The leukemogenic t(8;21) fusion protein AML1-ETO controls rRNA genes and associates with nucleolar-organizing regions at mitotic chromosomes

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Summary

RUNX1/AML1 is required for definitive hematopoiesis and is frequently targeted by chromosomal translocations in acute myeloid leukemia (AML). The t(8;21)-related AML1-ETO fusion protein blocks differentiation of myeloid progenitors. Here, we show by immunofluorescence microscopy that during interphase, endogenous AML1-ETO localizes to nuclear microenvironments distinct from those containing native RUNX1/AML1 protein. At mitosis, we clearly detect binding of AML1-ETO to nucleolar-organizing regions in AML-derived Kasumi-1 cells and binding of RUNX1/AML1 to the same regions in Jurkat cells. Both RUNX1/AML1 and AML1-ETO occupy ribosomal DNA repeats during interphase, as well as interact with the endogenous RNA Pol I transcription factor UBF1. Promoter cytosine methylation analysis indicates that RUNX1/AML1 binds to rDNA repeats that are more highly CpG methylated than those bound by AML1-ETO. Downregulation by RNA interference reveals that RUNX1/AML1 negatively regulates rDNA transcription, whereas AML1-ETO is a positive regulator in Kasumi-1 cells. Taken together, our findings identify a novel role for the leukemia-related AML1-ETO protein in epigenetic control of cell growth through upregulation of ribosomal gene transcription mediated by RNA Pol I, consistent with the hyper-proliferative phenotype of myeloid cells in AML patients.

Key words: Acute myelogenous leukemia, RUNX1, Ribosomal DNA transcription, RNA polymerase I, UBF1, Nucleolar organizing region

Introduction

The most frequent target of chromosomal translocations in acute myeloid leukemia (AML) is the Runt-related transcription factor RUNX1/AML1, a key regulator of hematopoiesis (Setoguchi et al., 2008; Pabst and Mueller, 2007; Growney et al., 2005; Ito, 2004; Nucifora and Rowley, 1995; Romana et al., 1995). RUNX1/AML1 directly regulates multiple distinct myeloid and lymphoid genes that are involved in hematopoietic lineage commitment (Huang et al., 2007; Otto et al., 2003; Frank et al., 1995; Nuchprayoon et al., 1994). The protein contains an N-terminal DNA-binding domain (runt-homology domain) and a C-terminal regulatory domain that contains a nuclear-matrix-targeting signal (NMTS) and several context-dependent transcriptional activation or repression domains (Wheeler et al., 2000; Meyers and Hiebert, 2000; Stein et al., 1999).

The 8;21 leukemic translocation fuses the RUNX1/AML1 gene to MTG8/ETO coding sequences resulting in the AML1-ETO fusion protein (Miyoshi et al., 1993; Erickson et al., 1992; Licht, 2001; Davis et al., 2003; Peterson et al., 2007a). AML1-ETO retains the DNA-binding function of the RUNX1/AML1 protein but does not contain the transactivation domain or the nuclear-matrix-targeting signal (NMTS) of RUNX1/AML1 (Zeng et al., 1997; McNeil et al., 1999). Previous studies have shown that exogenously expressed RUNX1/AML1 and AML1-ETO exhibit differential subnuclear targeting, which might be responsible in part for the aberrant function of the fusion protein (McNeil et al., 1999; Barseguian et al., 2002). Altered subnuclear targeting of AML1 in patients with the 8;21 translocation might contribute to the pathology of AML, because RUNX1/AML1 mutations that alter subnuclear routing and fidelity of transcriptional control result in a differentiation block and increase proliferation of myeloid progenitors (Vradii et al., 2005; Zaidi et al., 2007; Zaidi et al., 2005). In addition, a large number of co-factors interact with gene regulatory domains of RUNX1/AML1, including the C-terminus that is removed in AML1-ETO (Wotton et al., 1994; Giese et al., 1995; Hiebert et al., 1996; Rhoades et al., 1996; Petrovick et al., 1998; Rubnitz and Look, 1998; Osato et al., 1999). Through the recruitment of unique co-regulators that interact with the ETO moiety in lieu of the AML1 C-terminus, the AML1-ETO fusion protein antagonizes the transcriptional function of native RUNX1/AML1 (Hiebert et al., 2001). Thus, there are several plausible mechanisms by which the pathological formation of the AML1-ETO protein could block differentiation of myeloid progenitors and promote leukemia.

In addition to regulating hematopoiesis-specific genes, RUNX1/AML1 is also implicated in the regulation of cell-cycle genes, including $p21^{WAF1/CIP1}$, which encodes a cyclin-dependent kinase inhibitor important for checkpoint control and terminal differentiation (Lutterbach et al., 2000; Peterson et al., 2007b). RUNX1/AML1 controls cell cycle progression by shortening the G1-S phase in hematopoietic cells and is negatively regulated by cyclin D3 (Strom et al., 2000; Peterson et al., 2005). Levels of

RUNX1/AML1 increase as cells progress into S phase and are downregulated at the G2-M transition (Bernardin-Fried et al., 2004; Biggs et al., 2006). The closely related osteoblastic transcription factor RUNX2 also controls proliferation and is cell cycle regulated with maximal levels in G1 (Pratap et al., 2003; Galindo et al., 2005). Furthermore, the presence of RUNX2 in osteogenic mesenchymal cells during mitosis might reinforce cell fate through an epigenetic mechanism that retains phenotypic gene expression patterns after cell division (Young et al., 2007b; Young et al., 2007a). Because RUNX1/AML1 has also been detected during mitosis (Zaidi et al., 2003), it could perform an analogous function in hematopoietic cells.

In this study, we examined the biological functions of RUNX1/AML1 and AML1-ETO during mitosis and interphase in relation to their subcellular localization in hematopoietic cells. Among the main findings from our study is the observation that both proteins can associate with mitotic chromosomes and regulate transcription of ribosomal RNA genes, a fundamental process that supports the growth of cells and is tightly coupled with cell differentiation. Our results indicate that RUNX1/AML1 mediates epigenetic mechanisms that convey regulatory information to progeny cells and that these mechanisms are perturbed by the leukemia related AML1-ETO fusion protein.

Results

During interphase RUNX proteins localize in nuclear microenvironments where the transcriptional machinery is organized. As cells emerge from mitosis there is a stringent requirement for expression of RUNX1/AML1-responsive genes. Although RUNX1/AML1 is destabilized during the G2-M transition (Bernardin-Fried et al., 2004), at least a subset of RUNX1/AML1 remains associated with mitotic chromosomes (Zaidi et al., 2003). However, many AML patients express the AML1-ETO fusion protein and the important question arises whether this leukemia-associated protein is similarly capable of interacting with mitotic chromosomes.

Endogenous RUNX1/AML1 and AML1-ETO are targeted to distinct nuclear microenvironments

To begin examining the localization of AML1-ETO during interphase and mitosis, we used immunofluorescence microscopy to monitor the subcellular distribution of endogenously expressed RUNX1/AML1 and AML1-ETO in asynchronously growing Kasumi-1 cells that were derived from an AML patient with an 8;21 chromosomal translocation (Fig. 1). Double-label comparisons of immunofluorescence signals revealed the absence of a significant overlap in the distribution patterns of RUNX1/AML1 and AML1-ETO, and most sites were detected as distinct green or red immunofluorescence signals (Fig. 1). Biochemical fractionation of Kasumi-1 cells followed by immunoblotting validated the nuclear distribution of RUNX1/AML1 and AML1-ETO (data not shown). Hence, both RUNX1/AML1 and the AML1-ETO fusion protein are predominantly present in the nucleus, but are each directed to distinct subnuclear compartments. The distinct locations might reflect differences in the regulatory activities of these factors in leukemic cells.

