

# CTCF, a novel fusion partner of ETO2 in a post-transplant relapsed acute myeloid leukemia patient

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## Research

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## Abstract

## Background

ETO2 is a nuclear co-repressor, which plays a critical role in the regulation of the cell cycle, self-renewal capacity, and differentiation of hematopoietic progenitor cells.

## Methods

We identified novel fusion transcripts involving *ETO2* and *CTCF* by RNA-seq in a post-transplant relapsed case.

## Results

The *CTCF-ETO2* and *ETO2-CTCF* chimeric genes were validated by RT-PCR and Sanger sequencing. In addition, both transcripts apparently promoted cell proliferation which is beneficial to tumorigenesis.

## Conclusion

The novel fusions may have prognostic value and pathogenic mechanisms in acute myeloid leukemia.

## Materials And Methods

### Patient samples

This study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University following the Declaration of Helsinki. Informed consent was obtained from the patient according to the Declaration of Helsinki.

### Cytogenetic analysis

At the time of diagnosis, bone marrow cells were cultured for 24 h and analyzed for standard cytogenetic R-banding. The karyotype was described according to the International System for Human Cytogenetic Nomenclature (ISCN 2013).

### RNA sequencing

RNA-sequencing analysis was performed according to Roche instruction for users. The cDNA sample library was prepared by using the KAPA Stranded RNA-Seq Library Preparation Kits. The target regions were captured with SeqCap RNA choice probe. The library was sequenced by the Nextseq 550 (Illumina) for 151bp on each paired-end.

## Reverse transcription and Sanger sequencing

An aliquot of the RNA extracted for RNA sequencing was also reverse transcribed using random hexamers and standard techniques (Invitrogen). The primers used to detect the *CTCF-ETO2* transcript were *CTCF*-exon3: 5'-CGATTACGCCAGTGTAGAAGT-3' and *ETO2*-exon4: 5'-TGATGGCTGTTGGTGAGTG-3'. The primers used to detect the reciprocal *ETO2-CTCF* transcript were *ETO2*-exon 1: 5'-ATGTCCCAGACGCACCCT-3' and *CTCF*-exon 6: 5'-GCTGCTTTCGCAAGTGGA-3'. The length of the sequence amplified by the primers is 554bp and 109bp, respectively. Sanger sequencing was performed using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

## Cell lines and reagents

The 293T and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (Hyclone, SH30243.01); 32D cells was maintained in RPMI medium (Hyclone, SH30809.01); and all were supplemented with 10% fetal bovine serum (Gibco, 10099141) and antibiotic-antimycotic (Gibco, 15240-062). 10 $\mu$ M mL-3 (PeproTech, 9621313) are provided for 32D cells because they are IL-3 dependent.

## Plasmid constructs

The DNA sequence encoding *CTCF-ETO2* and reciprocal *ETO2-CTCF* were synthesized and inserted into both pcDNA3.1 expression vector (Genewiz) with Flag tag fused to their carboxy termini and VENUS-N-FLAG lentiviral vector (Genewiz). Wild-type *CTCF* and *ETO2* were also synthesized and inserted into pcDNA3.1 expression vector with Flag tag fused to their carboxy termini. The empty vector pcDNA3.1 and VENUS-N-FLAG were used as control vectors. All of the plasmids were purchased from Genewiz.

## Immunofluorescence

The HeLa cells was transiently transfected with Flag-tagged pcDNA3.1 expression plasmid by JetPEI transfection kit (Polyplus, 26032C1F). Then the cells were permeabilized and sequentially incubated with primary anti-Flag antibodies (CST, 14793), secondary antibodies (DyLight 549 goat anti-mouse IgG antibody; Abbkine, A23310), and 4-6-diamidino-2-phenylindole (DAPI; Beyotime, C1005). Images were obtained with a laser scanning confocal microscope (Leica SP2).

## Lentivirus production and transduction

The 293T cells were transfected with lentiviral vector constructs and pPACK packaging plasmids mix by calcium phosphate precipitation method. Cells were then infected with prepared lentivirus and sorted by BD FACS Aria II System for green fluorescent protein (GFP)-positive cells.

