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Modulation of FLT3 through decitabine-activated C/EBPa-PU.1 signal pathway in FLT3-ITD positive cells

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ABSTRACT

FMS-like tyrosine kinase 3 (FLT3)-mutant acute myeloid leukemia (AML) which occurs in approximately 30% of all AML patients still has a poor prognosis. This study aimed to examine the effect of decitabine (DAC) on FLT3-ITD positive AML. In our study, we found that expression of FLT3 and its downstream targets was decreased in FLT3-ITD mutant cell lines treated with DAC. DAC treatment could increase the percentage of apoptotic cells and CD11b positive cells tested by flow cytometry and upregulate the expression of cleaved caspase3, cleaved PARP, C/EBPa and PU.1 detected by western blot. To explore the effect of increased expression of PU.1 on FLT3 protein, we transiently transfected MOLM13 and MV4-11 cells with siRNA against PU.1 and a siRNA control. In both FLT3-ITD positive cells, the effect of DAC on downregulation of FLT3 was diminished in PU.1-konckdown MOLM13 and MV4-11 cells and there was a decrease of CD11b expression after PU.1 knockdown. Furthermore, the percentage of apoptotic cells was also decreased in PU.1-konckdown cells compared with siRNA control-expressing cells with the same dose of DAC. These findings indicated that DAC upregulated PU.1 to induce downregulation of FLT3 to trigger apoptosis. DAC was also found efficacious in mouse xenograft models of FLT3-ITD AML in our study. These findings may provide a novel theoretical basis for treatment of FLT3-ITD positive AML patients.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by clonal abnormal proliferation of hematopoietic stem or progenitor cells [1]. This dysregulation of blood cell leads to thrombocytopenia, neutropenia, and anemia, causing marrow failure and death within months or even weeks [2]. Although intensive chemotherapy has been used, the 5-year survival rate of adult AML patients is only about 50% and of patients older than 70 years is below 10% [3].

Mutant FMS-like tyrosine kinase 3 (FLT3) gene which presents mostly in form of internal tandem duplication (ITD) is one of the most common genomic mutations and occurs in approximately 30% of all AML patients [4]. FLT3 gene found in the chromosomal region 13q12.2 encodes a membrane-bound receptor tyrosine kinase (RTK) which belongs to RTK subclass III family [5]. FLT3 has been identified as an important marker in different hematological malignancies in recent years [6]. FLT3-ITD mutation is an independent predictor of worse overall survival (OS) and AML patients harboring FLT3-ITD mutation have a high leukemic burden and a higher relapse rate [7]. Cell proliferation, survival and differentiation are regulated by FTL3-ITD through constitutive activation of PI3K/AKT, STAT5, or MAPK/ERK canonical pathways and FTL3-ITD cooperates with other abnormalities to finally induce AML [8,9].

FLT3 inhibitors of first-generation are multi-kinase inhibitors of

Abbreviations: ACTB, β-actin; AML, acute myeloid leukemia; BCA, bicinchoninic acid; BSA, albumin from bovine serum; caspase, cysteinyl aspartate specific proteinase; C/EBPa, CCAATenhancer-binding protein a; cDNA, complementary deoxyribonucleic acid; DAC, decitabine; DMSO, Dimethyl sulfoxide; DNMT, DNA methyltransferase; ECL, Electro-Chemiluminescence; Ets, the Ezb transformation-specific sequence; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FCM, flow cytometry; FLT3-ITD, FMS-like tyrosine kinase 3 -Internal tandem duplication; HRP, horse radish peroxidase; MAPK/ERK, mitogen-activated protein kinase/extracellular regulated protein kinases; MDS, myelodysplastic syndromes; min, minute; MM, multiple myeloma; mRNA, messenger ribonucleic acid; PARP, poly ADP-ribose polymerase; PBS, optical density buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; PI3K/AKT, phosphatidylinositol 3-kinase/protein kinase B; PVDF, polyvinylidene difluoride; RT-qPCR, quantitative reverse transcription polymerase chain reaction; RIPA, radio immunoprecipitation assay; RTK, receptor tyrosine kinase; RT-PCR, reverse transcript polymerase chain reaction; STAT, signal transducing activator of transcription; TKIs, tyrosine kinase inhibitors

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VEGF, PDGFR, RAF-1, ERK, c-KIT and FLT3, while FLT3 inhibitors of second-generation are more targeted FLT3[10]. Despite the facts that FLT3 inhibitors can induce clinical remissions in majority of patients, drug resistance often occurs within few months of treatment [11]. Patients with FLT3-ITD still confer a very poor prognosis. TKIs combined with conventional chemotherapy are also underway for AML therapy. In recent study, it is reported that DNA methyltransferase inhibitor decitabine (DAC) combined with FLT3 TKI is confirmed a promising response rate in FLT3-ITD mutant AML patients.

DAC, the cytosine analogue 5-aza-2′-deoxycytidine, DNA methyltransferase inhibitor (DNMTi), is approved by the United States Food and Drug Administration (FDA) as a treatment of patients with refractory/relapsed leukemia and myelodysplastic syndrome (MDS)[12]. The effect of DAC on inhibition of proliferation, induction of apoptosis, and myeloid differentiation has shown anti-leukemic potential in several preclinical and clinical studies [13]. DAC treatment could induce the largest methylation decreases at CpG of CCAAT/enhancer-binding protein (C/EBP) promoter to promote cellular differentiation of AML cells [14]. Myeloid differentiation requires the induction of C/EBPa and PU.1 expression [15]. The expression of PU.1 is regulated positively by C/EBPa [16]. C/EBPa, a member of the C/EBP family, is found expressed in early myeloid progenitor cells [15]. Transactivation of granulocyte target genes of wild-type C/EBPa is blocked by the mutant C/EBPa in a dominant-negative manner to lead to a failure in granulocytic differentiation [15]. PU.1(encoded by the gene Sfpi1), the Ezb transformation-specific sequence (Ets) family transcription factor is expressed in monocytic, granulocytic and B-lymphoid cells and plays multiple roles in hematopoiesis [17]. Furthermore, expression of C/EBP and PU.1 is repressed by FLT3-ITD signaling [15,17] and PU.1 directly regulates FLT3 in a concentration-dependent manner in dendritic cell [17]. But the exact mechanism between C/EBPa-PU.1 and FLT3 is not clear. In our study, we examined the effect of DAC on C/EBPa-PU.1 in FLT3-ITD positive AML.

2. Methods

2.1. Ethics statement

The animal experiments were approved by the Animal Care Committee of Shanghai Jiao Tong University Affiliated Ren ji Hospital.

2.2. Cell culture and reagents

The MV4-11, MOLM13 and THP-1 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MV4-11 and MOLM13 cells were cultured in IMDM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) in humidified 5% CO2 at 37 °C; THP-1 cells were cultured in RPMI1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) in humidified 5% CO2 at 37 °C.

DAC was purchased from Beijing SL Pharmaceutical Co., Ltd. (Beijing, China). Bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diluent DMSO added to the medium served as control samples. Opti-MEM and Lipofectamine 2000 were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Anti-β-actin antibody was obtained from Abmart (Arlington, MA, USA). Anti-FLT3, anti-phosphorylated-FLT3(P-FLT3), anti-caspase-3, anti-PARP, anti-DNMT3a, anti-DNMT3b, anti-DNMT1, anti-STAT5, anti-phosphorylated-STAT5(P-STAT5), anti-AKT, anti-phosphorylated-AKT(P-AKT), anti-ERK and anti-phosphorylated-ERK(P-ERK) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). CD11b antibody was purchased from Biolegend.

