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Abstract	Internal tandem duplication in the *FLT3* gene (FLT3/ITD), which is found in patients with acute myeloid leukemia (AML), causes resistance to FLT3 inhibitors. We found that RUNX1, a transcription factor that regulates normal hematopoiesis, is up-regulated in patients with FLT3/ITD$^+$ AML. While RUNX1 can function as a tumor suppressor, recent data have shown that RUNX1 is required for AML cell survival. In the present study, we investigated the functional role of RUNX1 in FLT3/ITD signaling. FLT3/ITD-induced growth factor-independent proliferation and impaired G-CSF-mediated myeloid differentiation in 32D hematopoietic cells, coincident with up-regulation of RUNX1 expression. Silencing of RUNX1 expression significantly decreased proliferation and secondary colony formation, and partially abrogated the impaired myeloid differentiation of FLT3/ITD$^+$ 32D cells. Although the number of FLT3/ITD$^+$ 32D cells declined after incubation with the FLT3/ITD inhibitor AC220, the cells became refractory to AC220, concomitant with up-regulation of RUNX1. Silencing of RUNX1 abrogated the emergence and proliferation of AC220-resistant FLT3/ITD$^+$ 32D cells in the presence of AC220. Our data indicate that FLT3/ITD deregulates cell proliferation and differentiation and confers resistance to AC220 by up-regulating RUNX1 expression. These findings suggest an oncogenic role for RUNX1 in FLT3/ITD$^+$ cells and that inhibition of RUNX1 function represents a potential therapeutic strategy in patients with refractory FLT3/ITD$^+$ AML.

Keywords (separated by '-')	FLT3/ITD - AML - RUNX1 - AC220
Footnote Information	

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ORIGINAL ARTICLE

Internal tandem duplication of FLT3 deregulates proliferation and differentiation and confers resistance to the FLT3 inhibitor AC220 by Up-regulating RUNX1 expression in hematopoietic cells

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Abstract Internal tandem duplication in the *FLT3* gene (FLT3/ITD), which is found in patients with acute myeloid leukemia (AML), causes resistance to FLT3 inhibitors. We found that RUNX1, a transcription factor that regulates normal hematopoiesis, is up-regulated in patients with FLT3/ITD$^+$ AML. While RUNX1 can function as a tumor suppressor, recent data have shown that RUNX1 is required for AML cell survival. In the present study, we investigated the functional role of RUNX1 in FLT3/ITD signaling. FLT3/ITD-induced growth factor-independent proliferation and impaired G-CSF-mediated myeloid differentiation in 32D hematopoietic cells, coincident with up-regulation of RUNX1 expression. Silencing of RUNX1 expression significantly decreased proliferation and secondary colony formation, and partially abrogated the impaired myeloid differentiation of FLT3/ITD$^+$ 32D cells. Although the number of FLT3/ITD$^+$ 32D cells declined after incubation with the FLT3/ITD inhibitor AC220, the cells became refractory to AC220, concomitant with up-regulation of RUNX1. Silencing of RUNX1 abrogated the emergence and proliferation of AC220-resistant FLT3/ITD$^+$ 32D cells in the presence of AC220. Our data indicate that FLT3/ITD deregulates cell proliferation and differentiation and confers resistance to AC220 by up-regulating RUNX1 expression. These findings suggest an oncogenic role for RUNX1 in FLT3/ITD$^+$ cells and that inhibition of RUNX1 function represents a potential therapeutic strategy in patients with refractory FLT3/ITD$^+$ AML.

Keywords FLT3/ITD · AML · RUNX1 · AC220

Introduction

FMS-like tyrosine kinase 3 (FLT3), a class III receptor tyrosine kinase (RTK), has important roles in the survival, proliferation and differentiation of hematopoietic stem cells [1, 2]. The most common form of FLT3 mutation is Internal Tandem Duplication in the juxtamembrane domain of the FLT3 gene (FLT3/ITD) that induces constitutive activation of the FLT3-kinase by destroying the auto-inhibitory function of the kinase domain. FLT3/ITDs are detected in 10–15 % of children and 30 % of adult patients with acute myeloid leukemia (AML) and are associated with extremely poor prognoses [1, 2]. Although a number of antagonists against FLT3/ITD have been developed, few inhibitors are effective for the treatment of FLT3/ITD$^+$ AML because of the emergence of drug-resistant cells [3, 4]. For instance, AC220 (quizartinib), a second-generation class III tyrosine kinase inhibitor (TKI) used in phase II clinical trials [5, 6], is a very potent and specific inhibitor of FLT3/ITD compared with other TKIs; however, FLT3/ITD$^+$ cells can become refractory to AC220 [7]. These findings underscore the need to develop additional therapeutic strategies to overcome the resistance of FLT3/ITD$^+$ AML to TKIs. The mechanisms responsible for drug resistance include the acquisition of mutations in the FLT3 gene, activation of other pro-survival pathways, growth factors

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such as the FLT3 ligand, and microenvironmental-mediated resistance [8–12]. However, the additional mechanisms responsible for the drug resistance of FLT3/ITD$^+$ AML cells remain to be investigated.

While FLT3/ITDs are classified as a "class I mutation" that drives the proliferation of leukemia cells, they also impair the myeloid differentiation of hematopoietic cells by modulating transcriptional factor C/EBPα and Pu.1 [13, 14]. However, in addition to FLT3/ITDs, a "class II mutation" that impairs hematopoietic differentiation is generally required for the development of AML. A previous report demonstrated that the combination of FLT3/ITD and dominant negative RUNX1/ETO induces acute leukemia in mice [15]. RUNX1 is a core-binding transcription factor that plays an important role in hematopoietic homeostasis, particularly in differentiation and proliferation [16, 17]. RUNX1-deficient cells showed increased susceptibility to AML development in collaboration with MLL-ENL, N-Ras and EVI5 [18–20], suggesting that RUNX1 can function as a tumor suppressor in myeloid malignancies. In contrast, RUNX1 also promotes the survival of AML cells and lymphoma development and can function as an oncogene in cancer cells [21–24]. These data suggest the RUNX1 has a dual function that promotes and attenuates the proliferation of hematological malignant cells. In this study, we investigated the functional role of RUNX1 in aberrant cell proliferation, differentiation and drug resistance to an FLT3 inhibitor in FLT3/ITD$^+$ cells. Our data demonstrate that RUNX1 functions as a survival factor in the mouse FLT3/ITD$^+$ cell line.

