Targeting CDK1 promotes FLT3-activated acute myeloid leukemia differentiation through C/EBPα

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Mutations that activate the fms-like tyrosine kinase 3 (FLT3) receptor are among the most prevalent mutations in acute myeloid leukemias. The oncogenic role of FLT3 mutants has been attributed to the abnormal activation of several downstream signaling pathways, such as STAT3, STAT5, ERK1/2, and AKT. Here, we discovered that the cyclin-dependent kinase 1 (CDK1) pathway is also affected by internal tandem duplication mutations in FLT3. Moreover, we also identified C/EBPα, a granulopoiesis-promoting transcription factor, as a substrate for CDK1. We further demonstrated that CDK1 phosphorylates C/EBPα on serine 21, which inhibits its differentiation-inducing function. Importantly, we found that inhibition of CDK1 activity relieves the differentiation block in cell lines with mutated FLT3 as well as in primary patient–derived peripheral blood samples. Clinical trials with CDK1 inhibitors are currently under way for various malignancies. Our data strongly suggest that targeting the CDK1 pathway might be applied in the treatment of FLT3ITD mutant leukemias, especially those resistant to FLT3 inhibitor therapies.

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Introduction
In acute myeloid leukemia (AML), an immature cell can acquire genetic changes, such as chromosomal translocations, insertions, deletions, or point mutations, which lead to uncontrolled cell growth, protection against cell death, and differentiation arrest. Among the most common oncogenic mutations in AML are internal tandem duplications (ITD) or activating mutations in fms-like tyrosine kinase 3 (FLT3). FLT3 is normally expressed in early hematopoietic precursors and plays a role in their proliferation and differentiation (1, 2), but its aberrant activation contributes to the development of AML. FLT3ITD mutations occur in about 20%–30% of AML patients, and the majority of these mutations (over 70%) are located in the juxtamembrane domain of FLT3. A novel type of ITD mutation (over 28%) was recently identified within the first kinase domain of the receptor (3). Several amino acids in the kinase domain are also known to undergo activating point mutations, for example, mutations in aspartic acid 835, which are seen in about 7% of AML cases (4). The consequences of FLT3 mutations are self phosphorylation and ligand-independent activation of the FLT3 receptor, followed by activation of the downstream signaling pathways, mainly Stat5, Akt, ERK1/2, Pim-1/2, and SHP-1 (5–11). Patients with activating FLT3 mutations have a poor prognosis (1, 2, 4, 12–14); therefore, much effort is being put forth to develop specific therapies. Small molecule inhibitors that specifically inhibit the FLT3 activity are presently undergoing clinical trials (1, 2, 4, 12–16). We have previously demonstrated that one of the targets of the ERK1/2 kinase is C/EBPα, a transcription factor playing a critical role in granulocytic differentiation (17) and often inactivated in various subtypes of leukemia by multiple mechanisms, such as transcriptional and translational silencing, as well as genetic mutations and posttranslational modifications, which render C/EBPα protein nonfunctional. The importance of C/EBPα as a molecular switch is underscored by the fact that it is both necessary and sufficient for granulocytic differentiation (18, 19). Activity of C/EBPα can be modulated by phosphorylation, and a number of residues in the C/EBPα protein that are subject to modifications have been identified. However, until now, only phosphorylation of serine 21 has been shown to have clinical importance (20, 21). We have shown that this single amino acid modification by the ERK1/2 pathway inhibits the function of C/EBPα and is responsible for the differentiation block in FLT3ITD leukemic blasts (17, 21). Pharmacological or genetic abrogation of this phosphorylation event in leukemic cells, for example, treatment with MEK1 inhibitor or substitution with a nonphosphorylatable mutant of C/EBPα (S21A), permits granulopoiesis to proceed (17, 21). Phosphorylation of C/EBPα on serine 21 by p38 MAPK in hepatocytes, on the other hand, increases its transactivation potential on the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter and results in increased PEPCK expression (20). Thus, serine 21 phosphorylation in liver enhances gluconeogenesis and, therefore, may play a role in diabetes.

Interestingly, among FLT3ITD patients, only 39% demonstrated activation of MEK1, and thus the ERK1/2 pathway (22), yet C/EBPα can still be inactivated by phosphorylation on serine 21 (this study). Herein, we identified cyclin-dependent kinase 1 (CDK1, also known as CDC2) as an FLT3ITD-activated kinase, which is responsible for C/EBPα phosphorylation on serine 21 and the blocking of its function. Thus, we provide a molecular mechanism by which the consti-
tutively active FLT3 mutant receptor contributes to the pathogenesis of leukemia, and we propose the use of CDK1 inhibitors for the treatment of FLT3ITD leukemia.

