Global reduction of the epigenetic H3K79 methylation mark and increased chromosomal instability in CALM-AF10–positive leukemias

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Chromosomal translocations generating fusion proteins are frequently found in human leukemias. The fusion proteins play an important role in leukemogenesis by subverting the function of one or both partner proteins. The leukemogenic CALM-AF10 fusion protein is capable of interacting with the histone H3 lysine 79 (H3K79)–specific methyltransferase hDOT1L through the fused AF10 moiety. This interaction leads to local H3K79 hypermethylation on Hoxa5 loci, which up-regulates the expression of Hoxa5 and contributes to leukemogenesis.

However, the long latency of leukemogenesis of CALM-AF10 transgenic mice suggests that the direct effects of fusion oncogene are not sufficient for the induction of leukemia. In this study, we show that the CALM-AF10 fusion protein can also greatly reduce global H3K79 methylation in both human and murine leukemic cells by disruptng the AF10-mediated association of hDOT1L with chromatin. Cells with reduced H3K79 methylation are more sensitive to γ-irradiation and display increased chromosomal instability. Consistently, leukemia patients harboring CALM-AF10 fusion have more secondary chromosomal aberrations. These findings suggest that chromosomal instability associated with global epigenetic alteration contributes to malignant transformation in certain leukemias, and that leukemias with this type of epigenetic alteration might benefit from treatment regimens containing DNA-damaging agents. This study is registered with www.clinicaltrials.gov as NCT00266136. (Blood. 2009;114:651-658)

Introduction

The analysis of chromosomal translocations in leukemia has contributed greatly to our understanding of the pathogenesis of this disease.1 The translocation breakpoints pinpoint the location of genes that are critically altered in leukemogenesis. There are 2 principal mechanisms by which translocations alter gene function: (1) through the formation of fusion genes and (2) through deregulated expression of a gene close to the translocation breakpoint.2 It is well established that fusion genes act as oncogenes by ultimately activating signaling pathways that lead to proliferation and in many cases also to a block in differentiation.3,4 However, the results from many studies in which mouse models of fusion gene-driven leukemias were established showed that in most cases in animal models the expression of the fusion gene either did not result in the development of leukemia or showed a long latency until the animals developed leukemia (PML-RARA, ETv6-AML1). This observation can be explained by assuming that in addition to the expression of the fusion gene, other genetic alterations are required for the full leukemic phenotype. Whereas much work has focused on the direct mechanisms used by the various fusion proteins to activate or disrupt critical cellular pathways and on the identification of collaborating mutations, we now provide evidence that certain fusion proteins actively promote the acquisition of these additional mutations by causing genome-wide epigenetic changes that link to increased genomic instability.

The AF10 gene is involved in 2 leukemia-associated fusion genes. The t(10;11)(p12;q23) translocation results in the MLL-AF10 fusion, and the t(10;11)(p12;q14) translocation results in the CALM-AF10 fusion.5,6 Whereas the MLL-AF10 fusion is only observed in acute myeloid leukemia (AML),7 CALM-AF10 fusions have been reported in both acute lymphoblastic and AML, as well as in lymphoma.8 The protein encoded by CALM (the clathrin assembly lymphoid myeloid leukemia gene) binds to clathrin and is involved in clathrin-mediated endocytosis and the trafficking of vesicles between the trans-Golgi network and endosomes.9 Mutations in the CALM gene are responsible for defects in the hematopoietic system and iron metabolism in fit1 mice.10

The gene product of AF10 (ALL-1 fused gene from chromosome 10, or MLLT10) is a putative transcription factor containing an N-terminal plant homeo domain zinc finger motif and an octapeptide motif-leucine zipper (OM-LZ) region close to its C terminus.11 Whereas little is known about the role of AF10 in transcriptional regulation, it has been identified as an interaction partner of the histone methyltransferase hDOT1L.12 DOT1 is the sole enzyme responsible for the methylation at lysine 79 of histone H3 (H3K79),13,14 a modification conserved in yeast, chicken, and mammals.13,15,16 The Dot1 gene was first identified in a genetic screen in yeast as a disruption of telomeric silencing. Dot1 mutations affect telomere function due to the redistribution of SIR proteins