Materials and methods

Reagents

Recombinant murine granulocyte-colony stimulating factor (G-CSF) and IL-3 were purchased from R&D Systems (Minneapolis, MN, USA). Phycoerythrin (PE)-conjugated anti-mouse CD11b (Mac-1) and APC-conjugated anti-mouse Ly-6G and Ly-6C (Gr-1) antibodies were purchased from BD Biosciences (San Diego, CA, USA). PE-conjugated anti-Runx1 antibody and its isotype rat IgG2a were from eBioscience (San Diego, CA, USA). The FLT3/ITD inhibitor AC220 (quizartinib) and doxycycline were obtained from Selleckchem.com (Houston, TX) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Cell culture, retroviral transduction of FLT3/ITD and shRNA knockdown

The FLT3/ITD (N51)$^+$ 32D cells and FLT3/ITD$^-$ (wild-type FLT3$^+$) 32D cells were generated as described [25]. For shRNA knockdown of RUNX1, FLT3/ITD$^+$ 32D cells were electroporated with shRNA specific to RUNX1 cloned into the pSingle-tTS-shRNA vector (Clontech Laboratories, Mountain View, CA, USA) using a Nucleofector Kit V (Lonza, Basel, Switzerland). The shRNA sequence for RUNX1 was designed by Takara Biotechnology (Otsu, Japan) as follows: RUNX1-shRNA: 5-GTATTTACATAGACCCAAA-3; control shRNA: 5-TCTTAATCGCGTATAAGGC-3; tetracycline-inducible RUNX1 shRNA-A: 5-GGCAGAAACTAGATGATCA-3; RUNX1 shRNA-B: 5′-GTATTTACATAGACCCAAA-3′. These shRNAs did not show any homologous sequence over 75 % identity with the mouse mRNA database except for RUNX1. Stable transformants were selected with 1 mg/ml of geneticin and frozen for storage. To activate the RNA interference (RNAi) of RUNX1, 3 μg/ml of doxycycline (Dox) was added to the culture medium. The cells were cultured in RPMI-1640 plus 10 % Tet System Approved FBS (Clontech Laboratories, Mountain View, CA, USA). The reduction of RUNX1 mRNA and protein was validated by quantitative RT-PCR and intracellular flow cytometry. To generate the AC220-resistant cells, FLT3/ITD$^+$ 32D cells were exposed to stepwise increasing concentrations of AC220 starting with an initial dose of 0.5 nM and ending with a final dose of 10 nM. To determine the cell proliferation, the viable cells were counted with the FACSCalibur cytometer based on the light scatter. For the CFU assay, a total of 1×10^3 cells of FLT3/ITD$^-$ 32D cells and FLT3/ITD$^+$ 32D cells containing control or RUNX1 shRNA were plated on methylcellulose medium containing 30 % FBS in the absence of hematopoietic growth factors. The CFUs were enumerated on day 7 using microscopy.

cDNA microarray and quantitative RT-PCR

Ba/F3 cells containing wild-type FLT3, N51-FLT3/ITD and N78-FLT3/ITD were subjected to cDNA microarray as described [26]. The 40 modulated genes shared by FLT3/ITD$^+$ Ba/F3 cells, human FLT3/ITD$^+$ AML cells and human AML stem cells were classified based on their biological process as defined by Gene Ontology terms using the DAVID program [27], and the significantly enriched functional categories were connected and visualized using Cytoscape [28]. Quantitative RT-PCR was performed as described [26]. The primer sequences used for RT-PCR were as follows:RUNX1-F, 5′-CTACTCTGCCGTCCATCTCC-3′; RUNX1-R, 5′-CGAGGCGCCGTAGTATAGAT-3′; HPRT-F, 5′-TGGACAGGACTGAAAGACTTGCTCG-3′, and HPRT-R, 5′-GGCCACAATGTGATGGCCTCCC-3′.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM), and statistical significance was evaluated

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using the two-tailed Student *t* test in Microsoft Excel (Microsoft Corp., Seattle, WA, USA).

Results

FLT3/ITD up-regulates RUNX1 in human AML cells and mouse hematopoietic cells

We first identified genes that are potentially deregulated and associated with aberrant proliferation and/or differentiation by FLT3/ITD. A comparison of gene expression in Ba/F3 cells expressing different FLT3/ITDs (N51 and N78) [29] and those expressing wild-type FLT3 using microarray analysis identified 1264 (4.1 %) and 569 (1.9 %) mRNAs that are up-regulated and down-regulated by FLT3/ITD, respectively. Similar analyses of the expression of genes that are differentially regulated between FLT3/ITD$^+$ AML ($N = 78$) and FLT3/ITD$^-$ AML ($N = 190$) using a public gene expression profile database (www.ncbi.nlm.nih.gov/geo, accession number GSE1159, HG-U133A) [30] demonstrated that the expression of 2307 (17.4 %) genes was significantly higher and the expression of 1683 (12.7 %) genes was significantly lower in FLT3/ITD$^+$ AML compared to FLT3/ITD$^-$ AML samples. A comparison of the shared genes that are differentially regulated by FLT3/ITD in human AML samples and Ba/F3 cells identified 189 and 93 mRNAs that are up-regulated and down-regulated in both human AML and Ba/F3 cells by FLT3/ITD, respectively. Among these differentially regulated genes, 40 molecules were also deregulated in human AML stem cells compared with the normal hematopoietic stem cells (HSC) [31] (Fig. 1a; Table 1). The functional classification of the 40 genes using the Gene Ontology database demonstrated that they are significantly enriched for the regulation of mitosis, organ development, the immune system, lipid metabolic processes, cell cycle regulation and others (Fig. 1b). Among the 40 genes, RUNX1 mRNA expression was significantly higher in FLT3/ITD$^+$ AML (GSE1159, HG-U133A) and FLT3/ITD$^+$ Ba/F3 cells compared with their FLT3/ITD negative counterparts as well as in AML stem cells compared with HSCs. It is known that three variants of RUNX1 exist in humans (AML1), whereas four variants are present in mice. The Affymetrix Human Genome U133A Array that was used in GSE1159 contains 10 probes that detect either all three variants of RUNX1, 1b plus 1c or 1a alone. The array data demonstrated that all three splice variants of RUNX1 (AML1a, AML1b and AML1c) were up-regulated by in FLT3/ITD$^+$ AML samples compared to FLT3/ITD$^-$ samples (Fig. 1c, top). AML1a expression was elevated by 80 % and was the highest compared to other isoforms. Although the up-regulation of RUNX1 by FLT3/ITD was validated not only in Ba/F3 cells, but also in 32D cells by Q-RT-PCR (Fig. 1c, bottom panel), the sequence redundancy of the four variants of mouse RUNX1 mRNA made it infeasible to specify which isoforms were induced by FLT3/ITD in mouse cells. The overexpression of FLT3/ITD in the IL-3-dependent, non-leukemic, murine myeloid 32D cell line allowed for IL-3-independent proliferation that coincided with a significant increase in RUNX1 mRNA levels (Fig. 1d).

