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ORIGINAL ARTICLE

Pim kinases phosphorylate Chk1 and regulate its functions in acute myeloid leukemia

LL Yuan¹, AS Green², S Bertoli^{1,3}, F Grimal¹, V Mansat-De Mas^{1,4}, C Dozier¹, J Tamburini², C Récher^{1,3}, C Didier¹ and S Manenti¹

Phosphorylation by Akt on Ser 280 was reported to induce cytoplasmic retention and inactivation of CHK1 with consequent genetic instability in PTEN $-/-$ cells. In acute myeloid leukemia cells carrying the FLT3-internal tandem duplication (ITD) mutation, we observed high rates of FLT3-ITD-dependent CHK1 Ser 280 phosphorylation. Pharmacological inhibition and RNA interference identified Pim1/2, not Akt, as effectors of this phosphorylation. Pim1 catalyzed Ser 280 phosphorylation *in vitro* and ectopic expression of Pim1/2-induced CHK1 phosphorylation. Ser 280 phosphorylation did not modify CHK1 localization, but facilitated its cell cycle and resistance functions in leukemic cells. FLT3, PIM or CHK1 inhibitors synergized with DNA-damaging agents to induce apoptosis, allowing cells to bypass the etoposide-induced G2/M arrest. Consistently, etoposide-induced CHK1-dependent phosphorylations of CDC25C on Ser 216 and histone H3 on Thr11 were decreased upon FLT3 inhibition. Accordingly, ectopic expression of CHK1 improved the resistance of FLT3-ITD cells and maintained histone H3 phosphorylation in response to DNA damage, whereas expression of unphosphorylated Ser 280Ala mutant did not. Finally, FLT3- and Pim-dependent phosphorylation of CHK1 on Ser 280 was confirmed in primary blasts from patients. These results identify a new pathway involved in the resistance of FLT3-ITD leukemic cells to genotoxic agents, and they constitute the first report of CHK1 Ser 280 regulation in myeloid malignancies.

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Keywords: acute myeloid leukemia; checkpoint; Pim; CHK1; FLT3-ITD

INTRODUCTION

Eukaryotic cell cycle progression needs to be timely and spatially controlled in order to avoid abnormal cell divisions and consequent genetic instability. The ATR/CHK1 phosphorylation cascade is a master regulator of cell cycle progression that triggers cellular responses to different types of stress, for instance, DNA damage. The CHK1 Ser/Thr kinase phosphorylates different substrates, like p53, CDC25C, CDC25A or Wee1,¹ leading to CDKs inhibition and consequent cell cycle arrest. In addition to its role in the response to stress and DNA damage, CHK1 also displays central functions during unperturbed cell cycle, notably in DNA replication or during the completion of mitosis.^{2–6} Owing to its essential and varied roles, CHK1 activity must be finely regulated in order to ensure its correct function. The major mechanism of CHK1 regulation concerns its phosphorylation by ATR on the Ser 345 and Ser 317 residues, leading to its catalytic activation. Phosphorylation of these sites is currently considered as a general hallmark of CHK1 activation. In addition, autophosphorylation on the residue Ser 296 also contributes to CHK1 activation.⁷ Phosphorylations by CDK1 and CDK2 on Ser 286 and Ser 301 have also been reported to have positive effects on CHK1 functions at the G2/M transition and in response to DNA damage.^{8,9} Although the subcellular localization of the protein has not been extensively investigated, interesting studies point to the phosphorylation of CHK1 on Ser 280 by the Akt/PKB kinase in PTEN $-/-$ cells.^{10,11} This phosphorylation leads to mono-ubiquitination and subsequent retention of the protein in the

cytoplasmic compartment of these cells. This cytoplasmic relocation inhibits CHK1 functions in this model, leading to genetic instability caused by checkpoint deficiency.¹¹ More recently, the expression of an alternative splicing-derived short form of CHK1 was reported, and it was characterized as an inhibitory regulator of the full length kinase.¹²

Acute myeloid leukemia (AML) is a group of myeloid malignancies characterized by impaired differentiation occurring at different stages of the hematopoietic process and by excessive proliferation of leukemic progenitors. Mutations of the FLT3 tyrosine kinase are found in 25–30% of patients, FLT3-internal tandem duplication (ITD) being the most commonly detected. FLT3-ITD expression contributes to increased proliferation rates and cell death resistance, which are caused by constitutive activation of the receptor and various downstream signaling pathways like the PI3K/Akt signaling module or the STAT5 transcription factor and its targets.^{13–17} In particular, Pim1 Ser/Thr kinase is an important transcriptional target of STAT5, and the roles of Pim1 and Pim2 in the oncogenic process mediated by FLT3 have been documented.^{18,19} In fact, Pim kinases are frequently over-expressed in AML,²⁰ independently of FLT3 mutational status, and recent studies point to the importance of Pim2 in the regulation of the mTOR pathway in these pathologies.^{21,22} Pim kinases are constitutively active and their activity supports *in vitro* and *in vivo* tumor cell growth and survival through multiple substrates, including cell cycle regulators and apoptosis mediators.²³ In light of this, many reports provide new

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insights into their oncogenic action, and support the development of Pim-specific inhibitors as anticancer agents to increase the efficacy of conventional therapy.

The importance and the status of CHK1 in AML have been investigated by different groups.^{24,25} We recently described constitutive activation of this kinase in these pathologies, especially in patients with complex karyotype and poor prognosis,^{26,27} and we proposed that CHK1 inhibition may sensitize leukemic cells to genotoxic treatments.²⁸ However, the activation status of CHK1 in FLT3-ITD-positive leukemic cells remains unknown. In this work, we investigate the regulation of CHK1 in leukemic cells expressing FLT3-ITD, and demonstrate that Pim1/2 kinases are key regulators of its phosphorylation on Ser 280 and of its molecular and cellular functions in AML. Our current results provide new insight toward the development of targeted therapies in FLT3-ITD + AML.