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from the telomeric heterochromatin to the euchromatin caused by a loss of H3K79 methylation. In addition, yeast cells deficient in Dot1 function show increased radiation sensitivity, suggesting that H3K79 methylation is also involved in DNA repair. In mammalian cells, the local level of H3K79 methylation correlates with transcriptional activity. Aberrant H3K79 hypermethylation is associated with the activation of oncogenic Hox genes in mouse bone marrow cells expressing CALM-AF10 and MLL-AF10 fusions. This process depends on the recruitment of hDOT1L to the Hox loci, and the small OM-LZ region of AF10, which mediates the interaction of AF10 with hDOT1L.

However, studies using mouse leukemia models often show an extended latency for the development of malignancy. CALM-AF10 transgenic mice developed acute leukemia at a median age of 12 months. The transgenic mice were clinically healthy and had normal blood counts for the first 9 months. Interestingly, the Hoxa genes were up-regulated in both clinically healthy and leukemic CALM-AF10 mice. In another study using retroviral transduction of CALM-AF10, followed by bone marrow transplantation, the recipient mice developed leukemia after a median of 3 to 4 months posttransplantation. The long latency to malignancy in various leukemia models suggests that the direct effects of the fusion oncogenes are not sufficient for the induction of leukemia, and that the accumulation of additional genetic alterations is necessary for leukemogenesis in vivo. There is evidence that fusion proteins involving oncogenic tyrosine kinases, such as BCR-ABL, induce genomic instability, which may facilitate the acquisition of secondary genetic alterations. In contrast, it is currently unknown whether fusion proteins (such as CALM-AF10) involving transcriptional regulators alter genomic stability.

In this study, we report that the leukemic fusion CALM-AF10 causes global H3K79 hypomethylation by disturbing the association of hDOT1L with chromatin in human and murine cells. Cells with CALM-AF10–induced H3K79 hypomethylation became hypersensitive to radiation and displayed increased chromosomal instability. Moreover, leukemia patients carrying the CALM-AF10 fusion have a higher frequency of additional chromosomal aberrations than patients with an MLL-AF9 or a PML-RARA fusion gene. Our findings suggest a novel pathway for CALM-AF10 to exert its leukemogenic effect partially by increasing genome instability through alterations in epigenetic marks, and indicate that leukemias may have increased sensitivity to transcriptional regulators after genomic instability.

**Quantification of H3K79 methylation by mass spectrometry**

Relative quantification of H3K79 methylation was carried out by matrix-assisted laser desorption ionization–time of flight (Bruker, Autoflex) analysis of the peptides from the Arg-C digestion of histone H3. Histone H3 was purified from core histones by reverse-phase high-performance liquid chromatography using a C4 column, as previously reported.

**Short hairpin RNA-mediated stable inhibition of hDOT1L and AF10 expression in the T-REx-293 cell line**

Plasmid constructs encoding a short hairpin RNA targeting the DOT1L or AF10 gene were prepared, as previously described. The sequences of 2 pairs of complementary DNA oligonucleotides encoding short hairpin RNA were as follows (both annealed oligonucleotide duplexes of each pair have 5′-BglII and 3′-HindIII compatible overhangs). For DOT1L, the sense strand is 5′-GATCCCGCCGATAGAATGGATGATACGCGG-3′, the antisense strand is 5′-GATCCCGCCGATAGAATGGATGATACGCGG-3′. For AF10, the sense strand is 5′-GATC-CCGGGAGTTAGAAGAACTTGAGATTTTTA-3′, the antisense strand is 5′-GATCCGGGAGTTAGAAGAACTTGAGATTTTTA-3′, and the small OM-LZ region of AF10, which mediates the interaction of AF10 with hDOT1L, was purified using a C4 column, as previously reported.

