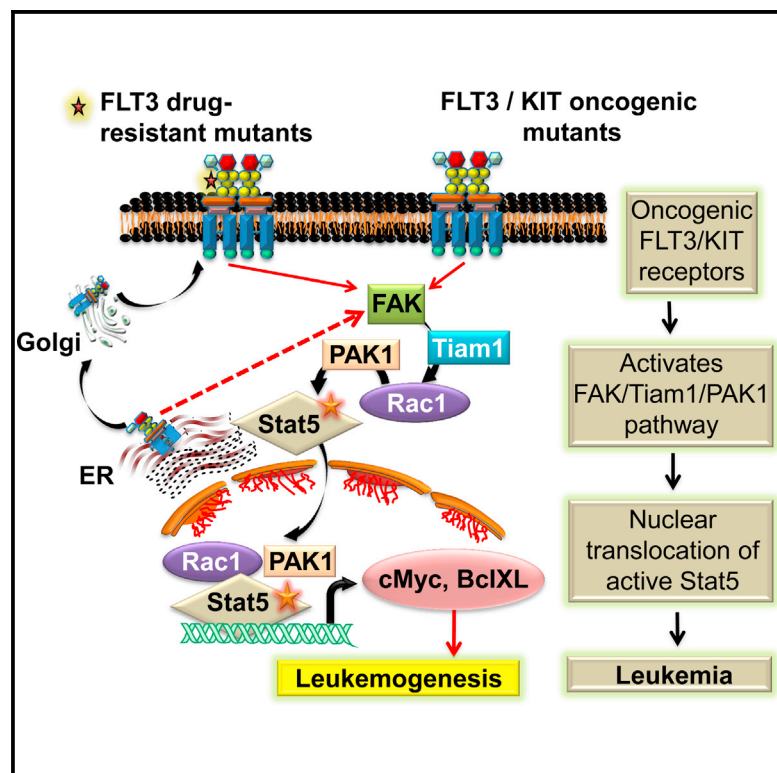# Regulation of Stat5 by FAK and PAK1 in Oncogenic FLT3- and KIT-Driven Leukemogenesis

Graphical Abstract

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In Brief

A significant impediment in treatment of leukemia, induced by oncogenic FLT3 and KIT receptors, is inadequate understanding of critical signaling pathways that lead to the development of this disease. In this study, Chatterjee et al. show an essential role of FAK/Tiam1/Rac1/PAK1 pathway in regulating nuclear translocation of Stat5 leading to leukemogenesis, in the context of oncogenic mutations of FLT3 and KIT, and provide multiple potential therapeutic targets to treat leukemia.

Highlights

FAK/Tiam1/Rac1/PAK1 regulate active Stat5 downstream from oncogenic KIT and FLT3

FAK/Tiam1/PAK1 inhibition prolongs survival of mice harboring KIT and FLT3 mutations

AC220-resistant mutants of FLT3 are sensitive to inhibition by FAK/Tiam1/PAK1 axis

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Regulation of Stat5 by FAK and PAK1 in Oncogenic FLT3- and KIT-Driven Leukemogenesis

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http://dx.doi.org/10.1016/j.celrep.2014.10.039

SUMMARY

Oncogenic mutations of FLT3 and KIT receptors are associated with poor survival in patients with acute myeloid leukemia (AML) and myeloproliferative neoplasms (MPNs), and currently available drugs are largely ineffective. Although Stat5 has been implicated in regulating several myeloid and lymphoid malignancies, how precisely Stat5 regulates leukemogenesis, including its nuclear translocation to induce gene transcription, is poorly understood. In leukemic cells, we show constitutive activation of focal adhesion kinase (FAK) whose inhibition represses leukemogenesis. Downstream of FAK, activation of Rac1 is regulated by RacGEF Tiam1, whose inhibition prolongs the survival of leukemic mice. Inhibition of the Rac1 effector PAK1 prolongs the survival of leukemic mice in part by inhibiting the nuclear translocation of Stat5. These results reveal a leukemic pathway involving FAK/Tiam1/Rac1/PAK1 and demonstrate an essential role for these signaling molecules in regulating the nuclear translocation of Stat5 in leukemogenesis.

