Epigenetically induced ectopic expression of UNCX impairs the proliferation and differentiation of myeloid cells

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ABSTRACT

We here describe a leukemogenic role of the homeobox gene *UNCX*, activated by epigenetic modifications in acute myeloid leukemia (AML). We found the ectopic activation of *UNCX* in a leukemia patient harboring a t(7;10)(p22;p14) translocation, in 22 of 61 of additional cases [a total of 23 positive patients out of 62 (37.1%)], and in 6 of 75 (8%) of AML cell lines. *UNCX* is embedded within a low-methylation region (canyon) and encodes for a transcription factor involved in somitogenesis and neurogenesis, with specific expression in the eye, brain, and kidney. *UNCX* expression turned out to be associated, and significantly correlated, with DNA methylation increase at its canyon borders based on data in our patients and in archived data of patients from The Cancer Genome Atlas. *UNCX*-positive and -negative patients displayed significant differences in their gene expression profiles. An enrichment of genes involved in cell proliferation and differentiation, such as *MAP2K1* and *CCNA1*, was revealed. Similar results were obtained in *UNCX*-transduced CD34$^+$ cells, associated with low proliferation and differentiation arrest. Accordingly, we showed that *UNCX* expression characterizes leukemia cells at their early stage of differentiation, mainly M2 and M3 subtypes carrying wild-type *NPM1*. We also observed that *UNCX* expression significantly associates with an increased frequency of acute promyelocytic leukemia with *PML-RARA* and AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* classes, according to the World Health Organization disease classification. In summary, our findings suggest a novel leukemogenic role of *UNCX*, associated with epigenetic modifications and with impaired cell proliferation and differentiation in AML.

Introduction

Homeobox (HB) genes encode transcription factors involved in key cellular processes such as body patterning, embryonic organogenesis, and cell-fate decisions.[1,2] A growing body of literature has highlighted the crucial role of HB genes in normal hematopoiesis and leukemogenesis.[3-6] Among HB genes, clustered *HOX* genes can promote the proliferation and inhibit the differentiation of hematopoietic progenitor cells and cause acute myeloid leukemia (AML)[7] and acute lymphoid leukemia.[8] Furthermore, several non-clustered HB genes, such as those belonging to the NKL subclass[2] or to the Parahox (CDX)[9] HB gene family, are critically involved in normal hematopoiesis and in leukemogenesis through their deregulation or ectopic expression. Notably, recent studies have highlighted a correlation between HB gene overexpression and mutations in epigenetic regulators.[8,10] Alterations of DNA methylation are now widely considered a hallmark of cancer,[11] although the precise leukemogenic mechanisms involving HB genes have still not been completely elucidated. Recently, Jeong *et al*. identified extended genomic regions exhibiting a low methylation level (≤10%), named "canyons", spanning conserved domains that frequently harbor transcription factor genes (mainly HB genes) in murine hematopoietic stem cells (HSCs).[10] Since several deregulated HB genes in AML fall inside canyons, this mechanism was proposed as a novel model for gene expression alteration in leukemogenesis.[10]

The starting point of this study was the identification of an M5 AML patient harboring a t(7;10)(p22;p14) translocation as the sole cytogenetic abnormality. This rearrangement was associated with the ectopic expression of the HB gene *UNCX* (NM_001080461, 7p22.3), likely as a result of a position effect.

UNCX has tissue-specific expression in the eye, brain, and kidney, and it encodes a transcription factor involved in somitogenesis[12,13] and neurogenesis.[14] The murine *Uncx* gene was shown to map within a large canyon (23 kb) entirely covered by the repressive H3K27me3 histone mark in HSCs.[10]

Notably, *UNCX* expression has never been associated with cancer. We thus investigated the ectopic expression of *UNCX* in an independent and extensive AML cohort and performed genomic and functional studies to investigate its contribution to leukemogenesis.

Methods

Patients, cell lines, and normal tissues

We studied 62 AML patients (Table 1), including Case 1 with the t(7;10)(p22;p14) translocation, 75 AML and 14 additional cancer cell lines, and 6 normal tissues (*Online Supplementary Table S1A* and *B*). The use of samples was approved by the Policlinico S. Orsola-Malpighi Ethics Committee (ref. n. 253/2013/O/Tess of 29/10/2013).

Assessment of *UNCX* expression levels in AML

UNCX expression was evaluated by RT-qPCR[15,16] using a TaqMan UNCX Gene expression assay (Applied Biosystems, Milan, Italy). The TBP Endogenous Control (Applied Biosystems) was used as reference and Case 1 at onset (1-Dx) as calibrator. We classified patients on a median value of *UNCX* expression level ($2^{-\Delta\Delta Ct}$=0.01300) as UNCX+ and UNCX–.

Methylation analysis of the *UNCX* canyon

DNA methylation ratios (MRs) of the *UNCX* canyon were determined through gene-specific amplification using *in vitro* transcription coupled with mass spectrometry (MassARRAY platform, Sequenom, San Diego, CA, USA).[17] Statistical significance was obtained by comparing MRs of *UNCX*+ and *UNCX*– cases by using Student's *t*-test; the Satterthwaite correction was applied when unequal variance was present. To clearly visualize the methylation differences, the MR values were submitted to angular transformation.

Methylation and correlation analysis of the *UNCX* canyon in AML samples from The Cancer Genome Atlas (TCGA)

We selected a total of 111 AML samples from the GDC Data Portal (*https://gdc.cancer.gov*), with both methylation and expression profile data. These subjects were divided according to the median value of *UNCX* expression (FPKM=0.0259) in *UNCX*-TCGA-positive (n=55) and *UNCX*-TCGA-negative (n=56). We compared the DNA methylation profile of *UNCX* as well as the whole genome (considering a minimum difference of 2-folds between groups) by the Mann-Whitney test. Spearman correlation was calculated between methylation and expression values within both sample sets. Correlation values were deemed significant at $P<0.05$.

DNMT3A mutational analysis

A full description of the analytical methods used is provided in the *Online Supplementary Methods*.

Gene-expression profiling and pathway analyses

Exon array was performed in two subgroups of our patient cohort [*UNCX*+ (ns. 1-Dx, 9, and 16) and *UNCX*– (ns. 13, 41, and 49)]. Similarly, 173 AML TCGA samples were divided according to the median value of *UNCX* expression in *UNCX*-TCGA-positive (n=47) and *UNCX*-TCGA-negative (n=126) cases. Pathway analysis was conducted on differentially expressed genes through QIAGEN's Ingenuity Pathway Analysis software (IPA, QIAGEN, Redwood City, CA, USA).