RUNX1/AML1 and AML1-ETO are specifically associated with metaphase chromosomes

To assess whether the leukemia-associated AML1-ETO fusion protein interacts with mitotic chromosomes, we investigated its presence in metaphase chromosomes by immunofluorescence microscopy (Fig. 2). We first examined the association of RUNX1/AML1 and AML1-ETO with mitotic chromosomes by transfecting actively growing HeLa cells with epitope-tagged expression constructs. Immunofluorescence microscopy reveals that ectopically expressed RUNX1/AML1 and AML1-ETO each associate with chromosomes in mitotic cells and are detected as paired foci (Fig. 2A). The pair-wise organization of these foci is similar to that reported for RUNX2 during mitosis (Young et al., 2007a).

To determine the endogenous cellular organization of RUNX1/AML1 and AML1-ETO proteins during mitosis, we prepared metaphase chromosome spreads for human Jurkat cells endogenously expressing RUNX1/AML1, HEL cells exhibiting physiological expression of ETO, and Kasumi-1 cells expressing the AML1-ETO translocation fusion protein. Endogenous RUNX1/AML1 (in Jurkat cells) and AML1-ETO (in Kasumi-1 cells) each show distinct paired foci on metaphase chromosome spreads, whereas ETO (in HEL cells) is not detected (Fig. 2B). Interestingly, RUNX1/AML1 cannot be detected on mitotic chromosomes in Kasumi-1 cells (data not shown), suggesting that AML1-ETO might exclude RUNX1/AML1 from its sites at mitosis (see below). Regardless of this possibility, our data clearly show that AML1-ETO specifically associates with mitotic chromatin in human leukemia cells, and thus provide the first indication that AML1-ETO interferes with the normal function of RUNX1/AML1 at mitosis.

RUNX1/AML1 and AML1-ETO each bind to human ribosomal DNA

The symmetrical pairing of large foci containing RUNX1/AML1 and AML1-ETO on mitotic chromosomes is reminiscent of previous results with osteoblasts showing mitotic association of RUNX2 with nucleolar-organizing regions (NORs) that represent sites containing

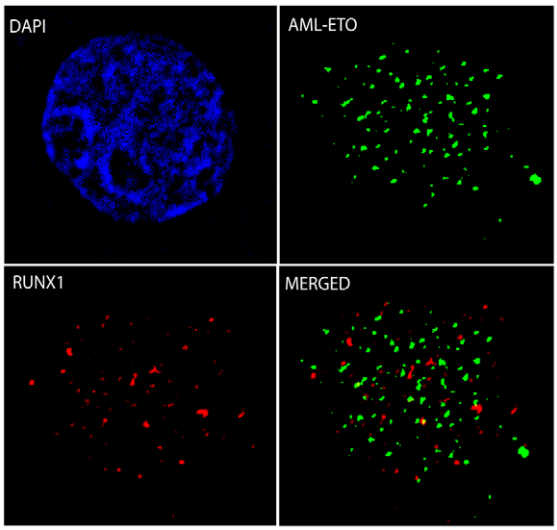

Fig. 1. RUNX1/AML1 and AML1-ETO are targeted to distinct subnuclear locations. Immunofluorescence microscopy for endogenous RUNX1/AML1 (red) and AML1-ETO (green) with DAPI staining (blue) in Kasumi-1 cells as well as merged images are shown. RUNX1/AML1 and AML1-ETO fusion protein are predominantly present in the nucleus but do not colocalize in the Kasumi-1 cells. An antibody detecting ETO was used to visualize the AML1-ETO fusion protein, whereas an antibody recognizing the C-terminal domain of RUNX1/AML1 was used to detect endogenous RUNX1/AML1.

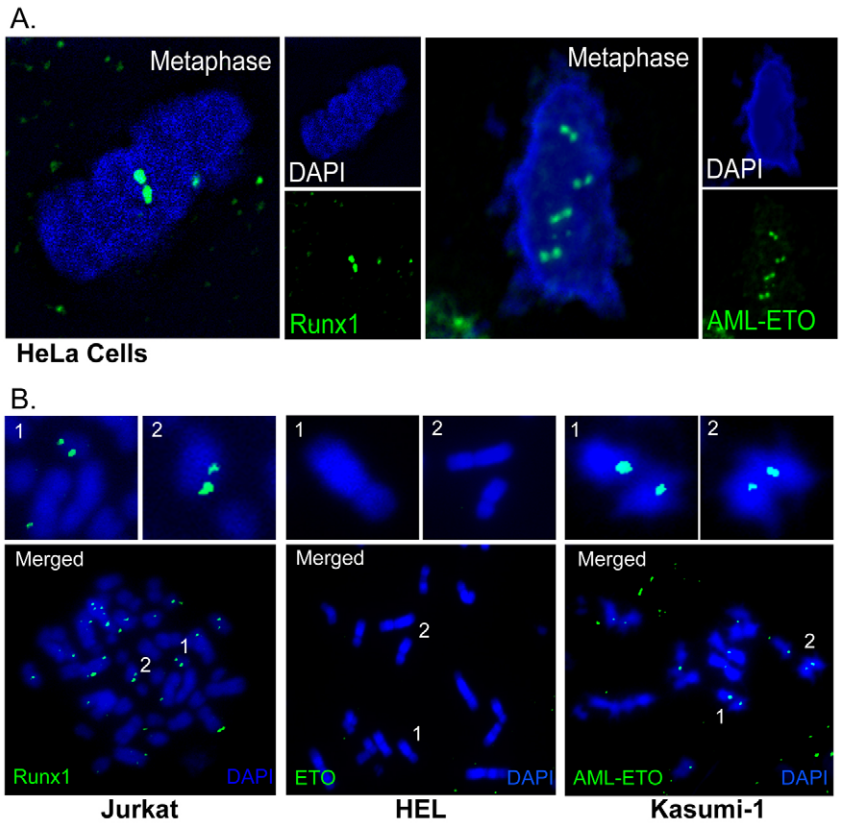

Fig. 2. RUNX1/AML1 and AML1-ETO associate with metaphase chromosomes in pairs. (A) Epitope-tagged RUNX1/AML1 and AML1-ETO were examined by in situ immunofluorescence in HeLa cells and metaphase cells were visually selected. RUNX1/AML1 and AML1-ETO foci (green) are associated with chromosomes (blue) in pairs (shown in deconvoluted images). (B) Mitotic chromosome spreads were prepared for human Jurkat, HEL and Kasumi-1 cells and processed for immunofluorescence microscopy using antibodies directed against the RUNX1/AML1, ETO and AML1-ETO proteins. RUNX1/AML1 and AML1-ETO show distinct foci on the mitotic chromosome spreads, whereas ETO is not detected on mitotic chromosomes. Panels above each image show enlargements of the areas labeled with numbers.