2.3. Western blot

Total proteins in the tumor tissue or cells were extracted using RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China). Equal amount of protein was then loaded in lanes of 10% gels (Beyotime Institute of Biotechnology, Shanghai, China), separated by electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore Corporation, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk and incubated with the relevant primary antibodies (1:1000) overnight at 4 °C. The membranes were washed three times and incubated with the relevant secondary antibodies (1:1000) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 1 h. The signals were visualized using ECL reagents (Millipore Corporation, Billerica, MA, USA).

2.4. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

The total RNA samples were extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using a PrimeScriptTM RT reagent Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Real-time PCR was performed with a 7500 Real-time PCR System (Biosystems, Foster City, CA, USA) using SYBR Premix Ex TaqTM II (Takara Bio Inc., Shiga, Japan). All data were normalized to human GAPDH. The PCR primers used are listed: PU.1-Forward CGT GCACAGCGAGTTCGA; PU.1-Reverse GCTCTGGAGCTCCGTGAAGT. GAPDH-Forward ACAGTCAGCCGCATCTTCTT; GAPDH-Reverse ACGA CCAAATCCGTTGACTC.

2.5. Flow cytometry analysis

The MV4–11 and MOLM13 cells were treated with indicated concentration of DAC. Then the Cells were collected 48 h later and washed twice with PBS, and the Annexin V-PI Kit (Nanjing Keygen, China) was used according to the manufacturer's guidelines. The MV4-11 and MOLM13 cells were resuspended in binding buffer and an aliquot of 5 μL Propidium Iodide (PI) and an aliquot of 5 μL Annexin V-Fluorescein Isothiocyanate (FITC) were added into the above suspension and co-cultured at room temperature away from light for 15 min.The detection was performed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

2.6. Cell morphology

MV4–11 and MOLM13 cells were treated with DMSO or DAC (1 μM and 2 μM) for 48 h respectively. For morphological observations, the cells were centrifuged onto slides by cytospin and stained with Wright's-Giemsa (1000×, magnification).

2.7. Detection of methylation level

The genomic DNA was extracted according to the instructions of DNA Extraction Kit of Sangon Biotech (Shanghai) Co., Ltd. The methylation primer (M) and non-methylation primer (U) of C/EBPa gene were synthesized by Sangon Biotech (Shanghai) Co., Ltd. C/EBPa-MF: TTAAAGGAGGGGCGTTTAATTAC and C/EBPa-MR: CCACTAACTCCTA CGCTACTAAACG; C/EBPa-NMF: TTTAAAGGAGGGGTGTTTAATTAT and C/EBPa-NMR: CCCACTAACTCCTACACTACTAAACA. The amplified products were detected by 40 g/L agarose gel electrophoresis at 100 V for 35 min.

2.8. Detection of CD11b marker

Expression of CD11b in MV4-11 and MOLM13 cells was assessed by

flow cytometry according to the instructions. The MV4-11 and MOLM13 cells were treated with indicated concentration of DAC. Then the Cells were collected 48 h later and washed twice with PBS. The MV4-11 and MOLM13 cells were resuspended in PBS and CD11b antibody was added to co-culture at room temperature away from light for 15 min. The detection was performed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

2.9. RNA interference experiments

PU.1-specific small interfering RNA (siRNA) or a nonspecific siRNA control (NS siRNA) were chemically synthesized at GenePharma (Shanghai, China). The sequence of the PU.1 siRNA was as follows: 5′-UAUAGAUCCGUGUCAUAG GGCACCA-3′. A 5 μL of aliquot of 20 μM siRNA was introduced into 1×10^5 MV4-11 and MOLM13 cells by using Lipofectamine 2000, according to the manufacturer's instructions. The transfected cells were incubated for 24 h, and the cells were collected to perform real-time qPCR analyses to detect PU.1 or to further experiments.

2.10. Isolation of patient-derived leukemic cells

Leukemic cells were obtained from peripheral blood of three AML patients with FLT3-ITD. The clinical investigation on patients was conducted according to the principles of the 1996 Declaration of Helsinki and was approved by the Joint Committee on Clinical Investigation of Ren Ji Hospital. The leukemic cells were washed and resuspended in RPMI-1640 with 20% FBS and treated with DAC, and then collected for apoptotic analysis and for western blotting analysis.

2.11. Tumor xenograft in nude mice

6-week-old female BALB/c-nu/nu mice (Slac, China) were employed in this study. MV4-11 cells (1×10^7) were suspended in 100 μL IMDM medium and injected subcutaneously into the flank of mice. At 100 mm^3 tumor volume, animals were randomly divided into two groups ($n = 5$/group). The control group was treated with vehicle alone, while the DAC group was treated with decitabine 0.1 mg/kg/day intraperitoneally for two weeks. Tumor size was calculated as: volume (mm^3) = $V = A \times B^2/2$ (A is the larger diameter and B is the smaller diameter). Tumors were harvested for further analysis.

2.12. Statistical analysis

All Data were reported as the mean ± standard error. We used the Student's *t*-test to determine the statistical significance of experimental results. Statistical analysis was performed using SPSS 13.0 or GraphPad Prism 5 software. *P*-value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. Decitabine inactivates FLT3 and its downstream pathways

As FTL3-ITD could modulate cell proliferation, survival and differentiation via constitutive activation of AKT, STAT5, or ERK canonical pathways to induce AML [8,9], we first examined expression and activity of FLT3 in the presence of DAC (1 μM and 2 μM) in MOLM13 and MV4-11 cells. After DAC treatment for 48 h, as expected, these cells displayed downregulation of FLT3 in a dose-dependent manner (Fig. 1A-B). DAC treatment significantly reduced expression of FLT3, but expression of phosphorylated FLT3 was not observed obvious change as shown in western blot. As for THP-1 cells harboring wtFLT3, we observed little change of both FLT3 and phosphorylated FLT3(Fig. 1C). The data suggested DAC may be more sensitive to FLT3-ITD. We next examined the activity of downstream targets (STAT5, AKT and ERK) of FLT3 in the presence of DAC in MV4-11 cells. Downstream targets of FLT3 either total expression of STAT5, AKT and ERK or phosphorylated expression of STAT5, AKT and ERK were inhibited after DAC treatment for 48 h especially at high dose of 2 μM (Fig. 1D). DAC at 1 μM and 2 μM had little effect on phosphorylated FLT3, but still could downregulate its downstream targets, it indicated that DAC downregulated STAT5, ERK, AKT directly rather than through inactivating FLT3. All these results suggested DAC was effective on downregulation of FLT3 and its downstream pathways.