**Results**

C/EBPα transcription factor can be phosphorylated on serine 21 by an ERK1/2-independent kinase. We reported previously that the granulocytic differentiation-promoting function of C/EBPα could be inhibited in FLT3ITD AML by ERK1/2-mediated phosphorylation of serine 21 (17, 21). It has also been reported that not every FLT3ITD mutant can constitutively activate the ERK1/2 pathway (22, 23). To test whether the differentiation blocker in FLT3ITD AML can be mediated by an ERK-independent phosphorylation of C/EBPα, we transiently coexpressed C/EBPα and FLT3ITD mutant N51 (24), known not to activate the ERK1/2 pathway (ref. 23 and Radomska, unpublished observations), or empty vector (MSCV-IRE-EGFP) in 293T cells and analyzed phosphorylation of C/EBPα on serine 21 by Western blot. Figure 1 shows that while activation of the ERK1/2 pathway by brief treatment with TPA in the absence of FLT3ITD N51 resulted in a subtle increase in phosphorylation of serine 21, a robust phosphorylation took place when C/EBPα was coexpressed with FLT3ITD N51. Consistent with the previous report (23), overexpression of FLT3ITD N51 in the absence of TPA stimulation did not activate the ERK1/2 pathway, and the treatment with MEK1/2 inhibitor PD98059 did not affect the serine 21 phosphorylation levels. In contrast, treatments with FLT3 inhibitors MLN518 and PKC412 led to a substantial decrease in serine 21 phosphorylation, while the treatment with MEK1/2 inhibitor showed no effect on the levels of active ERK1/2 kinase. The Western blot analysis revealed that the decrease in serine 21 phosphorylation of endogenous C/EBPα was decreased by about 60%. There was no change in C/EBPα protein expression, but there was an approximately 50% decrease in phosphoserine 21 containing C/EBPα protein species. Notably, CDK1 protein expression, measured by Western blot, was decreased upon release from arrest (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/jci43354ds1). Consistent with CDK1-mediated phosphorylation of C/EBPα, treatment of mitotic U937 cells with MEK1 or CDK2/CDK5 inhibitors had no effect on phosphorylation of serine 21, while inhibition of CDK1 did (Supplemental Figure 1B). To further prove the direct role of CDK1 in phosphorylating C/EBPα on serine 21, we performed a knockdown experiment. shRNA specifically targeting CDK1 was expressed from a retroviral vector in MOLM-14 cells. Following sorting of GFP+ cells, the effectiveness of CDK1 knockdown and its effect on phosphorylation of C/EBPα were measured by Western blot. As shown in Figure 2C, normalization for the β-actin levels demonstrated that CDK1 protein expression was decreased by about 60%. There was no change in C/EBPα total protein expression, but there was an approximately 50% decrease in phosphoserine 21 containing C/EBPα species. Notably, CDK1 knockdown had no effect on the levels of active ERK1/2 kinase. Taken together, our results identify serine 21 of C/EBPα as a substrate for CDK1 kinase.
CDK1 pathway is activated in FLT3ITD AML. Constitutively active FLT3ITD receptor kinase has been shown to stimulate a number of downstream pathways. To determine whether CDK1 can be super-activated by FLT3ITD as well, the CDK1 kinase complexes were immunoprecipitated from asynchronously growing FLT3ITD AML cell lines and used in vitro kinase reaction with histone H1 as a substrate. As shown in Figure 3A, all untreated and DMSO-treated cell lines exhibited high CDK1 activity. In contrast, the kinase activity was significantly repressed by the treatment with the FLT3 inhibitor MLN518 (Figure 3A). To ascertain whether the effect of FLT3ITD on CDK1 is direct or indirect, we examined the cell-cycle distribution of MOLM-14 cells treated with MLN518 or DMSO. Figure 3B shows that FLT3 inhibitor treatment led to a significant decrease in mitotic cells with enrichment of G0-arrested cells. Taken together, these data indicate that CDK1 is a downstream pathway activated by FLT3ITD mutant receptors in an indirect fashion.

Pharmacological and genetic inhibition of CDK1 activity in FLT3ITD AML relieves differentiation block. To determine the biological effect of CDK1 inhibition in FLT3ITD AML cells, we cultured MV4;11, MOLM-13, and MOLM-14 cells with small molecule inhibitors targeting CDK1: flavopiridol, roscovitine (both being presently tested in clinical trials; http://clinicaltrials.gov), and NU6102. For comparison, we also treated these cells with the FLT3 inhibitor MLN518, which we previously demonstrated as decreasing ERK1/2 activity and phosphorylation of C/EBPα (21), and herein we showed that it can also inhibit CDK1 activity (Figure 3). Figure 4A shows the Western blot results obtained for MOLM-14 cells; comparable results were found for MOLM-13 and MV4;11 cells (data not shown). The treatments with all CDK1 inhibitors tested as briefly as 18 hours resulted in substantial hypophosphorylation of C/EBPα. While exposure to 10 μM NU6102 decreased the levels of phosphoserine 21-C/EBPα by 40%–60% in all 3 cell lines (Figure 4A and data not shown), a decrease in serine 21 phosphorylation by 53%–86% was achieved with 100 nM flavopiridol (Figure 4A and data not shown) and 77%–89% by 25 μM roscovitine (Figure 4A and data not shown). Neither of these CDK1 inhibitors down-modulated the ERK1/2 activity (Figure 4A). In addition to affecting CDK1 activity, all 3 compounds are also known to exert inhibitory effect against a kinase highly homologous to CDK1, CDK2. To eliminate the involvement of CDK2 in phosphorylation of C/EBPα, we cultured the cells in the presence of another compound, PNU 112455A, which inhibits CDK2 and CDK5 (IC50 = 2 μM for both) but does not display activity against other kinases at concentrations as high as 100 μM. In contrast with NU6102, PNU 112455A had no effect on phosphorylation of C/EBPα in all cells (Figure 4A and data not shown).