tandemly repeated ribosomal DNA (rDNA) genes (Young et al., 2007a). Therefore, we postulated that RUNX1/AML1 and AML1-ETO may also bind to NORs and ribosomal repeats during mitosis in hematopoietic cells. We performed chromatin immunoprecipitation (ChIP) assays with primers spanning rDNA sequences to determine in vivo occupancy of rDNA repeats by RUNX1/AML1 and AML1-ETO in Kasumi-1 and Jurkat cells (Fig. 3A). The RNA Pol I regulatory protein upstream binding factor 1 (UBF1), which binds directly to rDNA and to mitotic NORs, was used as a positive control for ChIP assays. Quantitative PCR data showed that AML1-ETO occupies rDNA repeats during mitosis in Kasumi-1 cells. However, association of RUNX1/AML1 to the rDNA genes was barely detectable (Fig. 3B), consistent with the immunofluorescence data (see results above). For comparison, Jurkat cells that have high levels of RUNX1/ AML1 but do not express the AML1-ETO fusion protein showed rDNA occupancy by RUNX1/AML1 but not AML1-ETO, as expected (Fig. 3C). Thus, these biochemical data establish that RUNX1/AML1 (in Jurkat cells) and the AML1-ETO fusion protein (in Kasumi-1 cells) are each associated with rDNA repeats during mitosis and were visualized as NORs by immunofluorescence microscopy (see Fig. 2). Clear detection of the binding of AML1-ETO but not RUNX1/AML1 to mitotic chromosomes in Kasumi-1 cells indicates that AML1-ETO perturbs the normal function of RUNX1/AML1 during mitosis.

Because RUNX1/AML1 and AML1-ETO bind to rDNA repeats during mitosis, we used ChIP assays to examine whether these proteins are still capable of association during interphase in Kasumi-1, HEL and Jurkat cells. As positive controls, we showed that the RNA Pol I factor UBF1 interacted with rDNA repeats in all three cell types (Fig. 4). The native ETO protein endogenously expressed in HEL cells was not associated with the rDNA repeats, nor was ETO detected on rDNA repeats in Jurkat cells that expressed neither ETO nor the AML1-ETO fusion protein (Fig. 4A,B). Of note we observed that both RUNX1/AML1 and AML1-ETO occupied rDNA repeats in Kasumi-1 cells during interphase (Fig. 4C). The latter finding suggests that RUNX1/AML1 and AML1-ETO are concurrently bound to rDNA genes during interphase.

RUNX1/AML1 and AML1-ETO associate with UBF1 on rDNA repeats in vivo

Our finding that RUNX1/AML1, AML1-ETO and UBF1 each associate with rDNA repeats suggested that RUNX1/AML1 and AML1-ETO interact with UBF1 in vivo. We carried out co-immunoprecipitation assays to examine directly whether UBF1 is present in a protein complex with RUNX1/AML1 or AML1-ETO. AML1-ETO and RUNX1/AML1 were detected in immunoprecipitates obtained with a UBF1 antibody (Fig. 5A). To validate these results, reciprocal immunoprecipitation assays with antibodies against RUNX1/AML1 and AML1-ETO were performed. Indeed, UBF1 precipitated when immunocomplexes were prepared using either RUNX1/AML1 or ETO antibodies (Fig. 5B). We also observe colocalization, albeit limited, of both RUNX1/AML1 and AML1-ETO with nucleolar UBF1 during interphase using immunofluorescence microscopy (Fig. 5C). Thus, RUNX1/AML1 and AML1-ETO each associate with UBF1, consistent with the interaction of these proteins with the regulatory sequences of rDNA repeats.

To demonstrate that both RUNX1/AML1 and AML1-ETO bind directly to the rDNA repeats, we conducted electrophoretic mobility shift assays with a human rDNA probe spanning a RUNX-binding element and nuclear extracts from Kasumi-1 cells. Competition assays

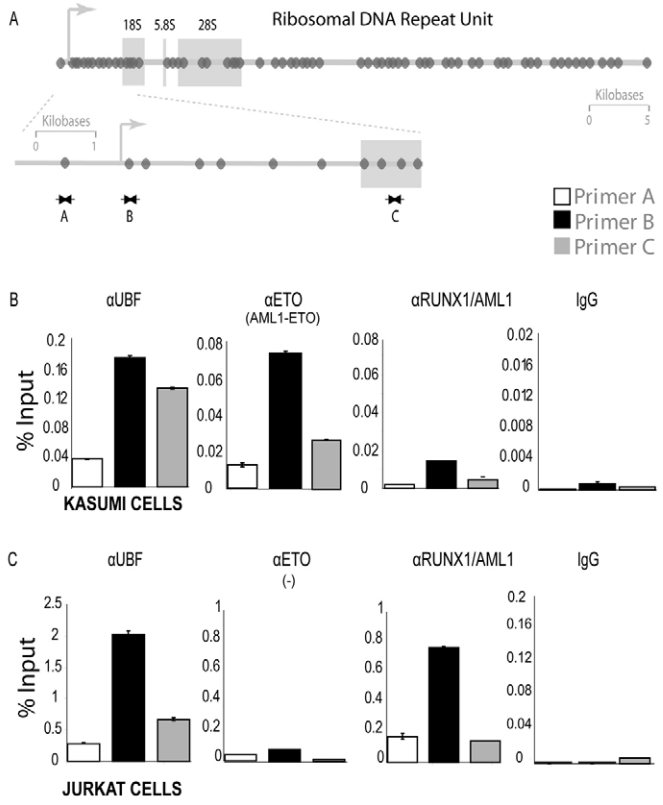

Fig. 3. AML1-ETO shows enhanced occupancy of rDNA repeats during mitosis. (A) Schematic of the RUNX consensus elements (ovals) in the human rDNA repeats depicting the locations of primers used for ChIP analysis. (B,C) ChIPs were done with antibodies for RUNX1/AML1, ETO and UBF1, as well as non-immune IgG in Kasumi-1 and Jurkat cells blocked in mitosis. An antibody detecting ETO was used to immunoprecipitate the AML1-ETO fusion protein, whereas an antibody recognizing the C-terminal domain of RUNX1/AML1 was used to pull down endogenous RUNX1/AML1. Quantitative PCR data are normalized to genomic DNA and denoted as percent input (note that *y*-axis scales vary).

