Helicase-like transcription factor is a RUNX1 target whose downregulation promotes genomic instability and correlates with complex cytogenetic features in acute myeloid leukemia

Chi Keung Cheng,[1] Natalie P. H. Chan,[1] Thomas S. K. Wan,[1] Lai Ying Lam,[1] Coty H. Y. Cheung,[1] Terry H. Y. Wong,[1] Rosalina K. L. Ip,[1] Raymond S. M. Wong,[2,3] and Margaret H. L. Ng[1,4]

[1]Department of Anatomical and Cellular Pathology, Prince of Wales Hospital, The Chinese University of Hong Kong; [2]Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong; [3]Sir Y. K. Pao Centre for Cancer, Prince of Wales Hospital, Hong Kong; and [4]State Key Laboratory in Oncology in South China, The Chinese University of Hong Kong, Cina

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Correspondence:
margaretng@cuhk.edu.hk

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ABSTRACT

Helicase-like transcription factor is a SWI/SNF chromatin remodeling factor involved in various biological processes. However, little is known about its role in hematopoiesis. In this study, we measured helicase-like transcription factor mRNA expression in the bone marrow of 204 adult patients with *de novo* acute myeloid leukemia. Patients were dichotomized into low and high expression groups at the median level for clinicopathological correlations. Helicase-like transcription factor levels were dramatically reduced in the low expression patient group compared to those in the normal controls (n=40) (P<0.0001). Low helicase-like transcription factor expression correlated positively with French-American-British M4/M5 subtypes (P<0.0001) and complex cytogenetic abnormalities (P=0.02 for ≥3 abnormalities; P=0.004 for ≥5 abnormalities) but negatively with CEBPA double mutations (P=0.012). Also, low expression correlated with poorer overall (P=0.005) and event-free (P=0.006) survival in the intermediate-risk cytogenetic subgroup. Consistent with the more aggressive disease associated with low expression, helicase-like transcription factor knockdown in leukemic cells promoted proliferation and chromosomal instability that was accompanied by downregulation of mitotic regulators and impaired DNA damage response. The significance of helicase-like transcription factor in genome maintenance was further indicated by its markedly elevated expression in normal human CD34$^+$ hematopoietic stem cells. We further demonstrated that helicase-like transcription factor was a RUNX1 target and transcriptionally repressed by RUNX1-ETO and site-specific DNA methylation through a duplicated RUNX1 binding site in its promoter. Taken together, our findings provide new mechanistic insights on genomic instability linked to helicase-like transcription factor deregulation, and strongly suggest a tumor suppressor function of the SWI/SNF protein in acute myeloid leukemia.

Introduction

SWI/SNF proteins are chromatin remodeling factors that use the energy of ATP hydrolysis to remodel nucleosomes and regulate transcription. These proteins are essential for lineage specification and stem cell maintenance.[1] Emerging evidence

has also suggested non-redundant roles of these proteins in normal and malignant hematopoiesis. It has been shown that BAF53a is required for hematopoietic stem/progenitor cell maintenance,[2] whereas BRG1 is necessary for erythroid and granulocytic differentiation.[3,4] On the other hand, *ARID1A* and *SNF5* are disrupted in Burkitt lymphoma and chronic myeloid leukemia, respectively.[5,6] The significance of SWI/SNF proteins in hematopoiesis is further exemplified by their interaction with key hematopoietic transcription factors to govern lineage-specific gene expression.[7]

Helicase-like transcription factor (HLTF) is a SWI/SNF protein initially identified as a transcription factor that binds to several gene promoters and enhancers.[8,9] However, growing evidence has indicated its involvement in DNA repair. HLTF has been suggested as the yeast Rad5 homolog, which governs postreplication repair of damaged DNA and its downregulation increases mutagenesis and chromosome abnormalities.[10,11] Consistent with this tumor suppressive function, *HLTF* is frequently silenced by promoter hypermethylation in colon and gastric cancer and its restoration inhibits proliferation.[12,13] In addition, HLTF expression is severely reduced in certain melanoma and lung cancer cell lines.[14] However, it has also been shown that HLTF upregulation is associated with tumor progression in hypopharyngeal and cervical cancers.[15,16] Accordingly, variable prognostic implications of *HLTF* expression have been reported.[17,18]

Unlike in solid tumors, the role of *HLTF* in hematological malignancies remains largely unclear. In this study, we sought to investigate potential *HLTF* deregulation and its clinicopathological and functional implications in acute myeloid leukemia (AML). We demonstrated that *HLTF* expression was reduced in adult AML patients with more aggressive disease phenotypes, including complex cytogenetic abnormalities that were recapitulated in *HLTF* knocked down leukemic cells exhibiting impaired mitotic and DNA repair functions. Further investigation of the *HLTF* transcriptional control delineated mechanisms involving altered RUNX1 functions that contribute to the *HLTF* downregulation. Our findings indicate *HLTF* as a RUNX1 target and that deregulated *HLTF* may represent a new molecular pathway underlying genomic instability in AML.

Methods

Patient samples

Diagnostic bone marrow (BM) samples from 204 adult patients (≥18 years old) with *de novo* AML were studied (Table 1). Acute promyelocytic leukemia, therapy-related AML, or AML arising from a prior myelodysplastic syndrome (MDS)/myeloproliferative neoplasm were excluded. Normal BM and peripheral blood samples were obtained from individuals who had no prior history of malignancy. All subjects gave informed consent for the study, which was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee and was in accordance with the Declaration of Helsinki. Treatment details, cytogenetic and mutational studies and immunophenotyping are provided in the *Online Supplementary*

Methods

Mononuclear cells were isolated from the patient and control samples for nucleic acid extraction.

The AML cohort from The Cancer Genome Atlas (TCGA) was used for data validation.[19]

Cell lines

OCI-AML3 was provided by Prof. M.D. Minden (Princess Margaret Cancer Centre, University Health Network, Toronto, Canada). U937T and U937T-AE lines were provided by Prof. D.E. Zhang (Department of Pathology, University of California, San Diego, USA). Other cell lines were obtained commercially. Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum.

Real-time quantitative PCR (RQ-PCR)

Total RNA was extracted and reverse transcribed. RQ-PCR was performed using TaqMan assays (Life Technologies). Each sample was measured in triplicate and expression levels were determined by $2^{-\Delta\Delta Ct}$. *GAPDH* was used for normalization. Primer/probe sequences are provided in the *Online Supplementary Table S1*.