We have previously reported that C/EBPα, when hypophosphorylated on serine 21, displays granulocytic differentiation-promoting activity (17, 21). We cultured FLT3ITD cell lines in the presence of CDK1 inhibitors for up to 3 days and monitored their morphology as well as the changes in maturation marker expression. As shown in Figure 4B, MOLM-14 cells treated with 10 μM NU6102 acquired granulocytic morphology as early as on day 2 of the culture, with more marked effect seen on day 3. Similar changes in cell morphology were also noted after 2 days of treatment with 12.5 μM roscovitine or 100 nM flavopiridol, although a rapid onset of apoptosis was more pronounced in those cultures (Figure 4B and data not shown). No morphological changes were observed when the cells were treated with a vehicle control, DMSO,
or CDK2/CDK5 inhibitor PNU 112455A (Figure 4B). Comparable results were obtained for MOLM-13 cells (data not shown), while for MV4;11 cells, morphological changes were less pronounced (data not shown) and not seen with non-FLT3ITD AML cells (U937, KG1a, and K562; data not shown). All 3 FLT3ITD cell lines treated with NU6102 also demonstrated downregulation of c-myc, which is indicative of myeloid maturation (Figure 4C and data not shown). In addition, NU6102 treatment led to a time-dependent increase in the number of surface CD11b–expressing MOLM-14 cells (up to 60% on days 3 and 4; Figure 4D), which is in accord with granulocytic differentiation. The ERK1/2 pathway was originally discovered to be responsible for phosphorylation of serine 21 (17, 21). Previously, we reported that inhibition of this pathway in MV4;11 cells, morphological changes were less pronounced (data not shown) and not seen with non-FLT3ITD AML cells (data not shown) and not seen with non-FLT3ITD AML cells (data not shown). All 3 FLT3ITD cell lines treated with NU6102 also demonstrated downregulation of c-myc, which is indicative of myeloid maturation (Figure 4C and data not shown). In addition, NU6102 treatment led to a time-dependent increase in the number of surface CD11b–expressing MOLM-14 cells (up to 60% on days 3 and 4; Figure 4D), which is in accord with granulocytic differentiation. The ERK1/2 pathway was originally discovered to be responsible for phosphorylation of serine 21 (17, 21). Previously, we reported that inhibition of this pathway in MV4;11 cells, morphological changes were less pronounced (data not shown) and not seen with non-FLT3ITD AML cells (U937, KG1a, and K562; data not shown). All 3 FLT3ITD cell lines treated with NU6102 also demonstrated downregulation of c-myc, which is indicative of myeloid maturation (Figure 4C and data not shown). In addition, NU6102 treatment led to a time-dependent increase in the number of surface CD11b–expressing MOLM-14 cells (up to 60% on days 3 and 4; Figure 4D), which is in accord with granulocytic differentiation. The ERK1/2 pathway was originally discovered to be responsible for phosphorylation of serine 21 (17, 21). Previously, we reported that inhibition of this pathway in MV4;11 cells, morphological changes were less pronounced (data not shown) and not seen with non-FLT3ITD AML cells (U937, KG1a, and K562; data not shown). All 3 FLT3ITD cell lines treated with NU6102 also demonstrated downregulation of c-myc, which is indicative of myeloid maturation (Figure 4C and data not shown). In addition, NU6102 treatment led to a time-dependent increase in the number of surface CD11b–expressing MOLM-14 cells (up to 60% on days 3 and 4; Figure 4D), which is in accord with granulocytic differentiation. The ERK1/2 pathway was originally discovered to be responsible for phosphorylation of serine 21 (17, 21). Previously, we reported that inhibition of this pathway in MV4;11 cells, morphological changes were less pronounced (data not shown) and not seen with non-FLT3ITD AML cells (U937, KG1a, and K562; data not shown). All 3 FLT3ITD cell lines treated with NU6102 also demonstrated downregulation of c-myc, which is indicative of myeloid maturation (Figure 4C and data not shown). In addition, NU6102 treatment led to a time-dependent increase in the number of surface CD11b–expressing MOLM-14 cells (up to 60% on days 3 and 4; Figure 4D), which is in accord with granulocytic differentiation. The ERK1/2 pathway was originally discovered to be responsible for phosphorylation of serine 21 (17, 21). Previously, we reported that inhibition of this pathway in MV4;11 cells, morphological changes were less pronounced (data not shown) and not seen with non-FLT3ITD AML cells (U937, KG1a, and K562; data not shown). All 3 FLT3ITD cell lines treated with NU6102 also demonstrated downregulation of c-myc, which is indicative of myeloid maturation (Figure 4C and data not shown).