Retrovirus-mediated *UNCX* expression in CB CD34$^+$ cells

Ectopic *UNCX* expression was achieved by retrovirus-mediated transduction of human cord blood (CB) CD34$^+$ cells.[18] Proliferation and differentiation rates were determined by colony forming cell (CFC) assays at 14 days after seeding. Flow cytometry analysis provided quantitative information regarding the maturation stage of infected cells.[18] Cell morphology was assessed by May-Grunwald-Giemsa staining.

Correlation between *UNCX* expression and clinical/molecular features in TCGA patients

A total of 161 out of 173 TCGA AML samples were analyzed for potential associations between *UNCX* and clinical/molecular features. *UNCX*-TCGA-positive and -negative patients (*Online Supplementary Table S2*) were compared using the χ^2 test or Fisher's exact test. Continuous variables and medians of distributions were analyzed by Mann-Whitney U test or Kruskal-Wallis test for multiple comparisons.

Results

A novel t(7;10)(p22;p14) translocation in AML is associated with ectopic expression of the HB gene *UNCX*

FISH results obtained in Case 1-Dx (Table 1) identified a breakpoint region on 7p22.3 within the fosmid clone

Table 1. Clinical, cytogenetic features, *UNCX* expression levels and *DNMT3A, FLT3*, and *NPM1* mutational status of the 62 acute myeloid leukemia patients included in the study.

	Case number	Age/Sex	FAB subtype	Karyotype	UNCX expression ($2^{-\Delta\Delta ct}$)	*DNMT3A* mut V328I, R882C	*FLT3* mut TKD	*NPM1* mut WT
UNCX+	1-Dx	46/M	M5	46,XY,t(7;10)(p22;p13)[18]/46,XY[2]	1.00000			
	3	42/M	M5	47,XY,+8[5]/46,XY,t(2;10)(q33;p13)[4]/46,XY[21]	0.13182	/	WT	WT
	4	20/F	M5	46,XX[20]	0.04966	/	ITD	WT
	7	44/F	M5	NA	0.01439	/	WT	WT
	9	34/M	M1	46,XY[13]*	4.04648	WT	WT	WT
	11	43/F	M5	46,XX [20]	0.01342	/	WT	WT
	14	54/M	M0/M1	46,XY[20]	0.08304	/	ITD	NA
	15	73/M	M2	47,XY,+8[3]/46,XY[15]	0.74484	/	WT	NA
	16	54/F	M3	46,XX,t(15,17)(q22,q12)[19]/46,XX[1]	4.95883	WT	WT	WT
	30	81/F	NI	46,XX[12]	0.10390	S708I	WT	MUT
	31	NA	NI	NA	0.04932	/	NA	NA
	33	NA	NI	NA	0.03751	R882H	ITD	WT
	34	83/M	NI	47,XY,+mar[20]	0.19981	L422L	WT	WT
	35	43/M	M4	46,XY,inv(16)[2]	0.02577	/	TKD	WT
	38	52/F	NI	46,XX,inv(16)[20]	0.01774	WT	WT	WT
	40	74/F	M3	46,XX,t(15;17)[9]/47,XX,+mar[13]	0.19843	/	WT	WT
	44	67/F	M4	47,XX,+8[3]/48,XX,+8,+21[13]	0.04565	/	WT	MUT
	46	49/M	M1	Complex karyotype	0.01305	/	NA	WT
	48	64/M	sAML to MDS	46,XY,+13[6]	0.02259	/	WT	WT
	51	57/M	M1	NA	0.02492	/	NA	WT
	52	50/M	M2	46,XY,t(3;?)(q21;?),add(7)(q34),der(7), del(14)(q23q32),add(16)(q22),-21,+1mar[4]/45, XY, t(3;?)(q21;?), add(7)(q34),-7, del(14)(q23q32), add(16)(q22),-21,+1mar[16]	0.06999	/	WT	WT
	56	77/F	NI	46,XX[8]	0.10236	/	WT	WT
	61	53/F	M2	46,XX[20]	3.72352	/	WT	WT
	1-Rem	46/M	M5	46,XY[30]	ND	V328I, R882C	WT	WT
UNCX−	2	24/M	M5	46,XY[10]	0.00037	/	WT	WT
	5	40/M	M5	46,XY[20]	ND	/	NA	NA
	6	50/F	M5	46,XX,inv(9)(p11p13)[20]	ND	WT	WT	MUT
	8	71/F	M5	46,XX,inv(9)(p11p13)[20]	ND	/	TKD	NA
	10	46/M	M5	46,XY[20]	ND	/	TKD	WT
	12	61/M	M5	47,XY,+8[3]/46,XY[5]	ND	/	TKD	MUT
	13	NA/M	M5	46,XX [20]	ND	WT	NA	NA
	17	33/F	M4	46,XX,t(11;21)(q13;q22)[4]/46, XX,add(21)(q22)[7]/46,XX[2]	0.00099	/	ITD	WT
	18	76/M	sAML to MDS	46,XY,del(5)(q13q31), del(11)(q21q23), add(15)(p13),-17,-18,+2mar[3]/46,XY, idem,del(4)(q27q33)[5]/46,XY[10]	0.00067	L422L	WT	WT
	19	77/M	sAML to MDS	46,XY[20]	0.00011	WT	WT	WT
	20	73/F	M0	46,XX[20]	ND	WT	WT	WT
	21	69/F	sAML to MDS	45,XX,t(1;3;13)(p34,q26,q14),-7[19]/46,XX[1]	ND	WT	WT	WT
	22	77/F	sAML to MDS	46,XX[20]	0.00065	L422L, R882C	WT	WT
	23	46/F	NI	46,XX,del(7)(q22q35)[3]	0.00022	/	WT	NA
	24	NA	NI	NA	0.00038	F732del	WT	WT
	25	50/F	M0	46,XX[23]/45,XX,-7[5]	ND	WT	WT	WT
	26	82/M	M0/M1	46,XY[20]	0.00031	S663L	NA	NA
	27	63/F	M4	46,XX,inv(16)(p13q22)[18]	ND	/	WT	WT
	28	61/F	M1	46,XX[7]/47,XX,+11[13]	ND	/	WT	WT
	29	NA	NI	NA	0.00512	WT	NA	NA