**RNA isolation and reverse transcription–polymerase chain reaction**

Total RNA was isolated with TRIzol reagent (Invitrogen). Reverse transcription into complementary DNA was performed using the Moloney murine leukemia virus reverse transcriptase (Invitrogen). Polymerase chain reactions (PCRs) were performed using the Takara Taq Hot Start Version. Primer information is available upon request.

**Establishment of stable cell lines**

T-REx-293 stable cell lines were established by culturing in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 5 μg/mL blasticidin (Invitrogen), and 220 μg/mL Zeocin (Invitrogen). The cell fractionation was performed as described. Two million cells were extracted in 200 μL cytoskeleton buffer on ice for 3 minutes, then centrifuged at 600 g for 3 minutes to separate soluble proteins in supernatant from cytoskeletal matrix and chromatin. Cell pellets from the centrifugation were resuspended and treated with 0.25 U/mL DNase I (cytoskeleton buffer with the NaCl concentration adjusted to 50 mM) for 30 minutes at RT. Then ammonium sulfate was added to a final concentration of 0.25 M. After incubation for 10 minutes at RT, the above centrifugation step was repeated to obtain the chromatin fraction in the supernatant and the cytoskeletal matrix in the pellet.

**Methods**

**Cell lines, constructs, and antibodies**

The T-REx-293 cell line was purchased from Invitrogen. The U937T cell line (provided by Dr Gerard Grosveld, St Jude’s Children’s Hospital, Memphis) is a derivative of U937 harboring the tet-VP16 fusion gene under the control of a tetracycline-inducible promoter. For inducible expression in T-REx-293 cell lines, all genes were cloned into pCDNA4-Flag derived from Dr Gerard Grosveld and stably transfected. The methyl-K79–specific antibody was described. The methyl-H3-K4, -K9, -K27, -K36, and histone H3 antibodies were purchased from Upstate Biotechnology.

**Metaphase chromosome immunofluorescence**

Metaphase chromosome spreads were prepared, as described previously. Twenty-four hours posttransfection, cells were treated with 0.5 μg/mL nocodazole (Sigma-Aldrich) for 16 hours, harvested, washed with phosphate-buffered saline, and centrifuged (200 g for 3 minutes). They were then resuspended and swollen in 75 mM KCl at 37°C for 20 minutes. The cells were then centrifuged (200 g for 3 minutes) and resuspended in ice-cold fixative (freshly prepared methanol and glacial acetic acid at a 3:1 ratio) on ice for 5 minutes. Further fixation was for 25 minutes on ice. Chromosomes were spread on ice-cold slides coated with polylysine. When completely dried, slides were blocked in blocking solution at room temperature (RT) for 30 minutes. Anti-Flag monoclonal antibody (Sigma-Aldrich) diluted at 1:1000 and anti-hemagglutinin (HA) polyclonal antibody (Santa Cruz Biotechnology) diluted at 1:500 were used as primary antibodies. The slides were mounted with Gel/Mount (M01; Biomedica) and observed with a Nikon fluorescence microscope (E6000). The objective lenses were x40 and x100.

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establishment of U937T stable cell lines was as described previously. Cotransfected pEGFP-C1 (BD Clontech) was used as selection marker, and resistant clones were isolated by limited dilution. Positive clones were identified by Western blotting for expression of genes of interest upon tetracycline withdrawal. The established U937T stable cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, 1 μg/mL tetracycline, 0.5 μg/mL puromycin, and 1 mg/mL G418.

**Colony formation assay**

The assay was performed, as described. Six thousand cells were plated in triplicate into a 6-well plate with 5 mL/well of 1.3% (wt/vol) methylcellulose (Sigma-Aldrich) containing RPMI 1640 medium and 10% FBS. To examine the sensitivity to γ-irradiation, cells were plated and incubated at 37°C for 1 hour, and subsequently treated with the indicated dosages of 137Cs irradiation (Gammacell 3000; MDS Nordion), followed by incubation for 10 days before colony counting.

