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

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Online Supplementary Information

Online Supplementary Methods

Patient treatment

Among the 204 adult patients with de novo AML, 162 of them (79.4%) received the standard cytarabine plus daunorubicin '7+3' induction chemotherapy.[1] Patients who achieved complete remission were then given consolidation treatment stratified by cytogenetics. Patients with non-favorable cytogenetics were referred for assessment for allogeneic stem cell transplantation. Patients who were not eligible for transplantation and those with favorable cytogenetics were given high-dose cytarabine-based chemotherapy for consolidation.[2] Twenty one patients received stem cell transplantation and their survival data had been censored at the time of transplantation.

Four patients received azacitidine-based treatment and 29 patients received palliative therapy due to underlying comorbidity or based on patients' decision. The remaining patients (*n*=9) had no follow-up data after the initial diagnosis.

Cytogenetic and mutational studies

G-banded cytogenetic studies were performed as described previously.[3] Cytogenetics were classified into favorable, intermediate and adverse according to the revised Medical Research Council classification.[4]

FLT3, *KIT*, *NPM1*, *CEBPA*, *IDH1*, *IDH2*, *WT1*, *DNMT3A* and *RUNX1* mutations were analyzed as previously described.[5-7]

Immunophenotyping

Flow cytometry was used to determine the expression of the following markers on leukemic cells: HLA-DR, CD2, CD3, CD5, CD7, CD13, CD14, CD15, CD19, CD20, CD33, CD34, cCD79a, and cMPO. A reaction was defined as positive when at least 20% of the

leukemic cells expressed the marker at a fluorescence intensity above cutoffs established using the corresponding isotype-matched control antibody.

Immunohistochemistry

Following deparaffinization, rehydration and antigen retrieval, sections (4μm-thick) were immunostained with a rabbit HLTF antibody (HPA015284 used at 1:100 dilution, Sigma-Aldrich**)** using the Leica Bond-Max automated stainer. Slides were then counterstained with hematoxylin and mounted. HLTF expression was assessed by the staining intensity (weak=1, moderate/strong=2) and the percentage of positively stained cells by two pathologists, who were blind to the results of *HLTF* mRNA expression. Erythroid cells were excluded from calculation.

Generation and analysis of stable HL-60 cell lines

HL-60 cells (2×10^6) were nucleofected with *HLTF* short hairpin RNA (shRNA) constructs (Sigma-Aldrich) or the control pLKO.1-puro vector following the manufacturer-optimized protocol (Amaxa). A transfection efficiency of 39±2% (mean±SD from 3 independent experiments) was achieved as determined by flow cytometric analysis of green fluorescent protein expression in the transfected cells. After 48 hours of nucleofection, cells were then selected with 1 μg/ml of puromycin for 4 weeks. Resistant lines were expanded and checked for HLTF knockdown by Western blotting.

Cell proliferation was measured by the WST-1 (Roche Life Science) and CytoSelect 96-well hematopoietic colony forming cell assays (Cell Biolabs). For the latter assay, cells were cultured in a semi-solid methylcellulose medium for 10 days and colonies were lysed and detected by the CyQuant GR Dye in a fluorescence plate reader.

G-banded metaphases were analyzed using the Ikaros karyotyping system (MetaSystems). A total of 23 metaphases were analyzed for each line at passage 20. The

karyotypes were described according to the International System for Human Cytogenetic Nomenclature [ISCN].

For studying histone H2AX serine 139 phosphorylation, cells were treated with the indicated concentrations of methyl methanesulfonate (MMS) (Sigma-Aldrich) for 1 hour. H2AX phosphorylation was measured by a cell-based ELISA assay kit (R&D Systems) following the manufacturer's protocol and the phosphorylation levels in each sample were normalized to total histone H2AX levels.

Western blotting

Western blotting was performed on whole cell lysates. Antibodies were obtained from LSBio (HLTF) or Cell Signaling Technology (cyclin B1, aurora kinase B (AURKB), STAG2, RAD21, SMC2, and RUNX1). RUNX1-ETO was analyzed by the same antibody detecting RUNX1 and CBFB-MYH11 by a CBFB antibody (Cell Signaling Technology). GAPDH served as the loading control.

Magnetic activated cell sorting (MACS)

$CD34^+$ and $CD34^-/CD11b^+$ cells from normocellular BM samples were purified on MACS columns using microbead-conjugated antibodies (Miltenyi Biotec).

DNA constructs

All promoter fragments were cloned into pGL3-Basic (Promega). pCMV-RUNX1-ETO and pCMV-CBFB-MYH11 were kindly provided by Prof. S.W. Hiebert (Department of Biochemistry, Vanderbilt-Ingram Cancer Center). The human RUNX1 expression plasmid (pCMV-based) was obtained from OriGene. The mutant *HLTF* promoter construct (-424_RUNX1mut) and the RUNX1_R201Q (equivalent to R174Q of

RUNX1b) plasmid were prepared using the GeneArt Site-Directed Mutagenesis System (Life Technologies) and verified by nucleotide sequencing.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using the MAGnify Chromatin Immunoprecipitation System (Life Technologies) with 10μg of anti-RUNX1 (N-20) or anti-ETO (C-20) antibodies (Santa Cruz Biotechnology). PCR primers are listed in Online Supplementary Table S1.

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

MS-HRM analysis was performed using the primers *HLTF*-HRM-F: 5'-CGTTTGAGGTAGGGAATTAAATAAAAT-3' and *HLTF*-HRM-R: 5'-TACGAAACCAAACCAATCAAAACACAA-3' (208bp) on a LightCycler 480 system (Roche Life Science). Each reaction contained 1×HRM master, 0.25μM of each primer, 3mM $MgCl_2$ and 10ng of bisulfite-modified DNA in a 20μl reaction. Samples were amplified using the default touchdown program and tested in duplicates. A series of methylation standards (100%, 10%, 5%, 2.5% and 0%) was prepared by mixing different amounts of DNA from a normocellular BM showing no *HLTF* promoter methylation by bisulfite sequencing with the CpG-methylated HeLa genomic DNA (New England Biolabs), which was also confirmed by bisulfite sequencing to be completely methylated.

Primers for bisulfite sequencing were *HLTF*-BS-F: 5'-TGAGGTAGGGAATTAAATAAAATAT-3' and *HLTF*-BS-R: 5'-AACTAACACTAAAAAAACCCAATCAC-3' (241bp). Amplified products were TA-cloned and at least 10 clones from each sample were sequenced.

MSP primers used for detection of methylated CpG sites in the duplicated RUNX1 site were *HLTF*-MSP-F: 5'-TAAATAAAATATCGGTATCGTAGGTATC-3' and *HLTF*-MSP-R: 5'-CTCTATAACTAACACTAAAAAAACCCA-3' (234bp) (33 cycles, 62°C annealing). For detection of unmethylated CpG sites, the same reverse primer and *HLTF*-USP-F: 5'-TAAATAAAATATTGGTATTGTAGGTATT-3' were used (234bp) (33 cycles, 57°C annealing).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed using the Gel Shift Kit (Affymetrix) with 5μg of nuclear extract from Jurkat cells (Santa Cruz Biotechnology) and 50nmol of biotin-labeled probes. The probe sequence was 5'-AAACACCGGCACCGCAGGCACCGCAGTCGCACTCC-3'. The same probe with 5-methyl deoxycytosines substituting for deoxycytosines at the four CpG sites (underlined) was synthesized from Gene Link.

HLTF mutation screening

All the 25 coding exons and exon-intron boundaries of *HLTF* were analyzed by direct sequencing. Primer sequences are provided in Online Supplementary Table S1. Sequence variants were checked using the dbSNP, Ensembl and COSMIC databases to identify novel variants.