3.2. Decitabine induces apoptosis in FLT3-ITD positive cells

To investigate whether DAC treatment resulted in apoptosis of FLT3-ITD positive cells, we examined the percentage of apoptotic cells by flow cytometry in MOLM13 and MV4-11 cells after treatment with DAC (1 μM and 2 μM) for 48 h. The percentage of apoptotic cells was increased in DAC-treated cells compared to the control cells (Fig. 2A). Evidence of cell apoptosis by morphologic assessment was present in DAC-treated FLT3-ITD positive cells (Fig. 2B). Caspase3 which plays an important role on apoptosis in many cells is the main executioner of caspase family and the levels of apoptosis are usually evaluated by the expression of caspase3[18]. In addition, cleaved-caspase3 can shear the downstream protein PARP into cleaved-PARP [19]. The expression of caspase3 and PARP was detected by western blot. Total expression of caspase3 and PARP wasn't observed obvious change in MOLM13 cells, but cleaved caspase3 and cleaved PARP were significantly increased in a dose-dependent manner (Fig. 2C). In MV4-11 cells, both total expression and cleaved expression of caspase3 and PARP were increased (Fig. 2C). Western blot demonstrated that increased activation of caspase3-PARP proapoptotic pathways was induced after treatment with DAC for 48 h.

3.3. Decitabine activates C/EBPa-PU.1 pathway

DAC inhibits the DNA methyltransferase (DNMT) thereby allowing to overcome the differentiation blockage in myeloid blasts [20]. MOLM13 and MV4–11 cells were treated with DAC (1 μM and 2 μM) for 48 h. A strong downregulation of DNMT1 and DNMT3b but little change of DNMT3a in western blot were shown after DAC treatment (Fig. 3A). Expression of C/EBPa and PU.1 was significantly increased after DAC treatment in both MOLM13 and MV4-11 cells (Fig. 3A). We also detected the mRNA expression level of PU.1 in MOLM13 and MV4-11 using RT-qPCR (Fig. 3B). The results indicated that the mRNA level of the PU.1 was increased in both leukemic cell lines compared with control cells which was consistent to the results of western blot. Taken together, these data showed that DAC induced expression of C/EBPa and PU.1 in FLT3-ITD positive cells. To test whether the effect of DAC on C/EBPa was due to demethylation, the methylation-specific PCR was performed. After 48 h treatment of DAC(2 μM), methylation level of C/EBPa was decreased in both MOLM13 and MV4–11 cells (Fig. 3C). DAC upregulated the expression of C/EBPa by demethylation, which in turn increased the expression of downstream PU.1. As C/EBPa and its downstream PU.1 were both important molecules to induce differentiation, we tested whether DAC treatment induced differentiation of MOLM13 and MV4-11 cells by detecting the marker of CD11b using flow cytometry. As results were shown, the percentage of positive CD11b was dramatically increased after DAC treatment for 48 h (Fig. 3D). In conclusion, DAC treatment activates C/EBPa-PU.1 pathway to upregulate expression of CD11b in FLT3-ITD positive cells.

3.4. Decitabine induces downregulation of FLT3-ITD by activating C/EBPa-PU.1 pathway to trigger apoptosis

Based on the apoptotic and differentiated effect observed for treatment with DAC in FLT3-ITD mutant cells, we further investigated

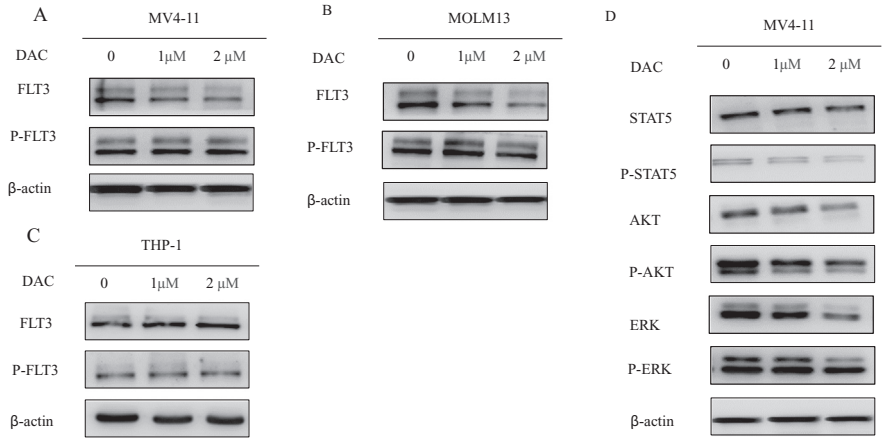

Fig. 1. Decitabine inactivates FLT3 and its downstream pathways. (A-C) Western blot analysis of FLT3 and P-FLT3 in treated MV4–11, MOLM13 and THP-1 cells. MV4–11, MOLM13 and THP-1 cells were treated with DAC (1 μM and 2 μM) for 48 h. DMSO-treated cells served as a control. (D) Western blot analysis of STAT5, P-STAT5, AKT, P-AKT, ERK and P-ERK were tested in MV4–11 cells treated with DAC (1 μM and 2 μM) for 48 h.