**Genomic instability assay**

T-REx-293 cells were induced to express proteins (CALM-AF10 or CALM-AF10ΔOM-LZ) for 3 days before irradiation with 2 Gy of gamma radiation (137Cs). Colcemid was then added, and the cells were harvested 1 hour later by standard cytogenetic methods and stained with Giemsa. Images of metaphases were captured with an Ikaros karyotyping system (Metasystems), and the chromatid gaps and breaks were counted by 2 persons in a blinded fashion. The metaphases were scored for each experimental condition.

**Patient data**

Leukemia patient samples were analyzed by standard cytogenetic techniques (G-banding) and, if required, by multicolor fluorescence in situ hybridization. The CALM-AF10, MLL-AF9, and PML-RARA fusion transcripts were also verified by reverse transcription-PCR in every case. The samples were obtained under study protocols approved by the ethics committees of the participating centers, and all patients provided written informed consent, in accordance with the Declaration of Helsinki.

**Statistical analysis**

Data were presented as mean plus or minus SEM (n = 3 or more). Statistical analysis was performed by Student t test. Values were considered statistically significant when P values were less than .05.

**Results**

**H3K79 methylation is reduced in leukemic cells from patients with a CALM-AF10 fusion**

The leukemic fusion protein CALM-AF10 is known to interact with the histone H3K79 methyltransferase hDOT1L, which leads to local H3K79 hypermethylation at the HOXA5 locus. To further analyze the impact of this fusion protein on hDOT1L function, we examined global H3K79 methylation levels in leukemic blasts from AML patients with the CALM-AF10 fusion. Western blotting with antibodies specific for dimethylated H3K79 revealed a drastic reduction in H3K79 methylation in samples from CALM-AF10–positive patients compared with samples from CALM-AF10–negative AML patients with a normal karyotype (Figure 1A). The reduction in H3K79 methylation was also obvious in the CALM-AF10–positive leukemia cell line U937.6 which shows local hypermethylation at the HOXA5 locus, but not in 2 other control cell lines (Figure 1B). Quantitative analysis of histone extracts using mass spectrometry confirmed the difference in methylation levels among these cell lines (supplemental Figure 1A, available on the Blood website; see the Supplemental Materials link at the top of the online article). Both H3K79 dimethylation and monomethylation were the lowest in U937 cells, whereas the nonmethylated form of H3K79 was more abundant. H3K79 trimethylation was rare and below the sensitivity of mass spectral detection (supplemental Table 1). No significant changes were observed in the level of histone methylation at other sites, including H4K20 (supplemental Figure 1B). These observations indicate that global H3K79 hypomethylation is a characteristic of cells expressing the CALM-AF10 fusion protein.