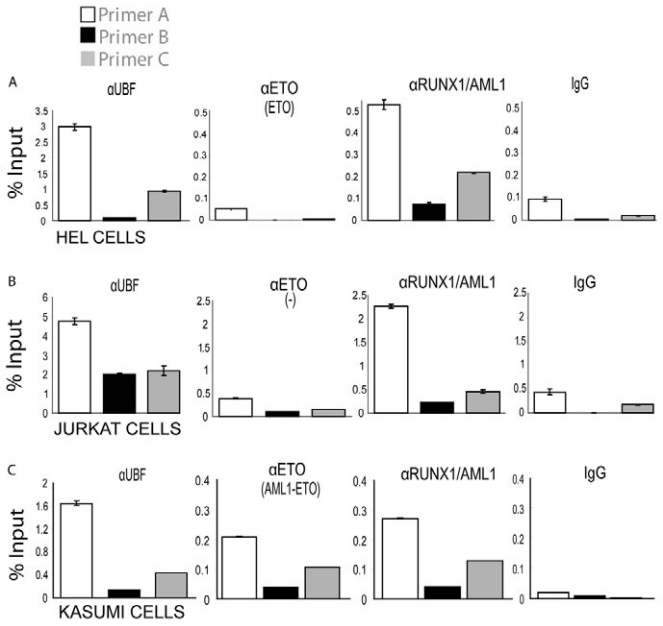

Fig. 4. Both RUNX1/AML1 and AML1-ETO occupy rDNA repeats in interphase Kasumi-1 cells. Chromatin immunoprecipitation was done in asynchronously growing HEL, Jurkat and Kasumi-1 cells using RUNX1/AML1, ETO, UBF1 and IgG antibodies. Three different PCR primer sets spanning RUNX consensus elements were used (see Fig. 3). (A,B) An antibody detecting ETO was used to immunoprecipitate the AML1-ETO fusion protein, whereas an antibody recognizing the C-terminal domain of RUNX1/AML1 was used to pull down endogenous RUNX1/AML1. Quantitative PCR data show that endogenous ETO does not bind to rDNA repeats in HEL cells nor in Jurkat cells (where ETO is not expressed), whereas UBF1 occupies the rDNA repeats in vivo in all the three cell lines tested. (C) RUNX1/AML1 and AML1-ETO both occupy rDNA repeats in Kasumi-1 cells. Quantitative PCR data are normalized to genomic DNA and denoted as percent input (note that *y*-axis scales vary).

with 100-fold molar excess of unlabeled wild type, mutant or RUNX consensus oligonucleotide were performed to establish the specific protein-DNA complex (Fig. 5D). Addition of antibodies against RUNX1 or AML1-ETO resulted in retarded mobility (supershift) of the complex (Fig. 5D). These results indicate that RUNX1 and AML1-ETO can interact with RUNX elements in the rDNA repeat.

To assess whether RUNX1/AML1 or AML1-ETO co-occupy rDNA repeats with the RNA Pol I transcription factor UBF1, ChIP-reChIP assays were performed in asynchronous Kasumi-1 cells. UBF1 antibody was used for the primary ChIP, followed by a second immunoprecipitation (reChIP) with either non-immune IgG (as a negative control) or antibodies against UBF1, RUNX1/AML1 or AML1-ETO. Quantitative PCR data showed that at least some UBF1-bound rDNA fragments are also bound to RUNX1/AML1 or AML1-ETO proteins (Fig. 5E). These results indicate that RUNX1/AML1 and AML1-ETO have a limited but significant presence on ribosomal DNA repeats that interact with UBF1 in vivo.

AML1-ETO occupancy of rDNA repeats is correlated with DNA hypomethylation and altered histone H3 methylation

It is well established that only a subset of rRNA genes are transcriptionally active at any one time (Grummt and Pikaard, 2003). We therefore investigated whether genomic occupancy of rDNA repeats by RUNX1/AML1 or AML1-ETO is linked to epigenetic chromatin modification. To monitor the association of RUNX1/AML1 and AML1-ETO at methylated and unmethylated rRNA genes, we used the ChIP-CHOP assay (Lawrence et al., 2004). Chromatin from Kasumi cells was immunoprecipitated with antibodies against RUNX1 or AML1-ETO (ChIP) and the resulting DNA was digested with McrBC enzyme (CHOP) prior to qPCR using rDNA primers. Only DNA that is methylated at two or more cytosines (within 55-3000 bp) was digested by McrBC. We found that rDNA regulatory regions associated with RUNX1/AML1 were sensitive to digestion with the McrBC enzyme (Fig. 6, primers A and B), whereas those associated with AML1-ETO are not. Interestingly, the 18S rRNA coding region detected by primer C was equally sensitive to McrBC in both RUNX1/AML1- and AML1-ETO-bound fractions. Taken together, our results indicate that rDNA repeats bound by RUNX1/AML1 are hyper-methylated relative to those that are bound by AML1-ETO (Fig. 6).

We next investigated the presence of AML1-ETO relative to post-translational modifications of nucleosomal histones. By immunofluorescence microscopy of Kasumi cells we observed significant colocalization of histone H3 dimethyl lysine 27 (H3K27me2) with AML1-ETO foci on mitotic chromosomes (Fig. 7A). There was limited colocalization with H3K9me2 and none