References:

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Online Supplementary Table S1. Primers used in this study.

Primer	Sequence (5' to 3')	Product size (bp)	Purpose
HLTF-TaqMan	Assay ID: Hs00172585_m1	75	*HLTF* RQ-PCR
SHPRH-TaqMan	Assay ID: Hs00542737_m1	63	*SHPRH* RQ-PCR
RUNX1-TaqMan	Assay ID: Hs00231079_m1	64	*RUNX1* RQ-PCR
RUNX3-QF RUNX3-QR RUNX3-TaqMan	TCAGCACCACAAGCCACTTC GGTCGGAGAATGGGTTCAGTT AGACCCCAATCCAAGGCACCTCG	78	*RUNX3* RQ-PCR
GAPDH-QF GAPDH-QR GAPDH-TaqMan	AGCCTCAAGATCATCAGCAATG CTTCCACGATACCAAAGTTGTCAT TCCTGCACCACCAACTGCTTAGCACC	91	*GAPDH* RQ-PCR
HLTF-ChIP-1F HLTF-ChIP-1R	GAGGCAGGGAATCAAACAAA CACAGAAGGGAGGGCAACT	181	ChIP PCR
HLTF-ChIP-2F HLTF-ChIP-2R	TGCTCAAAGGGAACAGTGAA TCCCAACTGCTCCTAATGCT	167	ChIP PCR
HLTF-E1F HLTF-E1R	GCGGCGACTTACCTTTCAGT GCCACATATGCGACCAACAG	313	*HLTF* mutation screening
HLTF-E2F HLTF-E2R	CCACTCACTGCAATTCCTGCT GATTAACCGCACACATCACCTG	463	*HLTF* mutation screening
HLTF-E3F HLTF-E3R	TGTGCAGTGTTTAGCAGTAGCATT GGCTCTAACAAGAAGCCCTAACAA	360	*HLTF* mutation screening
HLTF-E4F HLTF-E4R	TTTTTCTTTCAAGTCTGCCCAAC TTGTACCTTGGAGCCTTGAGC	398	*HLTF* mutation screening
HLTF-E5F HLTF-E5R	GGTTACAGGCATGAGCCACA GAAGACCACAAATACCCACACG	322	*HLTF* mutation screening
HLTF-E6F HLTF-E6R	TTATTGGCATGAGCCACCAC AGCCATTCCTTTTTCACATGC	315	*HLTF* mutation screening
HLTF-E7F HLTF-E7R	CAGGATCTGCATGTGAAAAAGG TCTAGTCCCAAACTGGTACCAACA	368	*HLTF* mutation screening
HLTF-E8F HLTF-E8R	TGGGAACGTTTTTCTGGTGA GAGACCATTCATCGCATTTCTG	282	*HLTF* mutation screening
HLTF-E9F HLTF-E9R	CTCCCCTTCAGTTGCTCCAG CCCAATTCAATCATCTCCTTCA	422	*HLTF* mutation screening
HLTF-E10F HLTF-E10R	AGACTGGGTCATAAAAATTTGAAGGT CGTGCCCAGCCTCTATCTCT	388	*HLTF* mutation screening
HLTF-E11F HLTF-E12R	GCTTTGGGTTGGTCCAAAAA CCATGGTTAGCTCCATGAAGAGTT	412	*HLTF* mutation screening
HLTF-E13F HLTF-E13R	GCTCTTACAATAGAGGGTGAAAATGA GGAAAATGCACCAAAAGGAAA	390	*HLTF* mutation screening
HLTF-E14F HLTF-E14R	TTCCCTGTTAAAAGTCCATCTGC TGAACTTAGATACGAACTGATGACCA	363	*HLTF* mutation screening
HLTF-E15F HLTF-E15R	TTTGGTCTAGACTGAAGGAAACTTGA TTGGAACACTCTAGAAACCTGTGG	369	*HLTF* mutation screening
HLTF-E16F HLTF-E16R	GCTTAAAGCAGTACCTAGCACATAG GGGGCAGAATTTACACCCACT	371	*HLTF* mutation screening
HLTF-E17F HLTF-E17R	CCTGGTACAGAGTGCCCATTT AAAGTGCCAACTGGTTCAAGC	317	*HLTF* mutation screening
HLTF-E18F HLTF-E18R	CAGATTGGTTAAAATCCCAAATAAGG CAGTGAATGGGAAACAAAGTAAACA	392	*HLTF* mutation screening
HLTF-E19F HLTF-E19R	CTGCTTTTCATTCTTGTTTTGGAG AGCAATCTCCATTTGACAGAACA	374	*HLTF* mutation screening
HLTF-E20F HLTF-E20R	TCCCTACTGGCAAGCGAAAC TTAAATCCAAGTGCAAAACTCCAT	374	*HLTF* mutation screening
HLTF-E21F HLTF-E21R	TGGAGTTCAGCATGTTCAGATGT ACAGGAATGAAAGCAGAATTACGA	394	*HLTF* mutation screening
HLTF-E22F HLTF-E22R	AGGGGAAATGGAATGGGTTC CTGAAAGTGAAAAACACTGTGGAACT	327	*HLTF* mutation screening
HLTF-E23F HLTF-E23R	TCCTGAATTTATCAGAAAATGACTGAC AAGTTTGCCTAAGAATTTTCACAGTT	478	*HLTF* mutation screening
HLTF-E24F HLTF-E24R	TGATTGGGAGGGTGGATTTT TTGATGAAGGAGGAAAAAGGTGA	390	*HLTF* mutation screening
HLTF-E25F HLTF-E25R	TTGCGGTTTTTGCCATTAGG AAATATGCCCCTTTTAGAAGACGTG	371	*HLTF* mutation screening

Online Supplementary Table S2. Relationship of *HLTF* mRNA expression with the 2008 revision of the World Health Organization (WHO) classification of AML.

	Entire cohort (*n*=204)	**Low *HLTF* (*n*=102)**	**High *HLTF* (*n*=102)**	***P***
AML with recurrent genetic abnormalities, n (%)				
AML with t(8;21)(q22;q22)	15 (7.7%)	10 (10.8%)	5 (5%)	0.179
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	12 (6.2%)	7 (7.5%)	5 (5%)	0.556
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	2 (1%)	2 (2.2%)	0 (0%)	0.229
AML with t(11;19)(q23;p13.3)	1 (0.5%)	1 (1.1%)	0 (0%)	0.479
AML with t(6;11)(q27;q23)	1 (0.5%)	1 (1.1%)	0 (0%)	0.479
AML with mutated *NPM1*	48 (24.7%)	27 (29%)	21 (20.8%)	0.244
AML with mutated *CEBPA* (double)	18 (9.3%)	4 (4.3%)	14 (13.9%)	0.026*
AML with mutated *CEBPA* (single)	5 (2.6%)	2 (2.2%)	3 (3%)	>0.999
AML with myelodysplasia-related changes, n (%)	32 (16.5%)	16 (17.2%)	16 (15.8%)	0.848
AML, not otherwise specified, n (%)	60 (30.9%)	23 (24.7%)	37 (36.6%)	0.088
Missing data	10	9	1	

* Statistically significant.

Online Supplementary Table S3. Comparison of immunophenotypes of leukemic blasts between AML patients with low and high *HLTF* mRNA expression.