Immunohistochemistry

Immunohistochemical staining was performed using BM biopsies from 24 AML patients, with 12 having low and the other 12 having high HLTF mRNA levels. Details are provided in the *Online Supplementary Methods.*

Generation and analysis of stable HL-60 cell lines

HLTF knockdown in HL-60 cells was performed using short hairpin RNA (shRNA). Details of cell line generation and the analysis of cell proliferation, G-banded metaphases and histone H2AX phosphorylation are provided in the *Online Supplementary Methods.*

DNA constructs, transient transfection and reporter gene assays

Details of the constructs are provided in the *Online Supplementary Methods*. Transient transfection was performed with Lipofectamine 2000 (Life Technologies). Each transfection contained 50ng of pGL3 reporter constructs, 150ng of pCMV expression plasmids, and 4ng of Renilla luciferase plasmid pRL-CMV (Promega). Luciferase activities were measured 24 hours after transfection using the Dual-Glo Luciferase Assay System (Promega).

Methylation-sensitive high resolution melting (MS-HRM) analysis, bisulfite sequencing and methylation-specific PCR (MSP)

Bisulfite-modified DNA was prepared using the EZ DNA Methylation Gold Kit (Zymo Research). Detailed procedures are provided in the *Online Supplementary Methods.*

Statistical analysis

To investigate the clinicopathological significance of HLTF expression, the median HLTF mRNA level was used as the cut-off to dichotomize the patient cohort into low and high expression groups. Unpaired *t* and Fisher's exact tests were used to analyze the relation with continuous and categorical variables between groups, respectively. Overall survival (OS) and event-free survival (EFS) were defined as previously described.[20] Kaplan-Meier curves were compared by log-rank test. Multivariate Cox regression analysis was performed to test the significance of HLTF levels with adjustment for other potential prognostic factors. Two-sided $P<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS 21.0 (IBM).

Other methods are provided in the *Online Supplementary Methods.*

Results

Adult AML patients with low HLTF expression have distinctive clinicopathological features associated with poorer prognosis

Compared to the normocellular BM group (n=40), *HLTF* mRNA levels were dramatically reduced ($P<0.0001$) in the low expression patient group but similar to the high expression patient group (*Online Supplementary Figure S1*). HLTF protein expression measured by immunohistochemical staining of BM biopsies correlated with HLTF mRNA expression in the 24 patients studied ($P=0.032$ by Pearson correlation) (*Online Supplementary Figure S2*). Low HLTF expression in the AML patients correlated positively with elevated lactate dehydrogenase levels ($P=0.02$) and the French-American-British (FAB) M4/M5 subtypes ($P<0.0001$), but negatively with *CEBPA* double mutations ($P=0.012$) (Table 1; *Online Supplementary Table S2*). Additionally, low HLTF expression was associated with CD14 ($P=0.003$), CD15 ($P=0.036$) and CD20 ($P=0.034$) positivity on leukemic blasts (*Online Supplementary Table S3*). Since HLTF downregulation has been shown to induce chromosome aberrations,[10] we investigated if HLTF expression correlated with specific cytogenetic features in AML. Notably, patients with low HLTF expression had more frequent multiple chromosome abnormalities (≥ 3

Table 1. Comparison of laboratory, cytogenetic and molecular features between AML patients with low and high HLTF expression.

Parameters	Entire cohort (n=204)	Low HLTF expression (n=102)	High HLTF expression (n=102)	P
Mean age, years (range)	52.3 (18-91)	52.0 (18-91)	52.6 (20-86)	0.756
Sex, n (% males)	107 (52.5%)	54 (52.9%)	53 (52%)	>0.999
Mean hemoglobin, g/dL (range)	8.1 (2.9-13.6)	8.1 (2.9-13.6)	8.1 (4.1-12.9)	0.926
Mean platelets, 10^9/L (range)	61.3 (2-328)	62.9 (8-328)	59.6 (2-247)	0.642
Mean WBC, 10^9/L (range)	43.3 (0.3-517)	48.5 (0.6-282.2)	38.1 (0.3-517)	0.242
Mean LDH, U/L (range)	705 (101-5860)	845 (132-5860)	572 (101-3180)	0.020*
Mean BM blast, % (range)	61 (12-98)	62 (12-95)	61 (15-98)	0.784
FAB subtypes[a], n (%)				
M0	8 (4%)	3 (3%)	5 (5%)	0.721
M1	48 (24.1%)	19 (19.2%)	29 (29%)	0.136
M2	57 (28.6%)	22 (22.2%)	35 (35%)	0.060
M4	38 (19.1%)	24 (24.2%)	14 (14%)	0.073
M5	42 (21.1%)	30 (30.3%)	12 (12%)	0.002*
M6	5 (2.5%)	1 (1%)	4 (4%)	0.369
M7	1 (0.5%)	0 (0%)	1 (1%)	>0.999
Missing data	5	3	2	
Cytogenetic abnormalities[b], n (%)				
t(8;21)	15 (8.2%)	10 (11.5%)	5 (5.3%)	0.178
inv(16)/t(16;16)	12 (6.6%)	7 (8%)	5 (5.3%)	0.555
Normal karyotype	100 (54.9%)	43 (49.4%)	57 (60%)	0.180
11q23	4 (2.2%)	3 (3.4%)	1 (1.1%)	0.350
inv(3)/t(3;3)	2 (1.1%)	2 (2.3%)	0 (0%)	0.227
+8	14 (7.7%)	6 (6.9%)	8 (8.4%)	0.785
+11	3 (1.6%)	0 (0%)	3 (3.2%)	0.247
+13	3 (1.6%)	3 (3.4%)	0 (0%)	0.107
-5/-5q	7 (3.8%)	5 (5.7%)	2 (2.1%)	0.262
-7/-7q	6 (3.3%)	5 (5.7%)	1 (1.1%)	0.606
-17	3 (1.6%)	1 (1.1%)	2 (2.1%)	>0.999
-X/Y	10 (5.5%)	7 (8%)	3 (3.2%)	0.105
del(9q)	3 (1.6%)	1 (1.1%)	2 (2.1%)	>0.999
Complex (≥3 unrelated abnormalities)	17 (9.3%)	13 (14.9%)	4 (4.2%)	0.020*
Complex (≥5 unrelated abnormalities)	11 (6.0%)	10 (11.5%)	1 (1.1%)	0.004*
Missing data	22	15	7	
Molecular markers, n (%)				
FLT3-ITD	43 (21.1%)	22 (21.6%)	21 (20.6%)	>0.999
FLT3-D835/I836	12 (5.9%)	6 (5.9%)	6 (5.9%)	>0.999
KIT mutations	9 (4.4%)	6 (5.9%)	3 (2.9%)	0.498
CEBPA double mutations	22 (10.8%)	5 (4.9%)	17 (16.7%)	0.012*
CEBPA single mutations	11 (5.4%)	6 (5.9%)	5 (4.9%)	>0.999
NPM1 mutations	52 (25.5%)	28 (27.5%)	24 (23.5%)	0.630
DNMT3A mutations	40 (19.6%)	23 (22.5%)	17 (16.7%)	0.378
WT1 mutations	12 (5.9%)	5 (4.9%)	7 (6.9%)	0.768
IDH1 mutations	12 (5.9%)	6 (5.9%)	6 (5.9%)	>0.999
IDH2 mutations	25 (12.3%)	10 (9.8%)	15 (14.7%)	0.394