**The CALM-AF10 fusion protein causes H3K79 hypomethylation**

We next examined whether the reduction in H3K79 methylation is indeed caused by the expression of CALM-AF10. To this end, we established cell lines in which the ectopic expression of Flag-tagged CALM-AF10 can be controlled by tetracycline. Western analysis showed that H3K79 methylation was reduced substantially in cells expressing the fusion protein induced by tetracycline, whereas expression of CALM or AF10 alone had no apparent effect (Figure 2A). Moreover, the original H3K79 methylation levels were fully restored in the hypomethylated cells by inhibiting the expression of CALM-AF10 through tetracycline withdrawal (Figure 2A, lane 3). In addition, H3K79 hypomethylation was also detected in murine leukemia cells from our previously described CALM-AF10 leukemia mouse model, in which CALM-AF10 is expressed retrovirally in transplanted bone marrow cells (Figure 2B). The expression of CALM-AF10 did not affect the transcript level of endogenous dDOT1L in the cell lines (supplemental Figure 2), which indicates that the H3K79 hypomethylation is not due to the down-regulation of the histone methyltransferase. These results demonstrate that the CALM-AF10 fusion causes global H3K79 hypomethylation in both human and murine cells.
This result indicates that AF10 is necessary for hDOT1L-mediated hycline to knock down the expression of either hDOT1L or AF10. Hypomethylation occurred when the cells were treated with doxycycline (Figure 4A, compare lanes 1 and 2). H3K79 methyltransferase was used as a positive control for H3K79 methylation (compare lane 4 with lane 3). The knockdown of the transfection construct was confirmed (top panel). Western analysis showed that a large portion of Flag-AF10, but not Flag-CALM-AF10, was located in the chromatin fraction when they were transfected separately (supplemental Figure 3B). Interestingly, hDOT1L-HA alone failed to bind to chromatin even though it is a histone methyltransferase. This observation is consistent with a previous report for the yeast homolog of hDOT1L.37 However, when expressed together with Flag-AF10, hDOT1L-HA was largely (30%-50%) in the chromatin fraction (Figure 4B). In contrast, the fraction of hDOT1L in the chromatin was much smaller (~1%) when Flag-CALM-AF10 was coexpressed (Figure 4B, compare lane 2 with lane 6). These observations suggest that AF10 can recruit hDOT1L to chromatin, and fusion to CALM dramatically reduces this recruitment. To further extend this finding, immunofluorescence staining was performed on metaphase chromosome spreads from transfected 293T cells. When transfected separately, Flag-AF10 was bound to chromosomes, whereas both hDOT1L-HA and Flag-CALM-AF10 were dispersed in the cell (supplemental Figure 2C). However, hDOT1L-HA was bound to chromosomes when cotransfected with Flag-AF10, but not Flag-CALM-AF10, indicating that CALM can inhibit hDOT1L binding to chromatin.

The hDOT1L interaction region of AF10 is necessary for induction of H3K79 hypomethylation by the CALM-AF10 fusion

The conserved OM-LZ domain in AF10 mediates its interaction with hDOT1L and is necessary for the transforming activity of AF10 fusion proteins.12,21,36 To determine whether the interaction between CALM-AF10 and hDOT1L is required for H3K79 hypomethylation, Flag-tagged CALM-AF10 and its OM-LZ deletion mutant (CALM-AF10ΔOM-LZ) were coexpressed with hDOT1L-HA in 293T cells. As expected, CALM-AF10ΔOM-LZ was unable to coimmunoprecipitate hDOT1L-HA (Figure 3A). Interestingly, expression of CALM-AF10ΔOM-LZ did not lead to reduced H3K79 methylation (Figure 3B). This observation strongly suggests that reduction of H3K79 methylation requires the interaction of the CALM-AF10 with hDOT1L.

AF10 regulates H3K79 methylation by targeting hDOT1L to chromatin, but fusion to CALM dramatically weakens the ability of this targeting