Antigens	Whole cohort	Low *HLTF* expression	High *HLTF* expression	*P*
HLA-DR	157/179 (87.7%)	79/89 (88.8%)	78/90 (86.7%)	0.821
CD13	166/179 (92.7%)	82/89 (92.1%)	84/90 (93.3%)	0.782
CD33	177/179 (98.9%)	88/89 (98.9%)	89/90 (98.9%)	>0.999
CD34	126/179 (70.4%)	59/89 (66.3%)	67/90 (74.4%)	0.255
CD14	46/175 (26.3%)	32/88 (36.4%)	14/87 (16.1%)	0.003*
CD15	87/156 (55.8%)	52/81 (64.2%)	35/75 (46.7%)	0.036*
MPO cytoplasmic	147/178 (82.6%)	75/89 (84.3%)	72/89 (80.9%)	0.693
CD79a cytoplasmic	23/178 (12.9%)	11/89 (12.4%)	12/89 (13.5%)	>0.999
CD19	14/179 (7.8%)	10/89 (11.2%)	4/90 (4.4%)	0.103
CD20	8/179 (4.5%)	7/89 (7.9%)	1/90 (1.1%)	0.034*
CD2	6/178 (3.4%)	2/88 (2.3%)	4/90 (4.4%)	0.682
CD3	5/179 (2.8%)	2/89 (2.2%)	3/90 (3.3%)	>0.999
CD5	13/179 (7.3%)	9/89 (10.1%)	4/90 (4.4%)	0.162
CD7	76/179 (42.5%)	34/89 (38.2%)	42/90 (46.7%)	0.291

* Statistically significant

Online Supplementary Table S4. Results of multivariate analysis for OS and EFS.

Patients with intermediate-risk cytogenetics (*n*=108)	**OS**			**EFS**		
Variable‡	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**
Age	0.037*	1.889	1.040-3.432	0.004*	2.251	1.293-3.920
WBC count	0.02*	1.005	1.001-1.009	0.027*	1.004	1.000-1.008
HLTF expression	0.093	1.618	0.922-2.839	0.032*	1.755	1.050-2.931
NPM1/*FLT3*-ITD	0.022*	0.423	0.202-0.884	0.001*	0.304	0.153-0.607
CEBPA	0.001*	0.201	0.076-0.529	0.001*	0.271	0.124-0.591

Patients with a normal karyotype (*n*=82)	**OS**			**EFS**		
Variable‡	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**
Age	0.081	1.868	0.926-3.771	0.055	1.903	0.986-3.673
WBC count	0.046*	1.005	1.000-1.009	0.024*	1.005	1.001-1.008
HLTF expression	0.384	1.363	0.679-2.737	0.287	1.402	0.753-2.613
NPM1/*FLT3*-ITD	0.056	0.469	0.216-1.019	0.004*	0.345	0.168-0.707
CEBPA	0.003*	0.152	0.044-0.531	0.005*	0.267	0.106-0.675

WBC, white blood cell; HR, hazard ratio; CI, confidence interval.

‡Age: >60 versus ≤60 years old; WBC: continuous variable; *HLTF* expression: low versus high; *NPM1*/*FLT3*-ITD: *NPM1*^{mut}/*FLT3*-ITD^{neg} versus other subtypes; *CEBPA*: with versus without mutation.

Hazard ratio >1 (or <1) indicates a higher risk (or lower risk) of an event for the first category listed for dichotomous variables.

*Statistically significant.

Online Supplementary Table S5. Karyotypes of the HL-60_control and HL-60_shRNA1-3 lines by analysis of G-banded metaphases.

HL-60 line	Karyotype	Subclone/ New clone	Changes vs HL-60_control
HL-60_control[a]	46,XX,dic(5;17)(q11;p11),del(9)(p21),der(10;13)(q10;q10),+13, add(14)(q24),add(16)(q?),der(16)t(5;16)(q31;q24),+18[12]/ 46,idem,1~8dmin[5]	/	/
HL-60_shRNA1	46,XX,dic(5;17)(q11;p11),del(9)(p21),der(10;13)(q10;q10),+13, add(14)(q24),add(16)(q?),der(16)t(5;16)(q31;q24),+18[15]/ 46,idem,6~11dmin[2]/ **45,X,-X,dic(5;17)(q11;p11),del(9)(p21),add(10)(p11.2), add(14)(q24),add(16)(q?),der(16)t(5;16)(q31;q24),+18[3]**	Subclone	• Gain of add(10)(p11.2) • Loss of der(10;13)(q10;q10) • Loss of trisomy 13 • Loss of X chromosome
HL-60_shRNA2	45,X,-X,dic(5;17)(q11;p11),ins(7;?)(q11.2;?),del(9)(p21), add(10)(p11.2),add(14)(q24),add(16)(q?),der(16)t(5;16)(q31;q24), +18[18]/67~90,<3n>,XX,-X,dic(5;17)(q11;p11),+7, ins(7;?)(q11.2;?)x2,del(9)(p21),add(10)(p11.2),+13,+14, add(14)(q24)x2,+15,add(16)(q?),der(16)t(5;16)(q31;q24)x2,-17, +18[cp2]	New	• Gain of ins(7;?)(q11.2;?) • Gain of add(10)(p11.2) • Loss of der(10;13)(q10;q10) • Loss of trisomy 13 • Loss of X chromosome • Hyperdiploid composite karyotype
HL-60_shRNA3	46,XX,dic(5;17)(q11;p11),del(9)(p21),der(10;13)(q10;q10),+13, add(14)(q24),add(16)(q?),der(16)t(5;16)(q31;q24),+18[8]/ 46,idem,2~34dmin[5]/ **92<4n>,XXXX,idemx2[2]**	Subclone	• Tetraploid

Cytogenetic subclones emerged in HLTF-knocked down lines are underlined. Commonly acquired chromosome abnormalities are shaded.

[a] Analysis of another control line revealed nearly identical karyotype as HL-60_control: 46,XX,dic(5;17)(q11;p11),del(9)(p21),der(10;13)(q10:q10),+13,add(14)(q24),add(16)(q?),der(16)t(5;16)(q31;q24),+18[13]/46,idem,4~50dmin[4].

Online Supplementary Table S6. Characteristics of the adult AML patients used for *HLTF* promoter methylation and mutation studies.

Parameters	**Adult patients with de novo AML (*n*=133)**
Mean age, years (range)	51.4 (18-86)
Sex, n (% males)	66 (49.6%)
Mean hemoglobin, g/dL (range)	8.4 (4.4-13.6)
Mean platelets, 10^9/L (range)	66.3 (2-328)
Mean WBC, 10^9/L (range)	44.7 (0.3-517)
Mean LDH, U/L (range)	691 (101-5860)
Mean BM blast, % (range)	62 (12-98)
FAB subtypes, n (%)	
M0	4 (3.1%)
M1	36 (27.7%)
M2	38 (29.2%)
M4	21 (16.2%)
M5	27 (20.8%)
M6	4 (3.1%)
M7	0 (0%)
Missing data	3
Cytogenetics, n (%)	
Favorable	16 (13.4%)
Intermediate – normal karyotype	67 (56.3%)
Intermediate – Abnormal karyotype	25 (21%)
Adverse	11 (9.2%)
Missing data	14
Molecular markers, n (%)	
FLT3-ITD	26 (19.5%)
FLT3-D835/I836	6 (4.5%)
KIT mutation	4 (3%)
CEBPA double mutation	16 (12%)
CEBPA single mutation	7 (5.3%)
NPM1 mutation	33 (24.8%)
DNMT3A mutation	26 (19.5%)
WT1 mutation	6 (4.5%)
IDH1 mutation	10 (7.5%)
IDH2 mutation	18 (13.5%)

WBC, white blood cell; LDH, lactate dehydrogenase; BM, bone marrow; FAB, French-American-British; ITD, internal tandem duplications.

Online Supplementary Table S7. Characteristics of the 100 adult AML patients from TCGA for validation of *HLTF* promoter methylation.