## Flow cytometry analysis

Transduced 32D cells were washed with PBS and stained with APC-conjugated mCD71 (Biolegend, 113820) antibody. For apoptosis, VENUS, *CTCF-ETO2* and *ETO2-CTCF* transduced 32D cells were treated with either 10ng/ml or 0 mL-3 for 24 hours, and then the cells were stained with Annexin-V (BD

Pharmingen, 550474) and 7-AAD (BD Pharmingen, 559925) according to the manufacturer's protocol. Antibody staining was monitored with a Novocyte flow cytometer. Data analysis was carried out using FlowJo software.

## Q-PCR

RNAs were extracted from cells using total RNA extraction (Trizol, Invitrogrn, 15596026). 0.5µg–1µg RNA was used to make cDNA library using Primescript RT master mix (Takara, RR036B). The resulted cDNA library was diluted five-fold and used for SYBR green based qPCR reactions (Takara, RR420B) on an ABI 7500 Real-Time PCR system. The following primers were used for mouse ζ-globinqF: 5'GAAGCCTGG GACAAGTTCAT-3'; ζ-globinqR: 5'GGGTTCAATAAAGGGGAGGA-3'; mβ1-globin qF: 5'GCTCTTGCCTGTGAACAATG-3'; β1-globinqR: 5' GTCAGAAGACAGATTT TCAAATG-3'; mβh1-globinqF: 5' TTGCCAAGGAATTCACCCCA-3'; βh1-globinqR: 5'CTCAATGCAGTCCCATGGA-3'; mε-globinqF: 5'GTTTTGGCTAGTCACTTCG G-3'; ε-globinqR: 5'CAAGGAACAGCTCAGTATTC-3'; GAPDH was used as the internal control: mGAPDHqF: 5'CATCACTGCCACCCAGAAGACTG-3'; mGAPDH qR: 5'ATGCCAGTGAGCTTCCCGTTCAG-3'. Gene expression levels were quantified with the  $2^{-\Delta\Delta Ct}$  method.

## CCK8 assay

Transduced 32D cells were rinsed with PBS for 3 times and treated with either 10ng/ml or 0 mL-3 in a 96-well cell culture plate. Approximately 15000 cells were seeded in each well. Three replicates were made for each measurement. Then, cells were incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> for 24 h, 48 h and 72h. Finally, 10 µL of the CCK-8 reagent (Donjindo, KR675) was added into each well, and OD at 450 nm was measured using a multifunction microplate reader (Infinite M200 Pro, Tecan) after incubation for 3 h at 37 °C. The fold each concentration accounted for of the control was presented as relative cell proliferation.

## Western blot

Cell lysates were separated by SDS–PAGE gel and transferred to PVDF membrane (Millipore). The membrane was probed with primary antibody and then with secondary antibody. Antibody binding was revealed by using an enhanced chemiluminescence reagent (GE Healthcare Biosciences). ImageJ was used to quantify the density and size of the blots.

C-myc antibody was purchased from Active Motif (61075). The following antibodies were obtained from Cell Signaling Technology: p-STAT3 (9145), STAT3(12640), p-STAT5(9359), STAT5(9363), p-AKT(4060), AKT(4691), p-Erk(4370), Erk(4695), p-JNK(9255), JNK(9252), BCL2(15071), P53(2527), CDK9(81464), Actin(4967). Other commercial antibodies: HOXA9(Abcam, ab140631), CyclinT1(Abcam, ab264326). Secondary antibodies for western blotting were from Beyotime: HRP goat anti-rabbit IgG (A0208), HRP goat anti-mouse IgG (A0216), HRP goat anti-rat IgG (A0192).

## Statistical analysis

Data were shown as mean  $\pm$  standard deviation (SD). The significance of differences between different groups was determined by ANOVA. Data analyses were performed using Graphpad Prism v6.0. Statistical significance threshold was set at 0.05; asterisks indicate significant differences (\* $P < .05$ ; \*\* $P < .01$ ; and \*\*\* $P < .001$ ).

## Background

In acute myeloid leukemia (AML) patients, cytogenetics represents the single most important prognostic factor for predicting remission rates, relapse risks and overall survival outcomes. In order to better understand the cytogenetic abnormality in a post-transplant relapsed AML patient who had no aberrant results by routine karyotype analysis and multiplex RT-PCR, we performed RNA sequencing and identified novel *CCCTC-binding factor (CTCF)- eight twenty-one 2 (ETO2)* and its reciprocal fusion transcripts.