We have previously reported that AF10 can interact with hDOT1L,12 but the physiologic function of this interaction is still unknown. To evaluate the role of AF10 in hDOT1L-mediated H3K79 methylation, AF10 expression was knocked down in T-REx-293 cells using inducible small interfering RNAs (siRNAs; Figure 4A, compare lane 4 with lane 3). The knockdown of the hDOT1L methyltransferase was used as a positive control for H3K79 hypomethylation (Figure 4A, compare lanes 1 and 2). H3K79 hypomethylation occurred when the cells were treated with doxycycline to knock down the expression of either hDOT1L or AF10. This result indicates that AF10 is necessary for hDOT1L-mediated H3K79 methylation. We next examined how AF10 might regulate the function of hDOT1L and the effect of the AF10 fusion with CALM. To this end, Flag-AF10, Flag-CALM-AF10, and hDOT1L-HA were expressed in 293T cells alone or in combination, and the cellular proteins were separated into soluble, chromatin, and matrix fractions (supplemental Figure 3A). Western analysis showed that a large portion of Flag-AF10, but not Flag-CALM-AF10, was located in the chromatin fraction when they were transfected separately (supplemental Figure 3B). Interestingly, hDOT1L-HA alone failed to bind to chromatin even though it is a histone methyltransferase. This observation is consistent with a previous report for the yeast homolog of hDOT1L.37 However, when expressed together with Flag-AF10, hDOT1L-HA was largely (30%-50%) in the chromatin fraction (Figure 4B). In contrast, the fraction of hDOT1L in the chromatin was much smaller (~1%) when Flag-CALM-AF10 was coexpressed (Figure 4B, compare lane 2 with lane 6). These observations suggest that AF10 can recruit hDOT1L to chromatin, and fusion to CALM dramatically reduces this recruitment. To further extend this finding, immunofluorescence staining was performed on metaphase chromosome spreads from transfected 293T cells. When transfected separately, Flag-AF10 was bound to chromosomes, whereas both hDOT1L-HA and Flag-CALM-AF10 were dispersed in the cell (supplemental Figure 2C). However, hDOT1L-HA was bound to chromosomes when cotransfected with Flag-AF10, but not Flag-CALM-AF10, indicating that CALM can inhibit hDOT1L binding to chromatin.
was dispersed in the cells when cotransfected with Flag-CALM-AF10 (Figure 4C). These data suggest that AF10 is involved in hDOT1L-mediated methylation by targeting hDOT1L to chromatin, and that CALM-AF10 greatly reduces hDOT1L recruitment, leading to global H3K79 hypomethylation.

**CALM-AF10 is a dominant-negative competitor of AF10 and affects H3K79 methylation by disrupting the AF10-mediated association of hDOT1L with chromatin**

Our previous report has demonstrated that CALM-AF10 can interact with hDOT1L in the nucleus, and this interaction does not appear to weaken the activity of hDOT1L per se because local H3K79 methylation at the *Hoxa5* gene is increased in CALM-AF10–expressing cells.21 In the presence of CALM-AF10, a small fraction of hDOT1L is still associated with chromatin, and this might account for local H3K79 hypermethylation (Figure 4B). We therefore reasoned that CALM-AF10 might compete with AF10 for hDOT1L interaction, and thus influence the recruitment of hDOT1L to chromatin. If the reduction of global H3K79 methylation in leukemia cells is really due to the hDOT1L-sequestering effect of the CALM-AF10 fusion protein, ectopic expression of AF10 might restore the function of the endogenous hDOT1L. To examine this, we established U937T cell lines (harboring the CALM-AF10 fusion) stably expressing Flag-AF10 or Flag-AF10 OM-LZ mutant had no obvious effect (Figure 5A left panel), although Flag-AF10 and Flag-AF10ΔOM-LZ were expressed at similar levels (Figure 5A right panel). The changes in H3K79 mono- and dimethylation levels were confirmed by quantitative analysis of histone extracts using mass spectrometry (Figure 5B). These results suggest that endogenous hDOT1L, which had been dissociated from chromatin by the endogenous CALM-AF10 in U937T, was retargeted to chromatin by the overexpressed AF10, thus restoring H3K79 methylation levels.

**Increased γ-irradiation sensitivity and chromosomal instability in cells with H3K79 hypomethylation**

Previous reports have demonstrated that Dot1 deletion or histone H3 lysine 79 substitution can lead to increased radiation sensitivity in *Saccharomyces cerevisiae*.18,19 To examine whether mammalian cells with hypomethylated H3K79 are more sensitive to radiation, T-REx-293 cell lines with inducible hDOT1L knockout and inducible CALM-AF10 expression were used. The knockdown of hDOT1L and the expression of CALM-AF10 had similar effect on H3K79 methylation and radiation sensitivity (Figure 6A). In both types of hypomethylated cells, survival at low-dose γ-irradiation (0.5 Gy to 3 Gy) dropped by 15% to 30% compared with control cells. The correlation of γ-irradiation sensitivity with H3K79 hypomethylation was also observed in the U937T leukemia cell line (Figure 6B), in which increased methylation by ectopic expression of Flag-AF10 (Figure 5) conferred a higher resistance to γ-irradiation. In support of a direct link between H3K79 hypomethylation and sensitivity to γ-irradiation, Dot1L-knockout mouse embryonic fibroblasts (MEFs) showed a 20% to 40% lower viability after γ-irradiation treatment than wild-type cells (Figure 6C).