Parameters	
Mean age, years (range)	56.1 (18-81)
Sex, n (% males)	56 (56%)
Mean hemoglobin, g/dL (range)	9.6 (6-13)
Mean platelets, 10^9/L (range)	66.5 (8-232)
Mean WBC, 10^9/L (range)	38.1 (1-297)
FAB subtypes, n (%)	
M0	11 (11%)
M1	23 (23%)
M2	24 (24%)
M4	22 (22%)
M5	15 (15%)
M6	3 (3%)
M7	2 (2%)
Cytogenetics-risk group*, n (%)	
Favorable	16 (16%)
Intermediate	53 (55%)
Poor	28 (29%)
Missing data	3

WBC, white blood cell; FAB, French-American-British.

* Acute Myeloid Leukemia Cancer and Leukemia Group B Cytogenetics Risk Category.

Online Supplementary Table S8. *HLTF* promoter methylation in the TCGA validation cohort.

		Mean beta-value ≤0.2*	**Mean beta-value >0.2**
CpG site	**Location**	**No. of patients (%)**	**No. of patients (%)**
cg04836786	5'-untranslated region	100 (100%)	0 (0%)
cg21926402	5'-untranslated region		
cg02398045	TSS200	100 (100%)	0 (0%)
cg15438497	TSS200		
cg24041269	TSS200		
cg05555455	Duplicated RUNX1 site	93 (93%)	7 (7%)
cg03678609	Duplicated RUNX1 site		

TSS200, within 200 bp of transcription start site.

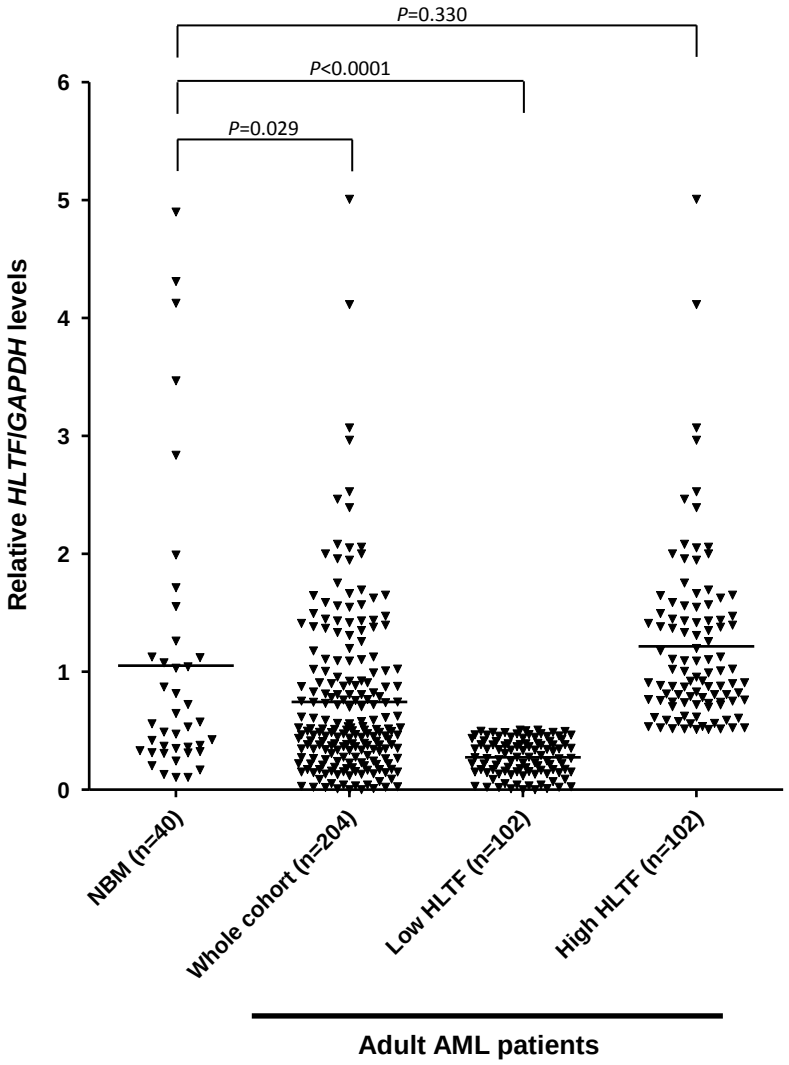
* Mean beta-values of CpG sites in each gene location were calculated. Values ≤0.2 and >0.2 were considered to be unmethylated and methylated, respectively.

Online Supplementary Table S9. *HLTF* sequence variations found in adult AML patients.

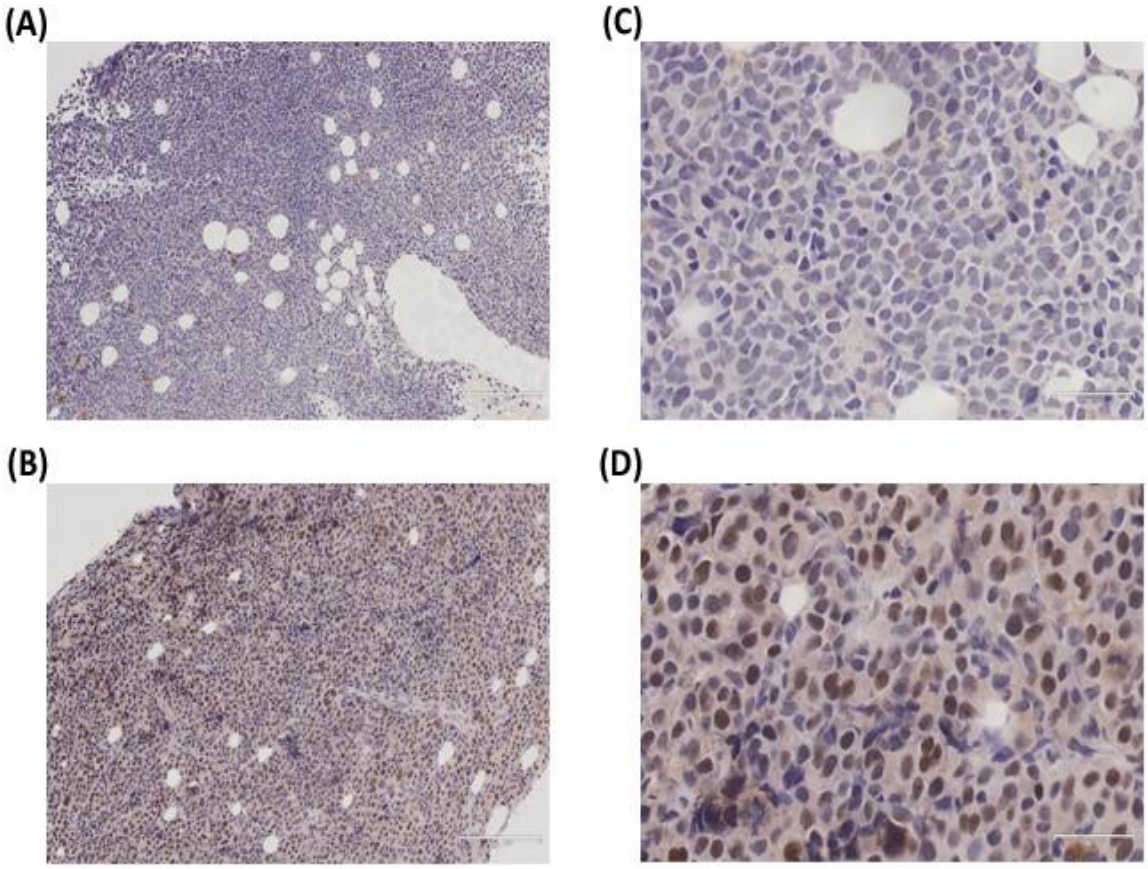
Nucleotide change	Exon	Predicted amino acid change	Mutation type	Age/Sex	FAB	Cytogenetics	Detected in germline or remission sample
c.2T>G*	1	p.M1_W3del	Deletion	60/F	M1	46,XX[20]	Yes
c.844G>A*	7	p.D282N	Missense	44/F	M4	46,XX,t(3;3)(q21;q26.2) [10]/45,idem,-7[10]	Yes
c.1013C>G	9	p.S338C	Missense	69/F	M2	NA	Yes
c.1393G>T*	14	p.G465W	Missense	64/F	M4	46,XX[20]	Yes
c.1746G>A*	16	p.W582*	Nonsense	61/M	M1	46,XY[27]	Yes
c.1921_1922insA*	18	p.T641N*fs*3	Frameshift	44/F	M1	46,XX,del(9)(q22)[20]	No
c.2586A>C*	22	p.T862T	Silent	57/F	M1	46,XX[20]	Yes
c.2980G>A	25	p.E994K	Missense	68/F	M5	47,XX,+8[16]/46,XX[6]	Yes