MATERIALS AND METHODS

Pharmacological inhibitors, reagents, cytokines and antibodies

The FLT3 inhibitor III²⁹, Akt inhibitor VIII, STAT5 inhibitor and PIM1/2 kinase inhibitors V and VI were purchased from Calbiochem (San Diego, CA, USA) and CHK1 inhibitor SCH-900776 from Active Biochem (Maplewood, NJ, USA). Etoposide (VP-16), nocodazole, protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3 were obtained from Sigma-Aldrich (Lyon, France). The FLT3 inhibitor AC-220 was obtained from Selleckbio (Selleck Chemicals, Houston, TX, USA). Recombinant Mouse Interleukin (IL)-3 was from R&D Systems (Lille, France). The following antibodies were used: monoclonal anti-CHK1 (G4), anti-Pim1 (12H8), polyclonal anti-Cdc25C (C-20), anti-Flt3/Flt2 (C-20) and anti-Akt1/Akt2 (H-136) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); polyclonal anti-phospho-Ser 280-CHK1 from Abgent (San Diego, CA, USA); monoclonal anti-phospho-STAT5 (Tyr694/699) from Millipore (Billerica, MA, USA); polyclonal anti-phospho-Ser 473-Akt, anti-phospho-Ser 317-CHK1, anti-phospho-Ser 345-Chk1, anti-phospho-Ser 216-Cdc25C, anti-STAT5, anti-phospho-Ser 235/236-RPS6, anti-RPS6 and anti-phospho-Thr11-Histone H3 from Cell Signaling Technology (Beverly, MA, USA); polyclonal anti-Pim2 from Atlas (Stockholm, Sweden); anti-HDAC1 and anti histone H3 from Abcam (Paris, France); anti-actin and anti- α -tubulin from Sigma (St Louis, MO, USA).

Cell lines and culture conditions

Human myeloid leukemia cell lines MV4-11, MOLM-13 and K562 (DSMZ, Braunschweig, Germany) were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). KG1 (DSMZ) cell line was grown in Iscove's Modified Dulbecco's Medium plus 20% FBS. Murine FLT3-ITD-expressing BA/F3 cells were cultured in RPMI medium supplemented with 10% FBS, and U2OS and HEK-293 cells (ATCC) were cultured in DMEM with 10% FBS. All cells were grown in the presence of 100 units/ml of penicillin and 100 μ g/ml of streptomycin at 37 °C and 5% CO₂.

Patient samples

Leukemic blasts from patients diagnosed with AML have been obtained after informed consent and stored at the HIMIP collection. According to French law, HIMIP collection has been declared to the Ministry of Higher Education and Research (DC 2008-307 collection 1) and a transfer agreement was obtained (AC 2008-129) after approbation by the 'Comité de Protection des Personnes Sud-Ouest et Outremer II' (ethical committee). Clinical and biological annotations of the samples have been declared to the CNIL (Comité National Informatique et Libertés that is, Data processing and Liberties National Committee). For some experiments, fresh leukemic blasts recovered at diagnosis were immediately treated with the inhibitors. In most cases, frozen cells were thawed in Iscove's Modified Dulbecco's Medium medium with 20% FBS and immediately processed for treatment with inhibitors.

Transfection of small interfering RNA and plasmids

Transfections of small interfering RNA (siRNA) in MV4-11 cells were carried out according to the manufacturer recommendations (Amaxa Nucleofector Kit L was used with program Q-001, and the kit was purchased from Lonza,

Cologne, Germany). For the transfection of plasmids, U2OS or HEK-293 cells were seeded 1 day prior to transfection. Cells were transfected with pcDNA3-Pim1, pcDNA3-Pim2 (kindly provided by Dr Shuchi Agrawal-Singh, Copenhagen University, Denmark) or pcDNA3-CHK1-Myc constructs (kindly provided by Ramon Parsons, Columbia University, USA) with Effectene Transfection Reagent (Qiagen, Courtaboeuf, France). For the co-transfection experiments, CHK1 and Pim plasmids were used in a ratio of 1:4. Twenty four hours post transfection, the cells were washed four times with PBS and harvested for experiments.

Lentiviral infections

We generated Chk1 Ser 280 mutated to Ala by using site-directed mutagenesis with forward primer 5'-CCCCGAGTCACTGCAGGTGGTGTGC-3' and reverse primer 5'-GACACACCACCTGCAGTACTCGGGG-3'. To generate lentiviral vectors expressing CHK1 proteins, WT or mutated S280A HA-CHK1 sequences were cloned into the pTrip-TAL-lres-GFP lentiviral vector. We used 293-T packaging cells that were co-transfected with lentiviral protein (GAG, POL and ENV)-encoding plasmids and plasmids containing GFP (as a control), CHK1 or CHK1 S280A genes, separately. Supernatants containing lentivirus were collected 48 h after transfection, during 3 consecutive days, and then stored at -80 °C. Murine FLT3-ITD-expressing BA/F3 cells (5 × 10⁵) were plated in 200 μ l of serum-free medium with 5 μ l of lentiviral supernatant. After 3 hours of incubation, cells were resuspended in 10% FBS medium. The percentage of infected cells was then determined by GFP detection in flow cytometry, and positive cells were sorted by flow cytometry.

In vitro phosphorylation assay

CHK1 was translated with the TNT7 Quick Coupled System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Two microlitres of the transcription/translation reaction mixture were incubated with 50 μ M of ATP and 5 ng of recombinant active Pim1 (Enzo Life Sciences, Lyon, France) in 20 μ l of kinase buffer (20 mM HEPES, pH 7.4, 8 mM MgCl₂, 5 mM dithiothreitol, 0.4 mM EDTA, 0.4 mM EGTA and 0.02% Tween 20). Reactions were performed at 30 °C for 1 h and terminated by the addition of Laemmli sample buffer.

Cytoplasmic and nuclear fractionation

MV4-11 cells were lysed in buffer A containing 20 mM HEPES, pH 7.6, 10 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.1% NP40, 1 mM Na₃VO₄, 1 mM dithiothreitol, 1.5 mM MgCl₂, 10 mM β -glycerophosphate, 50 mM NaF, protease inhibitor cocktail, phosphatase inhibitor cocktails 2 and 3, for 10 min on ice. After centrifugation (4 °C, 2000 r.p.m., 4 min), the supernatants were collected and stored at -80 °C. The pellets were then lysed in an equal volume of Laemmli sample buffer, sonicated and boiled for 3 min prior to western blot analysis.