32	62/M	NI	46,XX[2]/45,XX,t(3,21)(q26,q22), der(5q),-7,del(12)(p11,p13)[15]/45,XX,t(3,21)(q26,q22)der(5q),-7,del(11)(p13,p15), del(12)(p11,p13)[3]	0.01095	S714C	WT	WT
36	32/F	M1	46,XX[21]	0.00679	WT	WT	MUT
37	50/F	M1	46,XX[22]	0.01296	/	WT	WT
39	69/M	M5	43,XY,-7,hsr(11)(q13q23),-13, -17, del(20)(q11q13),-21-der(22)add(22)(p13), +1mar,1~3dmin[19]/44,XY,-7,hsr(11)(q13q23),-13,-17, del(20)(q11q13),-21-der(22)add(22)(p13), +2mar,1~3dmin[4]	0.00528	WT	NA	NA
41	NA	NI	46,XX [20]	ND	/	WT	WT
42	36/M	M0	NA	0.01141	/	ITD	MUT
43	65/F	M0/M1	46,XX[20]	0.00147	/	WT	WT
45	47/F	M2	46,XX[20]	ND	/	ITD, TKD	MUT
47	40/M	M5	46,XY,t(16;16)(p13;q22)[16]/46,XY, t(5;12)(q13;p13), t(16;16)(p13;q22)[4]	0.00514	G550V, C554S	WT	WT
49	64/F	M5	46,XX [20]	ND	WT	WT	WT
50	NA	NI	NA	0.00383	/	ITD	NA
53	NA	NI	NA	0.01296	/	NA	NA
54	NA	NI	NA	0.00372	/	WT	WT
55	67/F	M0	46,XX[20]	0.00737	/	ITD	MUT
57	NA	NI	NA	0.00034	/	TKD	MUT
58	62/F	M5	47,XX,+8,t(9;11)(p22;q23)[16]/48, XX,+8,+8,t(9;11)(p22;q23)[1]/50,XX,+8,+8, t(9;11)(p22;q23),+13,+19[3]	ND	/	WT	WT
59	59/F	sAML to MDS	NA	0.00040	/	WT	WT
60	65/M	NI	46,XY,t(3;12)(p22;q24),+4,-15,+mar[19]	0.00039	/	WT	MUT
62	72/F	M0/M1	46,XX,del(5)(q31q33)[2]	ND	/	WT	WT

AML: acute myeloid leukemia; NA: not available; NI: not identified; sAML: secondary AML. *Karyotype on peripheral blood sample. *UNCX*+: > 0.01300; *UNCX*–: ≤ 0.01300. Values are rounded to the nearest ten thousandth. 1-Dx: case 1 at the time of diagnosis; 1-Rem: case 1 at the time of remission. ND: not detectable = *UNCX* Ct value >40; WT: wild-type; /: not tested due to lack of DNA material.

G248P85449H7 (WI2-1959P13) that contained *UNCX* as the only target gene (*Online Supplementary Figure S1A and C*). A breakpoint region on 10p14 was mapped to the G248P8034H6 (WI2-3164O12) fosmid clone within the coding sequence of the housekeeping gene *UPF2*, which is involved in nonsense-mediated decay of mRNAs[19] and normally expressed in the bone marrow (BM) (*http://biogps.org*) (*Online Supplementary Figure S1B* and *C*). As a consequence of this rearrangement, *UNCX* was juxtaposed to the 3′ end of *UPF2* in the derivative chromosome 7 [der(7)], as shown by FISH (*Online Supplementary Figure S1*), and ectopically expressed in Case 1-Dx, as detected by RT-qPCR (Table 1 and Figure 1). In addition, Case 1-Dx presented mutations in *FLT3-TDK* (Table 1) and *WT1* (*data not shown*), which were not confirmed in Case 1 at complete remission (1-Rem), and wild-type *NPM1* (Table 1).

UNCX is ectopically expressed in a subset of AML patients and cell lines

To verify whether *UNCX* is expressed in AML independently of the t(7;10) translocation, *UNCX* transcript level was assessed by RT-qPCR in 61 additional AML cases. *UNCX* expression was detected in 37.1% (23 of 62) of our AML patient cohort (Table 1 and Figure 1A) and 8% (6 of 75) of the AML cell lines (Figure 1B and *Online Supplementary Table S1A*) at variable levels, regardless of the French-American-British (FAB) subtype. Interestingly, *UNCX* expression was not detectable in Case 1-Rem (Table 1). None of the other investigated cases (ns. 9, 16, and 61) or cell lines harbored the t(7;10)(p22;p14) balanced translocation, as confirmed by FISH, or mutations in *NPM1, FLT3* (Table 1), and *WT1* (*data not shown*).

UNCX expression was also detected in MEG-01 [chronic myeloid leukemia (CML)] and in brain cells. In normal cells, *UNCX* was not expressed in total BM, peripheral blood (PB), CB or BM $CD34^+$ stem-progenitor cells (*Online Supplementary Table S1B*), and in hematopoietic lineages at different stage of maturation, according to Blueprint Epigenome data portal (*http://blueprint-data.bsc.es/release_2016-08/#!/*) (*Online Supplementary Table S1C*).

In addition, we identified two alternative transcript isoforms for *UNCX* [UNCX-alternative 1 (UNCX-a1), GenBank KM587719 and UNCX-alternative 2 (UNCX-a2), GenBank KM587718], generated through the retention of distinct portions of intron II in both AML patients and cell lines (*Online Supplementary Figure S2* and further details in the *Online Supplementary Results*). Moreover, to evaluate UNCX expression at protein level, we tested two different polyclonal antibodies specific for the N- and the C-terminus of the UNCX protein (ab105966, Abcam, Cambridge, UK, and AV47546, Sigma, respectively) by Western blot

(WB). However, the lack of UNCX antibodies specificity (due to the detection of multiple non-specific bands) prevented the identification of the protein in HEK293T and Jurkat cell lines, indicated by the companies as WB positive controls, as well as in our AML cell lines. Notably, none of these antibodies has been referenced in any publication so far (further details in the *Online Supplementary Methods*).

UNCX ectopic expression is significantly associated with DNA methylation increase at *UNCX* canyon borders but is not correlated with *DNMT3A* mutations

To assess whether epigenetic changes were responsible for the ectopic expression of *UNCX* in AML patients, we analyzed DNA methylation. *UNCX* exon 1 and also a 2.5-kb region upstream of *UNCX* were significantly hypomethylated in all analyzed samples, regardless of *UNCX* expression level (*Online Supplementary Figure S3A*), as expected for genes within methylation canyons.[10]

Interestingly, we detected a significant increase of the MRs at *UNCX* 5′ and 3′ canyon borders in *UNCX*+ *versus* *UNCX*– samples. Specifically, three (Figure 2A, *Online Supplementary Table S3A* and *Online Supplementary Figure S3B-D*) and seven amplicons (Figure 2B, *Online Supplementary Table S3B* and *Online Supplementary Figure S3E-M*) at the 5′ and 3′ canyon borders, respectively, exhibited a significant increase in the average MR of CpGs.