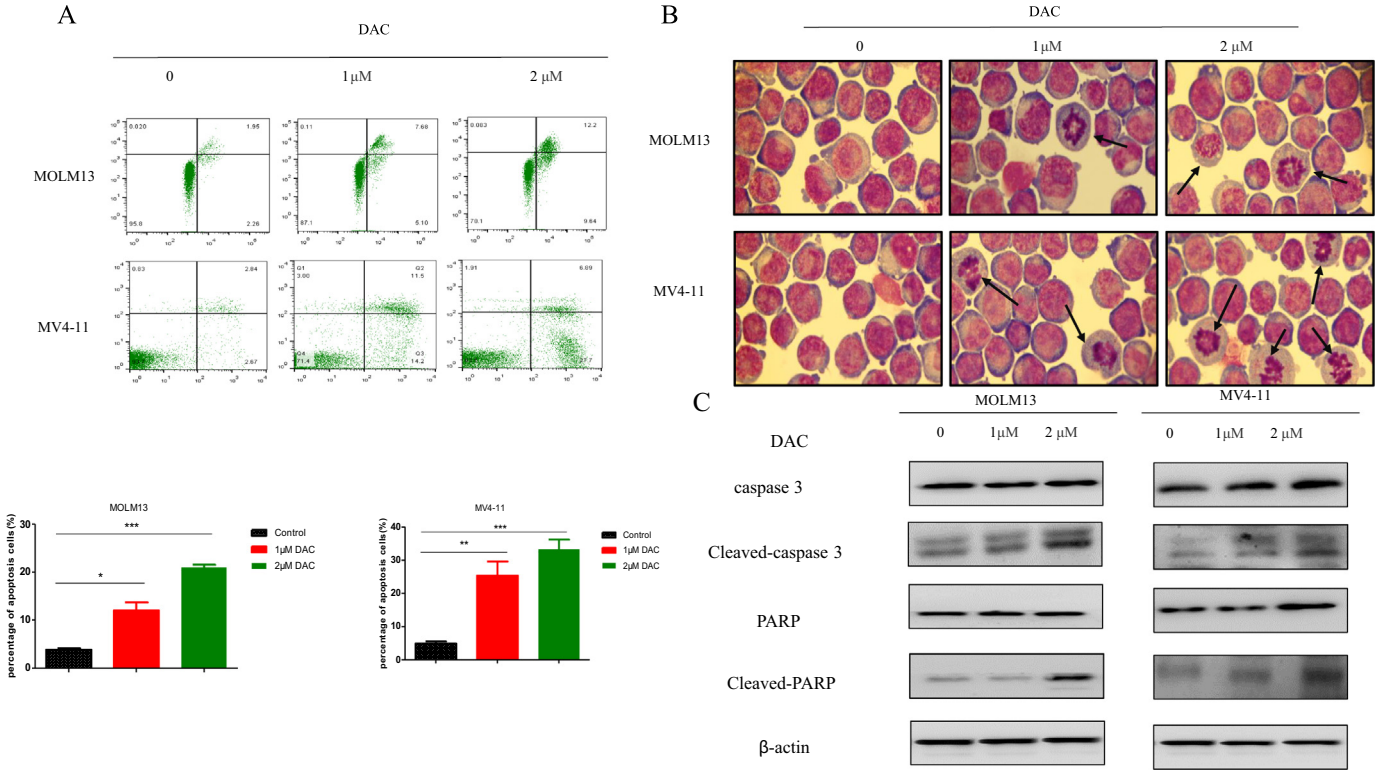

Fig. 2. Decitabine induces apoptosis in MOLM13 and MV4–11 cells. (A) MOLM13 and MV4–11 cells were treated with DAC (1 μM and 2 μM) for 48 h. DMSO-treated cells served as a control. The treated cells were double stained with annexin V-FITC and PI, cell apoptosis was analyzed by flow cytometry. Data are representatives of three independent experiments and expressed as the means ± standard deviation.*$P < .05$, **$P < .01$, ***$P < .001$. (B) Morphological changes in the MOLM13 and MV4–11 treated with DAC (1 μM and 2 μM) for 48 h were observed using light microscopy (Wright's-Giemsa staining; magnification, ×1000). Typical apoptotic characteristics was marked by arrows. (C) MOLM13 and MV4–11 cells were treated with DAC (1 μM and 2 μM) for 48 h. DMSO-treated cells served as a control. Western blot analysis of caspase3, cleaved caspase3, PARP and cleaved PARP in treated MOLM13 and MV4–11 cells.

the underlying molecular mechanism. Previous reports have demonstrated that PU.1 directly regulates FLT3 in a concentration-dependent manner in dendritic cell [17]. We have observed FLT3 protein is reduced and PU.1 is increased by DAC treatment. To explore the effect of increased expression of PU.1 on FLT3 protein, we transiently transfected MOLM13 and MV4-11 cells with siRNA against PU.1 and a siRNA control (NS siRNA). In both FLT3-ITD positive cells, the downregulation of PU.1 expression due to siRNA transfection was corroborated using RT-qPCR at 24 h. As the result was shown in RT-qPCR, PU.1 was effectively knocked down (Fig. 4A, Fig. 5A). MOLM13 and MV4-11 cells transfected with siRNA against PU.1 or a siRNA control were further treated with DAC at 2 μM for 48 h. The effect of DAC on FLT3 downregulation was diminished in PU.1-konckdown MOLM13 and MV4-11 cells. Taken together, the decreased FLT3 expression due to DAC treatment was reversed by knockdown of PU.1 (Fig. 4B, Fig. 5B). Consequently, upon treatment with DAC, there was a decrease of CD11b expression after PU.1 knockdown (Fig. 4C, Fig. 5C). To investigate the effect of PU.1-konckdown on the apoptosis of MOLM13 and MV4-11 cells, we further tested the percentage of apoptotic cells by flow cytometry. The results showed that the percentage of apoptotic cells was also decreased in PU.1-konckdown cells compared with siRNA control-expressing cells with the same dose of DAC (Fig. 4D, Fig. 5D). These findings indicated that DAC upregulated PU.1 which was the downstream signal of C/EBPa to induce downregulation of FLT3 to trigger apoptosis.

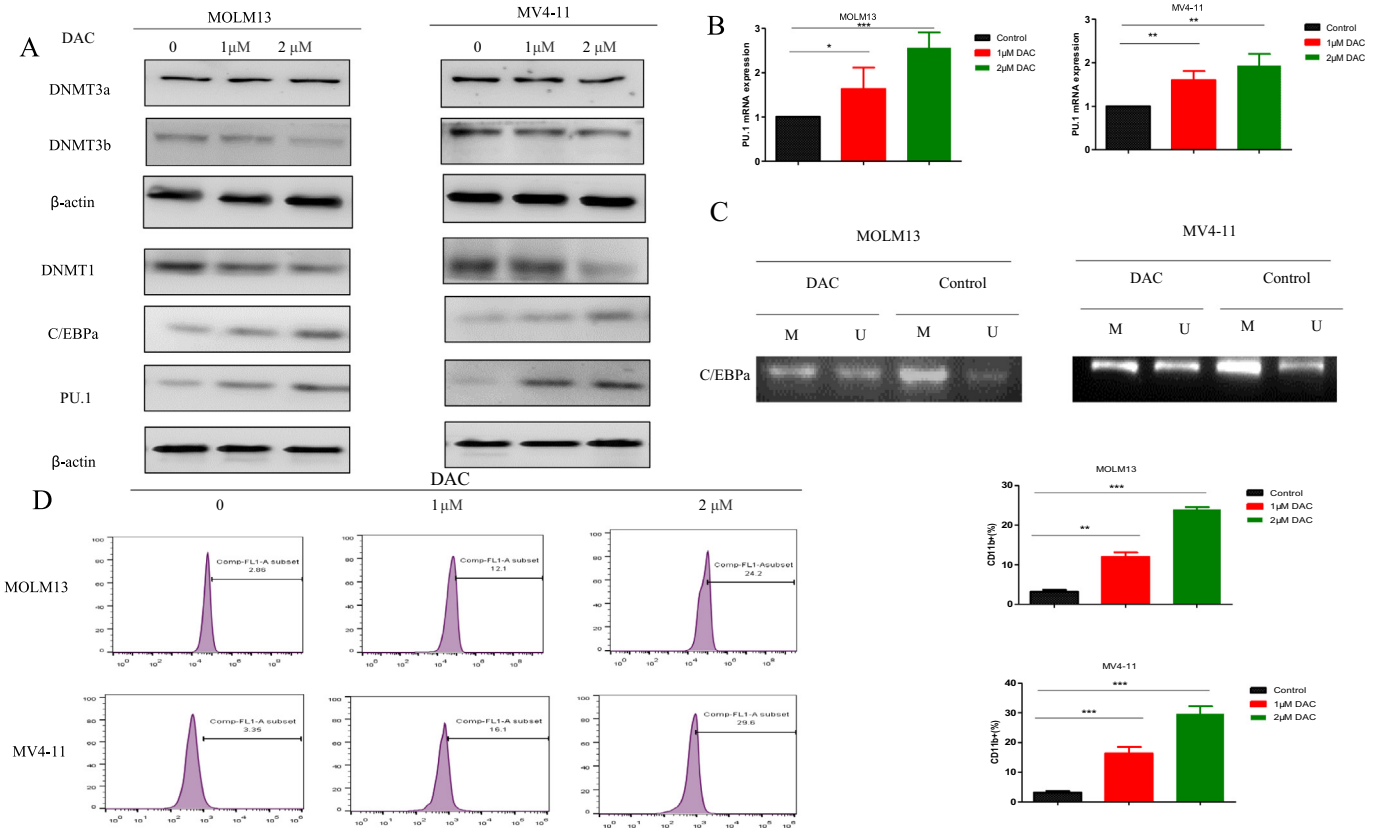

Fig. 3. Decitabine activates C/EBPa-PU.1 pathway. (A) Western blot analysis of DNMT1, DNMT3a, DNMT3b, C/EBPa and PU.1 in treated MOLM13 and MV4–11 cells. MOLM13 and MV4–11 cells were treated with DAC (1 μM and 2 μM) for 48 h. DMSO-treated cells served as a control. (B) mRNA expression of PU.1 was tested by quantitative real time PCR in MOLM13 and MV4–11 cells treated with DAC (1 μM and 2 μM) for 48 h. Data are representatives of three independent experiments and expressed as the means ± standard deviation, *P < .05, **P < .01, ***P < .001. (C) Detection of Methylation Level of C/EBPa in DAC-treated (2 μM) MOLM13 and MV4–11 cells. DMSO-treated cells served as a control. (D) MOLM13 and MV4–11 cells were treated with DAC (1 μM and 2 μM) for 48 h. DMSO-treated cells served as a control. CD11b expression was analyzed by flow cytometry at 48 h. Data are representatives of three independent experiments and expressed as the means ± standard deviation, **P < .01, ***P < .001.