FAB, French-American-British; NA, not available.

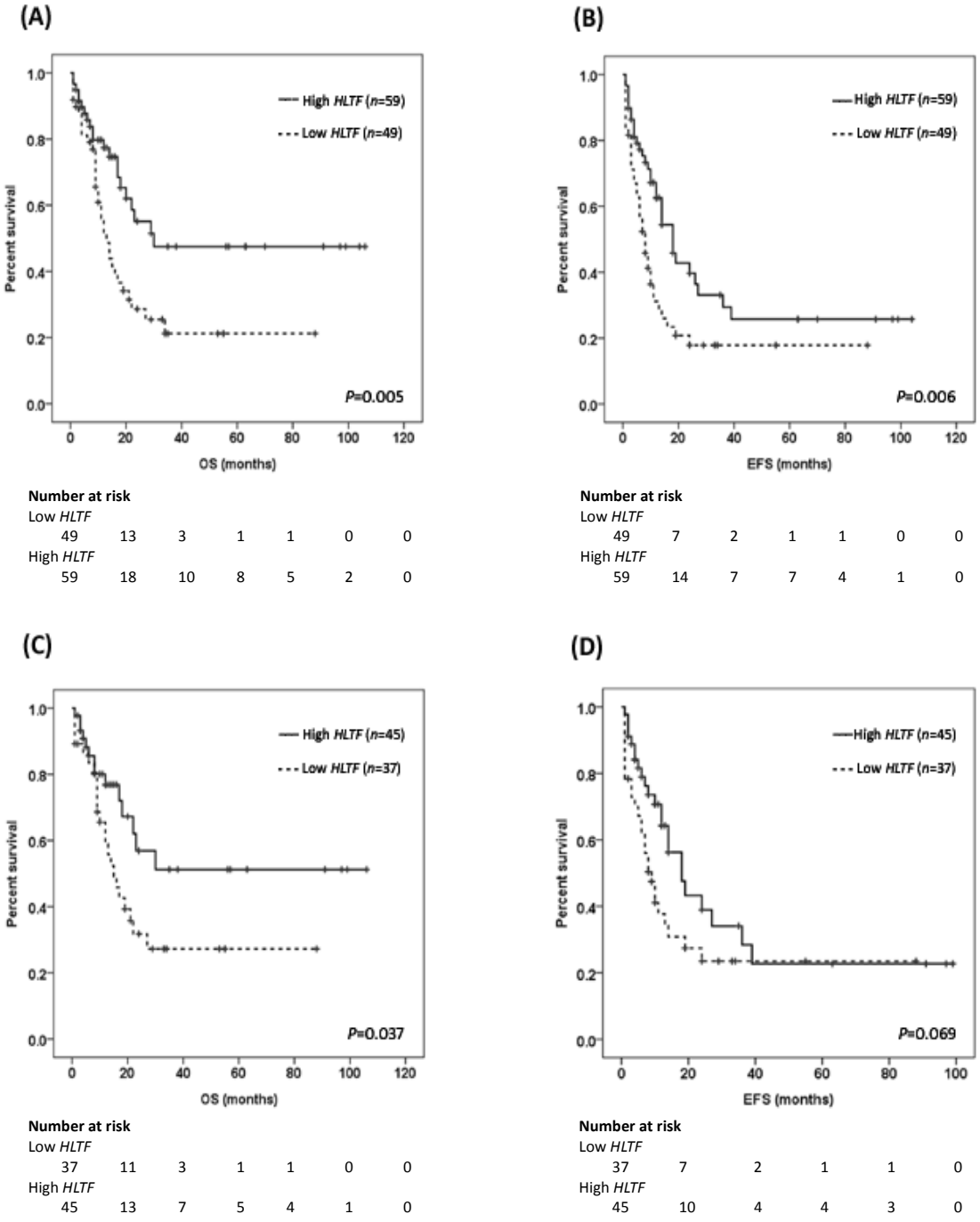
* indicates novel sequence variants.



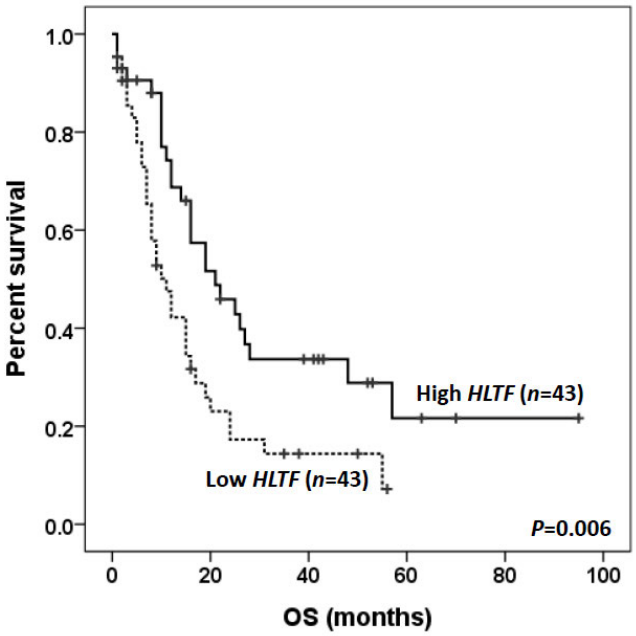
Online Supplementary Figure S1. *HLTF* mRNA levels in BM mononuclear cells from adult AML patients and normal controls. *HLTF* expression was determined by RQ-PCR and normalized to *GAPDH*. Expression levels were relative to the U937 myeloid cell line. Each triangle represents one subject, and the number of subjects in each group is shown. Horizontal lines indicate the mean *HLTF*/*GAPDH* levels. The median *HLTF* level among the patients was used as cutoff to dichotomize the cohort into low and high *HLTF* expression groups. NBM, normocellular bone marrow.



Online Supplementary Figure S2. HLTF protein expression in BM biopsies of AML patients. Representative immunohistochemical staining of HLTF protein expression in BM biopsies from two patients, with one having low *HLTF* mRNA expression (A and C) and the other having high *HLTF* mRNA expression (B and D). The slides were scanned using the Leica SCN400 Slide Scanner at magnification 10× and 40×, respectively. The images were captured using the Leica SlidePath Gateway software.