To corroborate this evidence, DNA methylation at *UNCX* canyon was found to be significantly higher ($P<0.0001$) in AML samples from TCGA expressing *UNCX* (*UNCX*-TCGA-positive) than in the *UNCX*-TCGA-negative cohort (Figure 2C). Intriguingly, there was a significant correlation between this DNA methylation increase and *UNCX* expression levels ($\rho_{rs}=0.649$, $P<0.0001$) (*Online Supplementary Table S3C*) in the *UNCX*-TCGA-positive set. Moreover, a genome-wide methylation analysis yielded 270 genomic regions containing 289 differentially methylated genes, differing from *UNCX*-TCGA-positive and *UNCX*-TCGA-negative samples by at least 2-fold (*Online Supplementary Table S3D*).

We then evaluated whether mutations in *DNMT3A* could explain the altered methylation status of our *UNCX*$^+$ cases. We detected approximately the same rate of *DNMT3A* mutation in *UNCX*+ and *UNCX*– patients from our internal cohort, with heterozygous lesions at position R882 and at other amino acid residues [3 of 8 (37.5%) in *UNCX*+, 6 of 19 (31.6%) in *UNCX*–] (Table 1). Notably, Case 1 showed the same mutations in both 1-Dx and 1-Rem samples (*Online Supplementary Table S3E*), as expected for pre-leukemic lesions.[20] In the TCGA cohort, we observed a significant association between mutations in *DNMT3A* and lack of *UNCX* expression (33.3% of mutant cases among *UNCX*-TCGA-negative *vs.* 9.1% among *UNCX*-TCGA-positive, $P=0.0022$ considering all patients' subtypes; 33.6% of mutant cases among *UNCX*-TCGA-negative *vs.* 13.3% among *UNCX*-TCGA-positive, $P=0.041$ considering non-M3 AML). By analyzing the mutational status of genes involved in DNA methylation (*DNMT1, 3A, 3B, IDH1/2, TET1/2, WT1*), we observed a significant association with *UNCX*-TCGA-negative cases (58.9% of mutated cases among *UNCX*-TCGA-negative, *vs.* 25% among *UNCX*-TCGA-positive AML considering all cases, $P=0.0002$; 59.3% of mutated cases among *UNCX*-TCGA-negative *vs.* 36.7% among *UNCX*-TCGA-positive AML, $P=0.0384$ considering non-M3 AML).

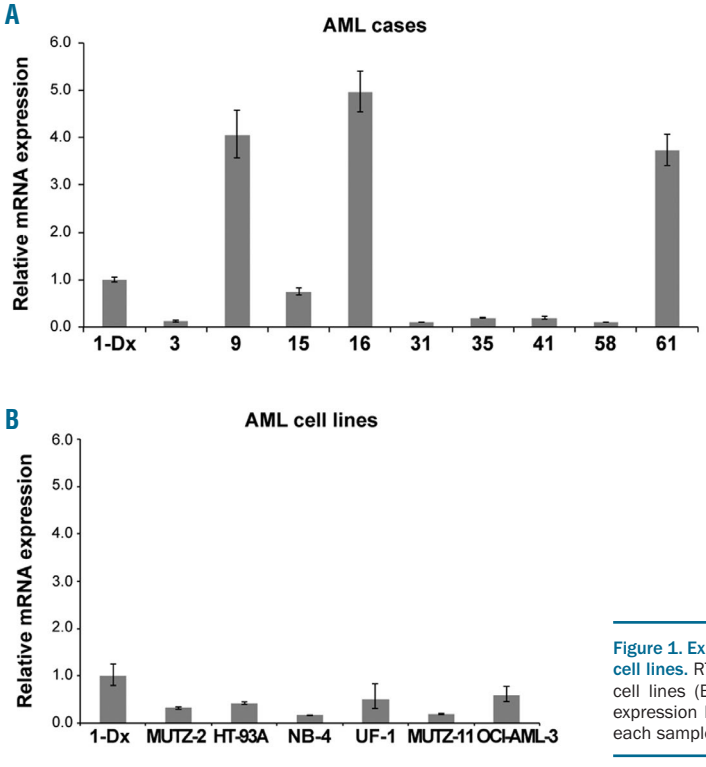


Figure 1. Expression levels of *UNCX* in acute myeloid leukemia (AML) patients and cell lines. RT-qPCR results showing *UNCX* expression in AML patients (A) and AML cell lines (B) in comparison to Case 1-Dx. Only positive samples exhibiting an expression level ≥0.10 are reported. The experiments were performed once and each sample was analyzed in triplicate.

Ectopic expression of *UNCX* in AML patients induces deregulation of genes involved in cell proliferation and differentiation

To characterize the transcriptional program associated with *UNCX* expression in AML, we performed gene expression profiling of *UNCX*+ (ns. 1, 9, and 16) and *UNCX*– (ns. 13, 41, and 49) cases. A total of 596 genes were differentially expressed (414 up-regulated and 182 down-regulated in *UNCX*+ cases; Array Express accession n. E-MTAB-4098) (*Online Supplementary Table S4A* and *Online Supplementary Figure S4A*). TCGA AML samples were divided into 47 (27.2%) *UNCX*-TCGA-positive and 126 (72.8%) *UNCX*-TCGA-negative patients, according to the *UNCX* median expression value, which was zero, since more than half (i.e. 126) the individuals did not express *UNCX* at all. To check whether the partitioning strategy applied to both data sets matched, we considered the set of *UNCX*-TCGA-negative patients and sought for a gene fingerprint that better transcriptionally discriminated them from a subset of 12 "extremely positive" patients, namely expressing *UNCX* over the 75th percentile. A fingerprint of 199 on 20,530 genes was found by means of the sPLS-DA. This set of genes was fed to a PCA that, when applied on the samples of our internal data set, differentiated perfectly *UNCX*+ from *UNCX*– individuals (*Online Supplementary Figure S4B* and *C*).

Analyses performed using Ingenuity Pathway Analysis software (IPA) (*Online Supplementary Table S4B-D*) identified a number of altered pathways, biological functions or diseases, and networks of genes involved in cell proliferation [mainly activation of mitogen-activated protein kinases (*MAPKs*), transforming growth factor-β (*TGF-β*), and phosphatidylinositol 3-kinase (*PI3K*) signaling pathways], cell-cycle regulation, hematopoiesis and hematologic disease, and cell death and survival. These results supported our hypothesis that *UNCX* could play an important role in differentiation (Figure 3A).