Silencing of RUNX1 expression partially abrogated the growth factor-independent proliferation and differentiation block toward the myeloid lineage induced by FLT3/ITD

Although the loss of RUNX1 functions as class II mutations that deregulate the differentiation of leukemia cells [16, 17], RUNX1 also promotes the survival of AML cells and can function as an oncogene in cancer cells [21–24]. Because RUNX1 expression is up-regulated by FLT3/ITD in AML cells, 32D cells and Ba/F3 cells, we investigated the FLT3/ITD-induced function of RUNX1 in aberrant proliferation, differentiation and drug resistance in 32D cells. 32D myeloid cells can differentiate into neutrophils in response to G-CSF [32] in contrast to pro-B Ba/F3 cells, which have little differentiation potential upon G-CSF stimulation. Furthermore, FLT3/ITDs are normally identified in patients with AML, but not ALL. We therefore performed the subsequent experiments using 32D cells.

Transduction of FLT3/ITD$^+$ 32D cells with RUNX1 shRNA inhibited RUNX1 mRNA and protein expression compared with those transduced with control shRNA (Fig. 2a, upper and middle panel), which coincided with a significant reduction in the number of proliferating cells in the absence of IL-3 (Fig. 2a, lower panel). Similarly, RUNX1 shRNA significantly reduced the number of colony-forming units (CFUs) of FLT3/ITD$^+$ 32D cells in the absence of growth factors (Fig. 2b, left panel). Moreover, RUNX1 shRNA inhibited the formation of secondary CFUs derived from the primary CFUs over-expressing FLT3/ITD (Fig. 2b, right panel).

Because RUNX1 also regulates the differentiation of HSCs [16, 17], we next compared the differentiation of FLT3/ITD$^+$ 32D cells transduced with shRNA against RUNX1 or control shRNA towards the myeloid lineage in the presence of 10 ng/ml of G-CSF. Because IL-3 interferes with differentiation towards the myeloid lineage, all of these experiments were performed in the absence of IL-3 [32]. After 3 days of incubation, the numbers of Gr-1$^+$ and/or Mac-1$^+$ cells, which represent differentiated myeloid cells, increased by 80 % following G-CSF stimulation in FLT3/ITD$^-$ (wild-type FLT3$^+$) 32D cells compared to those before stimulation (Fig. 2c, upper panel). However, G-CSF displayed little effect on the proportion

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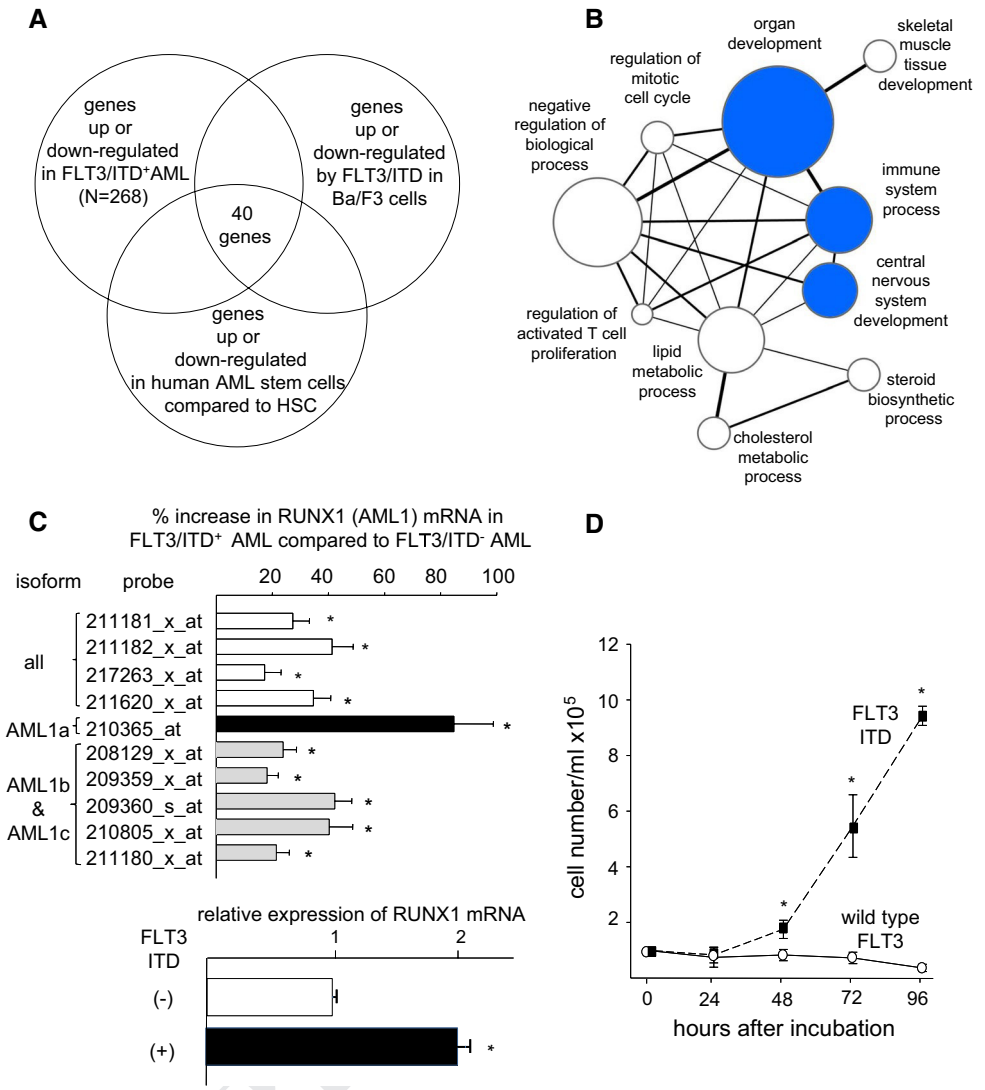