Next, we determined whether the differentiation-promoting effect of CDK1 inhibitors was dependent on C/EBPα expression. We designed an shRNA lentiviral construct specifically targeting CEBPA and demonstrated C/EBPα downregulation at the protein level (Supplemental Figure 2). In MOLM-14 cells, inhibition of the ERK1/2 pathway also led to differentiation with similar kinetics, but the effect of the inhibition of CDK1 was more potent, as measured by the downregulation of c-myc, upregulation of CD11b surface expression, increase in myeloperoxidase (MPO) and lysozyme mRNA expression, and morphological changes (Supplemental Figure 2).

To test whether specific knockdown of CDK1 protein expression would have the same effect as treatments with small molecule compounds, MOLM-14 cells were transduced with viral particles expressing CDK1 shRNA and EGFP. We anticipated that rapid inhibition of CDK1, which is necessary for cell-cycle progression through mitosis, may lead to growth arrest and apoptosis. We assumed that the expression level of CDK1 shRNA might parallel the level of EGFP expression and thus be in inverse correlation with the expression of the endogenous CDK1 protein. In order to provide the gradient of CDK1 knockdown, EGFP+ cells were sorted into 3 populations with low, medium, and high EGFP intensities. Each population was maintained in complete culture medium, and cell morphology was monitored daily. On day 4, we harvested 30,000 cells from each population, made lysates, and analyzed the degree of knockdown by Western blot. Day 4 was selected based on our previous knockdown experiment, showing that this was the earliest time point demonstrating detectable and significant decrease in CDK1 protein expression. Figure 6A shows that after normalization for the β-actin protein, there was not much difference between the inhibition of CDK1 protein expression in cells expressing medium and high levels of EGFP. Cells with low EGFP had a modest, but detectable decrease in CDK1. Morphological examination showed that cells sorted for high EGFP were enlarged in size, but did not show clear signs of myeloid differentiation. They also became growth arrested and died on day 7 (data not shown). Cells sorted for medium and low EGFP levels, on the other hand, acquired morphological changes consistent with granulocytic maturation on day 10 (Figure 6B), and this effect was stronger for low EGFP–expressing cells. Medium EGFP–expressing cells, in addition to myeloid maturation, were accompanied by severe cell death (data not shown).

Finally, primary FLT3ITD leukemic samples collected at diagnosis from the peripheral blood of patients were treated with NU6102. As expected, CDK1 inhibition led to a remarkable hypo-
phosphorylation of C/EBPα in the FLT3ITD patient cells in 3 out of 4 FLT3ITD patient samples (Figure 7). Figure 7 shows the Western blot analysis after 24 hours of treatment, although the effect was already noticeable after 10 hours. Phosphorylation of serine 21 was also observed in leukemic samples with the WT FLT3 gene (Figure 7), most likely due to constitutive activation of the ERK pathway. This is in agreement with the findings that the enhanced activity of this pathway was detected in over 80% of the AML samples tested, regardless of their FLT3 genotype status (26, 27). Nevertheless, the samples not harboring FLT3ITD mutations showed negligible or no effect upon treatment with the CDK1 inhibitor (Figure 7). In addition, 8 patient samples carrying FLT3ITD and 2 patient samples with the WT FLT3 receptor were also examined for the expression of mature (CD11b, CD11c, CD14, G-CSF-R, CD15, and CD16) and immature (CD33, CD34, CD38, and CD133) cell-surface markers following treatment with NU6102. Although each sample demonstrated different specific profiles of the response, all patient samples carrying FLT3ITD showed a general tendency to increase the expression of maturation markers and decrease that of immature markers (Figure 8A). In contrast, samples with WT FLT3 (patients C and D), which did not show a decrease in serine 21 phosphorylation, did not show any signs of differentiation (Supplemental Figure 4). Whenever we had enough material (patients A, F, and G), we also tested the mRNA expression of granulocyte-specific genes, such as CEBPE, CSF3R (coding for G-CSF receptor), neutrophil elastase, gelatinase A, and lysozyme. All 3 patient samples showed increases in CSF3R and CEBPE expression after treatment with CDK1 inhibitor; patient F demonstrated an increase in expression of all 5 genes (Figure 8B).

Moreover, the treatment of FLT3ITD-carrying specimens with NU6102 for 7 days was accompanied by morphological changes, suggesting granulocytic differentiation (Supplemental Figure 5). Since CDK1 inhibition was associated with substantial cell death, we wanted to make sure that lobing of the nuclei seen on cytopsin
preparations was caused by true differentiation, rather than apoptosis. Therefore, FLT3ITD leukemic samples treated with NU6102 were sorted for CD15+ cells and tested by the Wright-Giemsa method. As shown in Figure 8C, the same morphological forms were observed and importantly, CD15+ cells were nearly 90% viable (Figure 8C). In summary, inhibition of CDK1 in FLT3ITD cells led to an increase in C/EBPα function by its hypophosphorylation on serine 21 and the relief of the differentiation arrest.