Among the set of deregulated genes obtained from the analyses of both our cohort of samples and TCGA data, we tested the differential expression of 3 genes with an established role in leukemia (upregulation of *CCNA1* and *PIK3CB* and downregulation of *MAP2K1*) by RT-qPCR in all patients under study. We observed a statistically significant upregulation of *CCNA1* and downregulation of *MAP2K1* only in the 4 patients (ns. 1-Dx, 9, 16, and 61) showing the highest *UNCX* expression levels (UNCX≥1) (*Online Supplementary Figure S4D*), and not in the overall cohort of *UNCX*+ patients (*Online Supplementary Figure S4E*).

Similarly, *HOXA10* expression was significantly increased only in patients with UNCX ≥1 (*Online Supplementary Figure S4D*). Notably, *HOXA10* was not identified as a deregulated gene in the GEP analysis but was found to be up-regulated in LUNCXIΔN cells, as described below.

UNCX expression strongly affects the proliferation and differentiation of normal myeloid cells *in vitro*

To clarify the biological function of *UNCX* in hematopoietic cells, we ectopically expressed *UNCX* in CD34$^+$ CB cells (LUNCXIΔN cells). CFC assays showed a significantly reduced number of colonies in LUNCXIΔN cells compared with LXIΔN (cells transduced with an empty vector) or non-transduced (NT) cells (Figure 4A), reflecting an *UNCX*-mediated effect on cell proliferation. To further assess the role of *UNCX* in the proliferation of myeloid cells, LUNCXIΔN, LXIΔN, and NT cells were seeded at the same density and counted every two days after infection for eight days. The proliferation rate was already reduced at day 5 post-infection (PI) in LUNCXIΔN cells (Figure 4B). Flow cytometry analysis at days 7, 10, and 14 PI revealed that CD34 tended to increase in LUNCXIΔN cells (mean value 29.1%, 15.9%, and 7.4%, respectively) compared with control LXIΔN cells (mean value 20.9%, 3.4%, and 0.6%, respectively), and this increase became statistically significant at day 14 PI (Figure 5A). RT-qPCR analysis confirmed the differential expression of *CD34* at the transcript level; *CD34* mRNA levels were significantly increased starting from day 5 PI (*Online Supplementary Figure S5*) to day 14 PI (Figure 5B) in LUNCXIΔN cells compared with LXIΔN cells. We also evaluated the expression level of *HOXA10*, *KLF4*, and *MAFB*, which are master regulators of mono-macrophage differentiation.[21-24] We observed increasing levels of

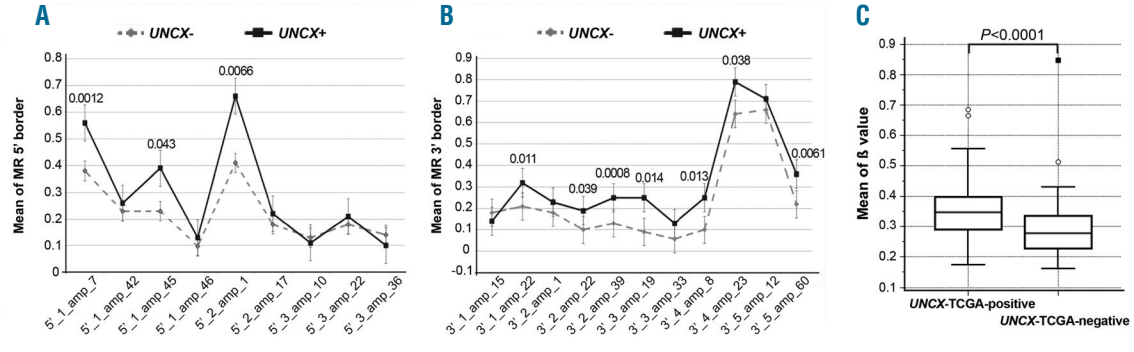


Figure 2. DNA methylation levels at *UNCX* canyon in our acute myeloid leukemia (AML) patient cohort and TCGA samples. Mean values of DNA methylation ratios (MRs) of MassARRAY amplicons obtained for each of the 20 amplicons tested at both 5′ (A) and 3′ (B) *UNCX* canyon borders; *P*-values are reported above each amplicon showing a significant increase in the average MR in *UNCX*+ *versus UNCX*– patients. (C) Box and Whisker plot showing DNA methylation β values at the *UNCX* canyon in *UNCX*-TCGA-positive (n=55, median 0.3471, interquartile range 0.2898-0.3981) and *UNCX*-TCGA-negative (n=56, median 0.2771, interquartile range 0.2273-0.3348) patients. Significant differences were identified using the Mann-Whitney U-test (*P*<0.0001).

