciferase activity of pRL-TK Renilla to correct for transfection efficiency. RUNX1/MTG8 alone induced down-regulation of endogenous IL-3, and

![Figure 3](image-url)

**Figure 3.** RUNX1/MTG8 and DNMT1 bind to RUNX1 target gene promoters in RUNX1/MTG8-positive cell lines and primary blasts. A, ChIP assay of the IL-3 promoter with the indicated antibody (Ab) using real-time PCR. RUNX1/MTG8, DNMT1, MeCP2, and HDAC1 were enriched on the IL-3 promoter in Kasumi-1 cells. B, ChIP assay of the IL-3 promoter done with the indicated antibody in 293T cells untransfected or transfected with RUNX1/MTG8 or/and DNMT1. C, ChIP assay of the IL-3 promoter done with the indicated antibody in primary blasts from bone marrow samples of a RUNX1/MTG8-positive and a RUNX1/MTG8-negative patient with AML. The exon 5 coding sequence in both samples was used as a negative control.
coexpression with DNMT1 seemed to further enhance the repression activity of the fusion protein (Fig. 5D). Of note, DNMT1 alone did not down-regulate IL-3, inasmuch as it did not bind to the promoter in the absence of RUNX1/MTG8 (Fig. 2B). All together, these findings indicated that a physical and functional interplay between DNMT1 and RUNX1/MTG8 on the IL-3 promoter is required for efficient gene silencing.

**Figure 4.** RUNX1/MTG8 physically interacts with DNMT1. A, RUNX1/MTG8 coimmunoprecipitated with DNMT1. 293T cells were cotransfected with RUNX1/MTG8 or/and Myc-tagged DNMT1 plasmids. Anti-MTG8 immunoprecipitation shows interaction of RUNX1/MTG8 with DNMT1. B, control for ectopic expression of RUNX1/MTG8 and Myc-tagged DNMT1 by immunoblotting in 293T cells. C, association of endogenous RUNX1/MTG8 with endogenous DNMT1 in Kasumi-1 cells. Cell lysates were immunoprecipitated with anti-DNMT1 and Western blot was done with antibody raised against MTG8 or DNMT1. The RUNX1/MTG8-negative myeloid THP-1 cell line was used as a control. WB, Western blot; IP, immunoprecipitation.

**Pharmacologic Modulation of the RUNX1/MTG8-DNMT1 Complex Bound to the IL-3 Promoter.** Depsipeptide (36) is a HDAC inhibitor that decreases levels of DNMT1 associated with the promoter of epigenetically silenced genes.5 Here, we investigated the kinetics of the RUNX1/MTG8-DNMT1 interaction on the IL-3 promoter upon drug exposure. Kasumi-1 cells were treated with depsipeptide and harvested at sequential time points (i.e., 0, 3, 6, and 24 hours). Using ChIP assays, we determined that both RUNX1/MTG8 and DNMT1, present in untreated cells, dissociated from IL-3 promoter at 24 hours (Fig. 6A). Importantly, dissociation of DNMT1 occurred early (at 3 hours) and preceded the release of RUNX1/MTG8 (Fig. 6B). Accordingly, dissociation of DNMT1 occurred early (at 3 hours) and preceded the release of RUNX1/MTG8, thus indicating that consistent with our ChIP results (Fig. 3B), a DNMT1 binding to the IL-3 promoter occurs in the presence of the fusion protein. The biological activity of depsipeptide was shown by histone H3 and H4 hyperacetylation of the IL-3 promoter (Fig. 6B), inhibition of HDAC activity (data not shown), and IL-3 reexpression (Fig. 6C).

In contrast, we showed treatment with decitabine resulted in concurrent stabilization of DNMT1 and RUNX1/MTG8 on the IL-3 promoter. 

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5 G. Marcucci, unpublished data.
promoter, consistent with the notion that this agent depletes DNMT1 activity by forcing DNA-protein covalent binding (Fig. 6D). The biological activity of decitabine was shown by IL-3 gene reexpression (Fig. 1A). Taken all together, the similar patterns of RUNX1/MTG8 and DNMT1 association and dissociation on the IL-3 promoter following pharmacologic modulation with HDAC and DNMT inhibitors further support the RUNX1/MTG8-DNMT1 interplay in vivo.

**Discussion**

Previous studies have showed that the oncogenic transcription factor RUNX1/MTG8 aberrantly recruits HDACs/mSin3A/N-CoR complex to block gene transcription mediated by RUNX1(9, 12–15, 19). Here, we addressed another crucial mechanism of the RUNX1/MTG8 oncogenic activity, the aberrant recruitment of DNMT1 for gene silencing.

We focused on IL-3 because this RUNX1 target gene is repressed in cells expressing RUNX1/MTG8, is methylated, synergistically repressed by the combination of HDAC and DNMT inhibitors (24), and has the capability of supporting proliferation and differentiation of hematopoietic cells (37). We confirmed that methylation of the IL-3 promoter is functionally relevant to the gene silencing in RUNX1/MTG8-positive cells by using an in vitro methylation assay (Fig. 2B). It is likely that additional CpG sequences other than those included in our construct might become methylated in vivo, thereby contributing to a more efficient IL-3 transcriptional repression than that observed in our experiment. Furthermore, we showed that the IL-3 expression depends on the RUNX1/MTG8 DNA binding by showing that RUNX1/MTG8 was bound to the silenced IL-3 promoter and induced a dose-dependent repression of this gene.