Immunoprecipitation

MV4-11 (20 × 10⁶) cells were lysed in 1 ml of cell lysis/IP buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, proteasome inhibitor cocktail, phosphatase inhibitor cocktails 2 and 3) for 20 min on ice. Lysates were pre-cleared by centrifugation for 10 min at 13 500 r.p.m. Then, 2 μ g of polyclonal phospho-S280-CHK1 antibody was incubated with the supernatant for 1 h. Red Protein A Affinity Gel (Sigma) was then added and incubated overnight at 4 °C. After centrifugation the pellets were washed twice with lysis buffer and the fractions were boiled for 3 min in Laemmli sample buffer. The immunoprecipitates were then analyzed by western blot with the anti-phospho-Ser 280-CHK1 antibody and biotinylated CHK1 (G4) antibody (biotinylation was done using BiotinTag Micro Biotinylation Kit from Sigma).

Western blot analysis

Cells (2 × 10⁶) were usually lysed in 100 μ l of Laemmli sample buffer, sonicated for 7 s and boiled for 3 min. Proteins were then resolved on 4–12% gradient SDS-PAGE and transferred to nitrocellulose membrane. Saturation of the membrane was done for 1 h in TBS with Tween 0.1% (TBS-T) containing 5% nonfat milk or 5% bovine serum albumin. Membranes were blotted with proper antibodies overnight at 4 °C or for 1 h at room temperature, washed thrice with TBS-T, and incubated for 30 min with HRP-conjugated secondary antibody (Promega) or ExtrAvidin-Peroxidase (Sigma) when biotinylated antibody was used. After three

additional washes, detection was achieved with Supersignal Westpico chemiluminescent substrate (Perkin Elmer, Shelton, CT, USA).

Apoptosis and cell cycle analysis

Cells were harvested and fixed in ice-cold 70% ethanol, permeabilized with 0.25% PBS Triton X-100 and stained with propidium iodide. Data were collected on a BD FACSCalibur cytometer and analyzed using the Cell Quest (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (Treestar, Ashland, OR, USA). Apoptotic cells were detected with Annexin V-FITC or Annexin V-APC (for the GFP-positive cell lines) detection kit from BD Pharmingen (San Diego, CA, USA) according to the manufacturer's instructions.

RESULTS

FLT3-dependent regulation of CHK1 Ser 280 phosphorylation in leukemic cell lines

To estimate the importance of FLT3-ITD on CHK1 activity, we first tested the effect of FLT3 inhibition on CHK1 Ser 317 and Ser 345 phosphorylations in leukemic cells. Pharmacological inhibition of FLT3 did not modify the levels of Ser 317 or Ser 345 phosphorylation in MV4-11 cells (Figure 1a), suggesting that FLT3 does not regulate CHK1 basal activation in these conditions. The efficiency of FLT3 inhibitor used in these experiments was assessed by western blot analysis of downstream FLT3 cell signaling pathways PI3K/Akt and STAT5 (Supplementary Figure 1).

We then investigated the phosphorylation of CHK1 on Ser 280, previously described to regulate CHK1 localization and function. Western blot analysis revealed that MV4-11 (FLT3-ITD), KG1 (FLT3wt) and K562 (FLT3-negative) leukemic cell lines all present detectable levels of Ser 280-phosphorylated CHK1 (Figure 1b). This level was higher in MV4-11 cells when considering the amount of CHK1 protein present in each cell line.

After treatment with FLT3 inhibitor III for 2 h, CHK1 phosphorylation on Ser 280 was reduced in MV4-11 cells, while it remained constant in KG1 and K562 cells (Figure 1b). Similar results were obtained with another FLT3 inhibitor (FLT3 inhibitor IV, data not shown). These data were also confirmed in the Ba/F3 murine cell line constitutively expressing the FLT3-ITD receptor (Figure 1c), further demonstrating that FLT3-ITD is an upstream regulator of CHK1 Ser 280 phosphorylation.

Signaling pathways influencing CHK1 phosphorylation in FLT3-ITD cells

As Akt was described to phosphorylate Ser 280, and is over-activated downstream of FLT3-ITD, we tested the impact of Akt pharmacological inhibition on Ser 280 phosphorylation. Surprisingly, complete Akt inhibition did not affect CHK1 Ser 280 phosphorylation in this model (Figure 2a).

The STAT pathway being a key effector of FLT3-ITD, we tested its involvement in CHK1 phosphorylation. Pharmacological inhibition of STAT5 efficiently reduced CHK1 Ser 280 phosphorylation in MV4-11 cells (Figure 2b), without impacting FLT3-ITD phosphorylation, suggesting the existence of an FLT3-ITD/STAT5/CHK1 axis in this model. These consequences of FLT3, Akt and STAT5 inhibition on Ser 280 phosphorylation were reproduced in MOLM-14, another human FLT3-ITD-expressing cell line (Supplementary Figure 2). siRNA-mediated STAT5 downregulation also reduced the level of CHK1 Ser 280 phosphorylation in MV4-11 cells, and in murine Ba/F3 cells expressing FLT3-ITD (Figure 2c). Importantly, siRNA-mediated or pharmacological inhibition of STAT5 induced downregulation of Pim1 and Pim2 proteins in these different models. Altogether, these data indicate that FLT3-ITD regulates CHK1 phosphorylation through STAT5 in leukemic cells.

Pim kinases are involved in Ser 280 phosphorylation downstream of FLT3-ITD

Pim1 kinase is a transcriptional target of STAT5 in leukemic cells and Pim kinases are frequently over-expressed in AML. As shown in Figures 2b and c, STAT5 inhibition indeed decreased the expression level of Pim1 in MV4-11 and Ba/F3-ITD cell lines. Quantitative PCR indicated that Pim1 mRNA was significantly reduced in these conditions (not shown), suggesting that this downregulation was transcriptional. As these kinases share structural and specificity characteristics with Akt, we assumed that they could be responsible for CHK1 phosphorylation. In consequence, we tested the effect of Pim1/2 pharmacological inhibition in MV4-11 cells, and found that it significantly reduced the level of Ser 280 phosphorylation (Figure 3a). Pim inhibition decreased the phosphorylation of the previously characterized Pim substrate RPS6, and did not modify the phosphorylations of FLT3-ITD and STAT5. Similar results were obtained in MOLM-14 cells (Supplementary Figure 2) and with another Pim inhibitor (Supplementary Figure 3).