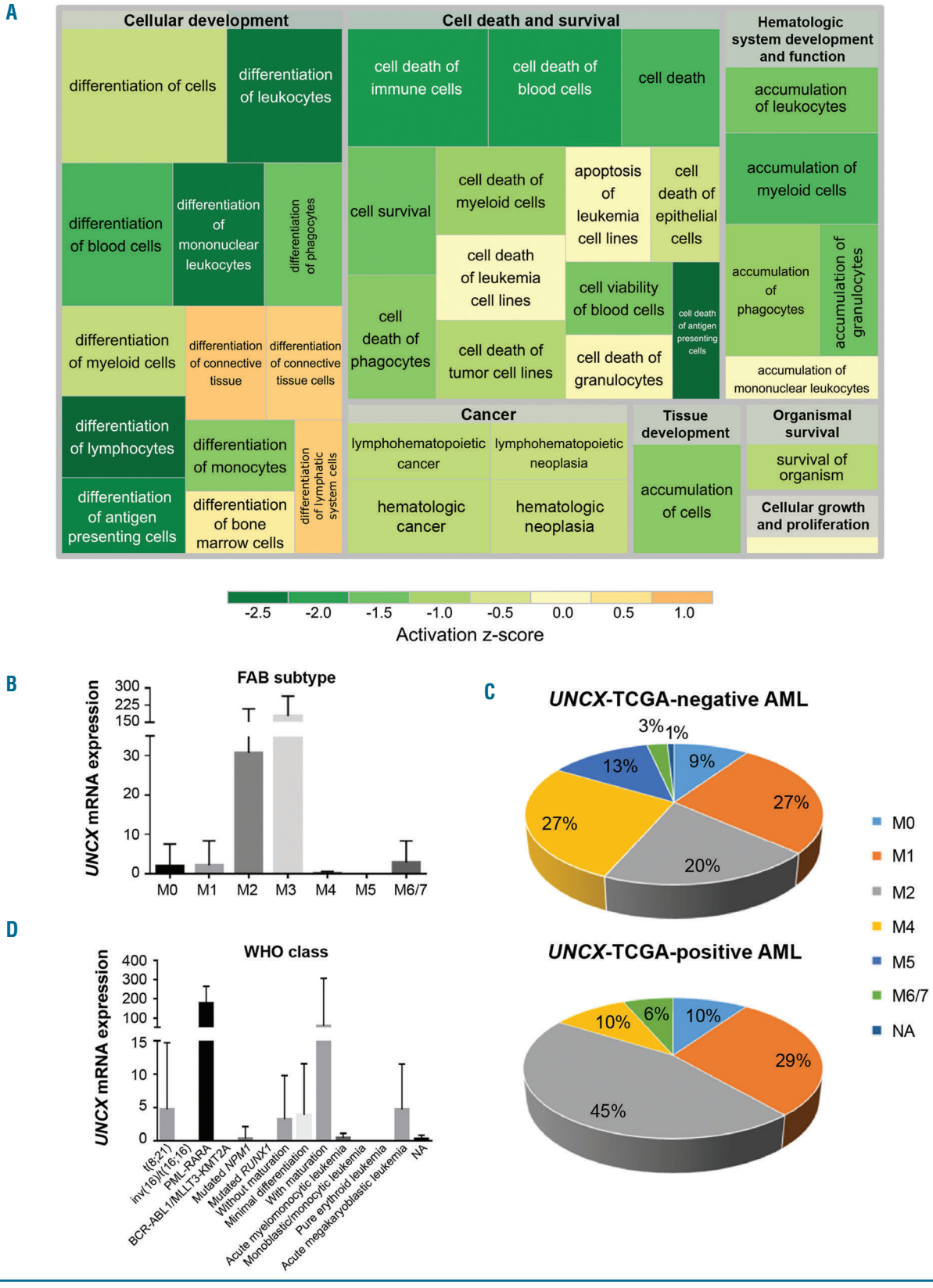


Figure 3. Association analysis of TCGA acute myeloid leukemia (AML) cohort. (A) Treemap showing the activation state of the enriched processes for commonly differentially expressed genes between exon array and TCGA data; green: reduced activity, orange: enhanced activity. Higher significance levels for a process are reflected by a larger enclosing rectangle. (B and D) *UNCX* transcript levels were obtained by TCGA RNASeq data. (B) Mean value and standard deviation across FAB types are shown. *UNCX* expression was significantly higher in M3 cases compared with the other FAB subtypes ($P<0.0001$, except for M6/7). (C) Graph showing the distribution of *UNCX*-TCGA-positive and *UNCX*-TCGA-negative AML cases across FAB types (excluding M3). Percentages are reported in the graphs. (D) Mean value and standard deviation across World Health Organization (WHO) classes (here reported with abbreviations) are shown (AML with *BCR-ABL1* and AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* are reported together due to low number of cases. *UNCX* expression was higher in acute promyelocytic leukemia (APL) with *PLM-RARA* and AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*.

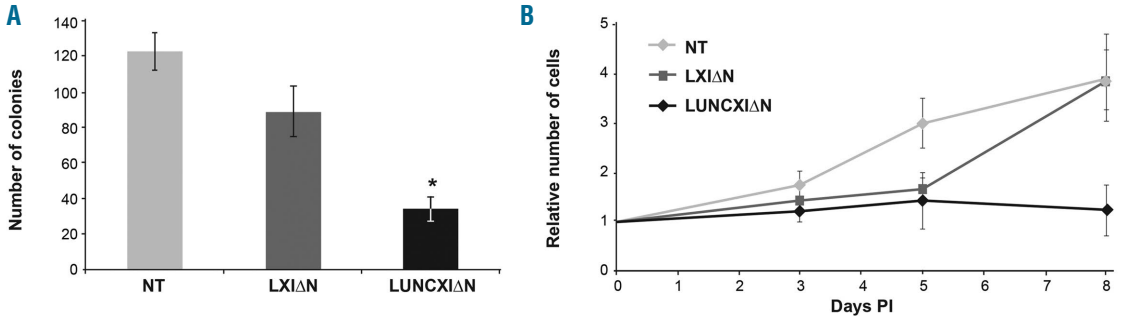


Figure 4. Colony forming cell (CFC) assay and cell-proliferation analysis of *UNCX*-transduced cells. Number of colonies obtained in CFC assays (A) and proliferation curves (B) of non-transduced (NT), LXIΔN, and LUNCXIΔN CD34$^+$ cells. All experimental data were verified in three independent experiments in triplicate.

HOXA10 and *KLF4* transcripts from day 5 to 14 PI (Figure 5B and *Online Supplementary Figure S5*). In the same cells, *MAFB* expression was elevated at days 5 and 7, but decreased after day 10 PI in LUNCXIΔN cells compared with control cells (Figure 5B and *Online Supplementary Figure S5*).

Notably, at day 14 PI, we observed a significant down-regulation of *MAP2K1* and upregulation of *CCNA1* in LUNCXIΔN cells (confirming what was observed in AML patients; see above), which could indicate an effect of *UNCX* activation on cell proliferation and cell cycle control (Figure 5B). Moreover, morphological analysis with May-Grunwald-Giemsa staining demonstrated a persistence of immature myeloid cells at day 14 PI, which particularly affected the mono-macrophage lineage in the LUNCXIΔN sample (5% *vs.* 1% in the LXIΔN control sample) (Figure 5C). The persistence of undifferentiated cells of mono-macrophage lineage, even after several days PI, confirmed the flow cytometry and RT-qPCR results.