*WBC: white blood cell; LDH, lactate dehydrogenase; BM: bone marrow; FAB: French-American-British; ITD: internal tandem duplications. [a]P<0.0001 when FAB M4 and M5 are combined. [b]All patients with a specific abnormality were counted irrespective of the presence of additional abnormalities. *Statistically significant.*

unrelated abnormalities) than patients with high HLTF expression (P=0.02) (Table 1). This association was even more obvious when at least 5 abnormalities were considered as complex (P=0.004). Complex karyotypes in AML have been consistently associated with a very poor prognosis, and defined as the presence of multiple chromosome abnormalities (≥3 or ≥5) in the absence of the prognostically favorable t(8;21), inv(16)/t(16;16) and t(15;17).[20] The association of low HLTF expression with a complex karyotype remained significant when patients with the core-binding factor (CBF) translocations were excluded (≥3 abnormalities: 13% *vs.* 5%, P=0.085; ≥5 abnormalities: 11% *vs.* 1%, P=0.011). Clonal heterogeneity has recently been identified as an additional unfavorable cytogenetic marker in AML.[21,22] The incidence of cytogenetic heterogeneity in the forms of distinct subclones or composite karyotypes was also significantly higher in the low *HLTF* expression group than in the high expression group (16% *vs.* 4%, P=0.012).

Survival data were available from 154 of the 204 AML patients who had received standard chemotherapy. Complete remission was achieved in 122 of them (79%). With a mean follow-up time of 24 months, patients with low *HLTF* expression tended to have worse OS (P=0.127) and EFS (P=0.168) than patients with high *HLTF* expression when all cases were analyzed. When stratified by cytogenetics, patients with intermediate-risk cytogenetics with low *HLTF* expression had significantly shorter OS (mean, 16 *vs.* 24 months, P=0.005) and EFS (mean, 11 *vs.* 20 months, P=0.006) than those patients with high *HLTF* expression (*Online Supplementary Figure S3*). Similar trends were also noted when only cytogenetically normal AML patients were analyzed (OS: mean, 17 *vs.* 23 months, P=0.037; EFS: mean, 13 *vs.* 18 months, P=0.069) (*Online Supplementary Figure S3*). The favorable and adverse cytogenetic risk groups were not analyzed separately because of small sample sizes. In multivariate analysis, low *HLTF* expression remained as an independent adverse prognos-

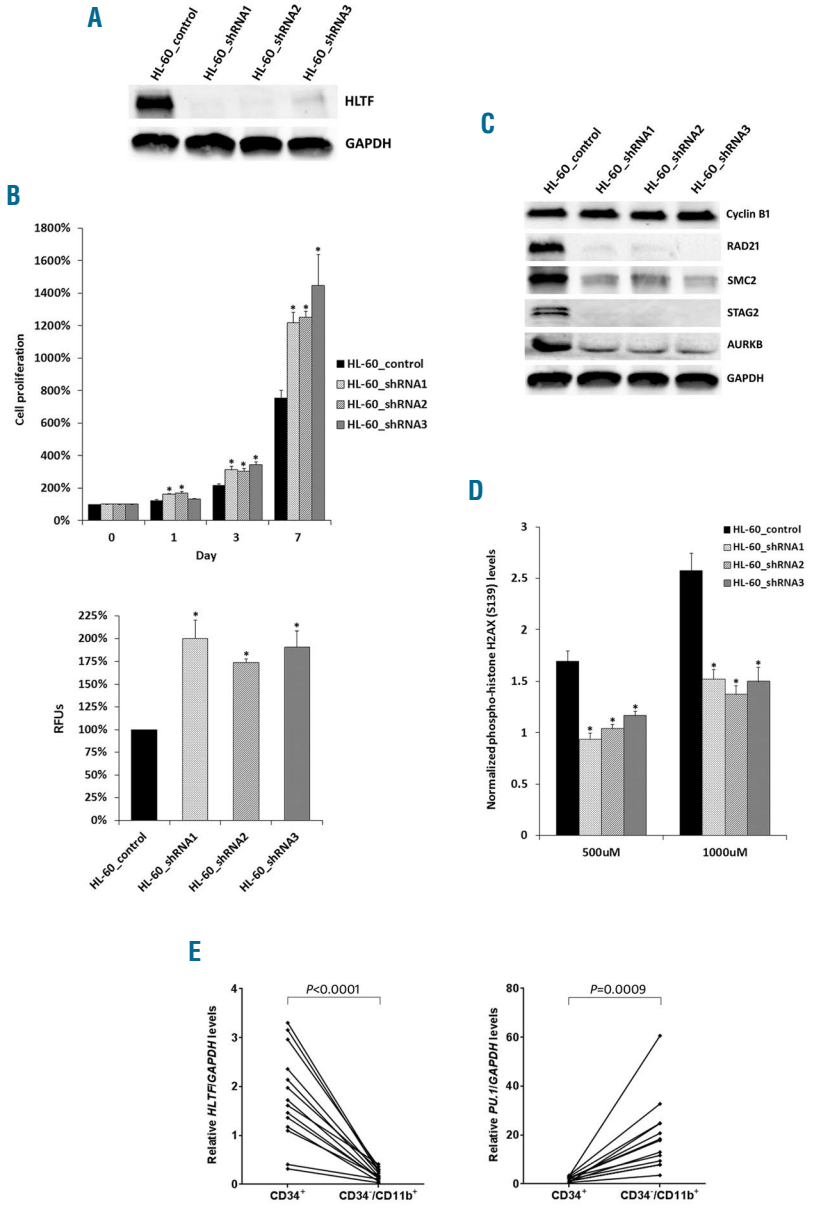

Figure 1. HLTF knockdown promotes proliferation and chromosomal instability in HL-60 cells. (A) Western blot analysis of HLTF expression in HL-60_control (empty vector control) and HL-60_shRNA1-3 lines. GAPDH served as a loading control. (B) WST-1 (top) and colony forming assays (bottom) for cell proliferation analysis of HL-60_control and HL-60_shRNA1-3 lines. For the latter assay, colonies were lysed and detected by the CyQuant GR Dye in a fluorescence plate reader after 10-day culture in a semi-solid methylcellulose medium. Results are expressed as mean ± SE from triplicate experiments. RFUs represent relative fluorescence units. (C) Western blotting of various mitotic regulators in HL-60_control and HL-60_shRNA1-3 lines. GAPDH served as a loading control. Representative blots from repeated experiments are shown. (D) Phosphorylation of histone H2AX at serine 139 in HL-60_control and HL-60_shRNA1-3 lines after 1-hour MMS (500μM and 1000μM) treatment. The phosphorylation levels were normalized to total histone H2AX levels in each sample. Results are expressed as mean ± SE from triplicate experiments and are relative to the untreated control in each cell line. (E) RQ-PCR analysis of HLTF expression in 14 pairs of normal human CD34$^+$ and CD34$^-$/CD11b$^+$ cell populations. Expression levels were relative to the U937 cell line. As expected, analysis of the same 13 pairs revealed a drastic increase in *PU.1* mRNA expression in the CD34$^-$/CD11b$^+$ mature myeloid compartment. * indicates P<0.05 *vs.* HL-60_control.