INTRODUCTION

Acute myeloid leukemia (AML) is a lethal disease characterized by uncontrolled growth of myeloid cells and is predominantly a disease of the elderly. Little progress has been made in terms of standard-of-care treatment for AML, which has essentially remained the same over decades. Long-term survival is observed in ~30% of younger patients and ~5% of older patients greater than 60 years of age. Internal tandem duplications (ITD), in-frame insertions, or duplication of amino acids near the juxtamembrane domain of FLT3 have been observed in ~25%–30% of all AML

patients and confer a poor prognosis (Kottaridis et al., 2001). Likewise, Gleevec-resistant activation loop mutations of KIT (KITD816V) are found in a number of patients with core-binding factor-AML and ~95% patients with systemic mastocytosis (SM) and confer poor overall survival (Beghini et al., 2004). Both FLT3ITD and KITD816V receptors are constitutively phosphorylated (Kiyoi et al., 2002; Spiekermann et al., 2003) and induce growth in a ligand-independent manner. Whereas effort has been devoted to the development of FLT3 and KIT inhibitors, as single agents, the efficacy of these inhibitors is limited and in some cases results in drug resistance (Smith et al., 2012). Given that direct targeting of FLT3ITD or KITD816V has met with little success, signaling pathways downstream from FLT3ITD/KITD816V provide attractive alternate targets for treating hematologic malignancies involving these receptors.