Ectopic expression of *UNCX* characterizes a specific subgroup of AML patients

The TCGA data showed that *UNCX* expression was associated with lower age (median age of *UNCX*-TCGA-positive patients 50 years; median age of *UNCX*-TCGA-negative patients 58.5 years; P=0.003) (*Online Supplementary Table S2A*), M3 FAB type (31.1% in *UNCX*-TCGA-positive patients; 0.9% in *UNCX*-TCGA-negative patients, with 93% of M3 cases expressing the gene; P<0.0001) (Figure 3B and *Online Supplementary Table S2A*), favorable prognosis according to karyotype-based classification (P=0.0001) (*Online Supplementary Table S2A*), and reduced fraction of bone marrow monocytes (median of *UNCX*-TCGA-positive patients 3.5%; median of *UNCX*-TCGA-negative patients 7.0%; P=0.0034) (*Online Supplementary Table S2A*). The same results were also confirmed by excluding M3 FAB cases from the analysis (*Online Supplementary Table S2B*), with a tendency towards increased *UNCX* expression in AML M2 cases compared to the other FAB types (P=0.0181). Accordingly, in the overall AML cohort M2 cases represented 45.1% of *UNCX*-TCGA-positive patients but only 20% of *UNCX*-TCGA-negative patients (P=0.0233) (Figure 3C and *Online Supplementary Table S2B*). The analysis of *UNCX* expression, according to the WHO disease classification, showed the highest *UNCX* levels in APL with *PML-RARA* and AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* classes (P<0.0001) (Figure 3D and *Online Supplementary Table S2A*). On the other hand, we observed an increased frequency of AML with inv(16)(p13.1q22), AML with mutated *RUNX1*, and AML with mutated *NPM1* among *UNCX*-TCGA-negative cases (*Online Supplementary Table S2A*). Indeed, *UNCX* expression showed an inverse correlation with *NPM1c* mutation, with 6.5% of *UNCX*-TCGA-positive and 32.1% of *UNCX*-TCGA-negative cases carrying the mutation (P=0.0029 excluding M3 cases). No association was observed with *FLT3*, *RAS*, and *IDH1* mutational status (*Online Supplementary Table S2C*). The results suggest that *UNCX* expression characterized a specific subgroup of AML patients, according to their biological and molecular features.

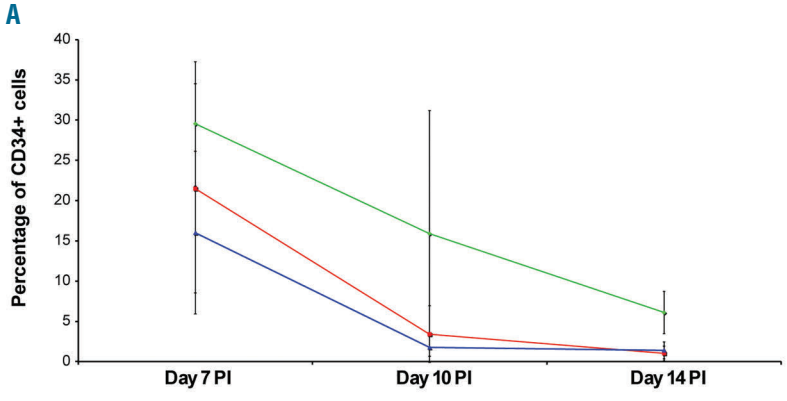
Discussion

We report for the first time the ectopic expression of *UNCX* in both 23 AML patients of our cohort (37.1%) and 8 AML cell lines (8%). Notably, the involvement of this HB gene and the identity of its downstream target genes in cancer have not previously been reported.

Induced expression of *UNCX* in normal CD34$^+$ CB cells *in vitro* markedly affected the proliferation and differentiation of the transduced cells. Indeed, *UNCX* expression resulted in both a reduction of the proliferation rate and an unprecedented persistence of CD34$^+$ cells at days 10 (15.9%) and 14 (7.4%) PI. This result is noteworthy because in similar experiments,[25] at day 10 PI, CD34$^+$HSCs were almost completely replaced by differentiated cells, and their contribution was reduced to approximately 7% of total cells. The observed reduction of CD34$^+$ cell proliferation and their differentiation arrest at a very immature stage supported the hypothesis that *UNCX* exerts a potential leukemogenic effect over the myeloid lineage, which was corroborated by the transcriptional increase of *HOXA10* and *KLF4*, along with the decrease of *MAFB* transcript levels. Notably, the expression of these genes, which are involved in mono-macrophage differentiation (*HOXA10* and *KLF4* in the early phase, *MAFB* in the late phase), was modulated along with *UNCX* activation *in vitro*. Upregulation of *HOXA10* was also observed in a subgroup of UNCX+ patients of our cohort (*UNCX* expression level ≥1). This result is in line with the recent work of

Yao *et al*. showing that *Hoxa10* overexpression in murine bone marrow cells suppresses differentiation and induces expansion of a cell population derived from the common granulocyte/monocyte progenitor population and results in the AML phenotype.[26] The identification of downstream target genes of *UNCX* will clarify its role in proliferation and differentiation.

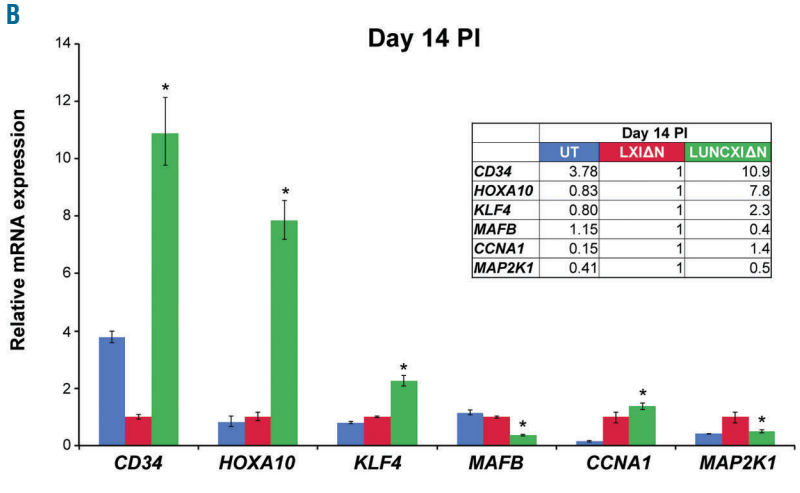
In parallel, the analysis of the TCGA AML cohort indicates that *UNCX* expression characterizes blast cells at early phases of differentiation, preferentially the M2 and M3 FAB types, which generally contain wild-type *NPM1*. Accordingly, *UNCX* positivity and *NPM1c* mutation are mutually exclusive both in our patient cohort and in the TCGA cohort. Moreover, the comparison between TCGA RNA-Seq data and our exon array data suggests that *UNCX* expression is associated with a differentiation arrest in AML blasts, as confirmed by forcing the ectopic expression of *UNCX* in stem-progenitor cells, which induces an accumulation of immature cells of the monomacrophage lineage. The different maturation stage of *UNCX*-TCGA-positive AML blasts and *UNCX*-transduced normal stem-progenitor cells may explain the differences in the transcriptional signatures associated with *UNCX* expression in the two settings. Our GEP data and pathway analyses clearly show that the 3 *UNCX*+ patients (*UNCX* expression level ≥1) displayed remarkable deregulation of genes involved in hemopoiesis, proliferation, and cell cycle, such as those implicated in