## Case Report

A 19-year-old female was admitted to our hospital on 2 September 2014 due to skin ecchymosis for 4 days and fever for 1 day. Blood tests showed a white blood cell count of  $132 \times 10^9/L$ , a platelet count of  $17 \times 10^9/L$ , and a hemoglobin level of 88 g/L. Bone marrow aspiration showed AML-M2a with 27.5% BM blast (Fig. 1A). Leukemic blasts were positive for CD13, CD34, CD64, CD38 and CD34 with partial weak positivity of HLA-DR and CD45 by flow cytometry. No abnormal results were identified by karyotype (Fig. 1B) analysis and multiplex RT-PCR. Therefore, the patient was diagnosed with AML and treated with induction regimens (idarubicin and cytarabine), which yield a complete remission. She went on to receive post-remission intensification and subsequent maintenance chemotherapy for a total of 2 years of chemotherapy. During maintenance therapy, the patient experienced first and second relapse, and underwent haploidentical transplants while in a non-complete remission status. *CSF3R*<sup>T618I</sup>, *RUNX1*<sup>G64R</sup> and *CEBPA*<sup>R297P</sup> mutations (detected by next-generation sequencing) occurred at that time. In addition, a leukemia-infiltrating breast mass was removed by surgery on 6 September 2016. Unfortunately, on October 2017, she relapsed again and died within 1 month.

Subsequent RNAseq on bone marrow samples collected before transplant identified novel *CTCF-ETO2* and its reciprocal fusion genes. RNAseq results revealed 1 breakpoint in intron 5 of *CTCF* (ENST00000401394.6) and 1 breakpoint in intron 1 of *ETO2* (ENST00000268679.9). Sanger sequencing of the RT-PCR products further confirmed in-frame fusions between *CTCF* (codon 125) and *ETO2* (codon 51) in both chimeric transcripts (Fig. 1C, D). *CTCF-ETO2* fusion transcript was formed by the fusion of *CTCF* exon 5 to *ETO2* exon 2, simultaneously, the reciprocal *ETO2-CTCF* chimeric transcript was composed of the fusion of *ETO2* exon 1 to *CTCF* exon 6. The main domains of *ETO2* and *CTCF* were preserved in fusion proteins (Fig. 1E).

Immunofluorescence analysis of subcellular distributions demonstrated that both *CTCF-ETO2* and *ETO2-CTCF* fusion proteins were localized at nuclear (Fig. 2A), to execute their functions in promoting cell proliferation. Significantly cell growth was detected via cell counting kit-8 assay in *CTCF-ETO2*

transduced 32D cell lines compared with vector group (Fig. 2C). Strikingly, the improved proliferative strength still had statistical significance when retreating murine IL-3 in mIL-3 dependent 32D cells. Similarly, Thirant et al found that ETO2-GLIS2 confers enhanced self-renewal of progenitor cells, and suggested that the nerve homology region 2 (NHR2) domain of ETO2 proteins in coordination with transcription factor ERG is essential for the self-renewal of ETO2-GLIS2 leukemia cells [1–3]. Besides, the activated signal pathway (eg, p-STAT3) and key molecules involving cell proliferation (c-myc) or cell cycle (CDK9, Cyclin T1) may partially account for this result (Fig. 2D). With regard to erythropoiesis, low erythroid-related gene expression in *CTCF-ETO2* transduced cells was detected (Fig. 2C), which probably results from dysregulation of *GATA1*. Besides, neither *CTCF-ETO2* nor *ETO2-CTCF* fusion gene had effect on cell apoptosis (Supplementary Fig), which is correspondent with *bcl2* expression in immunoblotting (Fig. 2D).

CTCF is a transcription factor that contains a DNA-binding domain composed of 11 highly conserved zinc fingers (ZF). The nuclear protein is encoded by the *CTCF* gene located on chromosome 16q22.1. As a transcription repressor or insulator, CTCF negatively regulates MYC, thus providing a mechanism for CTCF to promote erythroid differentiation[4, 5]. In addition, *CTCF* boundary remodels chromatin domain and drives aberrant *HOX* gene transcription in AML[6]. The present ETO2-CTCF fusion transcripts preserved ZF domain, consistent with previous study, down-regulated MYC protein and up-regulated HOXA9 protein was found in the western blot (Fig. 2D), and higher expression of erythroid gene was identified by Q-PCR (Fig. 2C).