Activity of CDK1 is controlled by a multiple-step process, but ultimately, CDK1 can be activated by binding to cyclin B1 (28, 29). To determine how the constitutively active FLT3ITD receptor affects CDK1 activity, MOLM-14 cells were untreated or treated with FLT3 inhibitor MLN518 or vehicle control DMSO and analyzed by Western blot staining with anti-cyclin B1 antibody. As shown in Figure 9A, treatment of MOLM-14 cells with FLT3 inhibitor led to a decreased expression of cyclin B1 protein. Conversely, introduction of FLT3ITD into Ba/F3 cells led to about a 2-fold increase in total cyclin B1 protein levels (Figure 9B). These data suggest a possible involvement of cyclin B1 in the activation of CDK1.

Discussion

Activating mutations in FLT3 receptor tyrosine kinase are among the most common mutations in AML and indicate poor prognoses (1, 2, 4, 12–14). Thus, development of small molecule inhibitors specifically targeting FLT3 seemed to be a promising approach for treating a large number of AML cases (15). Although different activating mutations in FLT3 exhibit divergent sensitivities toward the kinase domain of FLT3 (38, 39). To bypass this problem, a search for a new generation of FLT3 inhibitors is under way. In the meantime, combination therapies with selective FLT3 inhibitors and conventional cytotoxic chemotherapy are being investigated, but those have been characterized by unacceptable toxicity and poor tolerance (40). Alternative treatments of FLT3 mutant AML may involve inhibitors of the downstream pathways activated by FLT3ITD mutations. For example, we demonstrated before that inhibition of the ERK1/2 pathway in FLT3ITD-expressing AML cells leads to their differentiation (21). The multitude of downstream signaling pathways activated by mutant FLT3 receptors (5–11) may increase the repertoire of possible drug combinations.

A majority of the FLT3ITD downstream pathways control survival and apoptosis, while ERK1/2 signaling plays a role in the differentiation block by phosphorylating the C/EBPα transcription factor on serine 21 and inhibiting its function (17, 21). Interestingly, only a fraction of FLT3ITD patients exhibited activation of the ERK1/2 pathway (22). Of note, due to technical difficulties in examining the ERK activity in FLT3ITD leukemias, additional studies are needed to determine whether activation of ERK can serve as a specific biomarker of FLT3 signaling in primary leukemias (22). Moreover, we observed serine 21 phosphorylation on C/EBPα in cells with an FLT3ITD mutant receptor, which is disabled in ERK1/2 activation (mutant N51; refs. 23, 24). We hypothesized that a kinase other than ERK1/2 may be responsible for C/EBPα phosphorylation and differentiation block. In this report, we identified CDK1 (also known as CDC2) as the kinase specifically modifying C/EBPα on serine 21 in AML with FLT3ITD mutations. Thus, our data provide a potential molecular mechanism explaining the maturation block in FLT3ITD cases without activation of ERK1/2. However, in addition to ERK1/2, CDK1 is another modulator of C/EBPα differentiation function, and we cannot discard the contribution of other mediators to the differentiation block seen in FLT3ITD AML. Further, our observations do not rule out a potential interplay between ERK1/2 and CDK1 activity on C/EBPα function in certain FLT3ITD AML cases. The FLT3ITD and CDK1 connection was previously reported by Odgerel et al. (41). While they reported that CDK1 is partially inactivated in FLT3ITD AML cell lines, our work concludes that CDK1 can be activated by FLT3ITD mutations. This apparent contradiction could be explained by the use of different FLT3 inhibitors (PKC412 and MLN518, respectively) and the concentrations used, resulting in either effects in apoptosis (41) or differentiation (this study).

Several studies described modulation of C/EBPα activity by phosphorylation on various residues (17, 42–44). However, phos-
C/EBPα acts as a switch inhibiting neutrophilic differentiation of C/EBPα from phosphorylation of a single amino acid, serine 21, seems to have the most remarkable effect by shifting the activity of C/EBPα from a granulocytic differentiation-promoting factor (unphosphorylated) to a dominant negative form (phosphorylated) (17). P38MAPK-mediated phosphorylation of serine 21 in leukemic blasts blocks their maturation and may be responsible for disturbed neutrophilic development in severe congenital neutropenia (45). In liver cells, however, phosphorylation of serine 21 by p38 MAPK increases the activity of cyclin B1 rather than upregulation of CDK1 itself (49). While these data suggest that cyclin B1 is involved in the activation of CDK1, we cannot rule out that other CDK1 regulators, such as Myt1 and cdc25c, could also be involved (41). NU6102 demonstrated the best differentiation-promoting activity, perhaps because of the higher specificity against CDK1 versus other CDKs. The knockdown experiments are in accord with this hypothesis. Cells expressing lower levels of EGFP and presumably lower levels of shRNA showed more pronounced maturation, while the cells with higher levels of EGFP (and presumably the highest levels of shRNA) exhibited mainly apoptosis (this study).