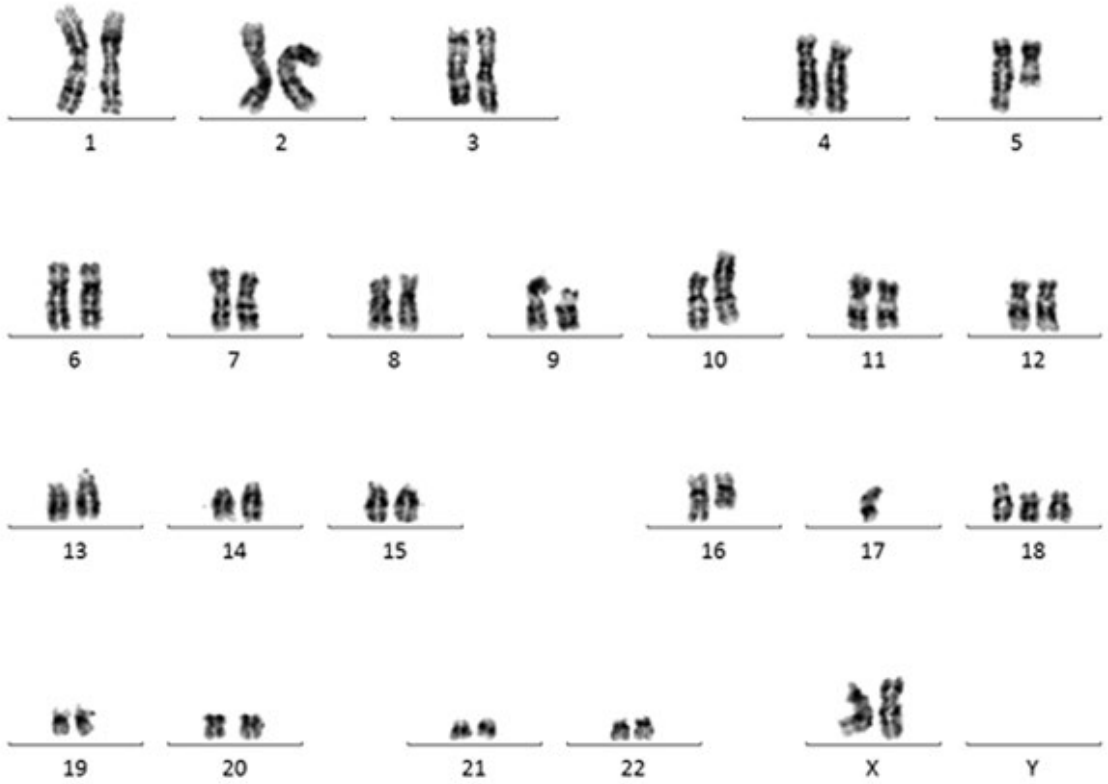


Online Supplementary Figure S3. Kaplan-Meier analysis of OS and EFS in our cohort. Kaplan-Meier curves for OS and EFS based on *HLTF* expression in adult AML patients with intermediate-risk cytogenetics (A and B) or a normal karyotype (C and D).

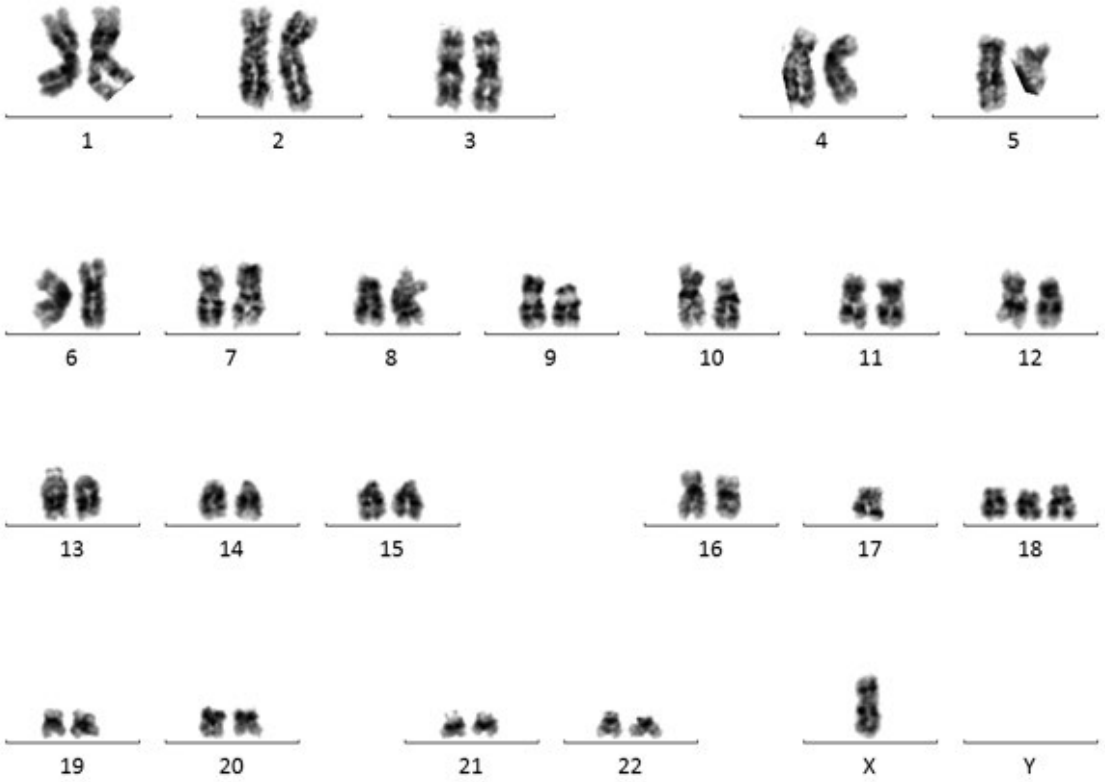


Number at risk					
Low *HLTF*					
43	8	3	0	0	0
High *HLTF*					
43	18	10	3	1	0

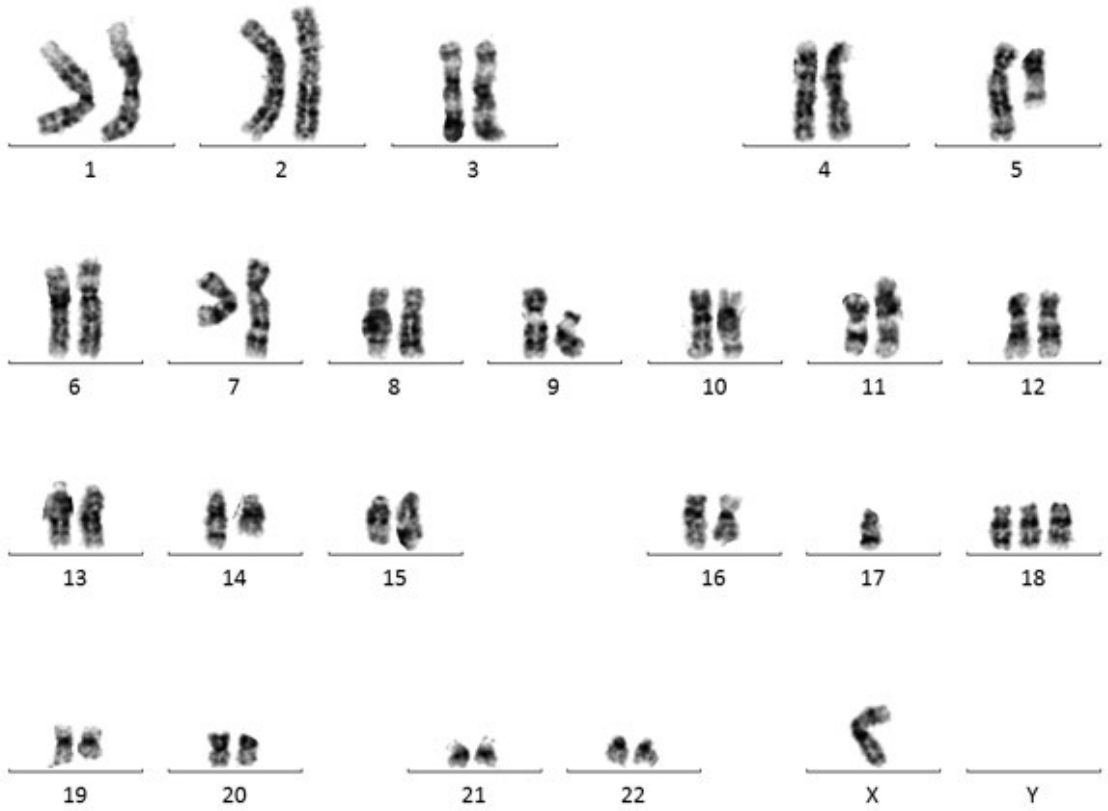
Online Supplementary Figure S4. Kaplan-Meier analysis of OS in the TCGA validation cohort. *HLTF* mRNA expression and clinical information from 86 cytogenetically-normal AML patients were obtained from TCGA. Patients were dichotomized into low and high *HLTF* expression according to the median mRNA level for the analysis.