To examine whether the radiation sensitivity in H3K79-hypomethylated cells is related to genomic instability, we examined the occurrence of chromosome breaks and gaps in T-REx-293 cells expressing the CALM-AF10 or CALM-AF10ΔOM-LZ fusion protein. In addition to reduced viability, cells with H3K79 hypomethylation showed an increased number of chromosome breaks and gaps after γ-irradiation. The mean number of breaks and gaps was 60% and 85% higher, respectively, in cells expressing
higher prevalence of complex aberrant karyotypes in patients and that this increased chromosomal instability is reflected in a sensitivity and subsequent chromosomal instability in cell lines, methylation caused by CALM-AF10 links to an increased radiation patients. Collectively, our data suggest that histone H3K79 hypomethylation, the occurrence of 3 and more rearrangements (a so-called additional chromosomal abnormalities (Figure 7B). Most strikingly, the occurrence of 3 and more rearrangements (a so-called complex aberrant karyotype) in CALM-AF10 patients was approximately 3- to 4-fold more frequent than in the other 2 types of patients. Collectively, our data suggest that histone H3K79 hypomethylation caused by CALM-AF10 links to an increased radiation sensitivity and subsequent chromosomal instability in cell lines, and that this increased chromosomal instability is reflected in a higher prevalence of complex aberrant karyotypes in patients with CALM-AF10–positive leukemias (Figure 7C).

**Discussion**

Although many leukemia-associated fusion genes involve chromatin-modifying genes, we report for the first time that a fusion protein can alter genome-wide histone modification (H3K79 methylation) levels, and that this alteration (hypomethylation) might relate to chromosomal instability.

We previously described that the CALM-AF10 and MLL-AF10 fusion proteins can recruit mouse Dot1L to Hoxa loci, causing local H3K79 hypermethylation and increased *Hoxa* expression, which was proposed to lead to leukemic transformation of mouse bone marrow progenitor cells. The importance of local H3K79 hypermethylation was recently shown to be very important for MLL-AF4–positive leukemias as well. We now show that the CALM-AF10 fusion protein, in addition to *Hox* gene up-regulation, causes global H3K79 hypomethylation and links to chromosomal instability. Global H3K79 hypomethylation as well as an increased number of complex chromosomal abnormalities were seen in CALM-AF10–positive patient samples as well. Interestingly, global hypomethylation and an increased number of complex chromosome aberrations were also found in MLL-AF10 patients (supplemental Figure 4). Considering that the transcriptional orientation of the *CALM* gene is the same as that of *AF10* (both are transcribed telomere to centromere), the complex chromosomal abnormalities in CALM-AF10–positive patients should not be due to the additional rearrangements that have to occur to generate a functional fusion protein as in the case of the MLL-AF10 (the *MLL* and *AF10* are transcribed in opposite orientations). Paradoxically, local hypermethylation and global hypomethylation of histone H3K79 coexist in leukemia cells, a situation reminiscent of the pattern of DNA methylation in tumors. Genome-wide DNA hypomethylation and local hypermethylation in the promoter regions of certain tumor suppressor genes are frequently found in various cancers. To confirm the coexistence of local hypermethylation and global hypomethylation of histone H3K79 in the same cells, chromatin immunoprecipitation experiments were performed in U937 (global H3K79 hypomethylation) and HL60 cells (normal H3K79 methylation). Consistent with our previous report, the HOXA5 gene was found to be up-regulated in U937 cells (CALM-AF10 positive) compared with HL60 cells (CALM-AF10 negative; supplemental Figure 5A). Chromatin immunoprecipitation data showed that some regions (amplicon b and e) of the HOXA5 locus have much more H3K79 methylation in U937 cells than those in HL60 cells (supplemental Figure 5C). This result directly supports the hypothesis that local hypermethylation and global hypomethylation of histone H3K79 can indeed coexist in the same cells.