To confirm these observations, we performed RNA interference experiments against Pim1 and Pim2. Downregulating either Pim1 or Pim2 did not modify Ser 280 phosphorylation (Figure 3b), but combined downregulation of both dramatically reduced the level of this phosphorylation (Figure 3c). These data demonstrate that Pim1 and 2 are key regulators of CHK1 phosphorylation in FLT3-ITD-positive cells, and that both kinases can compensate for each other to this respect, as previously reported.²³

Pim1 phosphorylates CHK1 *in vitro* and in cell cultures

In order to confirm this hypothesis, we performed *in vitro* phosphorylation of CHK1 by Pim1. Recombinant Pim1 was incubated with *in vitro*-translated CHK1 in the presence of ATP,

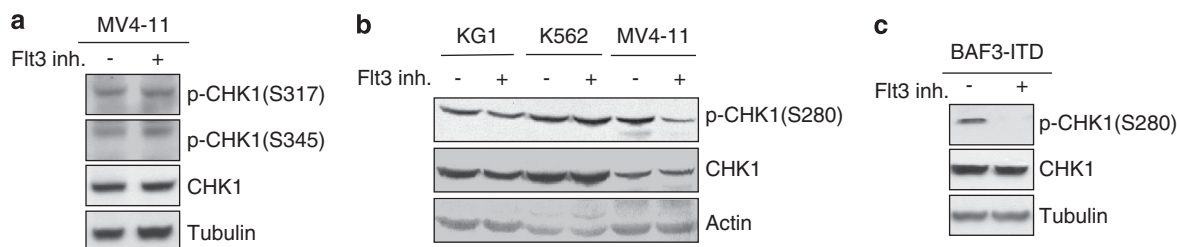


Figure 1. Phosphorylation of CHK1 on Ser 280 in leukemic cells. **(a)** Exponentially growing MV4-11 cells were treated for 2 h with Flt3 inhibitor III (100 nM). Western blot analyses of phospho-Ser 317 and phospho-Ser 345 or CHK1 were performed. Tubulin was used as a loading control in these experiments. **(b)** Exponentially growing leukemic cell lines K562 (FLT3-), KG1 (wtFLT3) and MV4-11 (FLT3-ITD) were treated with 100 nM of Flt3 inhibitor III (FLT3 inh) for 2 h, and western blot analyses of CHK1 and P-CHK1 Ser 280 were performed in the corresponding fractions. Actin was used as a loading control. This western blot is representative of three independent experiments. **(c)** Murine Ba/F3 cells constitutively expressing human FLT3-ITD were grown in the absence of IL-3 and processed for 4 h as in **a** for FLT3 inhibition and for western blot analysis.

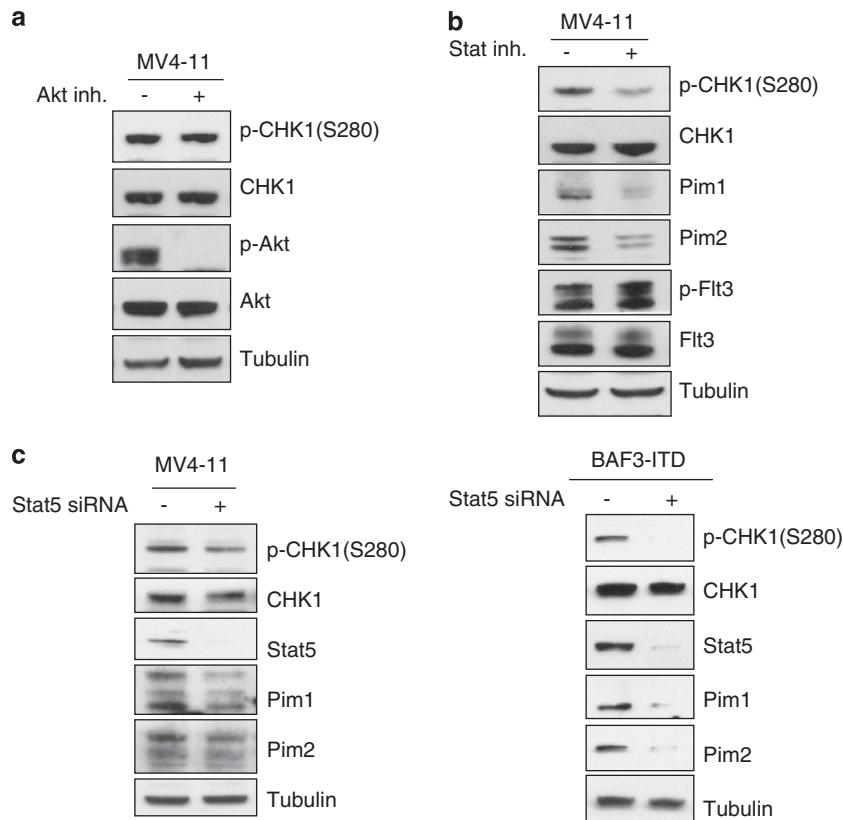


Figure 2. Cell signaling pathways involved in CHK1 phosphorylation in MV4-11 cells. Exponentially growing MV4-11 leukemic cells were treated for 2 h with 5 μM Akt inhibitor VIII (**a**) or 100 μM STAT5 inhibitor (**b**) and processed for western blot analysis as before. Phospho-Akt, Akt, FLT3, p-FLT3, Pim1, Pim2, p-CHK1 and CHK1 were detected by western blot. Data are representative of three independent experiments. (**c**) STAT5 was downregulated by RNA interference in MV4-11 (left panel) and Baf-3-ITD cells (right panel), and the effects on Ser 280 phosphorylation as well as on Pim1/2 and CHK1 expression were followed by western blot. Western blots against tubulin were used as loading controls in these experiments.