Fig. 1 FLT3/ITD up-regulates RUNX1 in human AML cells and mouse hematopoietic cells. **a** Aberrantly expressed genes by FLT3/ITD and AML stem cells were compared. The deregulated genes by N51-FLT3/ITD and N78-FLT3/ITD in Ba/F3 cells were compared to those differentially expressed between FLT3/ITD$^+$ AML ($N = 78$) and FLT3/ITD$^-$ AML patients ($N = 190$) that have been deposited in GSE1159. These shared genes were compared with the deregulated molecules in human AML stem cells [31], which led to the identification of 40 molecules. **b** The common 40 genes aberrantly regulated by FLT3/ITD and AML stem cells were functionally classified based on the biological process of Gene Ontology terms and visualized. The *circles and the lines* represent the functional groups and the presence of shared molecules between the functional groups, respectively. The *size of the circle* and the *thickness of the lines* demonstrate the number of genes in each group and those of the shared molecules, respectively. The *shaded circles* indicate the functional groups in which RUNX1 was identified. **c** The *top panel* indicates the percentage increase in RUNX1 mRNA in the patients with FLT3/ITD$^+$ AML ($N = 78$) compared to FLT3/ITD$^-$ AML ($N = 190$) that are deposited in GSE1159 [30]. The level of different isoforms (all isoforms, AML1a, AML1b plus AML1c) that had been quantitated using different probes are shown (*$P < 0.05$). The *bottom panel* demonstrates the relative expression of RUNX1 mRNA in FLT3/ITD$^-$ (wild-type FLT3$^+$) 32D cells and FLT3/ITD$^+$ 32D cells as determined by Q-RT-PCR (*$P < 0.05$). D: Proliferation of FLT3/ITD$^-$ (wild-type FLT3$^+$) 32D cells and FLT3/ITD$^+$ 32D cells cultured in the absence of IL-3 for 96 h (*$P < 0.05$)

of Gr-1$^+$ and/or Mac-1$^+$ FLT3/ITD$^+$ 32D cells (Fig. 2c, middle panel), indicating that FLT3/ITD inhibits G-CSF-induced myeloid differentiation. In contrast, transduction of RUNX1 shRNA into FLT3/ITD$^+$ 32D cells increased the proportion of Gr-1$^+$ and/or Mac-1$^+$ cells in the presence of G-CSF (Fig. 2c, lower panel), indicating that silencing RUNX1 expression partially abrogated the differentiation blockage toward myeloid lineage induced by FLT3/ITD.

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Table 1 Shared 40 genes that are deregulated by FLT3/ITD and AML stem cells

Symbol	Name of the gene	Up or down
Anp32b	Acidic leucine-rich nuclear phosphoprotein 32 family member B	Down
Cxcr4	Chemokine (C-X-C motif) receptor 4	Down
Flnb	Filamin B, beta (actin binding protein 278)	Down
Gprasp1	G protein-coupled receptor associated sorting protein 1	Down
Mbnl1	Muscleblind-like (Drosophila)	Down
Nap1l3	Nucleosome assembly protein 1-like 3	Down
Nfib	Nuclear factor I/B	Down
Ppt1	Palmitoyl-protein thioesterase 1	Down
Rasgrp1	RAS guanyl releasing protein 1	Down
Rgs2	Regulator of G-protein signaling 2, 24 kDa	Down
Stat5b	Signal transducer and activator of transcription 5B	Down
Stom	Stomatin	Down
Styk1	Serine/threonine/tyrosine kinase 1	Down
Vamp5	Vesicle-associated membrane protein 5	Down
Abhd2	Abhydrolase domain containing 2	Up
Cat	Catalase	Up
Ctsc	Cathepsin C	Up
Cul7	Cullin 7	Up
Ddx19a	DEAD (Asp-Glu-Ala-As) box polypeptide 19A	Up
Fdft1	Farnesyl-diphosphate farnesyltransferase 1	Up
Gart	Phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	Up
Glrx2	Glutaredoxin 2	Up
Gnl3 l	Guanine nucleotide binding protein-like 3 (nucleolar)-like	Up
Hsd17b7	Hydroxysteroid (17-beta) dehydrogenase 7	Up
Hspa4	Heat shock 70 kDa protein 4	Up
Hus1	HUS1 checkpoint homolog (*S. pombe*)	Up
Ide	Insulin-degrading enzyme	Up
Il1rap	Interleukin 1 receptor accessory protein	Up
Il2ra	Interleukin 2 receptor, alpha	Up
Kctd13	Potassium channel tetramerization domain containing 13	Up
Lrp8	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	Up
Lsm12	lsm12 homolog (*S. cerevisiae*)	Up
Lss	Lanosterol-synthase	Up
Pacsin2	Protein kinase C and casein kinase substrate in neurons 2	Up
Ptbp1	Polypyrimidine tract binding protein 1	Up
Rabl3	RAB, member of RAS oncogene family like 3	Up
Runx1	Runt-related transcription factor 1	Up
Slc7a6	Solute carrier family 7, member 6	Up
Spon2	Spondin 2, extracellular matrix protein	Up
Wdr74	WD repeat domain 74	Up

Silencing of RUNX1 expression enhances the cytotoxic effect of AC220 and abrogates the proliferation of FLT3/ITD$^+$ 32D cells refractory to AC220

Long-term exposure of AC220 or other TKIs can induce the emergence of drug-resistant FLT3/ITD$^+$ cells [3, 4, 7]. Because RUNX1 enhances the aberrant proliferation of FLT3/ITD$^+$ cells, we investigated whether RUNX1 is involved in the resistance to AC220 in FLT3/ITD$^+$ cells. The silencing of RUNX1 expression with shRNA resulted in a 70 % reduction of FLT3/ITD$^+$ 32D cells that proliferated in the absence of growth factors at 96 h (Fig. 3a).