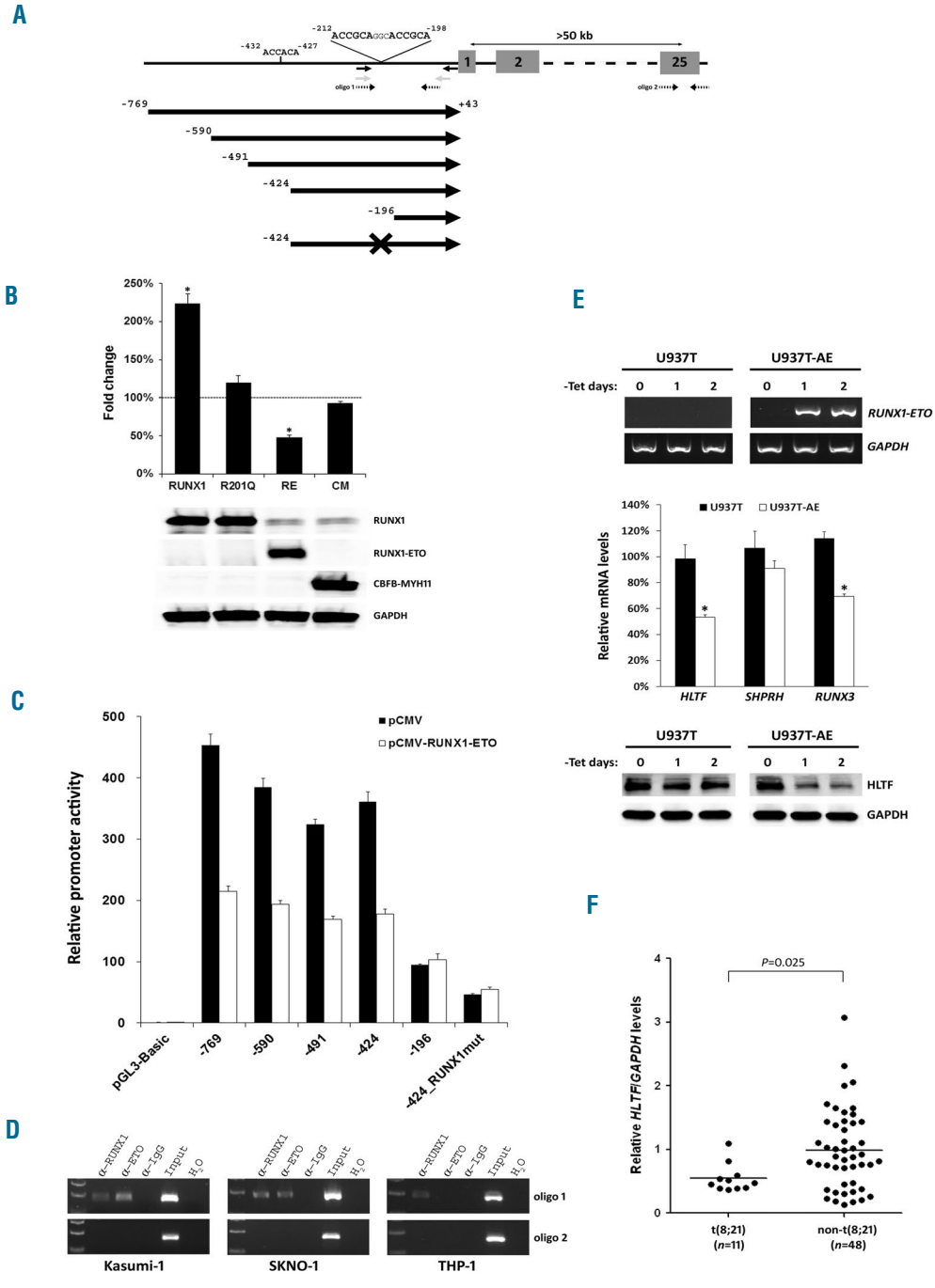

Figure 2. Transcriptional control of HLTF by RUNX1 and RUNX1-ETO. (A) A schematic representation of the human HLTF gene. The location of the duplicated and single RUNX1 binding sites on the HLTF promoter is shown. Dashed, filled and grey arrows indicate the primers used for ChIP assay, bisulfite sequencing and MS-HRM analysis, respectively. Filled boxes represent exons and the exon numbers are indicated. Horizontal arrows below the figure indicate the deletion and mutant promoter constructs analyzed in subsequent panels. The cross indicates a mutation introduced into the duplicated RUNX1 binding site. The first nucleotide of the HLTF mRNA (NM_003071.3) is assigned as +1. (B, top) HLTF (-769/+43) promoter-luciferase construct was co-transfected with RUNX1 (encodes AML1c), RUNX1_R201Q, RUNX1-ETO (RE) or CBFB-MYH11 (CM) expression plasmid together with pRL-CMV into K562 cells. Co-transfection with the same amount of empty pCMV vector was done in parallel. Results are presented as fold change by comparing the normalized firefly luciferase activity of the construct co-transfected with the expression plasmid with that co-transfected with the empty vector control. (B, bottom) Western blot analysis of RUNX1, RUNX1-ETO and CBFB-MYH11 expression in the K562 transfectants. GAPDH served as a loading control. (C) HLTF promoter-luciferase constructs were co-transfected with pCMV-RUNX1-ETO and pRL-CMV into K562 cells. Co-transfection with the same amount of empty pCMV was done in parallel. The duplicated RUNX1 site was mutated from 5'-ACCGCAGGCACCGCA-3' to 5'-GTCGACGGCGTC-GAC-3' in -424_RUNX1mut. Results are expressed as relative promoter activity by comparing the normalized firefly luciferase activity of the construct with the respective promoterless pGL3-Basic control. In all experiments in panels B and C, transfection efficiency was normalized according to the co-transfected pRL-CMV Renilla luciferase activity and results are expressed as mean ± SE from at least triplicate experiments. (D) Chromatin from t(8;21)-positive (Kasumi-1 and SKNO-1) and t(8;21)-negative (THP-1) cell lines was immunoprecipitated with the indicated antibodies and then analyzed by PCR using oligo 1 and oligo 2 primers. The oligo 1 primers amplified a 181-bp HLTF promoter fragment encompassing the duplicated RUNX1 binding site. The oligo 2 primers amplified a distal HLTF region lacking RUNX1 binding sites. Immunoprecipitation with anti-IgG was done as a negative control. (E, top) Confirmation of RUNX1-ETO induction following tetracycline withdrawal (-Tet) in U937T-AE cells by RT-PCR. Amplification of GAPDH was done as internal control. (E, middle) HLTF, SHPRH and RUNX3 levels were measured by RQ-PCR after 24 hours of tetracycline withdrawal in U937T and U937T-AE cell lines. Relative mRNA levels were calculated by comparing them with the expression levels before tetracycline withdrawal and results are presented as mean ± SE from triplicate experiments. (E, bottom) HLTF protein expression was analyzed by Western blotting following tetracycline withdrawal in U937T and U937T-AE cell lines. GAPDH served as a loading control. Representative blots from repeated experiments are shown. *indicates P<0.05. (F) HLTF mRNA levels in diagnostic BM samples from 59 patients with FAB-M2 AML were measured by RQ-PCR. Each circle represents one patient and the number of patients in each group is shown. Horizontal lines indicate the mean HLTF/GAPDH levels. Expression levels were relative to the U937 myeloid cell line.