In summary, we demonstrate that constitutive activation of the FLT3 receptor can lead to abnormal activation of multiple downstream signaling pathways (Figure 10), which are capable of inhibiting the function of C/EBPα, contributing to the differentiation block. Inhibiting either FLT3 receptor, MEK1 kinase, or CDK1 can restore the activity of C/EBPα and induce myeloid maturation of leukemic blasts.

Methods

Cell lines. Human AML lines carrying FLT3ITD mutations were kindly donated by Yoshinobu Matsuo (MOLM-13, Fujisaki Cell Center, Hayashibara Biochemical Labs, and the Kurashiki Medical Center, Kurashiki, Okayama, Japan), Neill Giese (MOLM-14; Calistoga Pharmaceuticals, Inc.), Stefan Heinrichs (MOLM-14; 50), and David Sternberg (Ba/F3-FLT3ITD, OSI Pharmaceuticals). MOLM-13, MOLM-14 (40), and their morphology was monitored daily on Wright-Giemsa–stained slides. Original magnification, ×40.
in RPMI 1640 with 10% FBS. The murine bone marrow–derived IL-3–dependent Ba/F3 cell line (51) was cultured in RPMI with 10% FBS and 10% WEHI-3B conditioned medium. A Ba/F3 stable line expressing the FLT3ITD mutant N51 (24) was maintained in RPMI/10% FBS/10% WEHI-3B conditioned medium and 750 μg/ml active G418. Human Embryonal Carcinoma 293T (HEK 293T; CRL 11268, ATCC) and Phoenix-Ampho Packaging (Orbigen) cell lines were cultured in DMEM with 10% FBS.

**Patient samples.** After informed consent was obtained, peripheral blood samples of AML patients were collected at the time of diagnosis before initiation of treatment. Blasts and mononuclear cells were purified by Ficolly-Hypaque (Nygaard) centrifugation and cryopreserved. Cells were thawed at 37°C, incubated on ice for 10 minutes, and washed twice in ice-cold HBSS. Cells were precultured in EX-VIVO (BioWhittaker) medium supplemented with 10 ng/ml human IL-3 (hIL-3), 10 ng/ml hIL-6, and 25 ng/ml hSCF at 37°C for 45 minutes on 150 mm Petri dishes to remove adherent cells. Suspension cells were collected and incubated at 37°C in the presence of 5 or 10 μM NU6102 or 0.1% DMSO (vehicle control).

**Reagents.** All inhibitors were prepared in DMSO. FLT3 inhibitors MLN518 (CT53518; Millenium Pharmaceuticals) and PKC-412 (Biomol) as well as CDK1 inhibitors NU6102 (Calbiochem/EMD Biosciences) and flavopiridol (Sigma-Aldrich) were reconstituted at 10 mM. Roscovitine was reconstituted at 25 mM. The CDK2/CDK5 inhibitor PNU112455A (Novagen). Following a 5-minute incubation at room temperature and centrifugation at 13,000 g for 20 minutes, the supernatant was collected and incubated with 0.2 ml of Glutathione Sepharose 4FF beads (Amersham) for 20 minutes, and bacterial pellets were subjected to a single cycle of freeze-thawing and centrifugation at 13,000 g for 10 minutes. Bacteria were then centrifuged at 1,500 × g for 10 minutes, and bacterial pellets were subjected to a single cycle of freeze-thawing. Lysed bacteria were suspended in 1 ml BugBuster Protein Extraction Agent (Novagen) and supplemented with 25 U of Benzonase Nuclease (Novagen). Following a 5-minute incubation at room temperature and centrifugation at 13,000 g for 20 minutes, the supernatant was collected and incubated with 0.2 ml of Glutathione Sepharose 4FF beads (Amersham) for 1 hour at room temperature. Beads were pelleted and washed 4 times with PBS and immediately suspended in in vitro kinase reaction mixture containing [γ-32P]ATP (10 Ci/mmol), purified CDK1 kinase (cat. P6020S; NEB), and supplied reaction buffer. The reactions were carried out at 30°C for 10 minutes, then stopped by adding 4 × Laemmli buffer and boiling samples at 100°C for 10 minutes. The products were resolved on SDS-PAGE, blotted to nitrocellulose membrane, and analyzed by autoradiography. To control for the amount of C/EBPα-GST protein, the same membrane was stained with the N-terminal anti-C/EBPα antibody.

**In vitro kinase assay.** FLT3ITD AML cells were treated with 10 μM MLN518 or 0.1% DMSO for 24 hours. Equal numbers of cells were harvested and plated (shown below Western blot; D, DMSO, N, NU6102). The numbers above bars indicate the percentage of phosphorylated C/EBPα species compared with samples treated with DMSO (set to 100%).
lysed in RIPA lysis buffer. Active CDK1 kinase complexes were immunoprecipitated with anti-CDK1 antibody and captured on Immobilized Protein A beads (IPA-300; Repligen). Following 3 washes with ice-cold PBS and 1 wash with ADBI CDK1 kinase reaction buffer (Upstate Biotechnology), the CDK1-containing beads were used in kinase reactions in ADBI containing purified histone H1 as a substrate and \[^{32}\text{P]}\text{ATP} (10 \text{ Ci/mmol}). The reactions were carried out at 30°C for 10 minutes, stopped by boiling in Laemmli buffer for 10 minutes, subjected to SDS-PAGE electrophoresis, and transferred onto nitrocellulose membranes. The incorporation of \[^{32}\text{P}\] into the substrate was examined by autoradiography, and the amounts of immunoprecipitated CDK1 kinase were compared by staining the same membrane with anti-CDK1 antibody.