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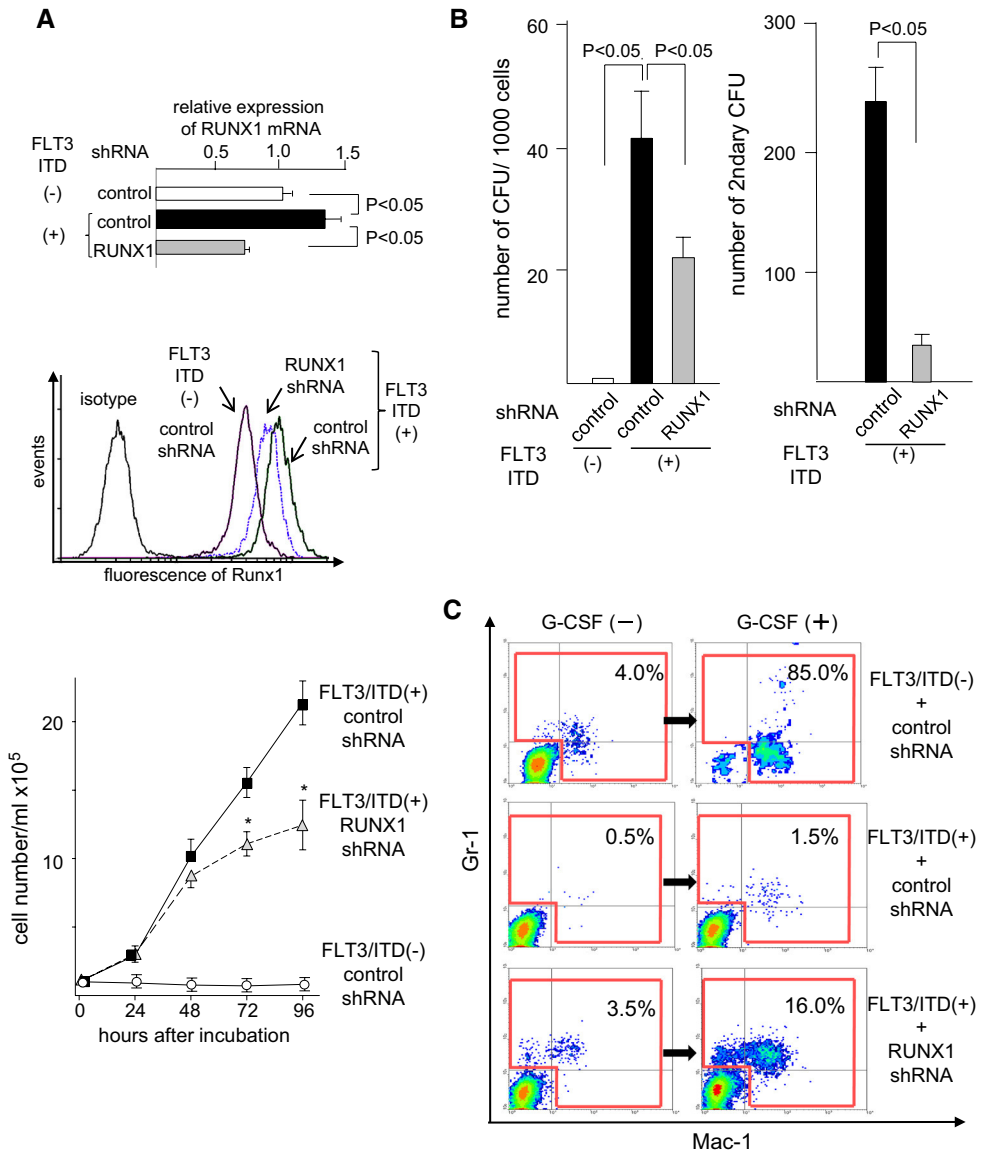

Fig. 2 Silencing of RUNX1 expression partially abrogated growth factor-independent proliferation and impaired differentiation toward the myeloid lineage induced by FLT3/ITD. **a** The proliferation of FLT3/ITD$^-$ (wild-type FLT3$^+$) 32D cells, FLT3/ITD$^+$ 32D cells containing RUNX1 shRNA and control shRNA was examined in the absence of IL-3 (lower panel, $*P < 0.05$). The *upper and middle panel* show the relative expression of RUNX1 mRNA and protein as determined by Q-RT-PCR and intracellular flow cytometry, respectively, in FLT3/ITD$^-$ 32D cells, FLT3/ITD$^+$ 32D cells containing control or RUNX1 shRNA. **b** A total of 1×10^3 cells of FLT3/ITD$^-$ 32D cells, FLT3/ITD$^+$ 32D cells containing control or RUNX1 shRNA were plated on methylcellulose medium containing 30 % FBS in the absence of hematopoietic growth factors. The CFUs were enumerated on day 7 by microscopy (*left panel*). The primary colonies were scrambled and subjected to a secondary colony-forming assay in the absence of growth factors as shown in the right panel. The colonies were enumerated on day 7. The data shown are representative of three experiments with identical results. **c** Cells stained with Gr-1 and Mac-1 antibodies were subjected to flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) and analyzed by CellQuest Pro software (BD Biosciences). The proportion of Gr-1$^+$ and/or Mac-1$^+$ cells was quantitated using flow cytometry in FLT3/ITD$^-$ or FLT3/ITD$^+$ 32D cells containing control or RUNX1 shRNA following incubation with 10 ng/ml of G-CSF for 72 h. The *left column* represents the cells prior to incubation with G-CSF, and the *right column* indicates those after incubation with G-CSF. The data shown are representative plots from 2 experiments with identical results

Similarly, incubation of the FLT3/ITD$^+$ 32D cells with 0.5 nM AC220 inhibited their factor-independent proliferation by 95 %, which was further increased to 99 % when combined with shRNA against RUNX1 (Fig. 3a). These data indicate that the silencing of RUNX1 enhances the cytotoxic effects of AC220 in FLT3/ITD$^+$ 32D cells. To determine the function of RUNX1 in AC220 resistance induced by FLT3/ITD, FLT3/ITD$^+$ 32D cells containing

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two different doxycycline-inducible shRNAs against RUNX1 and those harboring the control shRNA were exposed to AC220 along with doxycycline in the absence of IL-3. Incubation of the cells with 0.5 nM AC220 resulted in a gradual decline in the number of viable cells in all of the groups at 72 h, whereas FLT3/ITD$^+$ 32D cells containing the control shRNA re-proliferated after 72 h. In contrast, transduction of two different RUNX1 shRNAs decreased the expression of RUNX1 mRNA and protein and inhibited the emergence of AC220-resistant FLT3/ITD$^+$ cells (Fig. 3b). The data indicate that FLT3/ITD$^+$ 32D cells became resistant to AC220, whereas RUNX1 shRNA abrogated the emergence of AC220-resistant FLT3/ITD$^+$ 32D cells. Q-RT-PCR data demonstrated that the expression of RUNX1 in AC220-resistant cells was significantly higher compared to AC220 sensitive cells (Fig. 3c, left, $P < 0.05$). We therefore determined whether antagonizing RUNX1 could abolish the proliferation of refractory FLT3/ITD$^+$ cells to AC220. Incubation of the FLT3/ITD$^+$ 32D cells containing two different doxycycline-inducible shRNA for RUNX1 or control shRNA with stepwise increasing concentration of AC220 to 2 nM in the absence of doxycycline for 4 weeks allowed the cells proliferate in the presence of AC220 (Fig. 3d, top inset). These AC220-refractory cells were then incubated with 2 nM of AC220 in the presence of doxycycline to induce the shRNA against RUNX1 to decrease expression of RUNX1 mRNA and protein (Fig. 3b, inset). The number of FLT3/ITD$^+$ 32D cells containing control shRNA gradually increased as expected; however, the number of those with two different shRNAs against RUNX1 was significantly reduced (Fig. 3d), indicating that silencing RUNX1 abrogates the proliferation of AC220-refractory FLT3/ITD$^+$ cells. Moreover, escalating the dose of AC220 up to 10 nM allowed the refractory FLT3/ITD$^+$ 32D cells to proliferate; however, cells in which RUNX1 expression is silenced failed to grow in the presence of AC220 greater than 2 nM.