3.5. Decitabine is efficacious in primary FLT3-ITD AML cells and mouse xenograft models of FLT3-ITD AML

Primary FLT3-ITD AML cells were used to test for apoptosis after treatment with DAC. DAC treatment increased the percentage of apoptotic cells in all three primary FLT3-ITD AML cells (Fig. 6A). Furthermore, the level of FLT3 was decreased with increased level of PU.1 after treatment with DAC for 48 h and finally the level of cleaved-caspase3 was increased to trigger apoptosis (Fig. 6B). We next explored the efficacy of DAC treatment in mouse xenograft models of AML in vivo. A xenograft model was established by injecting MV4-11 cells into the flank of nude mouse. The control group was treated with vehicle alone, while the DAC group was treated with DAC(0.1 mg/kg/day) intraperitoneally for two weeks. The intraperitoneal administration of DAC could significantly inhibit the growth of tumor (Fig. 7A). Furthermore, the DAC-treated group exhibited a smaller tumor volume than the vehicle group (Fig. 7B). Western blot of the tumor tissue showed that the FLT3 and its downstream targets(STAT5, ERK, AKT) were downregulated in the DAC-treated group (Fig. 7C). Unlike in vitro results, DAC(0.1 mg/kg/day) have already downregulated phosphorylated FLT3. It suggested that DAC at the dose of 0.1 mg/kg/day in vivo downregulated FLT3 downstream targets (STAT5, ERK, AKT) not only via direct effect but also through inactivating FLT3. Treatment of DAC at 0.1 mg/kg/day for 2 weeks upregulated C/EBPa not as obvious as that of vitro, but still activated PU.1 which was the downstream target of C/EBPa, this indicated that in vivo moderate up-regulation of C/EBPa could activate PU.1(Fig. 7D) and upregulate expression of cleaved caspase3 and cleaved PARP (Fig. 7E). In conclusion, DAC is also efficacious in mouse xenograft models of FLT3-ITD AML.

4. Discussion

Our results first demonstrate that activating C/EBPa-PU.1 signal pathway by DAC is effective to induce FLT3-ITD downregulation in AML cells.

FLT3-ITD was first recognized as a frequently mutated gene in AML in 1996[21]. FLT3-ITD is expressed largely in human dendritic cells and hematopoietic progenitors and plays an essential role in leukemic cell proliferation, survival, and differentiation [22]. The prognosis of AML patients with a FLT3-ITD mutation remains generally poor. Several tyrosine kinase inhibitors (TKIs) targeting FLT3 have been developed and applied in clinical treatment but proven ineffective with worse OS [23].

DNA methyltransferase (DNMT) inhibitor is confirmed effectively in sickle cell anemia, MDS, chronic myeloid leukemia (CML) and AML [24]. FTL3-ITD induces AML mainly through constitutive activation of STAT5, PI3K/AKT, or MAPK/ERK canonical pathways. We first investigated the effect of DAC on downregulation of FLT3 and its downstream targets STAT5, AKT and ERK. DAC at 1 μM and 2 μM could significantly inhibit canonical pathways activated by FLT3-ITD in MV4-11 cells as shown in western blot (Fig. 1D). The percentage of apoptotic cells was also examined by flow cytometry in MOLM13 and MV4-11 cells after treatment with DAC (1 μM and 2 μM) for 48 h. The percentage of apoptotic cells was increased in DAC-treated cells compared to