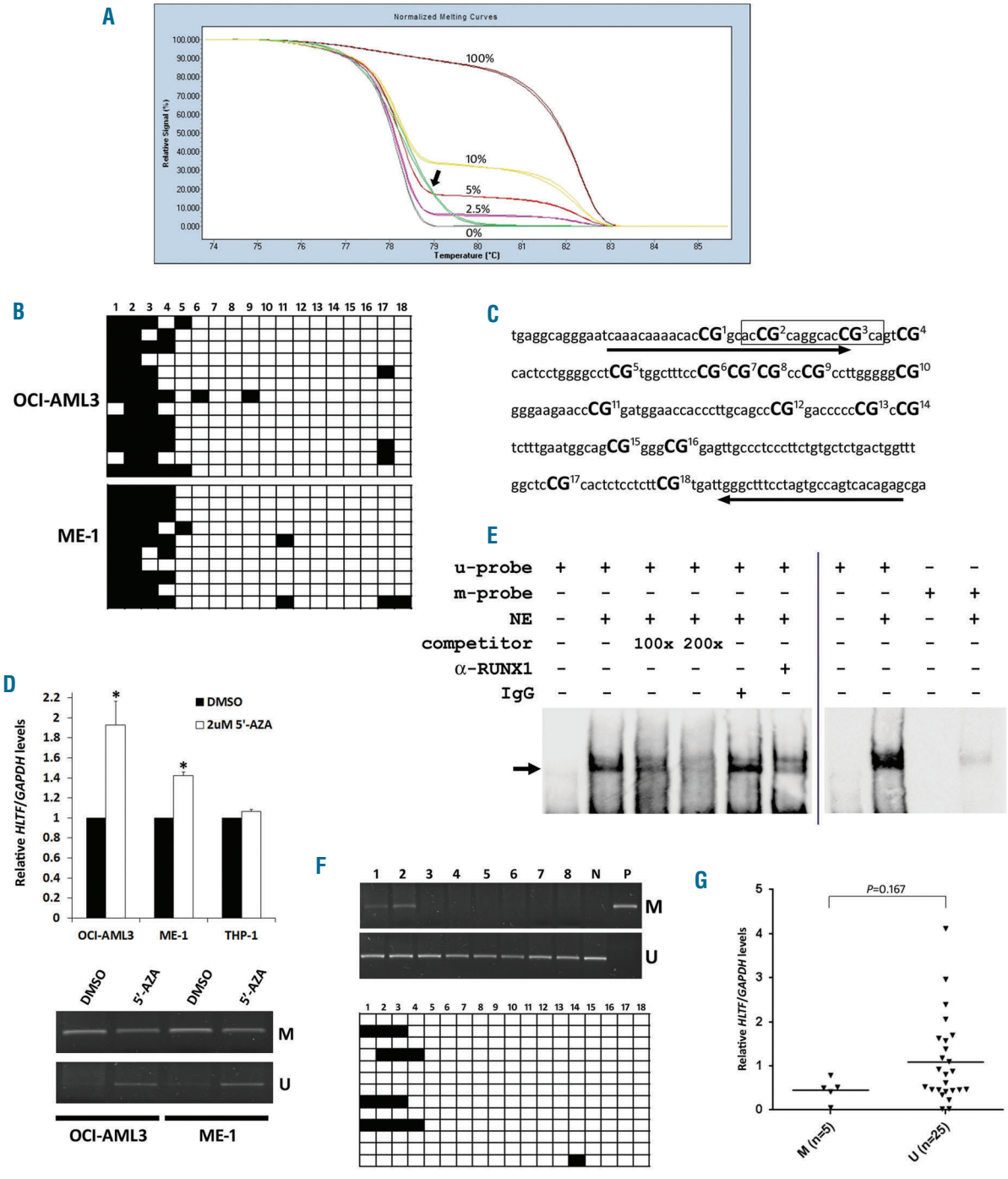

Figure 3. Aberrant CpG methylation at the duplicated RUNX1 binding site of the HLTF promoter causes HLTF repression in AML. (A) A representative normalized melting curve of MS-HRM analysis of HLTF promoter methylation in the ME-1 cell line (indicated by an arrow). The MS-HRM curves derived from the methylation standards (0%, 2.5%, 5%, 10% and 100%) are also shown. (B) Bisulfite sequencing of the HLTF promoter in OCI-AML3 and ME-1 cell lines. Each row of squares represents one PCR clone. Open and filled squares represent unmethylated and methylated CpG dinucleotides, respectively. Each CpG dinucleotide is numbered (no. 1-18). (C) Nucleotide sequence of the HLTF promoter region analyzed by bisulfite sequencing. The duplicated RUNX1 binding site is boxed. Arrows indicate the locations of the MSP primers. (D, top) RQ-PCR analysis of HLTF expression in AML cell lines after 4-day treatment with 2μM of 5'-AZA. Drugs were replenished at day 2 of the treatment. Results are presented as relative expression levels by comparing the normalized HLTF levels in the 5'-AZA-treated groups with those in the respective control group (DMSO-treated). Results are expressed as mean ± SE from duplicate experiments, each done in triplicate. *indicates $P<0.05$. (D, bottom) MSP analysis revealed partial demethylation of the duplicated RUNX1 site after 4-day 5'-AZA treatment (2μM) in OCI-AML3 and ME-1 cells. M and U represent PCR using primers specific for the methylated and unmethylated sequences, respectively. (E) EMSA analysis of RUNX1 binding to the HLTF promoter. Left, the specificity of the DNA-protein complex (indicated by an arrow) was confirmed by the addition of cold competitors and a RUNX1 antibody. For the supershift assays, nuclear extract (NE) was pre-incubated with 1μl of antibody at room temperature for 30 mins before probe addition. Right, the use of methylated EMSA probe (m-probe) abrogated RUNX1 binding to the HLTF promoter sequence. u-probe indicates the unmethylated probe. (F, top) Representative MSP analysis of CpG methylation of the duplicated RUNX1 site in the HLTF promoter in BM samples from 8 AML patients. N and P represent normocellular BM and the positive control OCI-AML3 cells, respectively. (F, bottom) Validation of the MSP results by bisulfite sequencing in an AML patient sample showing aberrant CpG methylation at the duplicated RUNX1 binding site. (G) HLTF mRNA levels in FAB-M1 patients with respect to the DNA methylation status of the duplicated RUNX1 binding site in the HLTF promoter. HLTF expression was determined by RQ-PCR and normalized to GAPDH. Expression levels were relative to the U937 myeloid cell line. Each triangle represents one patient, and the number of patients in each group is shown. Patients with RUNX1 mutations (n=6) were excluded from the analysis. Horizontal lines indicate the mean HLTF/GAPDH levels. M and U represent patients with and without methylation of the duplicated RUNX1 site, respectively.

tic factor for EFS in patients with intermediate-risk cytogenetics (*P*=0.032, hazard ratio=1.755, 95% confidence interval=1.05-2.931) (*Online Supplementary Table S4*). Low *HLTF* expression was also found to correlate with poorer OS in cytogenetically normal AML patients from the TCGA validation cohort (*P*=0.006) (*Online Supplementary Figure S4*).

HLTF knockdown promotes proliferation and chromosomal instability in HL-60 cells

To study the functional role of *HLTF* in AML, HLTF expression was stably knocked down in HL-60 cells. As shown in Figure 1A, HLTF protein levels were barely detectable in cells transfected with *HLTF* shRNAs (HL-60_shRNA1-3) as compared to the empty vector control (HL-60_control). Downregulation of *HLTF* expression significantly increased HL-60 proliferation in both WST-1 and colony-forming assays (Figure 1B). No apparent effect on HL-60 differentiation, as determined by May-Grunwald-Giemsa-stained morphological assessment, myeloperoxidase staining and flow cytometric analysis of cell surface marker expression (CD11b, CD14 and CD15), was observed following the knockdown (*data not shown*). Cytogenetic analysis of the HL-60_control and HL-60_shRNA1-3 lines revealed similar chromosome abnormalities including +18, del(9)(p21) and two rearrangements involving chromosome 5 as reported in the parental line (*Online Supplementary Table S5; Online Supplementary Figures S5-S8*).