Based on these results, we hypothesized that RUNX1/MTG8 interplays with DNMT1 to induce gene silencing and, therefore, we sought evidence for a physical and functional interaction of these two proteins. By ChIP we showed that both RUNX1/MTG8 and DNMT1 were bound to the IL-3 promoter in Kasumi-1 cells and RUNX1/MTG8-positive primary blasts and that the DNMT1 binding to the IL-3 promoter occurred only in the presence of RUNX1/MTG8 in cotransfected 293T cells. The physical association was further verified by communoprecipitation of RUNX1/MTG8 and DNMT1 in Kasumi-1 cells and, following plasmid coexpression, in 293T cells. We also proved that RUNX1/MTG8 and DNMT1 functioned as corepressors by demonstrating an enhanced down-regulation of the IL-3 promoter activity and endogenous IL-3 transcription in cotransfected 293T cells. Interestingly, transfection with DNMT1 alone increased, rather than decreased, endogenous IL-3 transcription. Although the mechanisms underlying this
finding remain unknown, it is conceivable that DNMT1 alone might modulate IL-3 expression indirectly, via activation or inhibition of different, unexplored pathways.

Taken together, these data provided the first evidence of a physical and functional interplay between RUNX1/MTG8 and DNMT1. Importantly, this was also verified by pharmacologic modulation with depsipeptide, a potent HDAC inhibitor investigated in clinical trials (36). In fact, by using ChIP assays, we determined that RUNX1/MTG8 and DNMT1 were concurrently released from the IL-3 promoter following depsipeptide treatment. Notably, DNMT1 was the first of the factors to be released, further supporting our hypothesis that the presence of RUNX1/MTG8 on the IL-3 promoter is required for DNMT1 binding.

Based on our data, we concluded that RUNX1/MTG8 interacts with DNMT1 to silence RUNX1 target genes. Because it has been established that RUNX1/MTG8 also recruits HDAC1, it is likely that this fusion protein ultimately acts as a master transcriptional repressor by promoting multiple mechanisms of gene silencing. Indeed, in our previous work, we showed that expression of IL-3, used as an example for RUNX1 target genes, was completely restored through a synergistic activity of DNMT and HDAC inhibitors (24). These data are fully consistent with the results from Cameron et al., who first reported the interaction of HDAC and DNMT inhibitors in inducing transcriptional reactivation of epigenetically silenced gene in transformed cells (23). Furthermore, the ability of leukemogenic fusion proteins to activate multiple mechanisms of transcriptional repression was shown by Di Croce et al. (29). This group recently reported that PML-RAR recruits DNMTs, in addition to HDACs, and increases methylation of the RAR2 promoter in vitro and in vivo (29). Of note, Esteller et al. suggested that frequent methylation of RAR2 can also occur in PML-RAR-negative AML cells (38). Although further investigation is required to reconcile these findings, one possible explanation is that methylation of genes targeted by specific oncogenic transcription factors is a frequent event in AML. This might occur either specifically through the aberrant recruitment of DNMTs by fusion proteins such as RUNX1/MTG8 or PML/RAR or through other secondary mechanisms yet to be identified.

Our findings raise the question of whether the interplay between RUNX1/MTG8 and DNMT1 occurred through a direct physical contact of these two proteins or rather is mediated by other factors present in the same multiprotein repressor complex.

**Figure 6.** Pharmacologic modulation of the RUNX1/MTG8-DNMT1 complex on IL-3 promoter. ChIP assay was done in Kasumi-1 cells using indicated antibodies. A, release of the RUNX1/MTG8-DNMT1 complex from the IL-3 promoter by exposure to the HDAC inhibitor depsipeptide (ChIP assay). B, hyperacetylation of histones H3 and H4 on the IL-3 promoter following depsipeptide treatment (Western Blot). C, IL-3 reexpression following depsipeptide treatment assessed by RT-PCR. D, trapping of the RUNX1/MTG8-DNMT1 complex on the IL-3 promoter following exposure to decitabine (ChIP assay).
complex. Whereas mutational analysis of RUNX1/MTG8 and DNMT1 will be necessary to identify conditions that could mediate a direct contact, it is interesting that both MeCP2 and HDAC1 were also found on the IL-3 promoter. This is consistent with previous studies showing that MeCP2 associates specifically with methylated DNA via its methyl-CpG binding domain (39, 40) while recruiting the mSin3A/HDAC complex via its transcriptional repression domain (41). Because RUNX1/MTG8 recruits HDACs, and MeCP2 interacts with DNMTs, one could argue that RUNX1/MTG8 and DNMT1 association does not occur directly, but via MeCP2 and HDAC1. The latter seems a likely model inasmuch as both wild-type MTG8 (Fig. 3A) and RUNX1 (not shown) did not seem to directly interact with DNMT1. Regardless, our findings indicate that the interplay between RUNX1/MTG8 and DNMT1 is functionally important because the RUNX1/MTG8-induced down-regulation of IL-3 was enhanced in presence of DNMT1.

In summary, in addition to the already reported interaction with HDACs, herein we show that RUNX1/MTG8 also recruits DNMT1 to silence RUNX1 target genes, thereby supporting the rationale for using the combination of HDAC and DNMT inhibitors as a therapeutic strategy in t(8;21) AML.

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