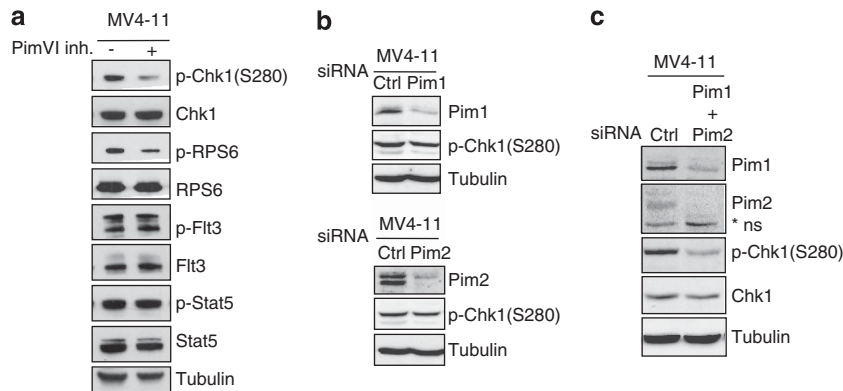


Figure 3. Pim1 and Pim2 kinases are involved in CHK1 phosphorylation on Ser 280. (**a**) Exponentially growing MV4-11 cells were treated for 4 h with 50 μM Pim inhibitor VI (Pim inh). Western blot analyses of p-CHK1, CHK1, p-RPS6 and RPS6 (an established substrate of Pim) were then performed. FLT3 and p-FLT3, as well as STAT5 and p-STAT5, were also detected by western blot, in order to verify the specificity of the Pim inhibitor VI. (**b**) MV4-11 cells were transfected with siRNA against Pim2 (higher panel), Pim1 (lower panel) or with control siRNA (Ctrl), and the levels of CHK1 Ser 280 phosphorylation (P-CHK1) and of CHK1 protein (CHK1), as well as the respective levels of the Pim2 and Pim1 proteins, were analyzed by western blot. (**c**) Same experiment as in **b**, but the cells were simultaneously transfected with Pim1 and Pim2 siRNAs. Tubulin was used as a loading control for these experiments.

and the product of the reaction was analyzed by western blot. As shown in Figure 4a, Pim1 efficiently phosphorylated CHK1 on Ser 280 *in vitro*. No signal was detected in the absence of ATP or with a Ser 280 Ala mutant of CHK1 (not shown).

We then performed transfection experiments of Pim1 and Pim2 in U2OS cells. As shown in Figure 4b, ectopic expression of Pim1 induced Ser 280 phosphorylation of endogenous CHK1, while Pim2 did not (Supplementary Figure 4). Moreover, when CHK1 and

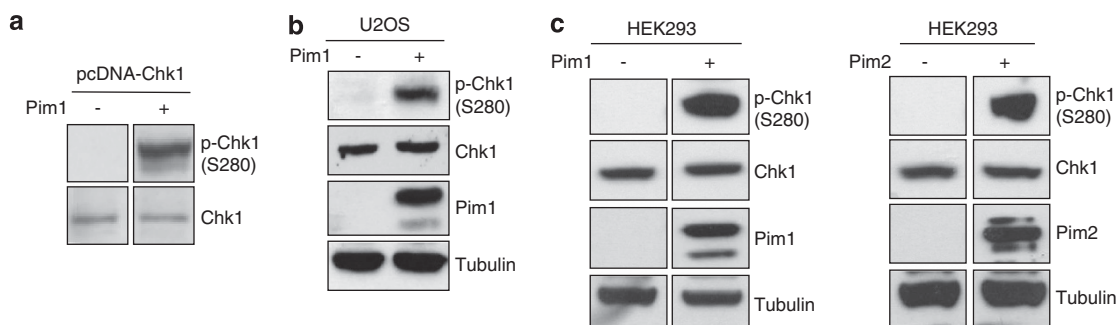


Figure 4. Pim1 and Pim2 phosphorylate CHK1 on Ser 280. (a) CHK1 was transcribed and translated *in vitro* in reticulocyte lysates, and the product of the reaction was incubated with 5 ng of recombinant active Pim1 and ATP at 30 °C for 30 min. The reaction was stopped with Laemmli buffer, and western blot analysis was performed with antibodies against CHK1 and p-Ser 280. (b) U2OS cells were transfected with Pim1, and western blot analyses of p-S280 (p-CHK1(S280)), CHK1 and Pim1 were performed. (c) HEK-293 cells were transfected with either Pim1 (left panel) or Pim2 (right panel), and the phosphorylation of CHK1 on Ser 280 was followed by western blot, as well as the protein levels of Pim1, Pim2 and CHK1. Tubulin was used as a loading control in these experiments.

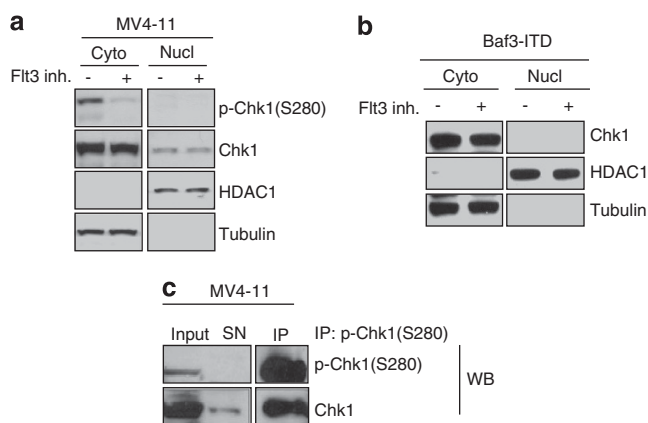


Figure 5. CHK1 localization in FLT3-ITD-expressing cells. (a) MV4-11 cells were treated for 2 h with FLT3 inhibitor III, and further fractionated into cytoplasmic (cyto) and nuclear (nucl) fractions. Western blot analysis of CHK1 and P-S280 was performed. HDAC1 and tubulin were used as markers of nuclear and cytoplasmic fractions, respectively. (b) Same experiment as in a, performed in FLT3-ITD-expressing Ba/F3 cells. (c) Exponentially growing MV4-11 cells were lysed in non denaturing RIPA buffer, and immunoprecipitation was performed with the anti P-Ser 280 antibody. The presence of CHK1 and of Ser 280-phosphorylated CHK1 was then tested by western blot in the initial fraction (input), in the supernatant of the immunoprecipitation (SN) and in the immunoprecipitated fraction (IP).

Pim2 were co-transfected, Pim2-dependent Ser 280 phosphorylation was detected on exogenously expressed CHK1 (Supplementary Figure 4). Ectopic expression of Pim1 or Pim2 also induced robust phosphorylation of endogenous CHK1 in HEK-293 cells (Figure 4c), confirming the results obtained in U2OS cells. These data demonstrate that Pim1/2 can phosphorylate CHK1 on Ser 280 in various cellular contexts.