Overexpression of focal adhesion kinase (FAK) in up to 50% of AML-patient-derived cells, but not in normal cells, has been observed, and FAK is hyperphosphorylated on Y397 in a number of patients. FAK+ AML cells show greater migration and resistance to daunorubicin compared to FAK− cells, and FAK expression correlates with high blast cell counts, early death, and shorter survival rate (Despeaux et al., 2011; Recher et al., 2004; Li and Hua, 2008). Presence of phosphorylated (p) pStat5 in newly diagnosed AML patients is also associated with poor overall survival (Brady et al., 2012). Constitutive activation of pStat5 is observed in 100% of SM patients bearing the KITD816V mutation (Baumgartner et al., 2009). A strong correlation between the presence of pStat5 and FLT3ITD mutations is seen in AML patients, and FLT3ITD expression results in constitutive Stat5 phosphorylation (Obermann et al., 2010; Spiekermann et al., 2003; Choudhary et al., 2005, 2007). Mutating the binding sites for Stat5 in the FLT3ITD abrogates the development of myeloproliferative neoplasms (MPNs) (Rocnik et al., 2006). Taken together, studies suggest that FLT3ITD/KITD814V, FAK, and Stat5 may be involved in regulating a critical pathway in AML and MPNs; however, the relationship between these signaling molecules in the context of leukemogenesis is not fully understood. Importantly,
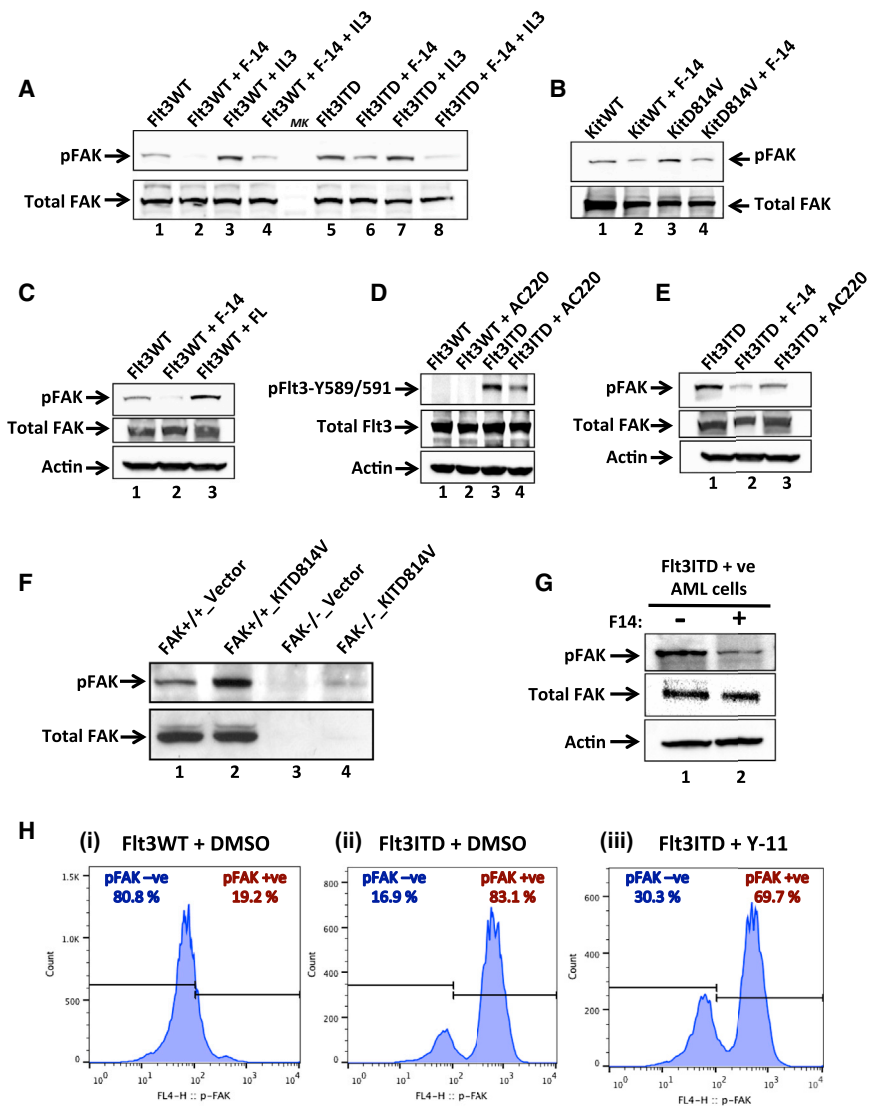
Figure 1. FAK Is Constitutively Phosphorylated in FLT3 and Activating KITD814V Oncogene-Bearing Cells

(A) Serum-starved 32D cells expressing FLT3ITD and FLT3WT were treated with DMSO (lanes 1 and 5), F-14 (lanes 2 and 6), IL-3 (lanes 3 and 7), or F-14 followed by IL-3 (lanes 4 and 8). An equal amount of protein was subjected to western blot analysis and probed with phospho-FAK (Y397) antibody (n = 3). "MK" denotes lane with protein ladder.

(B) 32D cells expressing KITWT or KITD814V were treated with F-14 (n = 2) and analyzed as described in (A).

(C) 32D cells expressing FLT3WT were treated with F-14 (lane 2) or stimulated with FLT3 ligand (FL) (lane 3) and analyzed as described in (A).

(D) 32D cells expressing FLT3WT or FLT3ITD were treated with FLT3ITD inhibitor AC220 and analyzed for activated FLT3 (pY589/591).

(E) 32D cells expressing FLT3ITD were treated with F-14 (lane 2) or with AC220 (lane 3; n = 2) and analyzed as above.

(F) Lysates from primary FAK−/−-deficient or WT BM cells expressing KITD814V or empty vector were analyzed for activated FAK.

(G) FLT3ITD+ve AML patient sample was analyzed for activated FAK (n = 2).

(H) 32D cells expressing FLT3ITD were treated with Y-11 and subjected to flow cytometry analysis. The percentage of cells show activated FAK under basal conditions in FLT3WT (i), FLT3ITD vehicle-treated cells (ii), and FLT3ITD treated with Y-11 (iii). n = 2.