Induction of RUNX1 in the AC220 resistant FLT3/ITD$^+$ 32D cells is mediated by a non-mutational mechanism

A previous report demonstrated that the resistance to AC220 by FLT3/ITD$^+$ AML cells was induced by the additional mutations in the kinase domain of the FLT3 gene [7–12]. Because AC220 resistance was mediated by RUNX1 expression, we investigated whether additional DNA mutations were responsible for the induction of RUNX1 expression in the resistant cells to AC220. The sequence of the kinase domain of ectopic FLT3 in the AC220 refractory FLT3/ITD$^+$ 32D cells was identical to the parental FLT3/ITD$^+$ 32D cells. Moreover, the RUNX1 gene sequence including all exons as well as exon/intron boundaries in AC220 refractory FLT3/ITD$^+$ 32D cells was identical to the parental FLT3/ITD$^+$ 32D cells and those reported in C57BL/6 J mice (NCBI: NC_000082). To address whether additional DNA mutations exist in the refractory cells, we next determined if induction of RUNX1 expression by the AC220 resistant cells was reversible upon withdrawal of AC220. If mutations were responsible for the up-regulation of RUNX1, its up-regulation should be irreversible even after withdrawal of AC220. While RUNX1 mRNA expression was significantly elevated in the AC220 refractory FLT3/ITD$^+$ 32D cells cultured in the presence of AC220 compared to parental FLT3/ITD$^+$ cells, AC220 withdrawal significantly decreased its expression in the AC220 refractory FLT3/ITD$^+$ 32D cells, which was comparable to those in parental FLT3/ITD$^+$ cells (Fig. 3c, right).

Discussion

The present study demonstrates that silencing RUNX1 in FLT3/ITD$^+$ cells partially normalized impaired differentiation, decreased aberrant proliferation and secondary colony formation and enhanced the cytotoxic effects of AC220 in FLT3/ITD$^+$ 32D cells. AC220 resistance was associated with a significant, but reversible elevation of RUNX1 expression in FLT3/ITD$^+$ cells. Moreover, the silencing of RUNX1 abrogated the emergence and proliferation of AC220-resistant FLT3/ITD$^+$ 32D cells, indicating that FLT3/ITD confers resistance to AC220 by up-regulating RUNX1 expression. These findings suggest that RUNX1 can function as an oncogene and that antagonizing RUNX1 may represent a potential therapeutic strategy in the patients with AC220-refractory FLT3/ITD$^+$ AML.

Our study identified the shared molecules that are deregulated by FLT3/ITD in patients with AML and in murine cell lines. A comparison of these molecules with those deregulated by human AML stem cells identified 40 molecules, which were associated with the immune system process, organ development, cell cycle, lipid metabolic process and other functional categories, suggesting that these functions may be crucial for FLT3/ITD$^+$ AML cells. RUNX1, a transcription factor that regulates hematopoietic homeostasis, particularly differentiation and proliferation, was up-regulated by FLT3/ITD in AML, Ba/F3, and 32D cells. Consistent with our data, up-regulation of RUNX1 by FLT3/ITD was also reported [33]. In human FLT3/ITD$^+$ AML analysis, all three variants of RUNX1 (AML1a, 1b and 1c) were up-regulated. However, AML1a expression, an antagonistic isoform for AML1b and 1c that negatively regulates proliferation and differentiation of normal hematopoietic cells [34], was higher compared to the other two isoforms. The dominant increase of AML1a in FLT3/ITD$^+$ AML may be associated with enhanced proliferation and/or blocked differentiation in AML cells.

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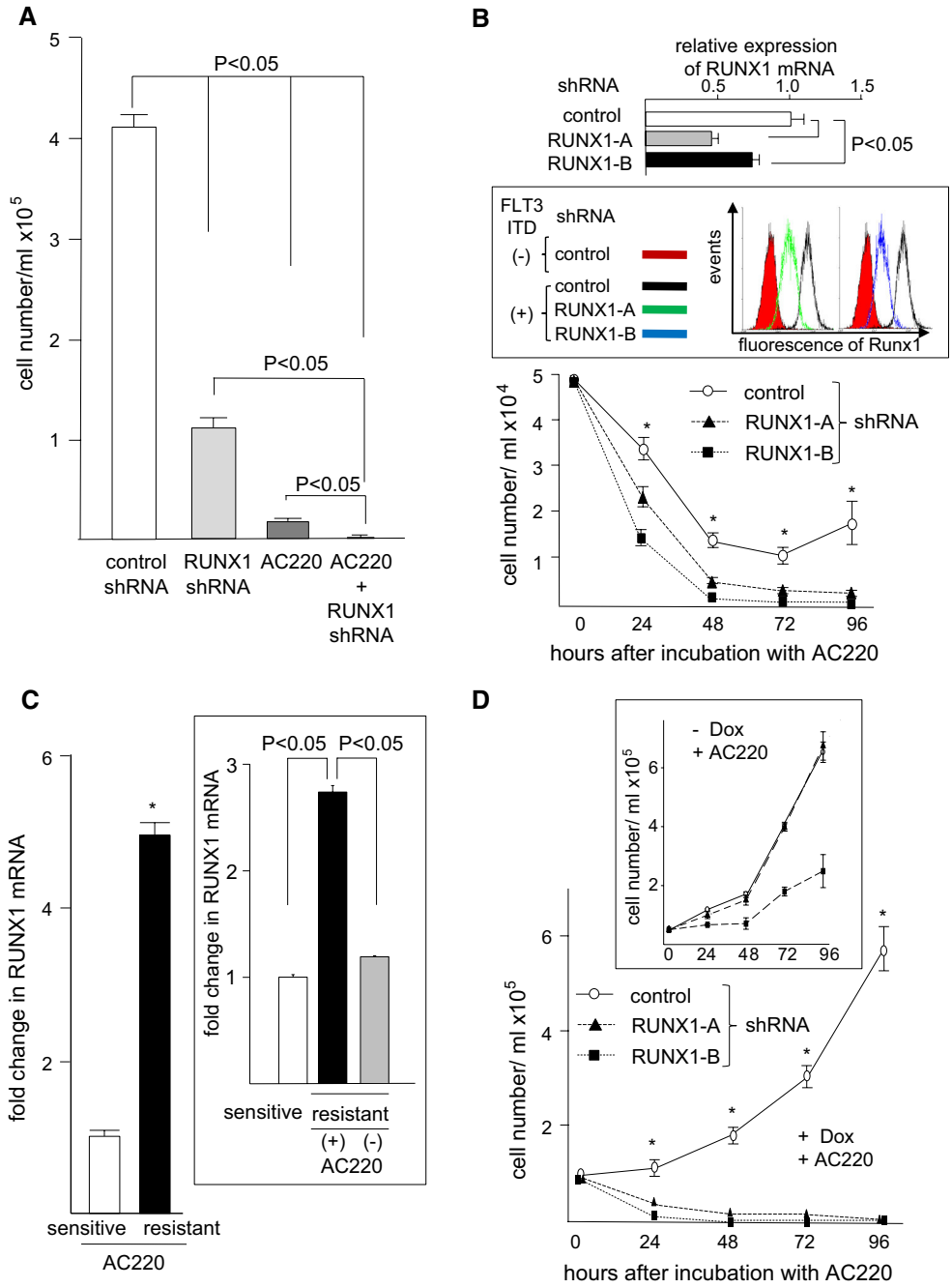