Ser 280 phosphorylation does not affect CHK1 subcellular distribution

We then investigated the status of CHK1 localization in MV4-11 cells. Although we failed to detect endogenous CHK1 using currently available immunofluorescence reagents (not shown), we performed cell fractionation showing that both total and Ser 280-phosphorylated CHK1 were mostly localized in the cytoplasmic fraction of these cells (Figure 5a). Upon FLT3

inhibition, Ser 280 phosphorylation was dramatically reduced, but CHK1 remained essentially cytoplasmic. Similar results were obtained in the Ba/F3 FLT3-ITD-expressing cell line (Figure 5b). As interpretation of these results could depend on the relative abundance of Ser 280-phosphorylated CHK1, we immunoprecipitated this phosphorylated form (Figure 5c). Only a few percent of the total protein remained in the supernatant in these conditions, demonstrating that CHK1 is in majority phosphorylated on Ser 280 in FLT3-ITD-expressing cells, but this phosphorylation does not influence its localization.

FLT3-ITD facilitates CHK1 functions

We then investigated whether FLT3-ITD activity governs CHK1 activation and functions. As CHK1 is activated in response to genotoxic stress, we treated MV4-11 cells with the topoisomerase II inhibitor etoposide (VP-16), and we followed CHK1 activation through phosphorylation of Ser 345 or Ser 317. Etoposide induced CHK1 phosphorylation on both residues after 1 h (Supplementary Figure 5A), but FLT3-ITD inhibition did not modify this phosphorylation. This suggests that FLT3-ITD does not influence activation of CHK1 by the upstream kinase ATR.

Hence, we reasoned that FLT3-ITD could modulate the activity of CHK1 against its substrates. To this end, we followed the phosphorylation of the CDC25C phosphatase on Ser 216. As CHK1 is not the only kinase phosphorylating CDC25C on this residue, we accumulated cells in mitosis with nocodazole to suppress the phosphorylation by other kinases operating during interphase. As can be seen in Figure 6a, phosphorylation of CDC25C on Ser 216 by CHK1 was detected when these mitotic cells were treated with etoposide. In these conditions, FLT3-ITD inhibition significantly decreased this phosphorylation, suggesting that FLT3 activity is important for CHK1-dependent phosphorylation of CDC25C (Figure 6a). As a biological marker of CHK1 activity, we also followed histone H3 phosphorylation on Thr11, a process inhibited by genotoxic stress in other models.³⁰ As shown in Figure 6b, etoposide treatment decreased the phosphorylation of histone H3 Thr 11 in MV4-11 cells, and FLT3-ITD inhibition further majorated this effect. Similar results were obtained in the murine FLT3-ITD-positive Ba/F3 cell line, and in the MOLM-13 human cell line (Supplementary Figure 5B and C respectively).

We then investigated whether FLT3-ITD activity was necessary for resistance against genotoxic agents and G2/M checkpoint, which are two well-described functions of CHK1. As shown in Figure 6c, FLT3 inhibition dramatically reduced the resistance of MV4-11 cells to etoposide treatment. Similar results were obtained in MOLM-14 cells (not shown). Accordingly, FLT3 inhibition also impaired the G2/M checkpoint induced by this genotoxic agent

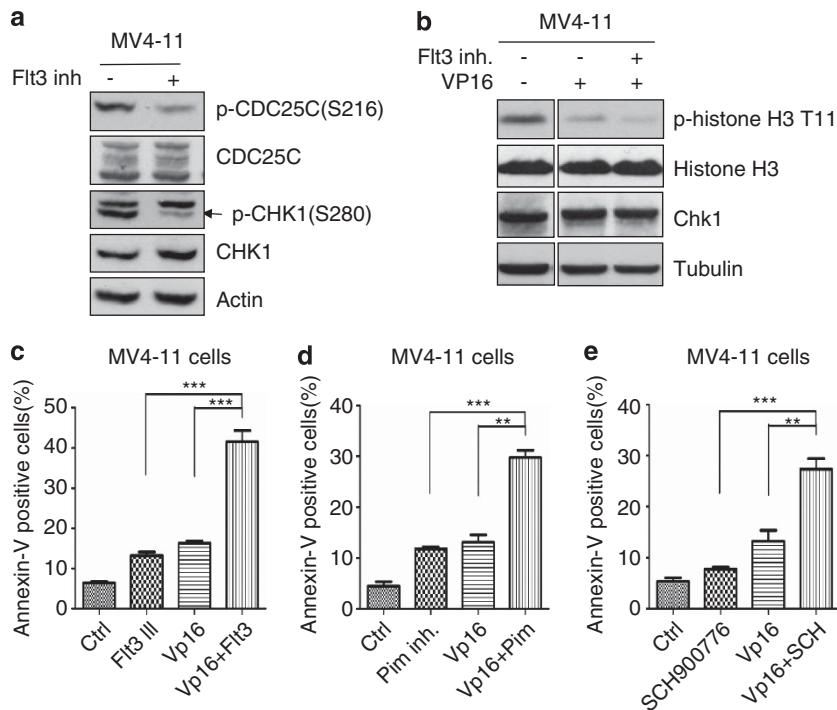


Figure 6. FLT3-ITD activity governs CHK1 molecular and cellular functions. **(a)** MV4-11 cells were blocked in mitosis with nocodazole for 24 h, and then treated with etoposide for 1 h, in the presence (+) or the absence (–) of FLT3 inhibitor III. Western blot analyses of CDC25C and of its phosphorylation level on Ser 216 were performed. Actin was used as a loading control in these experiments. **(b)** MV4-11 cells were treated for 1 h with etoposide (VP-16) in the presence or the absence of Flt3 inhibitor. Phosphorylation of histone H3 on Thr 11 (p-histone H3 T11), as well as the histone H3 protein and CHK1, were followed by western blot. Tubulin was used as a loading control in these experiments. **(c–e)** MV4-11 cells were incubated with etoposide alone or simultaneously with FLT3 (C; FLT3 III; 50 nM), PIM (D; PIM inh. VI; 50 μ M) or CHK1 (E; SCH-900776; 250 nM) inhibitors. After 24 h, flow cytometric analyses of apoptosis were performed by Annexin V staining. Results are mean \pm s.e.m. $n = 3$ independent experiments. ** $P < 0.01$; *** $P < 0.001$.

(Supplementary Figure 6A). In these conditions, cells accumulated in G1 and sub-G1 instead of G2/M, in good agreement with the highest rates of apoptosis observed in Figure 6c. When the same experiment was performed with PIM or CHK1 inhibitors, similar sensitization to etoposide was observed in terms of cell death induction (Figures 6d and e) and cell cycle distribution (Supplementary Figure 6B and C), further suggesting that the FLT3-ITD/Pim/CHK1 axis is involved in cell resistance in response to genotoxic agents. These results were reproduced in the murine Ba/F3-ITD cell line (Supplementary Figure 7A, B and C). In this cell type, siRNA-induced downregulation of CHK1 synergized with etoposide, confirming the results obtained through pharmacological CHK1 inhibition (Supplementary Figure 7D and E).