*ETO2* gene is located on chromosome 16q24.3 and encodes a member of myeloid translocation gene family which consists of *NHR1*, *NHR2*, *NHR3* and *MYND* (myeloid, nery, and DEAF-1) domains. As a nuclear co-repressor, ETO2 interacts with DNA-binding transcription factors and recruits a range of co-repressors to inhibit downstream gene expression, therefore, it plays a critical role in the regulation of the cell cycle, self-renewal capacity, and differentiation of hematopoietic progenitor cells[7]. ETO2 indirectly promoted leukemia stem cells transformation and guided a relapse gene program which induced dismal clinical outcomes in AML [8]. To our knowledge, five *ETO2* fusion genes have been reported in hematological malignancies up to date, including *inv(16)(p13.3q24.3)/ETO2-GLIS2*[2, 9], *t(1;16)(p31;q24)/NFIA-ETO2*[10] and *t(16;21)(q22;q24)/RUNX1-ETO2*[11] in AML, *t(9;16)(p13;q24)/PAX5-ETO2*[12] in ALL and *t(14;16)(q32;q24)/IGH-ETO2*[13] in lymphoma. Here we describe the first case presenting as AML with novel *CTCF-ETO2* and reciprocal chimeric genes.

Multiple relapses and extramedullary invasion, especially post-transplant relapse gave the patient fatal lethality in the present *CTCF-ETO2* and *ETO2-CTCF* positive AML case. Alternatively, Schuback et al found that positive *ETO2-GLIS2* fusion had significantly higher relapse rate, worse 5-year overall survival and event-free survival than negative fusion in 193 cytogenetically normal AML patients[14]. Micci et al described that three AML patients with *NFIA-ETO2* fusion had poor clinical outcome[10]. In contrast, four-year event free survival of *RUNX1-ETO2* tended to be higher compared with other AML patients (77% vs 51%,  $P = 0.06$ )[15]. It seems that the synergetic role of ETO2 and its partner would determine patient-

specific outcome. Better understanding of molecular and clinical characteristics of novel *CTCF-ETO2* fusion would be helpful for future treatment.

## Conclusions

In conclusion, *CTCF* has been reported as a translocation partner of *ETO2* in AML for the first time. Both chimeric transcripts, *CTCF-ETO2* and *ETO2-CTCF*, were located in the nuclei and could promote cell growth, but neither had effects on apoptosis. Further research is warranted to investigate functional characterization, prognostic value and potential therapy of this novel fusion in AML.

## Abbreviations

AML  
acute myeloid leukemia  
CTCF  
CCCTC-binding factor  
ETO2  
eight twenty-one 2  
NHR  
nerve homology region  
ZF  
zinc fingers

## Declarations

## Ethics approval and consent to participate:

This study was approved by the ethics committee in accordance with the Declaration of Helsinki protocol.

### Consent for publication:

All authors give consent for the publication of the manuscript.

### Availability of data and material:

All data obtained and analyzed in this study were available from the corresponding authors in a reasonable request.

### Competing interests:

The authors declare no conflict of interest.

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## Authors' contributions:

S.C. was the principal investigators. Z.S., Z.W., L.W. and X.B. performed most of the experiments. J.L., Y.X., Z.Z., J.Z., J.P., W.W. and L.Y. performed clinical analysis. S.Z., Z.W. and L.W. wrote the manuscript.