Mitotic arrest. U937 cells were arrested at mitosis by incubation in the presence of 100 μg/ml nocodazole for 16 hours. Cells were released from the block by washing twice and by subsequent culture in complete medium without nocodazole.

Figure 8
Granulocytic differentiation of FLT3ITD cells after inhibition of CDK1 activity. (A) Inhibition of CDK1 in patient samples with FLT3ITD induces granulocytic differentiation. Blood specimens from FLT3ITD AML patients (patient A and patient B; same as shown in Figure 7) were cultured in the presence of 0.1% DMSO or 10 mM NU6102 for 7 days. Cell aliquots were stained with anti-CD11c, anti-CD15, anti-CD14, anti-CD11b, anti-CD16, anti-G-CSF-R, anti-CD33, anti-CD133, anti-CD34, and anti-CD38 antibodies and analyzed by flow cytometry. The y axes indicate the percentage of positive cells. (B) Patient samples were treated as in A for 5 days and analyzed for mRNA expression of granulocytic cell-surface markers by quantitative RT-PCR. The y axes indicate relative expression to GAPDH. (C) CD15+ cells with granulocytic-like morphology are viable. CD15 expression on FLT3ITD AML patient A sample treated with DMSO (gray) or 5 mM NU6102 (white) during 10 days (left panel). Number indicates the percentage of CD15+ cells upon NU6102 treatment. CD15+ cells were sorted, cytocentrifuged, and stained with Wright-Giemsa method (middle panel). Original magnification, ×40. CD15+ cells were also stained with annexin V and PI to determine the extent of their viability (right panel). The numbers in each quadrant indicate percentages of viable (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic cells (upper left).
Western blot. Typically, 5 × 10⁵ cells were spun (1 K, 5 minutes), washed in PBS, lysed in 400 μl of 1× Laemmli Sample Buffer, and boiled at 100°C for 10 minutes. From 30 to 40 μl of each lysate was loaded on 7.5% SDS-PAGE gels and proteins transferred to nitrocellulose membranes. Following blocking in 5% milk/Tris-buffered saline (TBST; 25 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20), membranes were stained with primary antibodies diluted in 5% BSA/TBST/0.1% sodium azide overnight at 4°C and then with HRP-conjugated secondary antibodies at room temperature for 1 hour. Signals were detected by enhanced chemiluminescence and quantified by ImageQuant software (Molecular Dynamics). The primary antibodies were goat N-terminal C/EBPα (N-19; 1:1,000; sc-9315, Santa Cruz Biotechnology Inc.), rabbit phospho-Ser21-C/EBPα (1:1,000; #2841, Cell Signaling Technology), goat C-terminal C/EBPα (C-18, 1:1,000; sc-9314, Santa Cruz Biotechnology Inc.), rabbit C/EBPα (14AA, 1:1,000; sc-61, Santa Cruz Biotechnology Inc.), rabbit CDK1 (1:1,000; PC25, Calbiochem), rabbit phospho-(T202/Y204)-ERK1/2 (1:1,000; #9101, Cell Signaling Technology), goat C-terminal C/EBPα (C-19; 1:1,000; sc-596, Santa Cruz Biotechnology Inc.), rabbit phospho–Ser21-C/EBPα (1:1,000; #2841, Cell Signaling Technology), rabbit phospho-(T202/Y204)-ERK1/2 (1:1,000; #9101, Cell Signaling Technology), and rabbit β-tubulin (1:4,000 clone 2-28-33; T5293, Sigma-Aldrich). All secondary antibodies were HRP conjugated (Santa Cruz Biotechnology Inc.) and diluted 1:5,000 for rabbit-HRP, 1:3,000 for mouse-HRP, and 1:2,000 for goat-HRP.

After transfection, 293T cells were plated out at 2 × 10⁵ cells per well on 6-well plates and transfected by 5 μl of TransFectin (Bio-Rad) complexed with 2 μg of MSCV-IRES-EGFP or MSCV-FLT3ITD-IRES-EGFP (mutant N51; ref. 24) and 0.5 μg of pcDNA3-WT C/EBPα (21). Five hours later, FLT3 inhibitors (0.5 μM MLN518 and 0.2 μM PKC-412) were added and the cells were cultured for an additional 16 hours. PD98059 (at 100 μM) was added 1.5 hours before the cell harvest, and TPA (at 10 nM) was added for the last 15 minutes of the culture. At the end of the treatments, cells were collected and lysed in 600 μl of 1× Laemmli Sample Buffer. Then 30 μl per lane were loaded on PAGE/SDS gels (7.5%).