The reduction in the number of enhanced factor-independently proliferating FLT3/ITD$^+$ cells, in which RUNX1 expression is silenced, demonstrates that RUNX1 promotes the factor-independent proliferation of hematopoietic cells that are induced by FLT3/ITD, which supports a pro-survival role of RUNX1 in FLT3/ITD$^+$ cells. This is consistent with a recent report demonstrating that shRNA-mediated inhibition of RUNX1 reduced the colony-forming ability of human FLT3/ITD$^+$ MV4-11 cells, but not in THP-1 cells containing wild-type FLT3 [23], although this is in contrast with the accelerated death caused by loss of the RUNX1 gene in FLT3/ITD knock-in mice (FLT3/ITD/RUNX$^{fx/fx}$/Cre mice) [35]. While inactivating mutations of RUNX1, such as RUNX1-ETO, TEL-RUNX1 and RUNX1 point mutations are associated with leukemia [22, 36–38], recent reports demonstrated the dual role of RUNX1 in "promoting" and "attenuating" proliferation of leukemia or cancer cells. For instance, Down syndrome-related acute megakaryoblastic leukemia may be caused by an extra copy of RUNX1 because RUNX1 is located on chromosome 21 [39]. Amplification of RUNX1 was also reported in pediatric ALL and associated with a higher risk of relapse and

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◀ **Fig. 3** Silencing of RUNX1 expression enhances the cytotoxic effect of AC220 and abrogates the proliferation of FLT3/ITD$^+$ 32D cells refractory to AC220. **a** The cell number of FLT3/ITD$^+$ 32D cells containing RUNX1 shRNA and/or those incubated with 0.5 nM AC220 were compared with FLT3/ITD$^+$ 32D cells containing control shRNA incubated with DMSO control. The cells were incubated for 96 h in the absence of growth factors. Data are representative of three experiments with identical results. **b** The proliferation of FLT3/ITD$^+$ 32D cells containing control shRNA and FLT3/ITD$^+$ 32D cells transduced with two different tetracycline-inducible shRNAs for RUNX1 (**a**, **b**) was determined after incubation with 0.5 nM of AC220 and 3 μg/ml of doxycycline in the absence of IL-3 for 96 h (*$P < 0.05$). The *inset* shows the expression of RUNX1 mRNA and the protein that was analyzed by intracellular flow cytometry in the cells incubated with 3 μg/ml of doxycycline. The green and blue line represents the expression of RUNX1 protein in the cells transfected with Runx1-A and Runx1-B shRNA, respectively. **c** The expression of RUNX1 mRNA in AC220-resistant FLT3/ITD$^+$ 32D cells was compared with AC220-sensitive FLT3/ITD$^+$ cells using Q-RT-PCR (*$P < 0.05$). The *right inset* shows the expression of RUNX1 mRNA in the FLT3/ITD$^+$ cells that became refractory to 2 nM AC220, but incubated without AC220 for 96 h, compared to those maintained in 2 nM AC220. The RUNX1 mRNA expression in the AC220 sensitive cells is shown as the control. **d** Following incubation with escalating doses of AC220 in the absence of doxycycline and IL-3, FLT3/ITD$^+$ 32D cells that are refractory to 2 nM of AC220 were established, each of which contains control shRNA, RUNX1 shRNA-A or RUNX1 shRNA-B under the control of a tetracycline-inducible element. Their proliferation was quantitated in the presence of 3 μg/ml of doxycycline and 2 nM AC220 without IL-3 (*$P < 0.05$). The *inset* shows proliferation of the same cells incubated in the presence of AC220 without doxycycline

death [40–43]. Another study demonstrated that RUNX1 promotes lymphoma development and other types of cancers [24, 44]. Overexpression of RUNX1 correlates with poor prognosis of the patients with breast cancers [45]. Suppressing RUNX1 expression using shRNA inhibited the proliferation of MLL-AF4 leukemia cells [23] and AML cells [21]. These reports clearly demonstrate that in addition to acting as a tumor suppressor, RUNX1 has oncogenic functions and promotes the survival of hematopoietic malignancies.

Similar to human RUNX1, mouse RUNX1 mRNA contains 4 isoforms, although their functional role remains unknown. Some isoforms may antagonize others, as reported in human RUNX1 [34]. In this regard, complete deletion of RUNX1 in FLT3/ITD/RUNX$^{fx/fx}$/Cre mice can entirely eliminate the function of RUNX1, including inhibitory isoforms as well as antagonistic isoforms, whereas shRNA-mediated inhibition of RUNX1, which did not completely eliminate the expression of RUNX1, may alter the relative ratio of each isoform that may be mutually antagonistic. If this was the case, depending on the remaining level of each isoform, the overall function of RUNX1 may not be identical with the complete RUNX1 deficient cells. Although our shRNAs were designed to inhibit all 4 isoforms of mouse RUNX1 mRNA, their sequence redundancy did not allow quantitation of each variant before and after shRNA induction in the FLT3/ITD$^+$ cells. Alternatively, the differential phenotype may be explained by the dosage effect of RUNX1, as suggested in a previous report [21]. Complete loss of RUNX1 may transcriptionally abrogate the tumor suppressor gene expression or enhance the expression of genes that promote tumor progression. For instance, it was reported that knocking out RUNX1 deletion increases the expression of RUNX2 that could compensate for the loss of RUNX1, which may increase leukemia cell proliferation [21], whereas shRNA-mediated inhibition of RUNX1 may not be sufficient for the induction or suppression of other genes that can enhance cell proliferation. Finally, both shRNAs used in our experiments that target two different regions of RUNX1 mRNA successfully decreased RUNX1 protein and mRNA and displayed an identical inhibitory effect on proliferation of FLT3/ITD$^+$ cells. Although an off-target effect was not completely ruled out, searching the mouse mRNA database deposited in GenBank did not show any homologous sequence over 75 % identity with our RUNX1 shRNAs. Taken together, our data provide additional evidence that RUNX1 can function as an oncogene to support the proliferation and survival of FLT3/ITD$^+$ cells, although the detailed mechanism that confers its dual function remains a future subject of investigation.