LUNCXIΔN	I	II	MEAN	SEM
Day 7 PI	36.8	21.4	29.1	7.7
Day 10 PI	26.7	5.1	15.9	10.8
Day 14 PI	8.8	5.9	7.4	1.5

LXIΔN	I	II	MEAN	SEM
Day 7 PI	33.9	8	20	12.9
Day 10 PI	5.9	0.9	3.4	2.5
Day 14 PI	1.2	0	0.6	0.6

NT	I	II	MEAN	SEM
Day 7 PI	27.6	9.1	18.3	9.3
Day 10 PI	1.8	1.7	1.8	0.1
Day 14 PI	0.3	2.4	1.3	1.1



	Day 14 PI		
	UT	LXIΔN	LUNCXIΔN
CD34	3.78	1	10.9
HOXA10	0.83	1	7.8
KLF4	0.80	1	2.3
MAFB	1.15	1	0.4
CCNA1	0.15	1	1.4
MAP2K1	0.41	1	0.5

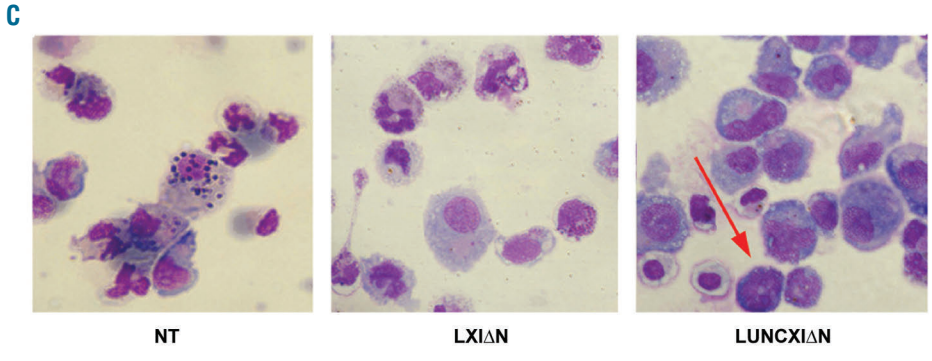


Figure 5. Delayed differentiation of CD34⁺ hematopoietic progenitor cells after retrovirus-mediated *UNCX* transfer. (A) Percentage values [including the calculated mean and the standard error of mean (SEM)] of CD34⁺ cells, measured in two independent *UNCX* transfer experiments (I and II), using flow cytometry at days 7, 10, and 14 post-infection (PI): non-transduced (NT) (blue line), LXIΔN (red line), and LUNCXIΔN (green line) CD34⁺ cells. *Two-tailed $P \leq 0.05$. (B) RT-qPCR results of the genes encoding specific surface markers and transcription factors implicated in hematopoiesis, including *MAP2K1* and *CCNA1*; the assay was performed on RNA extracted from cells at day 14 PI. *Two-tailed $P \leq 0.05$. (C) Morphological analysis after May-Grunwald-Giemsa staining at day 14 PI in NT, LXIΔN, and LUNCXIΔN CD34⁺ cells; the myeloid immature forms, characterized by a basophilic cytoplasm and a despiralized nucleus, are indicated with a red arrow.

MAPK, TGF-β, and *PI3K* signaling. All of these pathways play critical roles in proliferation, regulation of cell growth, differentiation, apoptosis, survival, and development in a wide range of cell types. Notably, although RT-qPCR analysis did not validate these data in the overall cohort of *UNCX*$^{+}$ patients, we observed *MAP2K1* downregulation and *CCNA1* upregulation in LUNCXIΔN cells, which showed *UNCX* expression level ≥1. Therefore, we speculate that abnormal levels of *UNCX* expression might be associated with specific transcriptional signatures and signaling pathway activation through a gene-dosage effect. However, further investigation is required to confirm this hypothesis. In AML patients (in both our and the TCGA cohorts), *UNCX* activation was associated and significantly correlated with a substantial increase in DNA methylation at both the 5′ and 3′ canyon borders. This methylation change might drastically affect *UNCX* transcription and result in its ectopic expression. However, here we found that *DNMT3A* mutations (both R882 and additional point mutations, including a few mutations not yet reported in the COSMIC database), did not associate with *UNCX* positivity, in contrast to what has been reported in mice where *Dnmt3a* mutations were directly correlated with shifts in DNA methylation at gene canyon borders.[10] Our results are in line with previous reports of somatic mutations in *DNMT3A* in approximately 20% of *de novo* AML cases and 36% of cytogenetically normal AML cases.[27,28] This result clearly suggests that *DNMT3A* mutations in humans are not directly correlated with *UNCX* canyon shrinkage and gene activation. In addition, analysis of the TCGA cohort showed the mutational status of alternative epigenetic modifier genes, classified according to the literature,[29-31] and we did not observe any significant association with *UNCX* expression. Hence, future analyses are mandatory to clarify the epigenetic mechanism behind the regulation of *UNCX*. Since the *Uncx* canyon was found to be enriched in the repressive histone mark H3K27me3,[10] we speculate that the increase in methylation might also induce a change in the histone mark configuration at the shrunk canyon of *UNCX*, which would result in changes in chromatin condensation and in a remodeling of nuclear architecture. This hypothesis, together with that concerning a possible role of hydroxymethylation in *UNCX* activation, could not be verified here due to the lack of vital frozen cells for our patients. Notably, the future development of efficient anti-UNCX specific antibodies will help to understand the role of UNCX at the protein level in leukemogenesis. In fact, commercially available antibodies failed to identify UNCX in tumor cell lines, even in those with a high expression of the gene (*Online Supplementary Table S1A and B*). This finding could be explained by the documented poor expression of homeodomain-containing proteins, as reported in several studies[32,33] and also for UNCX itself. Indeed, UNCX has been identified as a "missing protein" in the HEK293 cell line by means of bioinformatic predictions and high-throughput transcriptomic and proteomic technologies.[34]

In summary, our results suggest that ectopic activation of *UNCX* might represent an early epigenetic event in AML with a potential role in leukemogenesis because of its dramatic impact on the proliferation and differentiation of HSCs and progenitors. Our molecular and biological data indicate that *UNCX* expression may characterize a new subgroup of AML cases, which warrants further investigation. Moreover, although the molecular mechanisms underlying *UNCX* activation and its role in proliferation and differentiation remain to be clarified, this newly described alteration in AML, caused by an epigenetic modification affecting DNA methylation, introduces a previously undescribed scenario for the leukemogenic potential of epigenetic alterations.

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