[23] Interestingly, knockdown of HLTF expression led to the emergence of a cytogenetic subclone (HL-60_shRNA1) or new clone (HL-60_shRNA2) with the acquisition of common clonal aberrations such as add(10)(p11.2) as well as the loss of der(10;13) and trisomy 13 (*Online Supplementary Table S5; Online Supplementary Figures S5-S8*). Moreover, a tetraploid subclone developed in the HL-60_shRNA3 line. Western blot analysis showed that HLTF knockdown in the three HL-60 lines was associated with downregulated expression of STAG2, RAD21, SMC2 and AURKB (Figure 1C), which play important roles in chromosome packaging and segregation during cell division. A key early event in DNA damage response is phosphorylation of histone H2AX at serine 139, which marks the damaged sites and signals the recruitment of DNA repair machinery.[24] Methyl methanesulfonate (MMS), an alkylating agent, can induce DNA breakage and H2AX phosphorylation. As shown in Figure 1D, knockdown of HLTF expression attenuated H2AX phosphorylation in HL-60 cells after MMS treatment, indicating an impaired DNA damage response.

To gain insights into the role of *HLTF* in myelopoiesis, we measured *HLTF* mRNA levels in human $CD34^+$ hematopoietic stem cells (HSCs) and $CD34^-/CD11b^+$ cells isolated from 14 normocellular BM samples. In all the paired samples, *HLTF* expression was high in the HSC population but decreased dramatically (3.8- to 18.7-fold) in mature myeloid cells (Figure 1E). Using HemaExplorer,[25] we found that *HLTF* is also highly expressed in common myeloid progenitors, granulocyte-monocyte progenitors and promyelocytes. Consistently, *HLTF* expression drops sharply as promyelocytes differentiate into myelocytes and decreases further in polymorphonuclear cells.

HLTF is a RUNX1 target and transcriptionally repressed by RUNX1-ETO in AML

Since deregulated *HLTF* mRNA expression had significant clinicopathological implications in AML, we investigated the transcriptional control of *HLTF*. Previous studies using ChIP-sequencing have suggested HLTF as a putative RUNX1 target in t(4;11) leukemias.[26] Accordingly, analysis of the *HLTF* 5'-flanking sequences revealed a single and a duplicated RUNX1 consensus binding site in the proximal promoter region (Figure 2A). The leukemogenic RUNX1-ETO fusion protein associated with t(8;21)-AML interacts with the same consensus binding sites as RUNX1 and typically acts as a dominant mutant to repress RUNX1 targets.[27] To examine potential regulation of the *HLTF* promoter by RUNX1 and RUNX1-ETO, we constructed a luciferase reporter plasmid carrying an 812-bp *HLTF* promoter fragment (nucleotide (nt) -769 to +43) that encompasses the two RUNX1 binding sites. Overexpression of RUNX1 (the longest AML1c isoform) activated the *HLTF* promoter in K562 cells (Figure 2B). This activation was impaired when the RUNX1_R201Q mutant with defective transcriptional activity[28] was used (Figure 2B). On the other hand, ectopic expression of RUNX1-ETO repressed the *HLTF* promoter (Figure 2B) and similar results were obtained from HeLa cells (*data not shown*). In contrast, co-transfection with pCMV-CBFB-MYH11 encoding the related inv(16) fusion protein failed to repress the HLTF promoter (Figure 2B). Also, the co-transfection did not affect the RUNX1-mediated HLTF activation (*Online Supplementary Figure S9*). Deletion mapping showed that sequences between nt -769 and -424, which contain the single RUNX1 binding site, were not important for basal HLTF promoter activity and RUNX1-ETO repression (Figure 2C). However, further deletion to nt -196 or mutation of the duplicated RUNX1 site (-424_RUNX1mut) drastically reduced HLTF transcription and attenuated the RUNX1-ETO effect (Figure 2C). ChIP assays in t(8;21)-negative (THP-1) and t(8;21)-positive (Kasumi-1 and SKNO-1) cell lines confirmed the specific binding of RUNX1 and RUNX1-ETO to the HLTF promoter (Figure 2D).