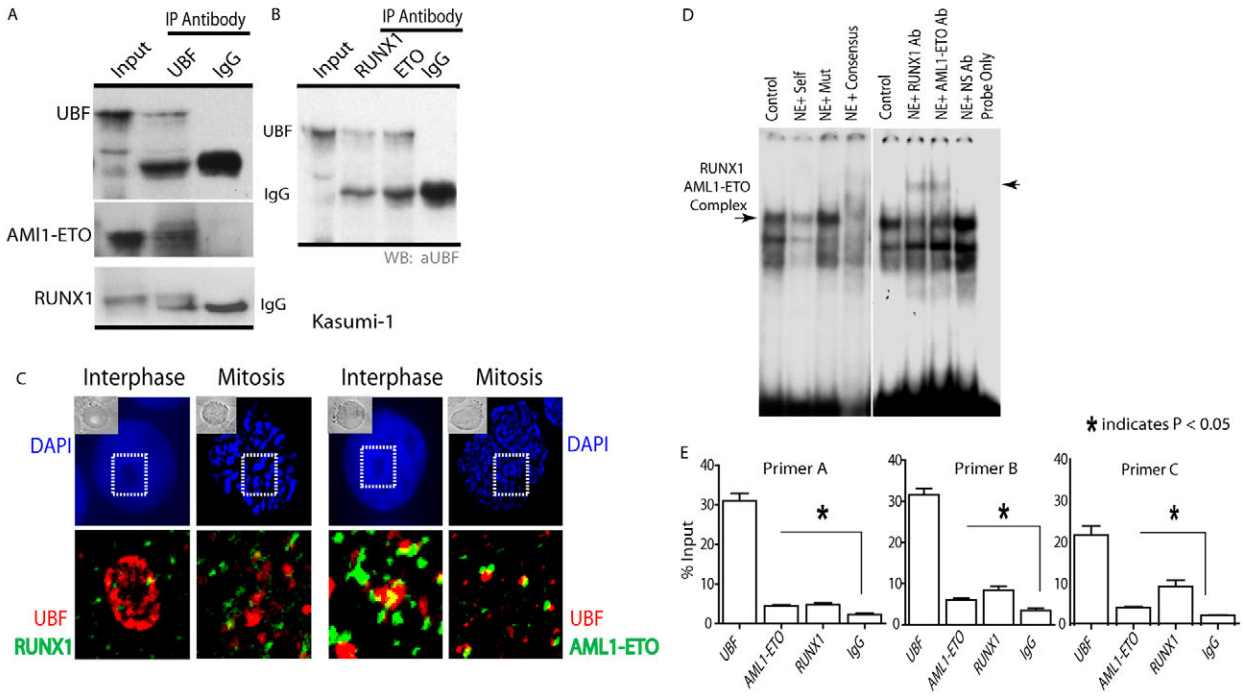

Fig. 5. Endogenous RUNX1/AML1 and AML1-ETO interact with UBF1 on the rDNA repeats. (A) Immunoprecipitation analysis was carried out with an antibody for UBF1 followed by western blotting with AML1-ETO- and RUNX1/AML1-specific antibodies. Both RUNX1 and AML1-ETO are detected in western blot analysis, however AML1-ETO shows greater interaction with UBF1 when compared with RUNX1/AML1. (B) Endogenous RUNX1/AML1 and AML1-ETO were immunoprecipitated from Kasumi-1 cells using rabbit polyclonal antibodies that specifically recognize the C-terminus of RUNX1/AML1 or the ETO moiety. A mouse monoclonal antibody was used to detect endogenous UBF1 by immunoblotting. UBF1 and IgG heavy chain are indicated. (C) Immunofluorescence microscopy was performed in Kasumi-1 cells to detect endogenous RUNX1/AML1 (green), UBF1 (red) or AML1-ETO (green) with DAPI staining (blue). There is colocalization, albeit limited, of both RUNX1/AML1 and AML1-ETO with nucleolar UBF1 during interphase. (D) Electrophoretic mobility shift assays were performed with a human rDNA probe spanning a RUNX-binding element and nuclear extracts from Kasumi-1 cells. Competition assays with 100-fold molar excess of unlabeled wild-type, mutant or RUNX consensus oligonucleotide were performed to establish the specific protein-DNA complex (left) as indicated. Super-shift immunoassays were performed by incubating binding reactions with the indicated antibodies (right). Normal IgG was used as a negative control (control). The arrow on the right indicates the supershift band. (E) ChIP-reChIP assays with endogenous proteins in interphase Kasumi-1 cells using UBF1 antibody (primary ChIP) and second immunoprecipitation (reChIP) with antibodies directed against UBF1, RUNX1/AML1, AML1-ETO or IgG. The re-ChIP data are plotted as a percentage immunoprecipitation of the primary ChIP (set as 100%). Each of the regions was immunoprecipitated with similar efficiency in the primary ChIP. These results show that RUNX1/AML1 and AML1-ETO each can co-occupy rDNA repeats with UBF1.

with H3K4me2. We also performed ChIP assays to monitor modified histones on rDNA repeats in Kasumi-1 cells that express AML1-ETO and RUNX1/AML1 or in Jurkat cells that express only RUNX1/AML1. We found that methylation of H3K4 was higher for rDNA repeats in Jurkat cells whereas the presence of AML1-ETO in Kasumi cells correlated with histone H3K27 methylation on the ribosomal genes (Fig. 7B). The function of H3K27 methylation in Pol I transcription remains to be established. Taken together, our results suggest that the presence of AML1-ETO at ribosomal genes results in epigenetic alterations that reflect the pathological consequences of the 8;21 translocation.

RUNX1/AML1 and AML1-ETO have opposing effects on ribosomal biogenesis

We addressed the functional relevance of rDNA occupancy by RUNX1/AML1 and AML1-ETO using RNA interference. Small-interfering RNAs (siRNAs) specifically targeted against the mRNA junction sequences of the AML1-ETO fusion protein (Heidenreich et al., 2003) selectively reduced AML1-ETO protein levels by 50-80% without affecting RUNX1/AML1 levels in Kasumi-1 cells (Fig. 8). Conversely, RUNX1/AML1 siRNAs specifically downregulated RUNX1/AML1 protein levels by 50-80% without any effect on AML1-ETO (Fig. 8A). Neither RUNX1 nor AML1-ETO protein was affected by control siRNAs. Quantitative analysis revealed that pre-rRNA synthesis is decreased significantly by depletion of AML1-ETO in Kasumi-1 cells. By contrast, downregulation of RUNX1/AML1 protein markedly increased pre-rRNA synthesis (Fig. 8B). In neither case did we observe major changes in the large pre-existing pools of total 28S rRNA. As a positive control, we assessed mRNA levels of the CDK inhibitor p21, which is upregulated by AML1-ETO (Peterson et al., 2007b), whereas RUNX1/AML1 represses transcription of the p21 gene (Lutterbach et al., 2000). Consistent with prior findings, our qPCR analysis indicates that p21 levels are downregulated upon depletion of AML1-ETO, whereas RUNX1/AML1 siRNA treatment increases p21 mRNA levels. To rule out an indirect cell cycle effect resulting from changes in p21 levels, we directly depleted p21 mRNA by RNAi and found no effect on pre-rRNA expression (Budde and Grummt, 1999) (also data not shown). Thus, both RUNX1/AML1 and AML1/ETO levels control pre-rRNA synthesis, but have opposing effects.

To further examine the role of RUNX1/AML1 and AML1-ETO in regulation of ribosomal genes, we electroporated epitope-tagged RUNX1/AML1 and AML1-ETO into mouse 32D myeloid progenitor cells. The expression of the exogenous proteins was confirmed by immunoblotting with specific antibodies (Fig. 8C).

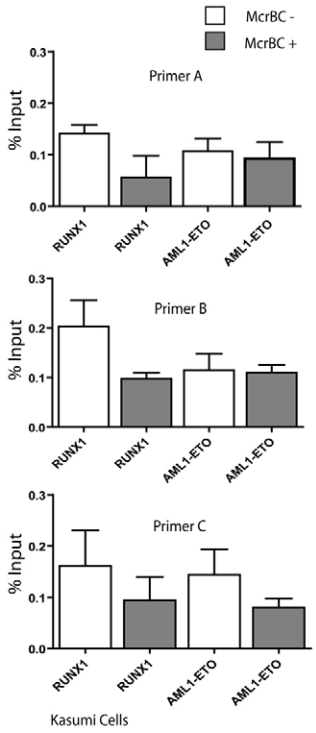

Fig. 6. RUNX1/AML1 associates with hypermethylated rDNA repeats. Chromatin from Kasumi cells was immunoprecipitated with antibodies against RUNX1/AML1 or AML1-ETO and the resulting DNA was digested with McrBC enzyme prior to qPCR using indicated rDNA primers. An antibody detecting ETO was used to immunoprecipitate the AML1-ETO fusion protein, whereas an antibody recognizing the C-terminal domain of RUNX1/AML1 was used to pull down endogenous RUNX1/AML1. Quantitative PCR data are normalized to genomic DNA and denoted as percentage input. These results indicate that rDNA repeats bound by RUNX1/AML1 are hypermethylated relative to those that are bound by AML1-ETO.

Total cellular RNA from transfected cells was isolated and quantified by RT-qPCR. The results reveal that pre-rRNA synthesis is significantly increased by exogenous expression of AML1-ETO, but forced expression of RUNX1/AML1 diminishes pre-rRNA levels (Fig. 8C). Together, the RNAi and forced expression studies demonstrate that RUNX1/AML1 negatively regulates rDNA transcription, whereas AML1-ETO is a positive regulator.

Discussion

Using immunofluorescence microscopy and chromatin immunoprecipitation, we have shown here that the leukemia-related AML1-ETO fusion protein and the native RUNX1/AML1 factor associate with ribosomal gene loci on mitotic chromosomes at nuclear-organizing regions, the precursors to interphase nucleoli. This principal finding provides the first indication that these regulatory proteins contribute to epigenetic control of ribosomal gene expression in pre-leukemic and leukemic cells in part by 'book-marking' these genes during mitosis. Decreased interaction of RUNX1/AML1 with mitotic chromosomes in AML-derived Kasumi-1 cells suggests that AML1-ETO interferes with RUNX1/AML1 function not only during interphase (Hiebert et al., 2001) but also during mitosis. Because regulation of ribosomal RNA genes is a fundamental process that supports the growth of cells and is tightly coupled with cell differentiation, our findings have immediate ramifications for the deregulation of growth control that is characteristic of cancer cells.