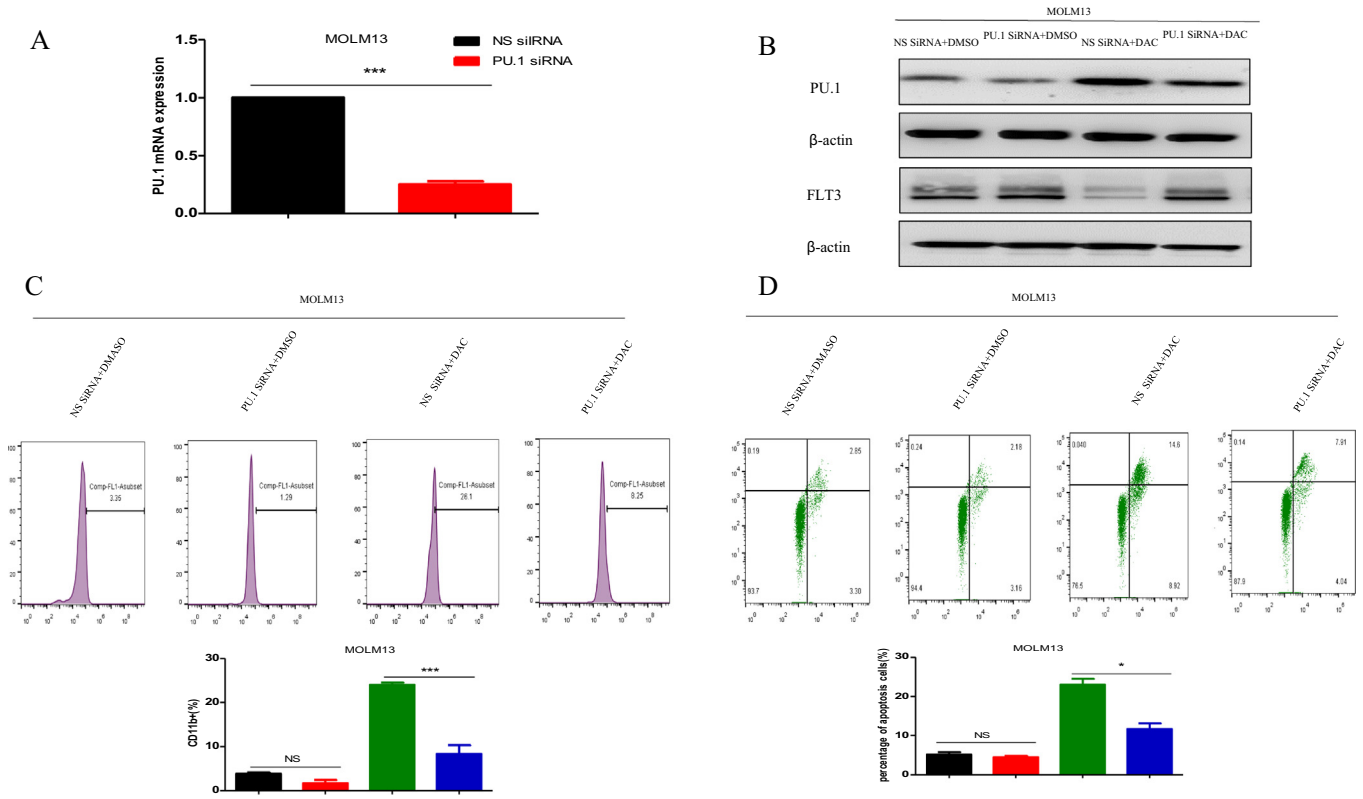

Fig. 4. Decitabine induces FLT3-ITD downregulation by activating C/EBPa-PU.1 pathway to trigger apoptosis in MOLM13 cells. (A)mRNA level of PU.1 due to siRNA transfection was corroborated using RT-qPCR at 24 h. ***P < .001. (B) Western blot analysis of PU.1 and FLT3 in treated MOLM13 cells. MOLM13 cells transfected with siRNA against PU.1 or a siRNA control were treated with DMSO or DAC (2 μM) for 48 h. (C) MOLM13 cells transfected with siRNA against PU.1 or a siRNA control were treated with DMSO or DAC (2 μM) for 48 h. CD11b expression was analyzed by flow cytometry at 48 h.***P < .001. (D) The treated cells were double stained with annexin V-FITC and PI, cell apoptosis was analyzed by flow cytometry. MOLM13 cells transfected with siRNA against PU.1 or a siRNA control were treated with DMSO or DAC (2 μM) for 48 h. Data are representatives of three independent experiments and expressed as the means ± standard deviation. *P < .05.

the control cells (Fig. 2A). Cleaved caspase3 and cleaved PARP was significantly increased in a dose-dependent manner to further confirm the treatment effect of DAC on inducing apoptosis of AML cells (Fig. 2C). That the effective suppression on FLT3-ITD suggests that DAC may serve as a potential drug to treat FLT3-ITD positive AML (Fig. 1A-B). Based on the apoptotic effect observed for treatment with DAC in FLT3-ITD mutant cells, we further investigated the underlying molecular mechanism.

Previous study has revealed that direct depletion of the DNA methyltransferase 1 (DNMT1) from the C/EBPa protein interactome activates terminal granulocytic fates [25]. As the inhibitor of DNMT, the decreased expression of DNMT1 upon DAC treatment on FLT3-ITD mutant MOLM13 and MV4-11 cells was observed (Fig. 3A). C/EBPa is a key myeloid regulator known to play a pivotal role in granulocytic and monocytic differentiation [26] and the expression of PU.1 can be regulated positively by C/EBPa [16]. C/EBPa, which belongs to the C/EBP family is located on chromosome 19 in humans as an intronless gene encoding a 42KD DNA-binding protein and on chromosome 7 in mice encoding a 30KD DNA-binding protein [27,28]. C/EBPa is initially recognized in adipogenesis and later found to be expressed in multiple tissues, not only hematopoietic cells but also the liver, lung, skin, mammary glands, acting as a tumor suppressor [29]. It has been demonstrated that transcription factor C/EBPa has great function in lineage specificity, cell differentiation and cell cycle control in cancer [30,31]. PU.1 is expressed in B cells at low or moderate levels and transiently in early erythroid precursors and also expressed in monocytes/macrophages at highest levels [32]. Moreover, the pathogenesis of various hematological malignancies, including AML [33], multiple myeloma (MM) [34] and MDS [35] is reported to associate with downregulation of PU.1. Several studies also report that PU.1 interacts with DNMT, and regulates its target genes through epigenetic modifications [36]. PU.1 as a novel biomarker to predict the differentiation and apoptotic effects of DAC has been demonstrated in K562 cells [36]. Based on above evidence, we further explored the role of C/EBPa-PU.1 in DAC-treated FLT3-ITD positive cells. After 48 h treatment of DAC, methylation level of C/EBPa was decreased in both MOLM13 and MV4-11 cells (Fig. 3C). DAC upregulated the expression of C/EBPa by demethylation, which in turn increased the mRNA level of PU.1 in RT-qPCR (Fig. 3B) and the expression of PU.1 in western blot (Fig. 3A). DAC treatment activated C/EBPa-PU.1 pathway to upregulate expression of CD11b in FLT3-ITD positive cells (Fig. 3D). To investigate the underlying molecular mechanism, we transiently transfected MOLM13 and MV4-11 cells with siRNA against PU.1 and a siRNA control. MOLM13 and MV4-11 cells transfected with siRNA against PU.1 or a siRNA control were further treated with DAC at 2 μM for 48 h. It is reported that FLT3 can be regulated by PU.1 in a concentration-dependent manner in dendritic cells [17]. In our experiment, we found that the effect of DAC on downregulation of FLT3 was diminished in PU.1-konckdown MOLM13 and MV4-11 cells (Fig. 4B, Fig. 5B). Consequently, upon transfection with siRNA against PU.1, there was a decrease of CD11b expression (Fig. 4C, Fig. 5C). It is found that PU.1 induces apoptosis in myeloma cells [37]. In FLT3-ITD mutant cells, the percentage of apoptotic cells was also decreased in PU.1-konckdown cells compared with siRNA control-expressing cells with the same dose of DAC (Fig. 4D, Fig. 5D). These findings indicated that DAC upregulated PU.1 to induce FLT3 downregulation to trigger apoptosis in FLT3-ITD mutant AML cells. DAC was also efficacious in primary FLT3-ITD AML cells and mouse xenograft models of FLT3-ITD AML. DAC