We next employed the inducible U937T-AE model[29] to study HLTF repression by RUNX1-ETO. The induction of RUNX1-ETO reduced the mRNA levels of HLTF and RUNX3 (a known transcriptional repression target of the fusion protein[30]) but not the related SHPRH (Figure 2E), which is another Rad5 homolog in human cells.[10] The downregulation of HLTF protein expression was also confirmed in U937T-AE cells after tetracycline removal (Figure 2E). We found no obvious changes in RAD21, SMC2, STAG2 and AURKB protein expression in U937T-AE cells upon RUNX1-ETO induction, suggesting that these genes may not be directly regulated by the fusion protein (*Online Supplementary Figure S10*). t(8;21)-AML is associated with the FAB M2 subtype. The comparison of HLTF mRNA expression revealed significantly reduced levels in t(8;21)-FAB M2 patients than non-t(8;21)-FAB M2 patients (*P*=0.025) (Figure 2F). Collectively, these findings indicate that RUNX1-ETO represses HLTF through direct binding to the duplicated RUNX1 site in the HLTF promoter.

The duplicated RUNX1 binding site in the HLTF promoter is aberrantly methylated in AML

Since *HLTF* is silenced by promoter hypermethylation in certain human cancers,[12,13] we evaluated this epigenetic change in AML using a MS-HRM approach with a sensitivity of about 2.5%. In total, we analyzed BM samples

from 133 adult AML patients in the original cohort (*Online Supplementary Table S6*), 7 AML cell lines and 10 normocellular BM samples. Aberrant HLTF promoter methylation was detected in one AML patient (~41% CpG methylation) (*Online Supplementary Figure S11*). None of the 10 normocellular BM samples exhibited the methylation. Among the 7 AML cell lines (THP-1, Kasumi-1, CMK, MV4-11, HL-60, ME-1 and OCI-AML3), two of them (OCI-AML3 and ME-1) also displayed abnormal MS-HRM melting profiles (Figure 3A). Intriguingly, in both cell lines, the methylation was restricted to four CpG sites (no. 1-4), which cluster along the duplicated RUNX1 binding site (Figure 3B,C). Treatment with 5-aza-2'-deoxycytidine (5'-AZA) increased *HLTF* mRNA expression in the methylated cell lines but not the unmethylated THP-1 cells (Figure 3D). MSP assays showed that the treatment caused partial demethylation of the duplicated RUNX1 site in both OCI-AML3 and ME-1 cells (Figure 3D). We next investigated whether such site-specific DNA methylation affects the binding of the cognate transcription factor RUNX1 using EMSAs. As shown in Figure 3E, a specific DNA-protein complex was formed when the duplicated RUNX1 site in the probe was unmethylated. However, the complex formation was profoundly inhibited when the four CpG sites clustered along the duplicated RUNX1 site were methylated (Figure 3E). Collectively, these findings indicate that methylation of the duplicated RUNX1 site blocks RUNX1 binding to the *HLTF* promoter and results in transcriptional inhibition of the *HLTF* gene.

Using the MSP assays, we found aberrant CpG methylation of the duplicated RUNX1 site in 4.5% (6 out of 133) of the adult AML patients (Figure 3F). All the positive cases belong to the FAB M1 subtype, accounting for 17% (6 out of 36) of the M1 cases. Such methylation was undetected in 16 normal (8 normocellular BM and 8 peripheral blood) samples examined. Those M1-AML patients with this site-specific methylation had reduced *HLTF* expression compared to those patients without the methylation, although the difference was not statistically significant in this small cohort (P=0.167) (Figure 3G). To validate our findings, we analyzed *HLTF* promoter methylation data (HumanMethylation450) in 100 AML patients from TCGA (*Online Supplementary Table S7*). Concordantly, the duplicated RUNX1 binding site was found to be methylated in 7 cases (*Online Supplementary Table S8*), with 6 of them being classified as FAB-M1 (representing 26% of the M1 cases in the validation cohort) and the remaining case as FAB-M0. Other CpG sites in the proximal promoter and 5'-untranslated region were unmethylated in all the samples.

Since *RUNX1* is mutated in AML and the mutations generally abolish RUNX1 transactivation potential,[28,31] we examined the correlation of *HLTF* expression with *RUNX1* mutations in the 133 adult AML patients. *RUNX1* mutations were detected in 12% (16 out of 133) of the cases. Patients with mutated *RUNX1* tended to have lower *HLTF* mRNA levels than patients with wild-type *RUNX1* (P=0.099) (*Online Supplementary Figure S12*).

HLTF is rarely mutated in AML

Since *SWI/SNF* mutations are found in human cancers,[1] we investigated whether *HLTF* is mutated in AML. Of the 133 adult AML patient samples tested for *HLTF* promoter methylation, 132 cases were available for mutation screening. Excluding 5 single nucleotide polymorphisms (p.D269N, p.T303T, p.N311S, p.A776A and p.T900S) detected, 8 *HLTF* sequence variations were identified with 6 of them being novel (p.M1_W3del, p.D282N, p.G465W, p.W582*, p.T641Nfs*3 and p.T862T) (*Online Supplementary Table S9*). Only the frameshift mutation (p.T641N*fs**3) was confirmed to be somatic as the change disappeared in the remission BM sample.

Discussion

Chromosomal abnormalities are considered the most significant prognostic determinants in AML, yet the underlying mechanisms have remained elusive. *TP53* (encoding p53) alterations have been regarded as an important pathway responsible for the marked genomic instability in complex karyotype AML, as they are frequent and correlate with genomic complexity in this AML subtype.[32] Herein we showed that reduced expression of the SWI/SNF gene *HLTF* was also closely associated with complex cytogenetic abnormalities in adult AML patients. Consistently, we showed that *HLTF* knockdown in leukemic cells induced structural and numerical chromosome defects. The increased chromosomal instability associated with HLTF downregulation may lead to clonal heterogeneity, with individual subclones developing drug resistance and causing relapse, thereby exacerbating AML outcomes. Complex cytogenetic abnormalities are also found in CBF-AML and have been shown to adversely affect long-term survival in some studies.[33,34] Interestingly, we observed that among our CBF-AML patients (n=27), lower HLTF expressors also had more secondary chromosome abnormalities (1.46 *vs.* 0.5) and higher chances of carrying a complex karyotype (23% *vs.* 0%) involving three or more additional aberrations than higher *HLTF* expressors. These observations suggest broad impacts of *HLTF* reduction on chromosomal instability across AML subtypes. Previous studies have shown that submicroscopic copy number alterations are present in about half of the cytogenetically normal AML patients,[35] and whether *HLTF* also impacts on these alterations warrants further investigation. Regarding prognostication, we revealed in this study an inverse correlation between reduced *HLTF* and CEBPA double mutations in adult AML patients. Since CEBPA double mutations are associated with a favorable prognosis,[36,37] these confounding interactions may partly contribute to the adverse prognostic effects of low *HLTF* expression in AML, particularly in the intermediate-risk cytogenetic subgroup, where CEBPA mutations are most prevalent.[37] Larger studies are needed to confirm these associations.