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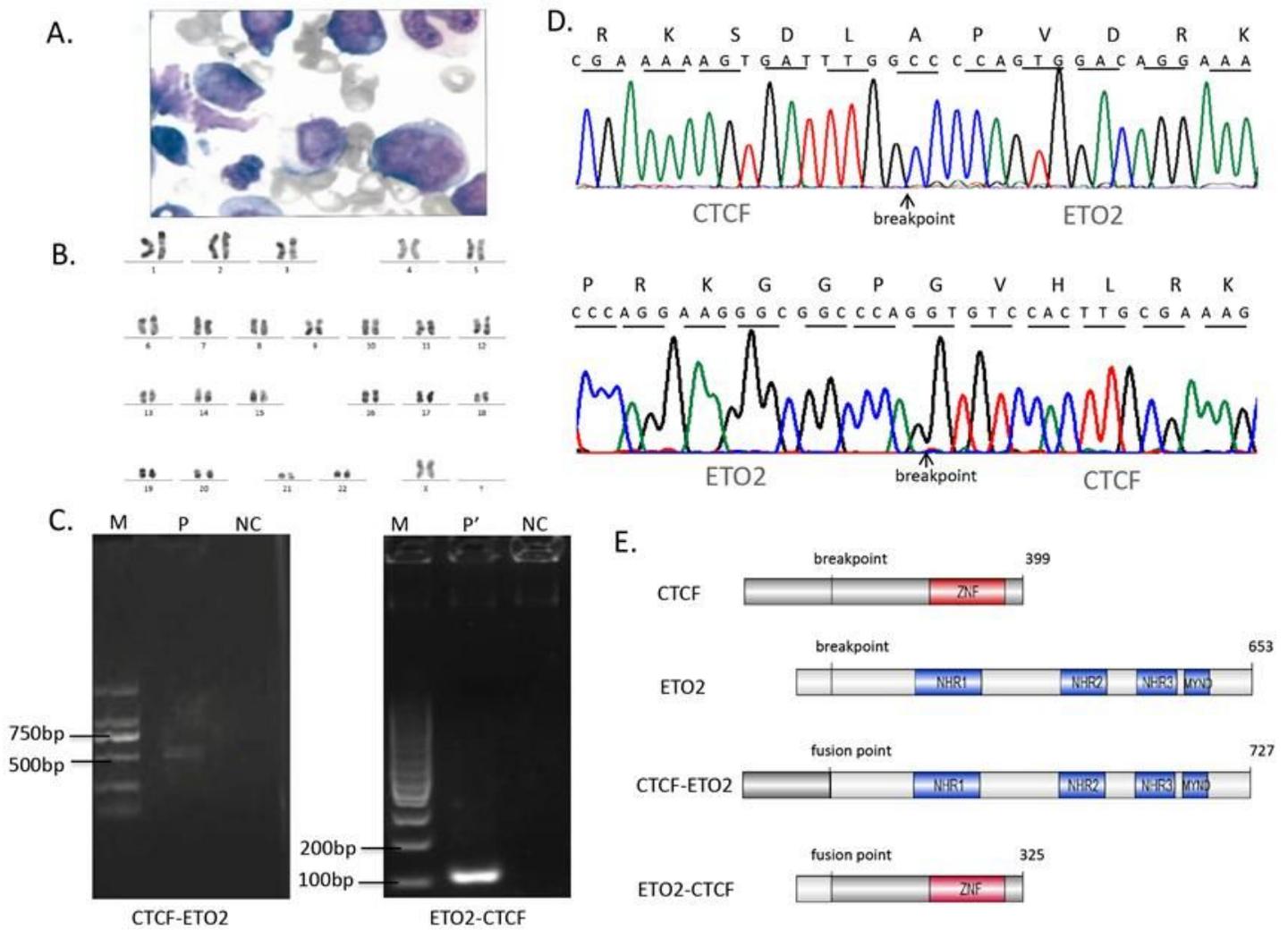
All the samples were from Jiangsu Biobank of Clinical Resources.