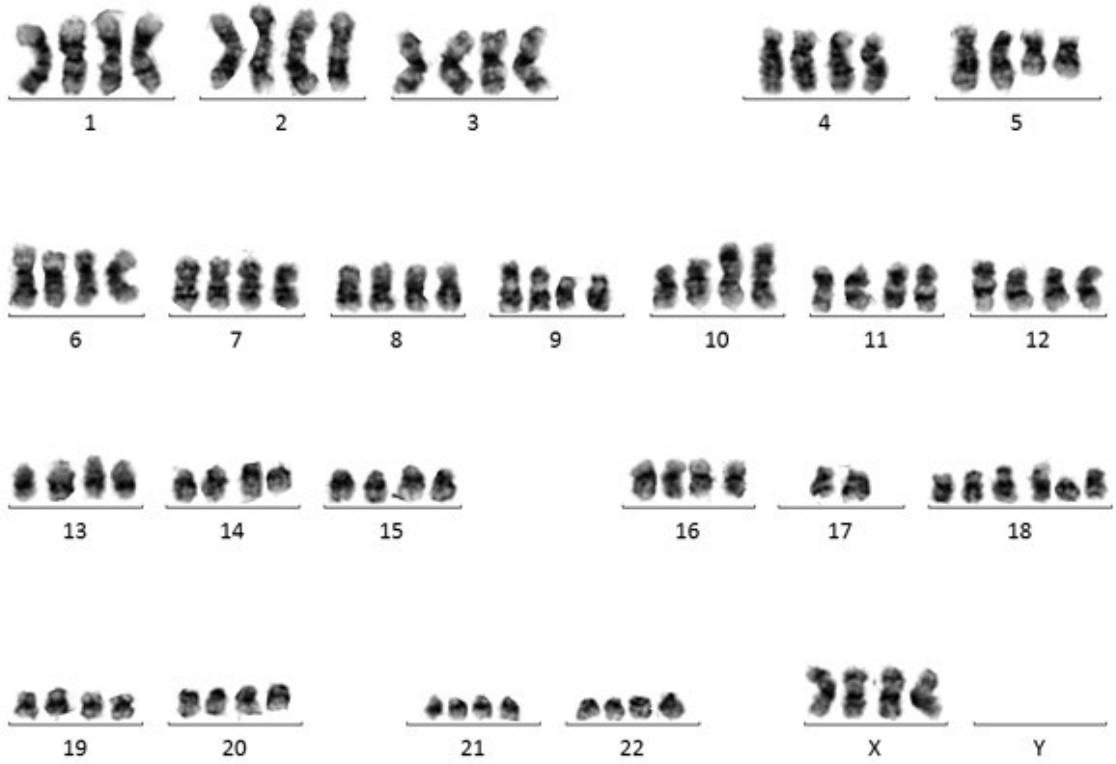
Online Supplementary Figure S5. G-banded karyotype of a representative metaphase of the HL-60_control line. The karyotype shown is 46,XX,dic(5;17)(q11;p11),del(9)(p21),der(10;13)(q10;q10),+13,add(14)(q24), add(16)(q?),der(16)t(5;16)(q31;q24),+18.



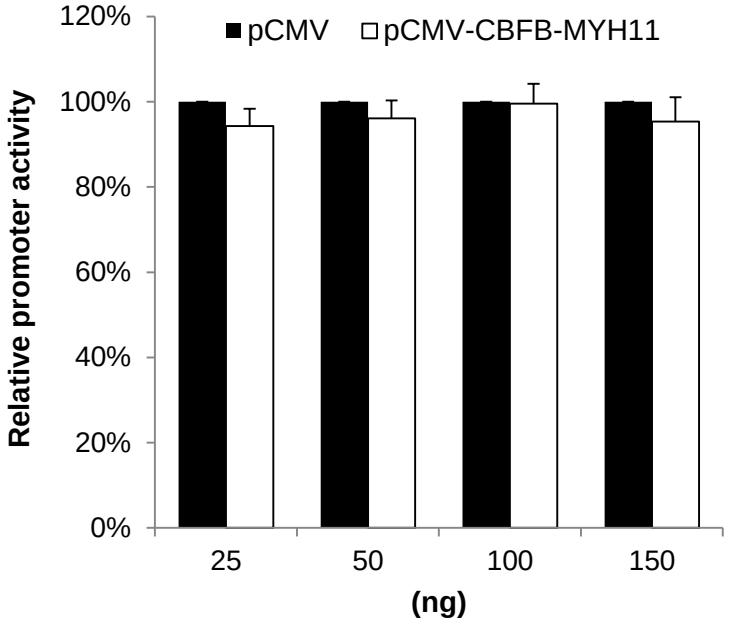
Online Supplementary Figure S6. G-banded karyotype of a representative metaphase of the new subclone emerged in the HL-60_shRNA1 line. The karyotype shown is 45,X,-X,dic(5;17)(q11;p11),del(9)(p21),add(10)(p11.2), add(14)(q24),add(16)(q?),der(16)t(5;16)(q31;q24),+18.



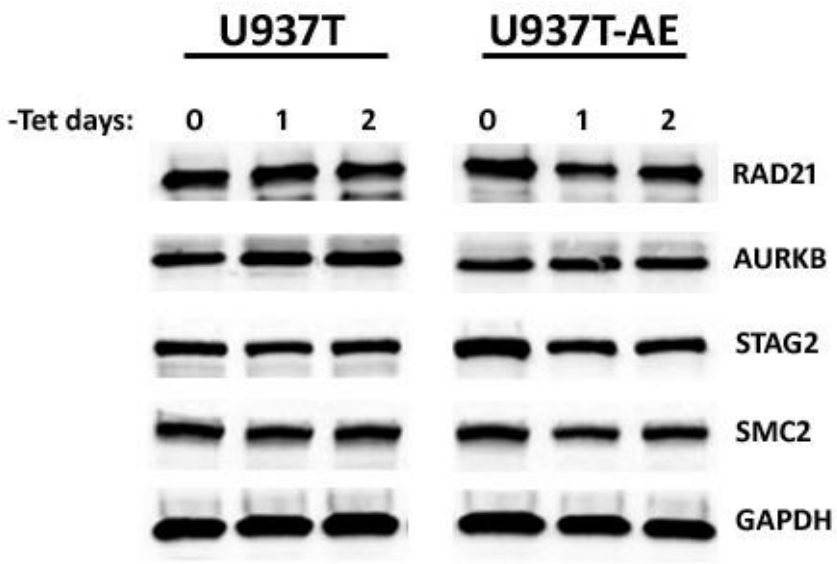
Online Supplementary Figure S7. G-banded karyotype of a representative metaphase of the HL-60_shRNA2 line. The karyotype shown is 45,X,-X,dic(5;17)(q11;p11),ins(7;?)(q11.2;?),del(9)(p21),add(10)(p11.2),add(14)(q24),add(16)(q?),der(16)t(5;16)(q31;q24),+18.