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although Stat5 has been implicated in regulating several hematologic malignancies, how precisely activation of Stat5 is regulated in the cytosol or in the nucleus of leukemic cells and what are the signaling molecules involved in its nuclear import in the context of AML or MPN remains unclear. Here, we reveal a leukemic pathway involving FAK/Tiam1/Rac1/PAK1 and demonstrate an essential role for these signaling molecules in regulating the nuclear translocation of Stat5 in leukemogenesis.

RESULTS

FAK Is Constitutively Phosphorylated in FLT3ITD- and KITD814V-Expressing Cells

32D cells expressing the wild-type (WT) FLT3 or KIT receptor (FLT3WT or KITWT) or its oncogenic version (FLT3ITD or KITD814V) were starved and treated with a FAK-specific inhibitor F-14 (Golubovskaya et al., 2008). Enhanced activation of FAK was observed in FLT3ITD-bearing cells compared to controls (Figure 1A, lane 1 versus 5), which was inhibited in the pres-

ence of F-14 (Figure 1A, lane 6 versus 5). Similar results were observed in cells expressing KITWT and KITD814V receptors (Figure 1B). To assess if activation of FAK was restricted to oncogenic FLT3- and KIT-receptor-expressing cells, same cells were stimulated with interleukin (IL)-3 to activate the IL-3 receptor and analyzed for FAK activation. As seen in Figure 1A (lane 3), IL-3 stimulation also resulted in activation of FAK, which was inhibited in the presence of F-14 (Figure 1A, lane 4). Similar results were observed upon treatment of cells with FLT3 ligand (FL) (Figure 1C, lane 1 versus 3). To assess the direct involvement of FLT3ITD in FAK activation, cells were treated with AC220, a potent FLT3ITD inhibitor (Smith et al., 2012). Treatment of FLT3ITD cells with AC220 inhibited the activation of FLT3ITD (Figure 1D, lane 3 versus 4) and also resulted in reduced FAK activation (Figure 1E, lane 1 versus 3). To rule out the nonspecific effects of F-14 on FAK inhibition, we utilized a genetic approach. WT bone marrow (BM) cells expressing KITD814V demonstrated increased levels of active FAK compared to controls, whereas FAK−/− BM cells showed absence of FAK expression (Figure 1F). FLT3ITD+ve AML-patient-derived cells also demonstrated constitutive FAK activation, which was inhibited in the presence of F-14 (Figure 1G). Figures 1D and S8D show the expression of total FLT3 and KIT receptors. We also performed intracellular staining to determine the effect of
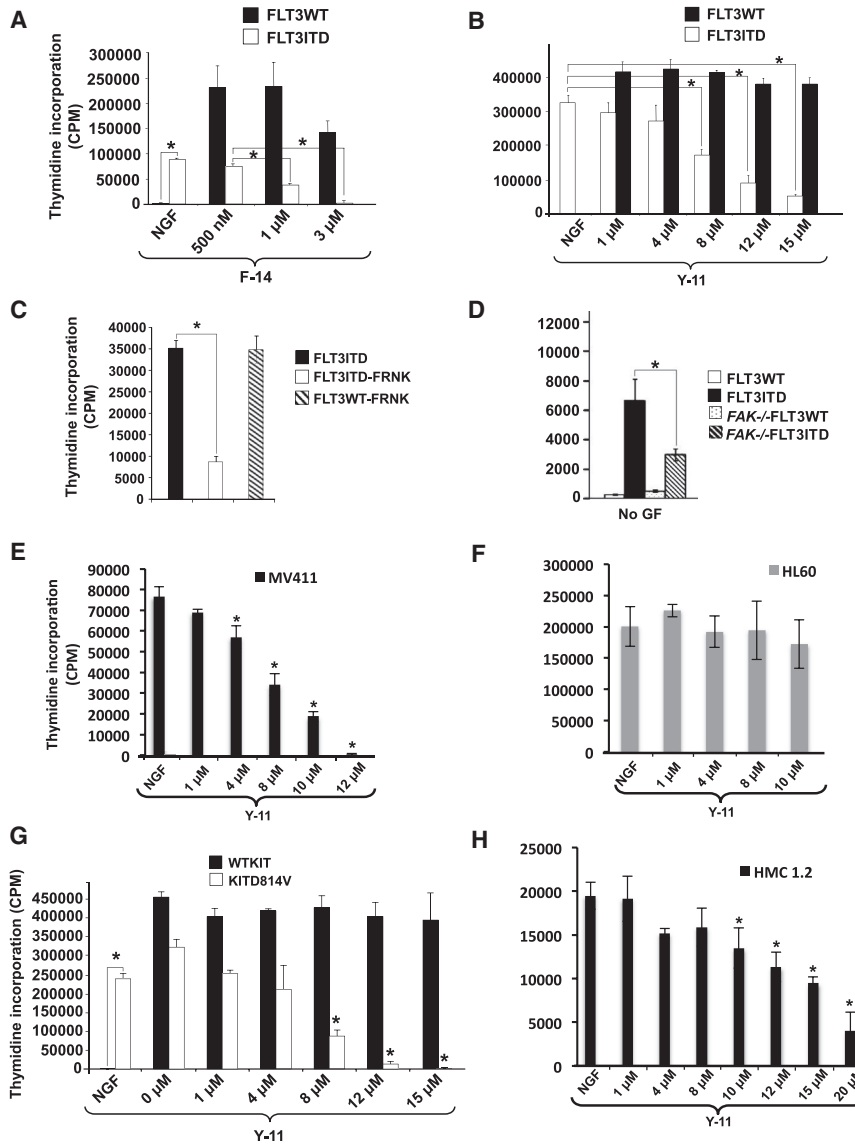
Figure 2. Inhibition of FAK Suppresses the Constitutive Growth of Oncogenic FLT3- and KIT-Bearing Cells