In addition to promoting cell proliferation, FLT3/ITD also inhibits the myeloid differentiation induced by G-CSF by down-regulating the expression of C/EBPα and PU.1, [13, 14]. Our data demonstrate that the knockdown of RUNX1 partially normalized the differentiation block toward myeloid lineage, indicating that FLT3/ITD inhibits myeloid differentiation through RUNX1 up-regulation. The inhibition of terminal differentiation mediated by RUNX1 in FLT3/ITD$^+$ cells is in contrast to the cell differentiation-inducing role of RUNX1 in normal hematopoiesis, suggesting that the function of RUNX1 in FLT3/ITD$^+$ cells may be distinct from normal hematopoietic cells. Alternatively, the shRNA in the present study might have altered the relative expression level of isoforms that may be mutually antagonistic, which may be associated with the opposing phenotype in FLT3/ITD$^+$ cells versus normal hematopoietic cells. A significant decrease in secondary colonies derived from primary FLT3/ITD$^+$ CFU by the silencing of RUNX1 suggests that RUNX1 facilitates the self-renewal of FLT3/ITD$^+$ cells, another important biological behavior of AML associated with disease progression. The enhancement of self-renewal by RUNX1 may represent one of the mechanisms responsible for its involvement in blocking the terminal differentiation of FLT3/ITD$^+$ cells.

Although AC220 is one of the most effective TKIs for FLT3/ITD$^+$ AML that is used in phase II clinical trials [5, 6], FLT3/ITD$^+$ 32D cells developed resistance to AC220 in our experiments, which is consistent with a previous

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report [7–12]. Our data indicate that antagonizing RUNX1 not only increases the sensitivity of FLT3/ITD$^+$ cells to AC220, but also inhibits the emergence of FLT3/ITD$^+$ 32D cells that are refractory to AC220, indicating that the FLT3/ITD-mediated up-regulation of RUNX1 confers resistance to AC220 in FLT3/ITD$^+$ cells. Interestingly, the expression of RUNX1 in AC220 was significantly elevated in the AC220-resistant FLT3/ITD$^+$ 32D cells compared to the sensitive cells. A previous report demonstrated that the resistance to AC220 by FLT3/ITD$^+$ AML cells was induced by the additional mutations in the kinase domain of FLT3 gene [7–12]; however, our data suggest that RUNX1 up-regulation by AC220 resistant cells was unlikely to be a consequence of additional mutations because up-regulation of RUNX1 by the AC220 refractory cells was reversible. In this regard, an epigenetic mechanism is likely involved in the up-regulation of RUNX1 by the AC220-refractory cells. Similar to FLT3/ITD, FLT3-ligand may also induce expression of RUNX1, which is one of the factors related to drug resistance [11]. Given that RUNX1 is a transcription factor, it is highly likely that down-stream molecules of RUNX1 are also responsible for the resistant phenotype. Consistent with the up-regulation of RUNX1 expression in the resistant cells, the silencing of RUNX1 abrogated the proliferation of AC220-resistant FLT3/ITD$^+$ 32D cells cultured in the presence of AC220, indicating that RUNX1 inhibition can sensitize the AC220-resistant FLT3/ITD$^+$ AML cells to AC220. However, the IC50 of AC220 in the patients' plasma may be higher than 2 nM. While FLT3/ITD$^+$ cells became resistant to 10 nM of AC220 in in vitro culture, cells in which RUNX1 expression is silenced failed to proliferate at any concentration of AC220 over 2 nM. These data suggest that the resistance of FLT3/ITD$^+$ cells to a higher concentration of AC220 is also mediated by RUNX1 and that a higher dose of AC220 unlikely overcomes the resistance that is mediated by RUNX1. In this regard, RUNX1 may represent a novel therapeutic target for FLT3/ITD$^+$ AMLs (Fig. 4). However, targeting RUNX1 may cause several adverse effects in vivo [46]. Although loss of RUNX1 minimally impacts long-term HSC [47], deletion of RUNX1 in adult HSCs causes multi-lineage blocks in B and T lymphoid development and megakaryocyte maturation [16]. Because of the possible adverse effects that may be caused by RUNX1 inhibition, targeting the transcriptional targets of RUNX1 may represent an alternative therapeutic strategy.

In summary, FLT3/ITD blocks myeloid differentiation, enhances aberrant proliferation and self-renewal, and confers resistance to AC220 by up-regulating RUNX1 expression (Fig. 4). Our data suggest that RUNX1 functions as a survival factor in FLT3/ITD$^+$ cells and that targeting RUNX1 may represent an additional therapeutic strategy for patients with FLT3/ITD$^+$ AML, in particular for those who become resistant to AC220 or other TKIs.

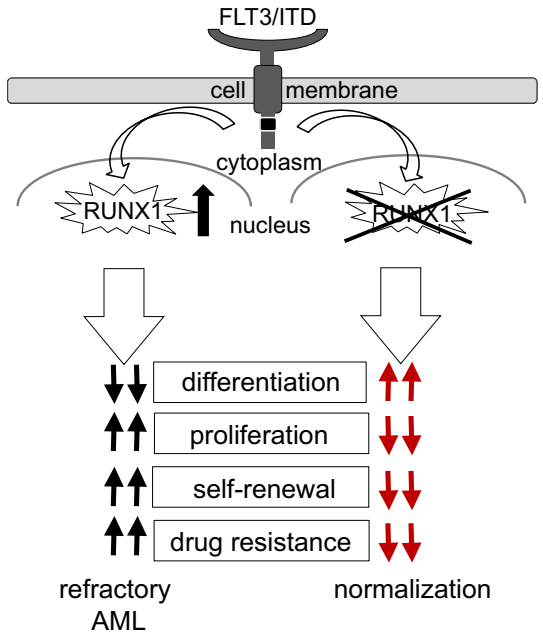

Fig. 4 Suggested model for the refractory phenotype of FLT3/ITD$^+$ cells regulated by RUNX1. Our data demonstrate that FLT3/ITD blocks differentiation toward the myeloid lineage, enhances factor-independent proliferation, promotes self-renewal and increases resistance to AC220 by up-regulating RUNX1 expression, all of which are associated with the refractory phenotype of AML. Conversely, antagonizing RUNX1 expression facilitates differentiation and decreases the proliferation, self-renewal and AC220-resistance of FLT3/ITD$^+$ cells

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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