Transcription by RNA polymerase I regulates the rate of ribosome biogenesis and the biosynthetic potential of the cell (Moss, 2004; White, 2005). RNA Pol I activity is also tightly linked to the signals that control cell growth, and a number of physiological and pathological stimuli affect the rate of RNA Pol I transcription (Russell and Zomerdijk, 2006). It is clear that cancer involves significant changes to transcription factors, such as p53, that interact with the Pol I complex (Grummt, 1999). The fact that elevated rRNA synthesis has recently been shown to accelerate proliferation of transformed cells (Zhao et al., 2003) provides further reason to believe that ribosomal biogenesis has a profound impact on cancer biology.

We evaluated the functional roles for RUNX1/AML1 and the leukemia-related AML1-ETO in rDNA transcription. Although immunoprecipitation and immunofluorescence microscopy results indicate that both proteins interact with UBF1 in interphase cells, the combined results from our studies suggest that AML1-ETO and RUNX1/AML1 perform opposing activities in control of ribosomal gene transcription. Indeed, downregulation of RUNX1/AML1 or AML1-ETO by RNA interference in Kasumi-1 cells reveals that RUNX1/AML1 negatively regulates rDNA transcription, whereas AML1-ETO is a positive regulator. Consistent with a negative role for RUNX1/AML1 in ribosomal RNA synthesis we found that it binds to highly methylated rDNA regulatory sequences. Additionally, Kasumi-1 cells expressing the AML1-ETO fusion protein and Jurkat cells that express only AML1 differ in post-translational epigenetic marks of histone proteins at the rDNA repeats. Hence, our findings suggest a novel pathological role for the leukemogenic AML1-ETO protein in epigenetic regulation of cell growth through control of RNA-Pol-I-mediated ribosomal gene transcription.

We also have observed that endogenous RUNX1/AML1, ETO and AML1-ETO proteins are directed to distinct subcellular compartments. In interphase cells, the AML1-ETO fusion protein is localized in the nucleus but targeted to nuclear microenvironments distinct from those containing endogenous RUNX1/AML1 protein. Thus, as we previously reported for ectopically expressed RUNX1/AML1 and AML1-ETO proteins (McNeil et al., 1999; Barseguian et al., 2002), localization of the leukemia-related fusion protein is deregulated both during interphase and mitosis. The altered subnuclear location of AML1-ETO is a direct consequence of the elimination of the RUNX1/AML1 targeting signal and the addition of specific determinants residing in ETO that are fused to AML1 during the t(8;21) chromosomal rearrangement. By contrast, unlike the AML1-ETO protein, native ETO endogenously expressed in HEL cells is excluded from mitotic chromosomes. Thus, the runt-homology DNA-binding domain that is fused to ETO in the chimeric AML1-ETO protein supports the recruitment of the ETO moiety to the rDNA repeats to deregulate rDNA transcription.

RUNX1/AML1 is a scaffolding protein that recruits many co-regulatory transcription factors to focally organized nuclear microenvironments (Zaidi et al., 2007; Lian et al., 2004). Many of these cofactors interact with the C-terminus of RUNX1/AML1 and can support either repression or activation by RUNX1/AML1 (e.g. TLE/Groucho, LEF1, CBP, p300 and MOZ) (reviewed by Durst and Hiebert, 2004). The loss of the RUNX1/AML1 C-terminus in the AML1-ETO fusion protein thus precludes the recruitment of a large group of possible co-factors to rDNA repeats and other RUNX1/AML1 target genes. Furthermore, the acquisition of ETO-related protein-coding sequences in AML-ETO-expressing cells will mediate the interactions of a distinct group of co-factors during interphase and mitosis. For example, the AML1-ETO fusion protein has been shown to aberrantly recruit co-repressor complexes (e.g.

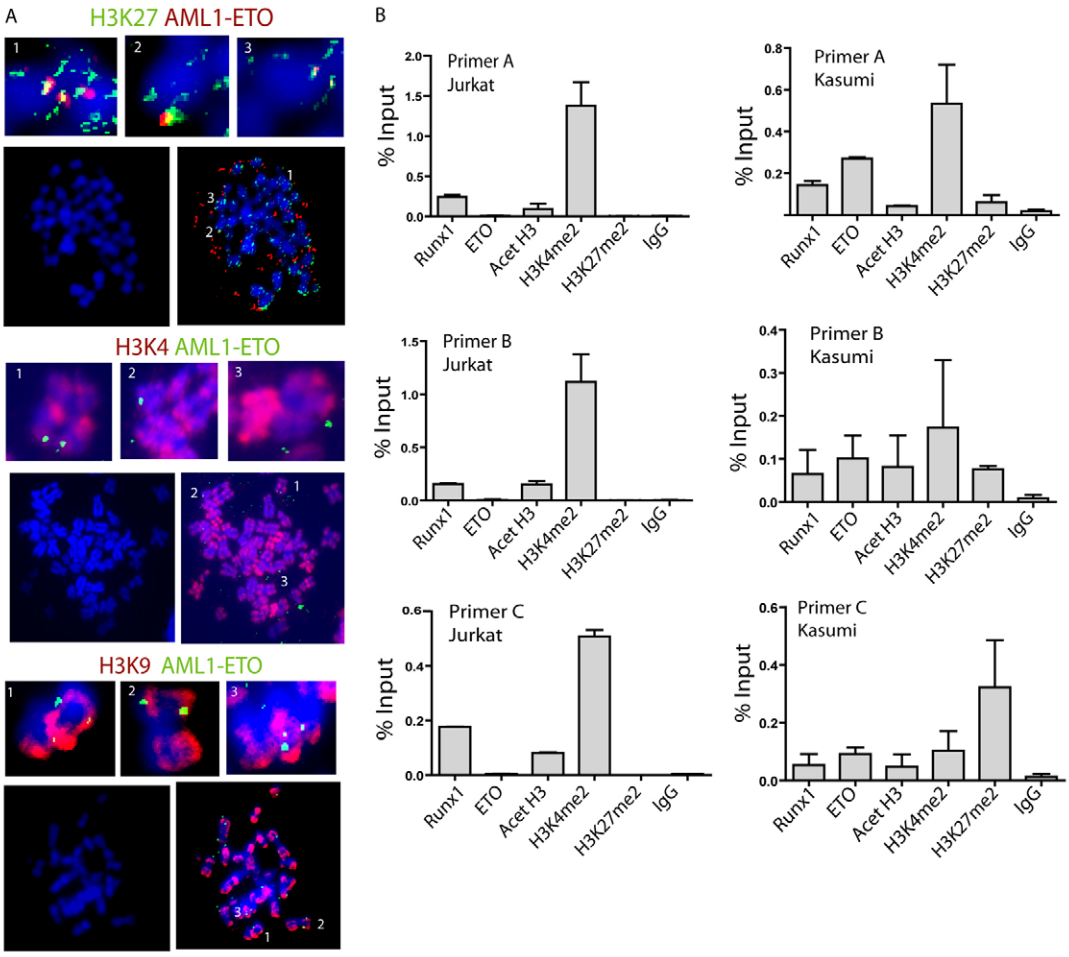

Fig. 7. AML1-ETO presence on rDNA repeats correlates with altered histone H3 methylation. (A) AML1-ETO colocalizes with histone H3 dimethyl lysine 27 (H3K27) on mitotic chromosomes. Immunofluorescence microscopy with antibodies against post-translational modifications of nucleosomal histones in metaphase spreads prepared from Kasumi-1 cells. The antibodies used to detect histone modifications were as follows: H3K9me2, H3K4me2 and H3K27me2. Each group of five panels shows merged images co-stained with antibodies against AML1-ETO. The lower left panels in each group show DAPI staining only. The top three panels in each group (indicated by numbers) show enlargements of the areas numbered on the lower right of each group. (B) AML1-ETO occupancy of rDNA repeats is associated with histone H3 lysine 27 methylation (H3K27me2). ChIPs were performed with RUNX1/AML1, AML1-ETO, IgG and histone modification antibodies were done in Jurkat (left panels) and Kasumi-1 cells (right panels). The antibodies used to detect histone modifications were as follows: Acetylated histone H3 (Acet-H3), H3K4me2 and H3K27me2. Presence of AML1-ETO in Kasumi-1 cells correlates with elevated histone H3K27 methylation on the rDNA repeats compared with Jurkat cells, which express only RUNX1/AML1.