(A and B) BaF3 (A) or 32D (B) cells expressing FLT3WT or FLT3ITD were cultured for 48 hr in the presence or absence of F-14 or Y-11 in replicates of four and subjected to a thymidine incorporation assay. CPM, counts per minute.

(C) BaF3 cells coexpressing FRNK and either FLT3ITD or FLT3WT were subjected to thymidine incorporation assay as in (A) and (B).

(D) WT or FAK−/− BM cells expressing FLT3ITD or FLT3WT were subjected to proliferation assay in the absence of growth factors as described in (A) and (B).

(E and F) MV4-11 cells expressing endogenous levels of FLT3ITD (E) or HL60 cells harboring FLT3WT (F) were subjected to thymidine incorporation assay in presence of Y-11.

(G and H) 32D cells expressing KITD814V or WTKIT (G) and HMC1.2 human leukemic cells line harboring KIT (D816V + G560V) mutations (H) were cultured in the absence or presence of Y-11 and subjected to thymidine incorporation assay.

Thymidine incorporation is depicted on y axis as mean ± SD; *$p < 0.05$. NGF/No GF, cells grown in presence of no growth factors/cytokines. Data are representative of at least three independent experiments.

Y-11; another FAK-specific inhibitor (Golubovskaya et al., 2012) on FLT3ITD mediated repression of FAK. As seen in Figure 1H, the percentage of cells showing activated FAK was significantly higher in FLT3ITD-expressing cells (83.1%, middle panel) as compared to WT (19.2%; left versus middle panel). Treatment of FLT3ITD cells with Y-11 inhibited the activation of FAK (69.7%, right panel) and correspondingly increased the levels of unphosphorylated FAK (30.3%) in comparison to vehicle treated (16.9%; right panel versus middle panel). These results suggest that FAK is hyperactive in FLT3 and KIT oncogene-bearing cells and pharmacologic inhibition or genetic loss of FAK can repress the activation of FAK in these cells.