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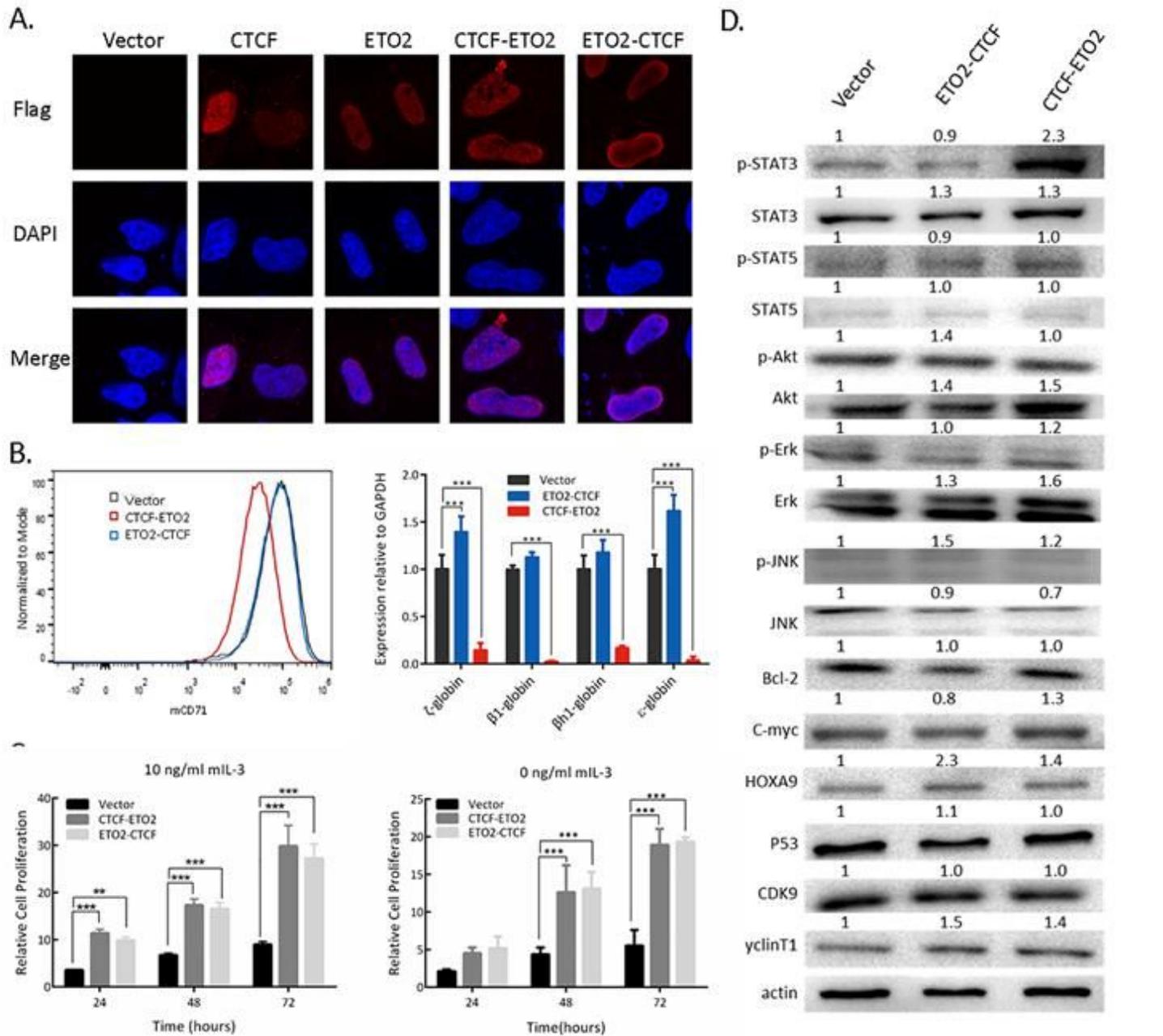
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## Figures



**Figure 1**

Identification of CTCF-ETO2 fusion transcript and reciprocal ETO2-CTCF fusion transcript from a M2 patient. A. The May-Giemsa staining of BM films. Leukemic myeloblasts admixed with myelocytes, bands and segmented neutrophils. B. Karyotype analysis: 46, XX. C. RT-PCR analysis of CTCF-ETO2 fusion gene and ETO2-CTCF chimeric transcript. NC (negative control). D. Partial sequence analysis of fusion transcripts. E. Schematic diagram of CTCF, ETO2, CTCF-ETO2 and ETO2-CTCF protein. ZNF (zinc finger), NHR (nerve homology region), MYND (myeloid, nervy, and DEAF-1).



**Figure 2**

The subcellular localization and proliferative effect of fusion proteins. A. Immunofluorescence analysis of CTCF-ETO2 and ETO2-CTCF fusion protein in vector HeLa cells transfected with expression plasmids. B. CTCF-ETO2 fusion protein played a role in down-regulating erythroid gene expression in 32D cells. Expression levels of ζ-globin, β1-globin, βh1-globin and γ-globin were assessed by qPCR and mCD71 (immature erythroid surface marker) was shown by flow cytometry. C. Both CTCF-ETO2 and ETO2-CTCF fusion genes promoted 32D cell proliferation. Relative proliferation of 32D cells treated by 0 or 10 ng/ml mL-3 was detected via CCK-8 assay. D. Western blot analysis of 293T cells expressing pcDNA3.1-tagged vector and fusions. Protein levels of STAT3, phosphorylated (p-) STAT3, STAT5, p-STAT5, AKT, p-AKT, ERK, p-ERK, JNK, p-JNK, BCL2, c-MYC, HOXA9, P53, CDK9 and cyclinT1 were calculated by ImageJ.

## Supplementary Files

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