Retroviral transductions. Retroviruses were produced by transfecting Phoenix A cells. Virus-containing supernatants were collected at 48 and 72 hours after transfection, filtered through 0.45-μm filter, and concentrated using a Centrifuge Plus-70 100000 MWCO column (Millipore). Retroviral transduction was performed in culture dishes (Falcon 1008; BD) coated with 12 μg/ml RetroNectin during 2 consecutive days using a MOI between 2.5 and 5. The EGFP-expressing cells were enriched by sorting on day 3 and cultured for up to 8 additional days.

Lentiviral transductions. 293T cells were cotransfected using Lipofectamine 2000 with C/EBPα shRNA in pGhU6 vector or the shRNA control and lentiviral constructs Gag-Pol and Env. Virus was harvested and concentrated using a Centrifuge Plus-70 100000 MWCO column (Millipore). A single lentiviral transduction was performed in the presence of polybrene (8 μg/ml) (Sigma-Aldrich). MOLM-14 cells were infected with an MOI of 5. One day after transduction, cells were treated with either 0.01% DMSO control or 5 μM NU6102. Infected cells were determined by EGFP flow cytometry analysis.

Morphological examination. About 10⁴ cells were spun at 500 g for 5 minutes onto glass slides and Wright-Giemsa stained with Diff-Quik solution (Dade Behring).
Flow cytometry. Cells were washed once in PBS and blocked in 2% FBS/PBS on ice for 15 minutes. Surface staining was performed on ice for 30–40 minutes followed by 2 washes with PBS. Antibodies used were as follows: PE-conjugated anti-human CD11b (555388, BD Biosciences – Pharmingen), PE-Cy5-conjugated anti-human CD11b (used in MOLM-14 cells transduced with pGhU6; #301308, Biolegend), FITC-conjugated anti-human CD11c (#11-0116-73, ebioScience), FITC-conjugated anti-human G-CSR-F/CD114 (# FAB831F; R&D Systems), APC-conjugated anti-human CD14 (# 561383, BD Biosciences), Pacific Blue–conjugated anti-human CD15 (#57-0159-73; ebioScience), eFluor650NC-conjugated anti-human CD16 (#93-0168-41, ebioScience), PE Cy7–conjugated anti-human CD33 (#25-0338-42, ebioScience), APC-conjugated anti-human CD34 (#343150, Biolegend), PE-Cy7–conjugated anti-human CD38 (#25-0389-41, ebioScience), and biotin-conjugated anti-human CD133 (#13-1338, ebioScience). Exclusion of dead cells was done by addition of DAPI.

Cell sorting was performed using a FACSAria cell sorter, and immunophenotyping was done on an LSRII flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Treestar Inc.).

Quantitative RT-PCR. RNA was isolated by TRI Reagent (MRC Inc.), treated with DNase, and reverse-transcribed to cDNA (Invitrogen). Quantitative RT-PCR was performed using qSYBR Green Supermix (BioRad). Amplification was done with a Corbett Rotor Gene 6000 (QIAGEN) using the following parameters: 95°C (10 minutes), 45 cycles of 95°C (15 s) and 60°C (1 minute). Primer sequences were as follows: human GAPDH F: 5′-CCACATCGTGACACACT-3′; human GAPDH R: 5′-GACGGGCCCAATACG-3′; human G-CSF-R F: 5′-TTTCGAAACTCTTTCGACCCAGAA-3′; human G-CSF-R R: 5′-CAGGCCAGGCTCAGTTTTC-3′; human C/EBP F: 5′-CTCCGATCTCTTGGCGTGA-3′; human C/EBP R: 5′-TGGGCAGAAGTTATGTTGA-3′; human gelatinase A F: 5′-GTTGGACAAAGCAGCCTACAT-3′; human gelatinase A R: 5′-GTCGTCCTCTCCATGAGTT-3′; human neutrophil elastase F: 5′-CCACCCCGGACGTGTTTC-3′; human neutrophil elastase R: 5′-GTGGCCAGCCGCTTGAG-3′; human MPO F: 5′-AGACCCTGGTGGAGGAGAA-3′; human MPO R: 5′-CGCCAGCCGCTTGACTTG-3′; human lysozyme F: 5′-GCTGAGAGATACATCCTG-3′; human lysozyme R: 5′-CCATGCTCTAATGCTTG-3′.

Apoptosis assay. The analysis of apoptotic cells was performed using annexin V–FLUOS kit (Roche) according to the manufacturer's protocol. Simultaneous labeling with propidium iodide (PI) was used for exclusion of the necrotic cells.

Cell-cycle analysis. Cells were suspended in phosphate-citrate buffer solution with 0.02% saponin for permeabilization and then incubated with 20 μg/ml Hoechst 33342 (Invitrogen) and 1 μg/ml Pyronin Y (Sigma-Aldrich). Incorporation of Hoechst 33342 and Pyronin Y were measured by flow cytometry.

Study approval. Patients' informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board: Committee on Clinical Investigations of Beth Israel Deaconess Medical Center.

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