Online Supplementary Figure S8. G-banded karyotype of a representative metaphase of the new tetraploid subclone emerged in the HL-60_shRNA3 line. The karyotype shown is 92<4n>,XXXX,dic(5;17)(q11;p11)x2,del(9)(p21)x2, der(10;13)(q10;q10)x2,+13,+13,add(14)(q24)x2,add(16)(q?)x2, der(16)t(5;16)(q31;q24)x2,+18,+18.

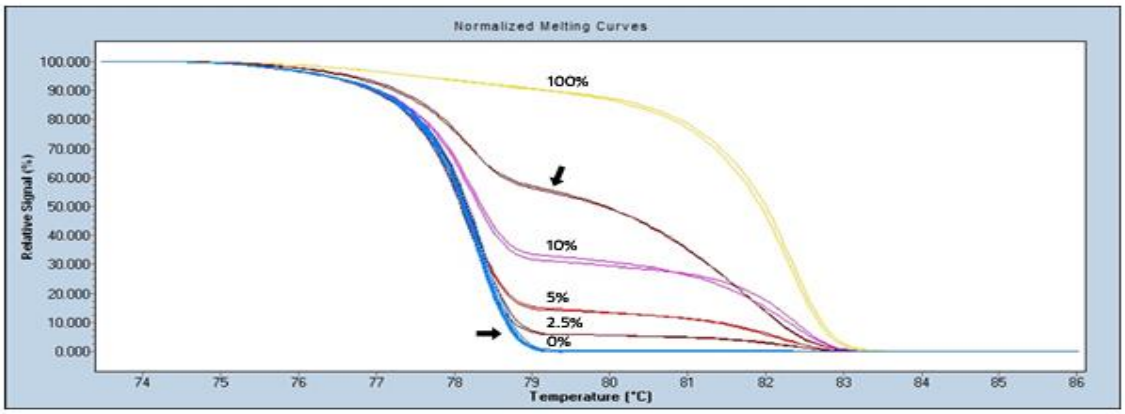


Online Supplementary Figure S9. CBFB-MYH11 did not affect the RUNX1-mediated activation of the *HLTF* promoter. Fifty nanograms of the *HLTF* (-769/+43) promoter-luciferase construct and 150ng of the RUNX1 expression plasmid were co-transfected with an increasing amount of the CBFB-MYH11 expression plasmid (pCMV-CBFB-MYH11) into K562 cells. Co-transfection with the same amount of empty pCMV vector was done in parallel. Transfection efficiency was normalized according to the co-transfected pRL-CMV *Renilla* luciferase activity and results are expressed as mean±SE from triplicate experiments. Results are presented as relative promoter activity by comparing the normalized firefly luciferase activity of the construct co-transfected with pCMV-CBFB-MYH11 with that co-transfected with the empty vector control.

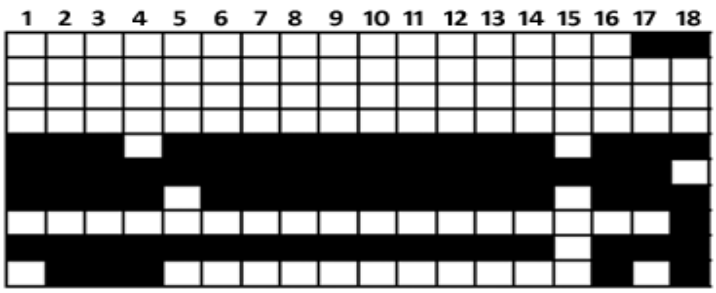


Online Supplementary Figure S10. RAD21, AURKB, STAG2, and SMC2 expression in U937T and U937T-AE cells in response to tetracycline withdrawal (-Tet). The expression of the proteins was analyzed by Western blotting. GAPDH served as a loading control. Representative blots from repeated experiments are shown.

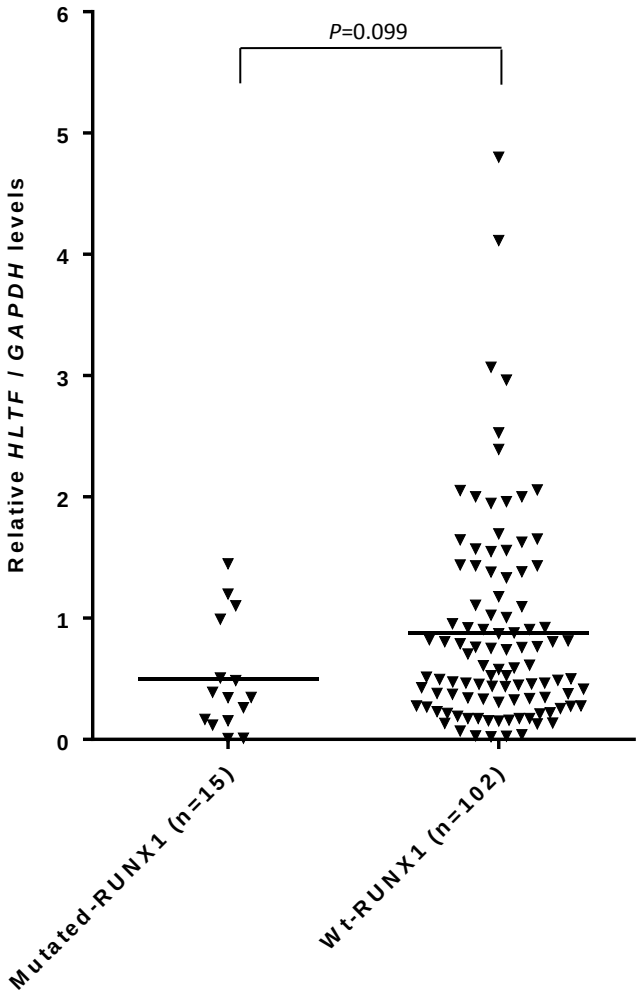
(A)



(B)



Online Supplementary Figure S11. DNA methylation analysis of the *HLTF* promoter in AML patient samples. (A) Representative normalized melting curves of MS-HRM analysis of *HLTF* promoter in BM samples from AML patients. The MS-HRM curves derived from unknown samples (indicated by arrows) are plotted against those derived from standards with known concentrations of methylated and unmethylated templates. Six patient samples are shown, including one case with aberrant DNA methylation (brown) and five cases without methylation (blue). (B) Bisulfite sequencing of the patient sample showing aberrant MS-HRM melting curve. Each row of squares represents one PCR clone. Open and filled squares indicate unmethylated and methylated CpG dinucleotides, respectively. Each CpG dinucleotide is numbered (no. 1-18). The methylation density of the *HLTF* promoter in this sample was about 41%.



Online Supplementary Figure S12. *HLTF* mRNA levels in adult AML patients with respect to *RUNX1* mutation status. *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 aberrant *HLTF* promoter methylation (*n*=7) or t(8;21) (*n*=9) were excluded for the analysis because of their confounding effects on *HLTF* repression. Horizontal lines indicate the mean *HLTF*/*GAPDH* levels. Wt, wild-type.