In our previous report, we found that CALM-AF10 and hDOT1L could colocalize in the nucleus when they were coexpressed. Yet, this colocalization apparently does not allow CALM-AF10 to target hDOT1L to chromatin as efficiently as AF10, due to the poor chromatin-binding ability of CALM-AF10 itself (Figures 4B-C and supplemental Figure 2). But CALM-AF10 might still be able to target hDOT1L to a limited number of chromatin sites (Figure 4B, lane 6) through binding to specific target genes leading to local H3K79 hypermethylation. However, the majority of CALM-AF10 proteins in the nucleus could have a dominant-negative effect in competing with the endogenous AF10 for hDOT1L binding to cause disruption of chromatin association of hDOT1L, and thus global H3K79 hypomethylation. One has to be cautious about the molecular mechanism leading to H3K79 hypomethylation because most of our observations are based on experiments with overexpressed proteins and may not be easily extrapolated to the leukemogenesis process in vivo. For example, one contributing factor in vivo could be that the CALM-AF10 fusion moves endogenous hDOT1L out of nucleus (because the CALM-AF10 shuttles between nucleus and cytoplasm), which would cause the transcription elongation complex to function less efficiently and lead to reduced H3K79 methylation because of a smaller nuclear hDOT1L pool.
In the present study, we demonstrated that global histone H3K79 hypomethylation clearly increases γ-irradiation sensitivity in association with chromosomal instability. Consistent with our findings of a high proportion of CALM-AF10 patients with complex aberrant karyotypes, it was reported recently that Dot1L-deficient mouse embryonic stem cells lack H3K79 methylation and also show chromosomal instability (chromosome aneuploidy). However, the mechanism linking genome instability to H3K79 hypomethylation is not firmly established at the moment. Especially the controversy regarding the binding of the damage sensor protein 53BP1 to methylated H3K79 or methylated H4K20 at sites of DNA damage still needs to be resolved. Because our Western blot analysis and mass spectrometry data indicate that the expression of CALM-AF10 does not alter H4K20 methylation levels (supplemental Figure 1B and data not shown), the radiation sensitivity of cells with global H3K79 hypomethylation is unlikely to be due to altered H4K20 methylation. The relevance of H3K79 hypomethylation in the control of genome stability warrants further investigation.

Because H3K79 methylation is important for transcriptional elongation, global hypomethylation could also lead to a reduced expression of a great number of genes. Thus, an interesting scenario about the contribution of CALM-AF10 in leukemogenesis can be envisioned whereby the CALM-AF10 fusion, in addition to up-regulating specific oncogenes, may promote leukemogenesis by interfering with multiple cellular pathways through global hypomethylation of H3K79.

The work presented in this study suggests that CALM-AF10 fusion-mediated leukemogenesis is not only dependent on the transcriptional deregulation of specific target genes, but that a second mechanism, namely chromosomal instability, might be involved in leukemogenesis. Chromosomal instability is well known to play an important role in both tumor initiation and progression, and its contribution of CALM-AF10 in leukemogenesis can be envisioned whereby the CALM-AF10 fusion, in addition to up-regulating specific oncogenes (eg, HOXA5 genes) via local hypermethylation, might promote leukemogenesis by interfering with multiple cellular pathways through global hypomethylation of H3K79.

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Authorship

Contribution: G.-L.X., S.K.B., Y.Z., and Y.-H.L. designed the experiments and the study; G.-L.X., S.K.B., and Y.-H.L. wrote the manuscript; Y.-H.L., P.M.K., S.K.B., and K.-L.Z. performed experiments and collected and analyzed data; and Y.C., Y.-Q.L., A.J.D., C.B., and Y.Z. provided important reagents and reviewed the manuscript.

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Global reduction of the epigenetic H3K79 methylation mark and increased chromosomal instability in CALM-AF10–positive leukemias

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