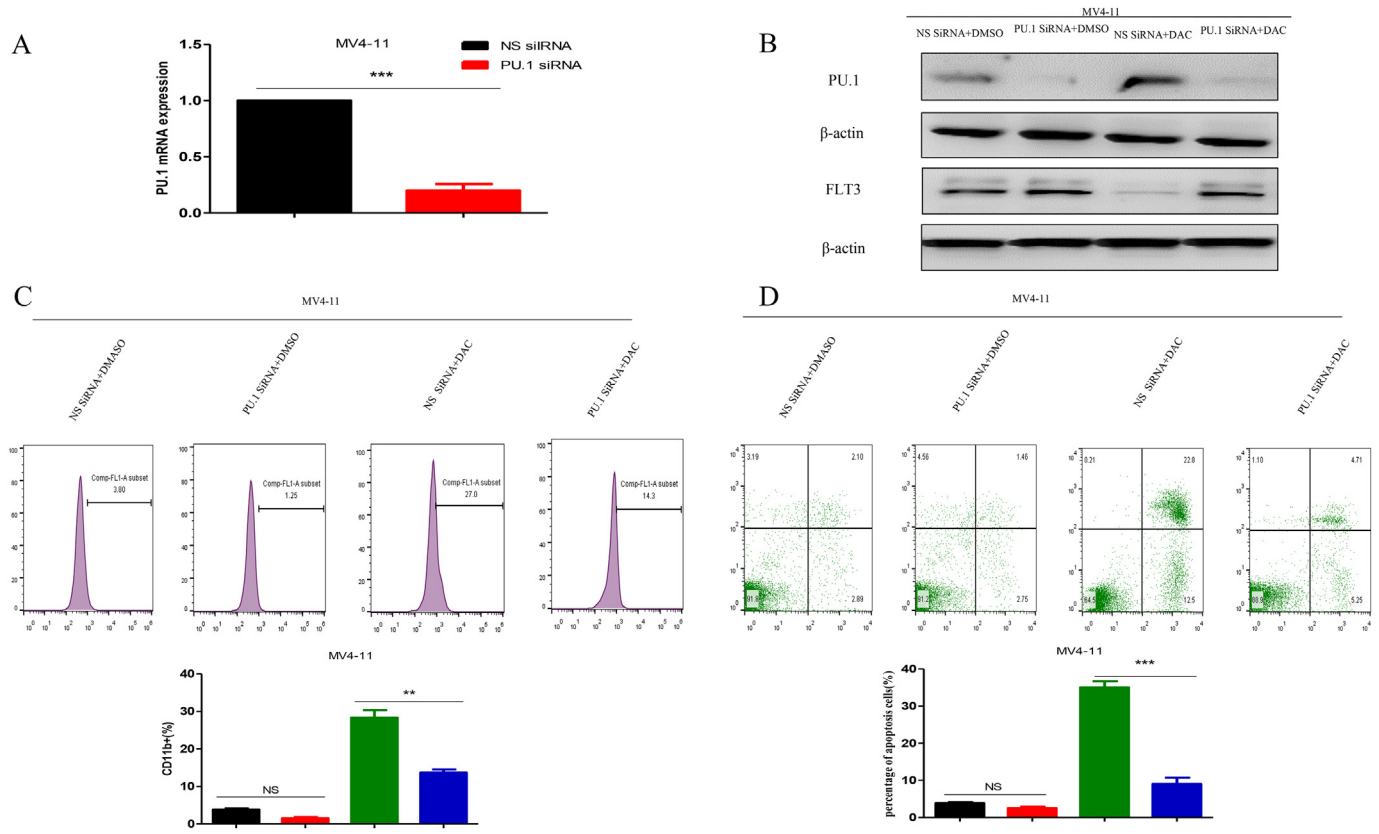

Fig. 5. Decitabine induces FLT3-ITD downregulation by activating C/EBPa-PU.1 pathway to trigger apoptosis in MV4–11 cells. (A) mRNA level of PU.1 due to siRNA transfection was corroborated using RT-qPCR at 24 h. ***P < .001. (B) Western blot analysis of PU.1 and FLT3 in treated MV4–11 cells. MV4–11 cells transfected with siRNA against PU.1 or a siRNA control were treated with DMSO or DAC (2 μM) for 48 h. (C) MV4–11 cells transfected with siRNA against PU.1 or a siRNA control were treated with DMSO or DAC (2 μM) for 48 h. CD11b expression was analyzed by flow cytometry at 48 h.**P < .01. (D) The treated cells were double stained with annexin V-FITC and PI, cell apoptosis was analyzed by flow cytometry. MV4–11 cells transfected with siRNA against PU.1 or a siRNA control were treated with DMSO or DAC (2 μM) for 48 h. Data are representatives of three independent experiments and expressed as the means ± standard deviation. ***P < .001.

treatment increased the percentage of apoptotic cells in all three primary FLT3-ITD AML cells (Fig. 6A). The level of FLT3 was decreased with increased level of PU.1 after treatment with DAC for 48 h and finally the level of cleaved-caspase3 was increased to trigger apoptosis (Fig. 6B).The intraperitoneal administration of DAC could significantly inhibit the growth of tumor (Fig. 7A-B). Western blot of the tumor tissue showed that the FLT3 and its downstream targets(STAT5, ERK, AKT) were downregulated in the DAC-treated group in vivo (Fig. 7C).

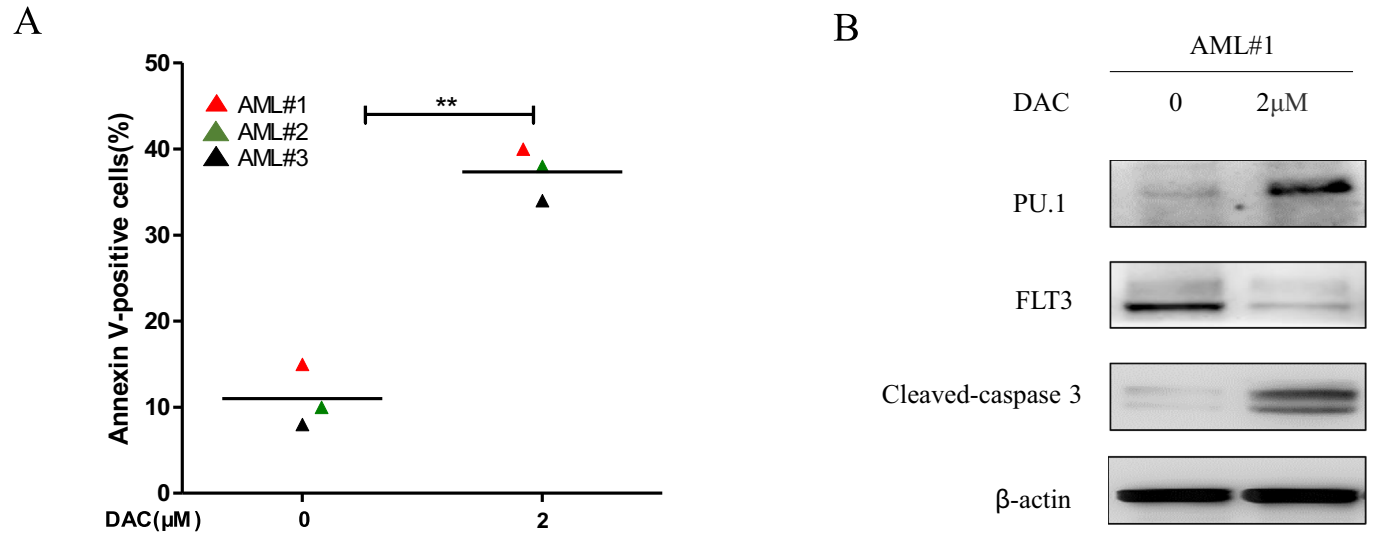

Fig. 6. Decitabine is efficacious in primary FLT3-ITD AML cells. (A) Primary FLT3-ITD leukemic cells from 3 AML patients were treated with DAC for 48 h. Apoptotic cells were detected with Annexin V–FITC using flow cytometry, **P < .01. (B) Western blot analysis of PU.1, FLT3-ITD, Cleaved-caspase 3 and β-actin in sample AML#1.