Altogether, these data indicate that FLT3 signaling is critical for the protection of cells against genotoxic agents in a CHK1-dependent way.

Ser 280 phosphorylation is involved in CHK1 functions in FLT3-ITD cells

To investigate the importance of CHK1 Ser 280 phosphorylation in its functions, we expressed HA-tagged wild type or Ser 280A mutant in Ba/F3-ITD cells (Figure 7). Expression levels of these two forms of CHK1 were similar (Figure 7a); exogenous CHK1wt was phosphorylated on Ser 280, and pharmacological inhibition of FLT3 suppressed this phosphorylation (Figure 7b). In consequence, we tested the influence of this expression on histone H3 phosphorylation in response to etoposide. As shown in Figure 7a, CHK1wt expression maintained histone H3 phosphorylation upon treatment with etoposide, while expression of the Ser 280A mutant did not. These data demonstrate that Ser 280 phosphorylation is an important determinant of CHK1 substrate

phosphorylation in response to genotoxic agents in FLT3-ITD-expressing cells. We then asked whether Ser 280 phosphorylation was involved in CHK1-dependent resistance in response to genotoxic stress. For this, endogenous CHK1 was downregulated with 3' UTR siRNA in Ba/F3-ITD cells expressing either wild type or mutant HA-tagged CHK1 (Figure 7c). In these conditions, resistance to etoposide became essentially dependent on ectopic CHK1. As depicted in Figure 7d, downregulating endogenous CHK1 did not modify the sensitivity of CHK1wt-expressing cells to etoposide. By contrast, in cells expressing the Ser 280A mutant, downregulating endogenous CHK1 increased the sensitivity to this drug. These data demonstrate that Ser 280 phosphorylation is an important step of CHK1-dependent resistance in FLT3-ITD-expressing cells.

Pim regulates Ser 280 phosphorylation in primary cells from patients

To confirm these data from established cell lines, we used primary cells from patients expressing either wild-type FLT3 or FLT3-ITD. Cells were treated for 4 h with Pim inhibitor VI, and Ser 280 phosphorylation was assessed by western blot. Pim inhibition in these cells induced Ser 280 dephosphorylation in five patient samples tested (Figure 8a). Interestingly, positively responding patients included both wild type and FLT3-ITD genotypes. Similar results were observed in four fresh samples from other patients, demonstrating that the process of freezing and thawing did not influence the cellular response (data not shown). These data confirm that Pim is a key regulator of CHK1 in these pathologies, and they suggest that this regulation is not dependent on FLT3 status. To test this hypothesis, two series of primary cells were treated with two different FLT3 inhibitors: FLT3 inhibitor III and AC-220, recently used in a phase II clinical trial in AML.³¹ As shown

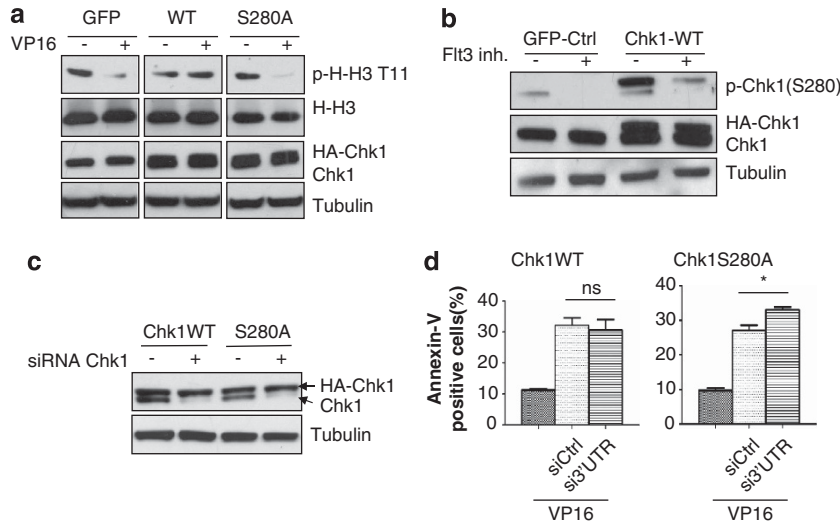


Figure 7. Ser 280 phosphorylation regulates CHK1 functions in FLT3-ITD cells. Ba/F3 FLT3-ITD cells expressing either GFP as a control, wild-type CHK1 or CHK1 S280A, were generated by lentiviral infection as described in the Material and Methods section. **(a)** The three cell types (GFP, WT and S280A) were treated with 10 μ M of the genotoxic agent etoposide (VP-16) for 1 h, and histone H3 phosphorylation on threonine 11 (p-H-H3) was followed by western blot analysis. The amounts of histone H3 (H-H3) and CHK1 proteins were also analyzed, and tubulin was used as a loading control. **(b)** Control (GFP) or CHK1-expressing Ba/F3-ITD cells were treated with FLT3 inhibitor (100 nM) for 2 h, and Ser 280 phosphorylation, as well as CHK1 and tubulin levels were followed by western blot. **(c, d)** Ba/F3-ITD cells expressing either CHK1wt or S280A mutant were treated or not with 3' UTR siRNA, allowing the specific downregulation of endogenous CHK1 as judged by western blot analysis **(c)**. They were then subjected to etoposide (VP-16) treatment for 24 h, and apoptosis was detected by annexin V staining **(d)**. Results are mean \pm s.e.m. $n = 3$ independent experiments * $P < 0.05$ ns, non-significant.