N-CoR–Sin3–HDAC1) to actively shut down transcription from RUNX1/AML1 target genes important for normal hematopoiesis (Hiebert et al., 2001). However, AML1-ETO does not always function as a transcriptional repressor. For example, expression of AML1-ETO has been shown to transactivate the BCL-2 and MDR1 promoters in reporter gene assays, although binding to a RUNX element might not be necessary (Klampfer et al., 1996; Hines et al., 2007; Burel et al., 2001). Additionally, AML1-ETO upregulates C/EBPε to induce the expression of the G-CSF receptor and synergistically activates the M-CSF receptor promoter in combination with AML1 (Rhoades et al., 1996; Shimizu et al., 2000). We propose that modified association of cofactors with rDNA genes activates rDNA transcription in leukemia cells expressing the 8;21 fusion protein. However, the actual mechanism by which AML1-ETO compromises fidelity of rDNA transcription may be complex. An indirect mechanism that is not ruled out by our data is that AML1-ETO binds to the regulatory element of another gene that is responsible for activation of rDNA transcription.

In conclusion, there are two major implications of the results presented in our study. First, the AML1-ETO fusion protein not only affects RNA Pol II gene regulation but also RNA-Pol-I-mediated ribosomal RNA transcription during interphase. Second, AML1-ETO perturbs normal functions of RUNX1/AML1 in mitotic cells to alter hematopoietic lineage-specific control of ribosomal RNA genes in progeny cells. The current findings suggest that AML1-ETO deregulates multiple gene regulatory pathways that control growth, proliferation and lineage identity. The coordinated deregulation of these intricate and biologically linked processes might clarify the potent properties of AML1-ETO in altering normal hematopoiesis and promoting development of leukemia.

Materials and Methods

Cell culture

The human erythroleukemia (HEL) cells and the t(8;21)-carrying cell line Kasumi-1 were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were

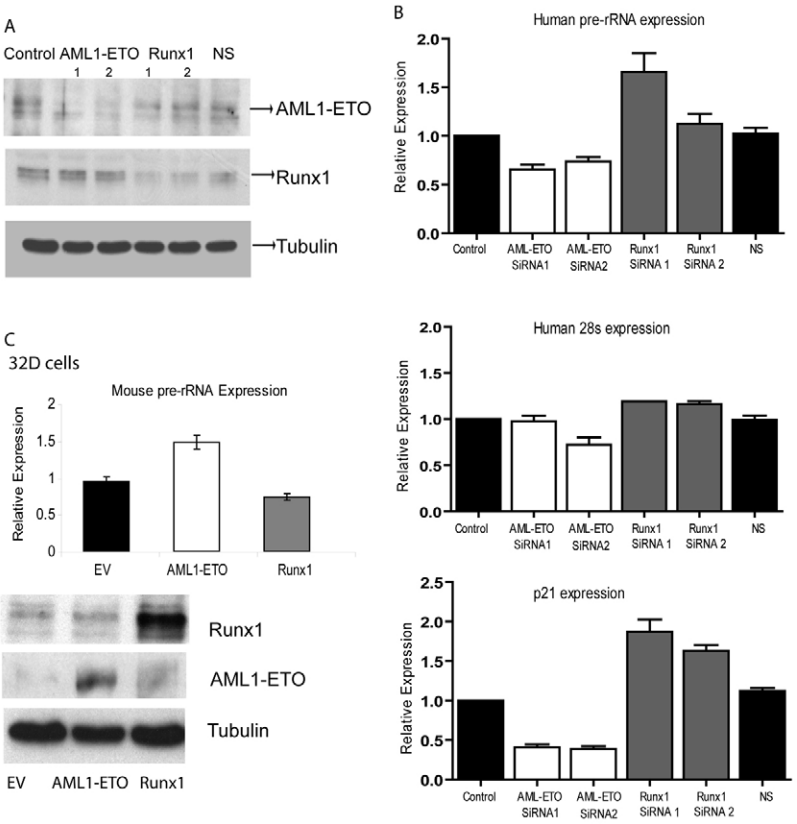

Fig. 8. RUNX1/AML1 or AML1-ETO deficiency alters rRNA synthesis. Kasumi-1 cells were transfected with two independent RUNX1/AML1 or AML1-ETO siRNAs or non-silencing (NS) siRNA. (A) To check the efficiency of knockdown, protein expression of RUNX1/AML1, AML1-ETO and α-tubulin was examined by western blot analysis. (B) Expression of unprocessed rRNA (pre-rRNA synthesis), 28S RNA and p21 was examined by RT-PCR analysis. Bars represent expression levels relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (± s.e.m.) from three independent experiments performed in triplicate. (C) Epitope-tagged RUNX1/AML1 and AML1-ETO were each ectopically expressed in mouse 32D myeloid progenitor cells. Pre-rRNA synthesis was measured by RT-qPCR relative to GAPDH in equal numbers of cells (top). Expression of the exogenous proteins was analyzed by western blot analysis (bottom). EV, empty vector.

maintained at 37°C in a humidified atmosphere with 95% air, 5% CO_2 at a concentration between 0.5×10^6 and 1×10^6 cells/ml.

Plasmid constructs and transfection experiments

Constructs pCMV5-HA-AML1/ETO and pCMV5-HA-RUNX1/AML1 were used in this study (Barseguian et al., 2002). HeLa cells were seeded in six-well culture plates at a density of $1.5\text{-}2.0\times10^5$ cells/well, and transient transfections were performed 24 hours later using FuGENE 6 according to the manufacturer's instructions (Roche, Indianapolis, IN). Cells were harvested 24 hours after transfection for immunofluorescence microscopy. Amaxa nucleofection of HEL and Kasumi-1 cells was performed according to the protocol suggested by the manufacturer (Amaxa, Gaithersburg, MD). The immunostaining was performed using anti-HA antibody.

Metaphase spread preparation

For metaphase spread preparation, cells were incubated with colcemid (Invitrogen Corporation, Carlsbad, CA) to a final concentration of 0.05 μg/ml at 37°C for 3-4 hours. Chromosome spreads were generated by incubating mitotic cells in 0.075 M KCl solution for 20 minutes at 37°C, fixed with methanol to acetic acid (3:1 vol/vol), dropped onto frosted glass microscope slides and air-dried.