Previous studies have revealed that the mRNA expression of cohesin and condensin genes is downregulated in *Hltf*-deficient cells,[38] suggesting that HLTF may regulate these genes at the transcriptional level. The consensus HLTF DNA-binding sequence has been reported to be 5'MCWTDK3'.[39] Interestingly, the 5'-flanking region of *STAG2, RAD21* and *SMC2* contains multiple *HLTF* binding sites. Moreover, we observed a strong positive correlation between the mRNA expression of *HLTF* and the three genes in 173 AML patient samples from TCGA. These findings suggest that HLTF may transcriptionally regulate *STAG2, RAD21* and *SMC2* in a coordinated manner to govern genome functions. Also, our present data suggested that HLTF regulates DNA damage response as its down-

regulation impairs H2AX phosphorylation in the presence of DNA damage. This defect will likely compromise DNA repair and induce mutations and/or chromosome aberrations, thereby promoting genomic instability. Thus, it is conceivable that AML leukemic cells deficient in HLTF are prone to acquiring genetic alterations. ATM and ATR are two major kinases that phosphorylate H2AX,[24] and interestingly, their expressions are found to be reduced in *Hltf*-depleted cells.[40] Of note, the cohesin and condensin complexes are also strongly implicated in other processes including DNA repair and gene regulation, indicating that *HLTF* deregulation has diverse effects on genome functions. It could be postulated that the high-level *HLTF* expression in normal human $CD34^+$ cells is required for HSC functions by preventing genomic instability and malignant transformation.

Herein we demonstrated that *HLTF* is a direct transcriptional target of RUNX1. Like HLTF, RUNX1 impairment has been linked to an increase in genomic instability.[41] Interestingly, emerging evidence has indicated a functional interplay between RUNX1 and p53. It has been shown that RUNX1 can stimulate p53 activity and is recruited onto p53 target promoters in response to DNA damage.[42] Likewise, RUNX1 and p53 synergistically activate the transcription of *GADD45A*, encoding a sensor of DNA stress, and MDS/AML patients with mutated *RUNX1* show decreased *GADD45A* expression.[43] These observations are further corroborated by the inability of RUNX1 to induce cellular senescence in murine fibroblasts with defective p53.[44] The role of HLTF in genome maintenance and its intimate relationship with RUNX1 raise the possibility that a RUNX1/HLTF axis may collaborate with p53 in AML leukemogenesis.

It has been shown that the RUNX1-ETO fusion protein induces a mutator phenotype by downregulating DNA repair genes mainly in the base excision repair pathway.[45] Reduced *HLTF* expression has been shown to increase spontaneous and damage-induced mutagenesis.[10,11] Our findings that *HLTF* is transcriptionally repressed by RUNX1-ETO thus provide further mechanistic insights on how the fusion protein predisposes to the acquisition of additional mutations. On the other hand, our data suggested that the duplicated RUNX1 binding site in the *HLTF* promoter was not repressed by the related CBFB-MYH11 protein. In fact, it was recently found that CBFB-MYH11 cooperated with RUNX1 and a distinct set of regulators to preferentially activate gene expression.[46] More interestingly, our data revealed differential patterns of *HLTF* promoter methylation in solid tumor and blood cancers. While the methylation spans along the entire *HLTF* promoter in solid tumors,[12,13] *HLTF* promoter methylation in AML clusters along the duplicated RUNX1 binding site. Importantly, such methylation seems sufficient to abrogate RUNX1 transcriptional functions and thus may represent a novel mechanism deregulating RUNX1 in AML. Although the mechanisms underlying this site-specific DNA methylation are unclear, it was noted that all the six M1-AML cases with the methylation harbor *IDH1/2* mutations as compared to only 16.7% mutation in M1-AML cases lacking the methylation. Since *IDH1/2* mutations induce DNA hypermethylation,[47] it is possible that the aberrant *HLTF* methylation is related to mutant IDH1/2 activities.

RUNX1 activates transcription by recruiting coactivators including p300.[48] Interestingly, a putative p300 binding site is found adjacent to the duplicated RUNX1 site. Mutation of this p300 site repressed the *HLTF* promoter, whereas p300 overexpression activated the promoter (Cheng *et al.*, 2016, unpublished observations). These findings strongly suggest that RUNX1 and p300 cooperatively regulate *HLTF* transcription. It is worth noting that *p300* and *MOZ* (another RUNX1 coactivator) are predominantly disrupted in FAB-M4/M5 AML.[49] Since HLTF expression is also reduced in these AML subtypes, the reduction may be due to altered RUNX1 coactivator activities. On the other hand, the reduction may be due to *microRNA*-mediated gene repression as the 3'-untranslated region of *HLTF* contains two putative binding sites for microRNA-155, which is specifically over-expressed in FAB-M4/M5 AML.[50] These observations suggest that mechanisms other than altered RUNX1 functions also contribute to *HLTF* downregulation in AML.

While SWI/SNF mutations are common in certain human cancers,[1] *HLTF* is rarely mutated in AML and only detected in 1 out of 132 (~0.8%) adult AML patients. The mutation is expected to produce a truncated protein lacking the RING domain and the last three helicase domains. Since the RING domain is implicated in proliferating cell nuclear antigen ubiquitination,[10] the loss of this domain may impair the postreplication repair function. Interestingly, the nonsense c.1746G>A variant (*Online Supplementary Table S9*) also seems to affect the DNA repair function by protein truncation. However, the significance of other identified variants is unclear. It remains to be determined whether HLTF variants affect leukemia risk and phenotype.

In conclusion, our findings indicate that *HLTF* is a novel RUNX1 target and a potentially important regulator of genomic stability in AML. HLTF contains multiple discrete domains, in particular a unique DNA-binding domain that is absent in most SWI/SNF proteins.[8] Characterizing the molecular functions of these domains shall help decipher its role in leukemogenesis and identify targets for therapeutic intervention.

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