Inhibition of FAK Suppresses the Constitutive Growth of Oncogenic FLT3- and KIT-Expressing Cells

We assessed the functional significance of constitutive activation of FAK in FLT3ITD-expressing cells. As seen in Figure 2A,

treatment of BaF3 cells with F-14 significantly repressed the ligand-independent growth of FLT3ITD-bearing cells in a dose-dependent manner, with minimal effect on FLT3WT-expressing cells. Similar growth repression was seen in 32D cells treated with Y-11 (Figure 2B). Expression of FRNK, a dominant negative version of FAK (Zhao and Guan, 2009), also repressed the ligand-independent growth of FLT3ITD-expressing cells (Figure 2C), which was partly a result of reduced survival (Figures S1A and S1B). Expression of FRNK in FLT3 cells is shown in Figure S8D. Although these results suggest an essential role for FAK in ligand-independent growth of FLT3ITD-expressing cells, a direct role of FAK was ascertained by complete ablation of FAK. As seen in Figure 2D, expression of FLT3ITD in WT BM cells (FAK+/+) demonstrated ligand-independent growth, which was significantly repressed in FAK−/− cells. We also assessed whether targeting hyperactive FAK in cell lines derived from human leukemic patients shows similar effects. We used MV4-11 and HL60 cells that express the FLT3ITD and WT receptors, respectively. Treatment of MV4-11 cells with Y-11 showed a dose-dependent repression of constitutive growth (Figure 2E), whereas no such effect was observed in HL60 cells (Figure 2F).

To assess whether FAK plays a similar role in cells bearing an oncogenic form of KIT (KITD816V in humans and KITD814V in mouse), we used 32D cells expressing WTKIT or KITD814V. As seen in Figure 2G, treatment of these cells with Y-11 resulted
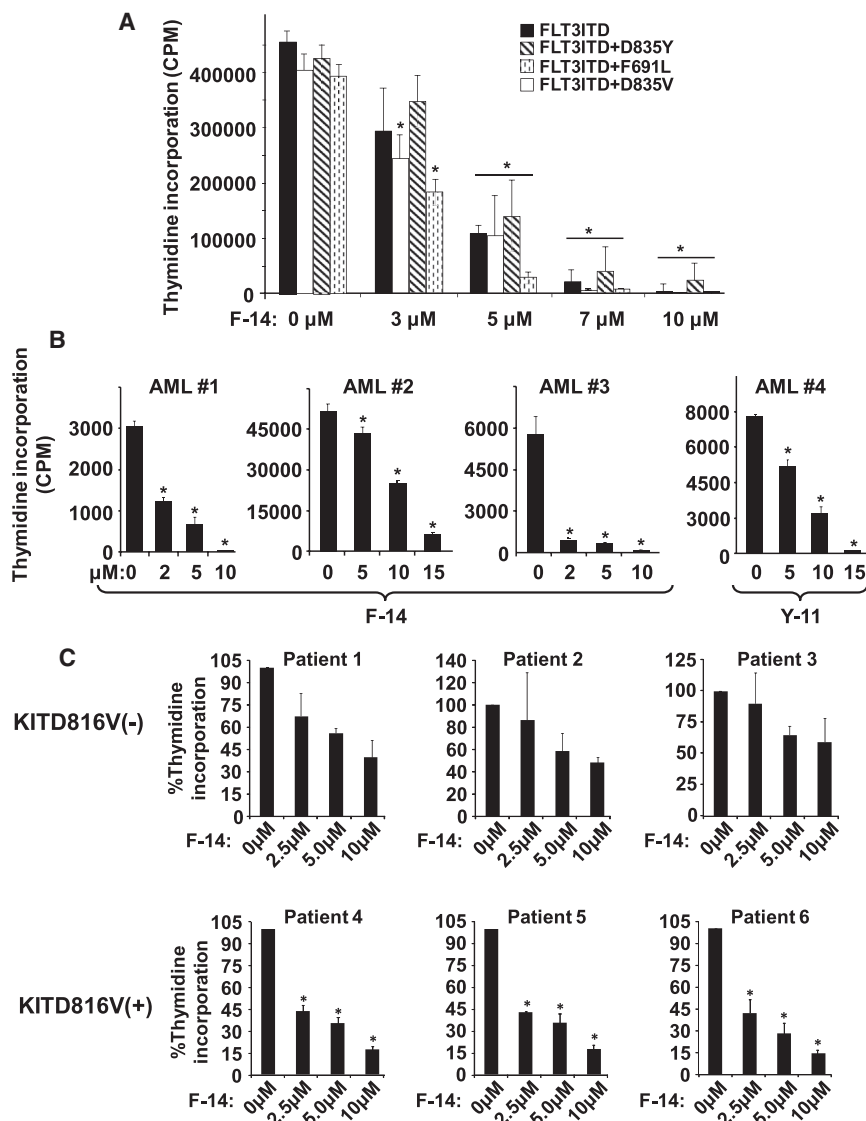
A