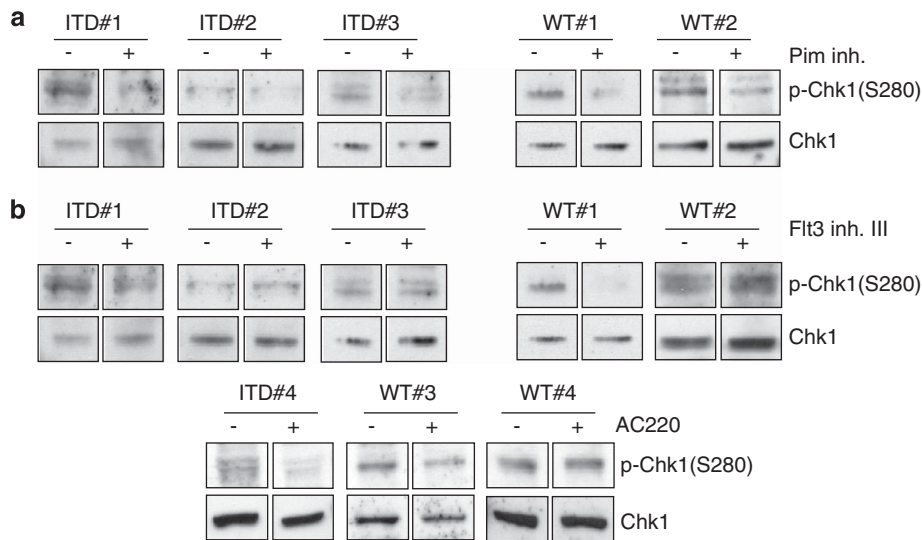


Figure 8. Pim and FLT3 regulate CHK1 phosphorylation in primary cells from patients. Primary cells from patients were thawed as described in Materials and Methods, and treated for 2 or 4 h with Pim inhibitor VI (50 μ M) **(a)**, with FLT3 inhibitor III (100 nM) **(b, upper panel)** or with AC-220 (5 nM) **(b, lower panel)**. Western blot analysis of p-Ser 280 and CHK1 were then performed.

in Figure 8b, CHK1 Ser 280 phosphorylation was reduced by these two inhibitors in some patients, independently of the FLT3 receptor status. In addition, in several primary samples, FLT3 inhibition did not modify CHK1 Ser 280 phosphorylation. Altogether, these data confirm that Pim regulates Ser 280 phosphorylation, and they suggest that this may occur downstream of FLT3, or through other yet unidentified mechanisms.

DISCUSSION

In this work, we investigated the status of CHK1 phosphorylation on Ser 280 in leukemic cells, and its impact on the function of the protein in this model. Interestingly, we observed that Ser 280

phosphorylation level was high, and that the major CHK1 population was cytoplasmic in FLT3-ITD cells. This is in apparent good agreement with previous studies describing Ser 280 phosphorylation-dependent cytoplasmic retention of CHK1.^{10,11} However, our results argue against this hypothesis, as inhibition of Ser 280 phosphorylation by different ways did not correlate with CHK1 localization changes in leukemic cells, suggesting that this phosphorylation is not a determinant for CHK1 nucleocytoplasmic distribution in this model. The reasons for this discrepancy remain unclear, but recent studies argue for the absence of localization difference between wild-type CHK1 and S280A mutant in certain circumstances and in different cell lines.^{32,33}

Our study also reveals that the Ser/Thr kinases Pim1/2 regulate CHK1 Ser 280 phosphorylation in leukemic cells, whereas Akt does not. This is reminiscent of a recent work describing phosphorylation of Ser 280 by the Ser/Thr kinase p90^{RSK} in RPE1 epithelial cells.³⁴ This suggests that different key signaling kinases can catalyze this phosphorylation, depending on the cell type and/or the microenvironmental context.

To our knowledge, we bring the first demonstration that Pim1 and Pim2 directly regulate CHK1, although these kinases have been involved in the regulation of other cell cycle proteins.²³ This is important as Pim kinases are key factors of the oncogenic capacity of FLT3-ITD,^{18,19} and can be considered as potential therapeutic targets in these pathologies. Although our data clearly argue for Pim kinases being direct regulators of CHK1, they do not distinguish between the relative importance of Pim1 and Pim2 to this respect. Indeed, our transfection experiments suggest a higher capacity of Pim1 to phosphorylate CHK1 in certain but not all cell types, but the experiments were performed in U2OS and HEK-293 cells, in which key signaling pathways may be different from leukemic cells. Further experiments will be needed to answer this question.

Interestingly, we observed that Ser 280 phosphorylation level was also relatively high in the KG1 and K562 leukemic cell lines, which do not express FLT3-ITD. This suggests that depending on the cell context and on the activated signaling pathways, various mechanisms can lead to CHK1 Ser 280 phosphorylation. Identifying the oncogenes and the downstream pathways involved in this process in the different leukemic cell types would be of interest, regarding CHK1 regulation as a potential therapeutic target in leukemia.

This is also in good accordance with the results obtained with primary leukemic cells, which point to the fact that Pim inhibition results in Ser 280 dephosphorylation in some samples expressing wild-type FLT3. Whether this reflects decoupling between Pim-dependent regulation of CHK1 and FLT3 mutational status or the fact that wild-type FLT3 is active in some primary samples, due to the presence of FLT3 ligand for instance, remains unclear. To this respect, one may also notice that the wild-type FLT3 receptor was found to be over-expressed in a majority of AMLs. This is also the case of Pim2, independently of the FLT3 status.²⁰ These data must be taken into account when analyzing the results obtained in primary cells.

Although FLT3 inhibition does not seem to affect CHK1 activation by itself, as judged by the activating phosphorylation of Ser 345 by ATR, we cannot rule out that its catalytic activity is indeed modified by Ser 280 phosphorylation. *In vitro* kinase activity measurement of CHK1 immunoprecipitated from leukemic cells should be necessary in order to confirm that no modification of the catalytic activity occurs upon Ser 280 dephosphorylation.

Interestingly, our data suggest that this phosphorylation influences the action of CHK1 on its substrates, CDC25C and histone H3, in response to genotoxic stress. This may be explained by various hypotheses, but one interesting possibility could be that subtle modifications of CHK1 localization occur upon phosphorylation of this residue, more complex than a simple nucleo-cytoplasmic equilibrium, but having an impact on the accessibility of the kinase to its substrates. Alternatively, changes in the capacity of CHK1 to interact with these substrates could also be hypothesized.

Our results introduce the idea that targeting the FLT3/STAT/Pim/CHK1 axis may constitute a potential therapeutic strategy for AML treatments. Although we did not detect any relationship between FLT3 activity and CHK1-activating phosphorylation (Ser 345), previous studies from our team^{26,27} described constitutive activation of CHK1 in AML blasts, more specifically in cells from patients with complex karyotype. Whether a specific status of Ser 280 phosphorylation exists in these cells would be

interesting to test, in order to better understand CHK1 functional (de)regulations in these patients with a negative prognosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)