Immunofluorescence microscopy

Cells were grown in regular growth medium for 1-2 days and then processed for in situ immunofluorescence. 500 μl cell suspension were deposited onto glass slides in a Shandon Cytospin 2 centrifuge. Cells were rinsed with ice-cold phosphate-buffered saline (PBS) and fixed in 3.7% formaldehyde in PBS for 10 minutes on ice. After rinsing once with PBS, the cells were permeabilized in 0.25% Triton X-100 in PBS, rinsed twice in PBSA (0.5% bovine serum albumin in PBS) and stained with antibodies.

The following primary antibodies and dilutions were used: UBF rabbit polyclonal (H-300), UBF mouse monoclonal (both 1:100; Santa Cruz Biotechnology); AML1 rabbit polyclonal (1:100; Active Motif), AML1 mouse monoclonal (1:100; 2B5 generous gift from Yoshiaki Ito, National University of Singapore, Singapore); ETO rabbit polyclonal (1:100; Calbiochem, San Diego, CA). For localization of antigen-antibody complexes, we used the following complementary fluorescent secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, and Alexa Fluor 594 goat anti-mouse IgG (1:800; Molecular Probes/Invitrogen).

Staining of cell preparations and chromosome spreads was recorded with a CCD camera attached to an epifluorescence Zeiss Axioplan 2 (Zeiss, Thorwood, NY) microscope. For interphase studies single image planes were acquired and deconvoluted using the Metamorph Imaging Software (Universal Imaging, Dowingtown, PA). For metaphase spreads Z-series image stacks were acquired at 0.25 μm intervals with 67 nm/pixel (*x-y*). Restoration of images was carried out by 3D deconvolution using a measured point-spread function as described previously (Carrington et al., 1995).

Chromatin immunoprecipitation and analysis

Chromatin immunoprecipitation assays (ChIPs) were performed by crosslinking asynchronously growing cells with 1% formaldehyde in RPMI for 10 minutes at room temperature. Crosslinking was quenched by adding glycine to a final concentration of 250 mM for 10 minutes. Cells were collected and washed twice with PBS. Cell pellets were resuspended in 2.5 ml lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40, 25 μM MG-132, and 1× Complete® protease inhibitor cocktail (Roche). After 10 minutes on ice, cells were sonicated to obtain DNA fragments of ~500 bp as determined by agarose gel electrophoresis with ethidium bromide staining. Protein-DNA complexes were isolated by centrifugation at 15,000 rpm for 20 minutes. Supernatants with protein-DNA complexes were incubated for 16 hours with 3 μg rabbit polyclonal antibody directed against each protein. The following primary antibodies were used: UBF rabbit polyclonal, ETO rabbit polyclonal (Santa Cruz Biotechnology) and AML1 rabbit polyclonal (Active Motif). The antibodies used to detect histone modifications were as follows: Acetylated histone H3, dimethyl H3K4 and dimethyl H3K27 (Upstate Biotechnology, Lake Placid, NY). Antibody-protein-DNA complexes were further incubated with 50-60 μl of 30% protein A/G beads (Santa Cruz Biotechnology) to isolate antibody-bound fractions of chromatin. Immunocomplexes were washed with the following buffers: low salt (20 mM Tris-HCl pH 8.1, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1× complete protease inhibitor), high salt (20 mM Tris-HCl, pH 8.1, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA), LiCl (10 mM Tris-HCl, pH 8.1, 250 mM LiCl, 1% deoxycholate, 1% NP-40, 1 mM EDTA) and twice in TE (10 mM Tris-HCl pH 8.1, 1 mM EDTA). Protein-DNA complexes were eluted in 1% SDS and 100 mM $NaHCO_3$. Crosslinks of pull-down fractions and inputs (2% of total IP fraction) were reversed by incubation overnight in elution buffer and 0.2 M NaCl. DNA then was extracted, purified, precipitated and resuspended in TE for qPCR. ChIP-reChIP experiments were carried out essentially as described (Sinkkonen et al., 2005). Briefly, UBF1 ChIP complexes were eluted in 10 mM DTT buffer for 30 minutes at 37°C, diluted 1:40 in ChIP lysis buffer and subjected to a second immunoprecipitation (i.e. re-ChIP) as described above. Quantitative PCR was done to quantify the immunoprecipitated DNA as described previously (Frank et al., 2001). For ChIP-CHOP PCR (Lawrence et al., 2004), 10% of the immunoprecipitated DNA was

digested with 10 U McrBC (New England Biolabs) in reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 1 mM GTP pH 7.9) at 37°C for 2-3 hours. Quantitative PCR was done to quantify the immunoprecipitated DNA as described previously. All ChIP and ChIP-CHOP PCR experiments were repeated at least twice.

Co-immunoprecipitation analysis

Kasumi-1 cells (50-70% confluent) were used for co-immunoprecipitation studies as described previously (Hassan et al., 2004). Equal amounts of cell lysate were immunoprecipitated with antibodies for UBF1 (F-9 or H-300), ETO (H-54, Santa Cruz Biotechnology) and AML1 (39000, Active Motif), overnight in phosphate-buffered saline with 5 mM EDTA. After a 2 hour incubation with protein A/G beads followed by three washes with PBS, the immunocomplexes were separated by 10% SDS-PAGE and western blotted with the indicated antibodies.

Electrophoretic mobility shift assay

Asynchronously growing Kasumi cells were harvested in ice-cold PBS buffer, cell pellets were lysed, and nuclear extracts prepared. The following oligonucleotides (double-stranded) representing wild-type (WT) and mutant (MT) RUNX binding elements of the human rDNA promoter were synthesized: WT, GGCTATCT-ATTTTGTGGTTAGAATAAAGTT; MT, GGCTATCTATTTTGTACTTAGAA-TAAAGTT. The probes were end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (New England Biolabs). Consensus and mutant oligonucleotides were used as competitors. Nuclear protein extracts (5 μg) were incubated for 30 minutes at room temperature with 1 μg nonspecific competitor DNA poly (dI·dC) (Pharmacia, Piscataway, NJ) and 80,000 cpm of labeled oligonucleotides. Competition assays were performed by mixing 100-fold molar excess of unlabeled oligonucleotides (wild type or mutant) with nuclear extracts before addition of probes. RUNX1 and ETO antibodies (2 μg each; Santa Cruz Biologicals) were used for super-shift experiments. Normal rabbit IgG was used as a nonspecific control. Protein-DNA complexes were visualized by autoradiography of 5% polyacrylamide gels.

RNA interference

Exponentially growing Kasumi-1 cells were electroporated using Amaxa Nucleofector (Amaxa Biosystems) according to the manufacturer's protocol, with siRNAs against RUNX1/AML1 (Dharmacon, Lafayette, CO) or AML1-ETO, as described previously (Heidenreich et al., 2003). A non-silencing siRNA (Qiagen) were used as a negative control. Total RNA and protein were isolated for further analysis.

Quantitative reverse transcription-PCR (qRT-PCR)

RNA was extracted from all samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Purified total RNA was subjected to DNase I digestion, followed by column purification using the DNA-free RNA Kit (Zymo Research, Orange, CA). Eluted total DNA-free RNA was quantified by spectrophotometry and 1 μg was added to a reverse transcription reaction using the iScript cDNA synthesis kit (Bio-Rad) with a mixture of random hexamers and oligo(dT) primers. Relative quantification was determined using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) measuring real-time SYBR Green supermix fluorescence. The relative level of each mRNA was determined using the comparative C_T method for relative quantification using GAPDH or mCox as an endogenous reference. Primer sets used here have been described previously (Young et al., 2007a).

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