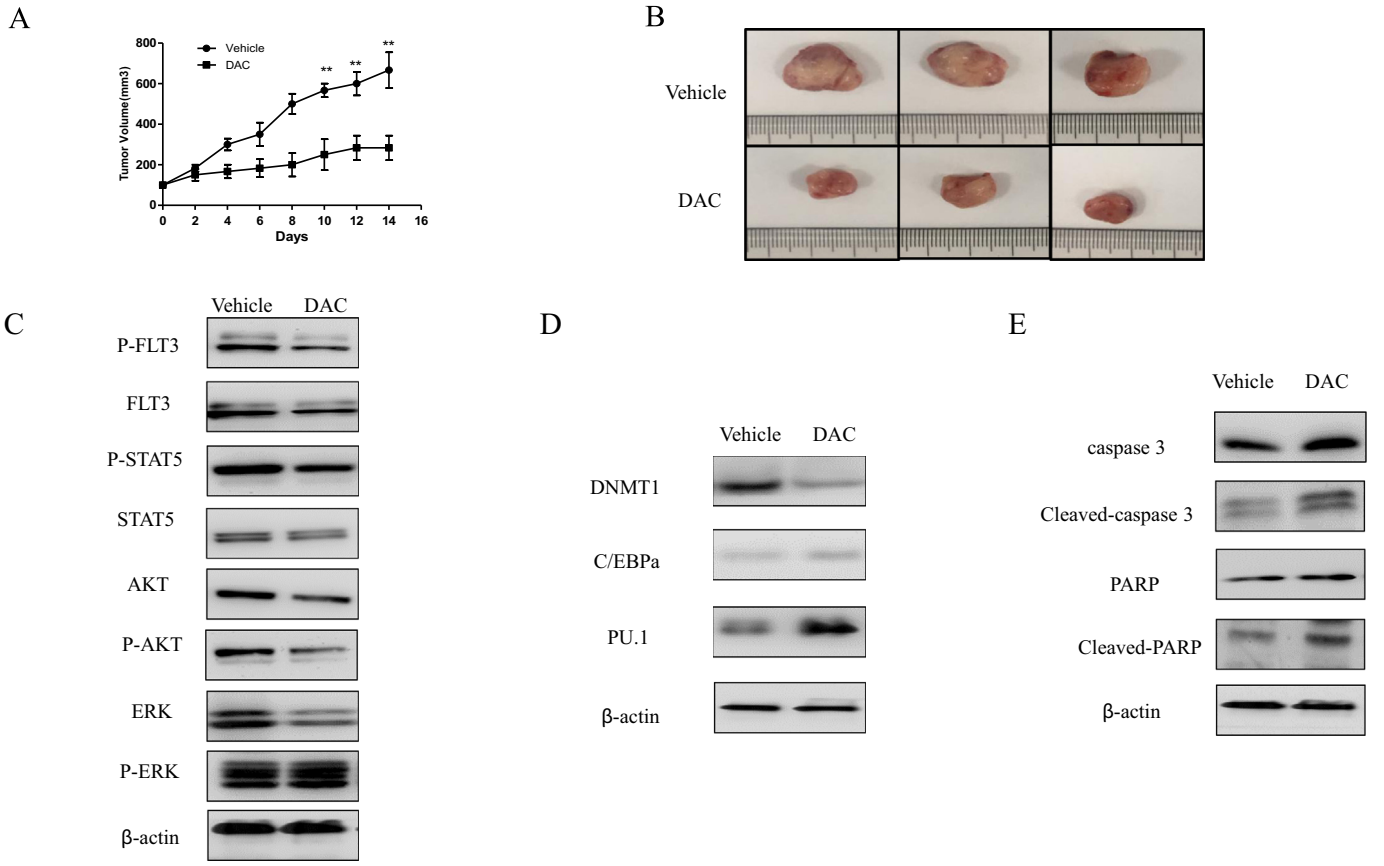

Fig. 7. Decitabine is efficacious in mouse xenograft models of FLT3-ITD AML. (A-B) Six-week-old female nude mice were subcutaneously injected in the flank with 1×10^7 MV4–11 cells to establish a xenograft model of AML. When tumors were established, mice were treated with daily vehicle or intraperitoneal injections of DAC(0.1 mg/kg/d) for 2 weeks. Tumor growth was evaluated by measuring tumors with a caliper. **P < .01.(C) Western blot analysis of FLT3, P-FLT3, STAT5, P-STAT5, AKT, P-AKT, ERK and P-ERK in tumor tissue. (D) Western blot analysis of DNMT1, C/EBPa and PU.1 in tumor tissue. (E) Western blot analysis of caspase3, cleaved caspase3, PARP and cleaved PARP in tumor tissue.

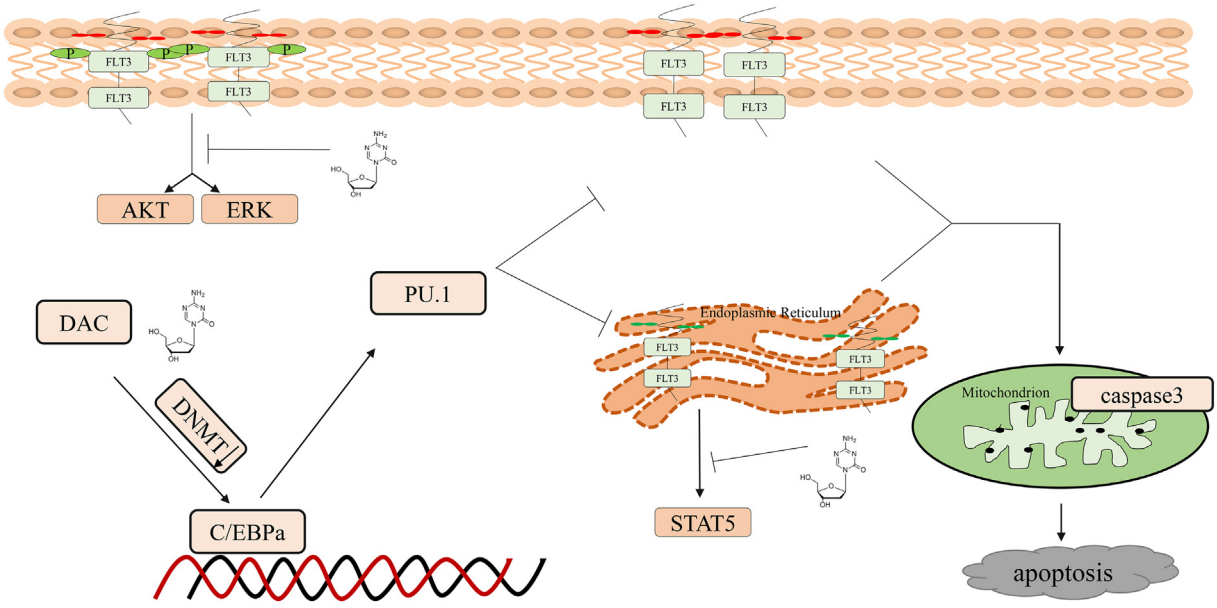

Fig. 8. A schematic model of decitabine (DAC) modulating FLT3 through C/EBPa-PU.1 signal pathway.
DAC blocks constitutive activation of AKT, ERK or STAT5 abnormal pathways triggered by FTL3-ITD. Furthermore, DAC upregulates the expression of C/EBPa by downregulating DNMT to promote demethylation, which in turn increases the expression of downstream PU.1 to induce downregulation of FLT3-ITD to trigger apoptosis.

DAC treatment activated the C/EPBa-PU.1 pathway (Fig. 7D) and up-regulated expression of cleaved caspase3 and cleaved PARP (Fig. 7E).

Our results first demonstrated that DAC activated C/EBPa-PU.1 signal pathway to modulate FLT3 in FLT3-ITD positive cells. The effect of DAC on downregulation of FLT3 is diminished in PU.1-konckdown MOLM13 and MV4–11 cells in PU.1 siRNA experiment. Our research proposes that the effect of PU.1 on FLT3 through induction of differentiation is an interesting point deserved more focus in further study. Furthermore, the percentage of apoptotic cells is decreased in PU.1-konckdown cells compared with siRNA control-expressing cells with the same dose of DAC, but the further mechanisms between downregulation of FLT3 and induction of apoptosis is still worthy to explore (Fig. 8).

In conclusion, DAC induced FLT3-ITD downregulation by activating C/EBPa-PU.1 pathway to trigger apoptosis in FLT3-ITD mutant AML cells and was efficacious to reduce the tumor volume in mouse xenograft models of FLT3-ITD AML. These findings may provide a novel theoretical basis for treatment of FLT3-ITD positive AML patients.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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