B

C

KITD816V(-)

KITD816V(+)

as a driver mutation and a critical therapeutic target in AML. We assessed whether these mutants were sensitive to FAK inhibition. As seen in Figure 3A, AC220-resistant FLT3-kinase-domain-mutant (D835Y, F691L, and D835V)-induced growth is inhibited by F-14.

AML FLT3ITD+ and KITD816V+ SM-Patient-Derived Cells Are Sensitive to FAK Inhibition

Next, we assessed whether inhibition of FAK in primary FLT3ITD+ AML cells inhibits their growth. We examined cells derived from 16 independent patients. Data from four representative patients are shown. In Figure 3B, a dose-dependent reduction in the growth of all FLT3ITD+ AML cells was observed in the presence of F-14 and Y-11. Likewise, SM-patient-derived cells positive for KITD816V mutation also demonstrated significantly greater growth reduction relative to patients lacking the expression of KITD816V (Figure 3C). These results

in growth repression of KITD814V-expressing cells. Similar results were observed upon Y-11 treatment of HMC1.2 cells derived from a human mastocytosis patient bearing the activating KIT mutation (Figure 2H). These results show that FAK plays an essential role in supporting the constitutive growth of FLT3 and KIT oncogene-bearing hematopoietic cells, which is modulated by pharmacologic or genetic inhibition of FAK.

AC220-Resistant "Driver" Mutations of FLT3 Are Sensitive to FAK Inhibition

Recent translational studies validated FLT3ITD mutations in AML to function as "driver," but not "passenger," mutations (Smith et al., 2012). Smith et al. demonstrated the presence of point mutations at three residues within the kinase domain of FLT3ITD that conferred resistance to AC220, an inhibitor of FLT3 and KIT. Acquisition of AC220-resistant substitutions at two of these residues was observed in all FLT3ITD+ AML patients with acquired resistance to AC220, thus validating FLT3ITD to function

demonstrate that FAK is indeed hyperactive in FLT3ITD- and KITD816V-expressing cells and its inhibition is associated with enhanced apoptosis and growth repression.

FAK and Rac1 Modulate the Nuclear Translocation of Active Stat5 in FLT3 and KIT Oncogene-Expressing Cells

In an effort to identify downstream targets of FAK that might contribute to FLT3ITD-induced growth and enhanced survival, we examined the activation of phosphatidylinositol 3-kinase (PI3K), ERK mitogen-activated protein kinase (MAPK) as well as Rac guanosine triphosphatases (GTPases). In nonhematopoietic cell types, all three pathways have been shown to be regulated by FAK (Gabarra-Niecko et al., 2003; Yin, 2011). We found constitutive and enhanced activation of Rac1 in FLT3ITD-bearing cells relative to FLT3WT-bearing cells (Figure 4A; lanes 1 and 2 versus 4 in control [CT] panel). Expression of FRNK in FLT3ITD-expressing cells significantly inhibited the
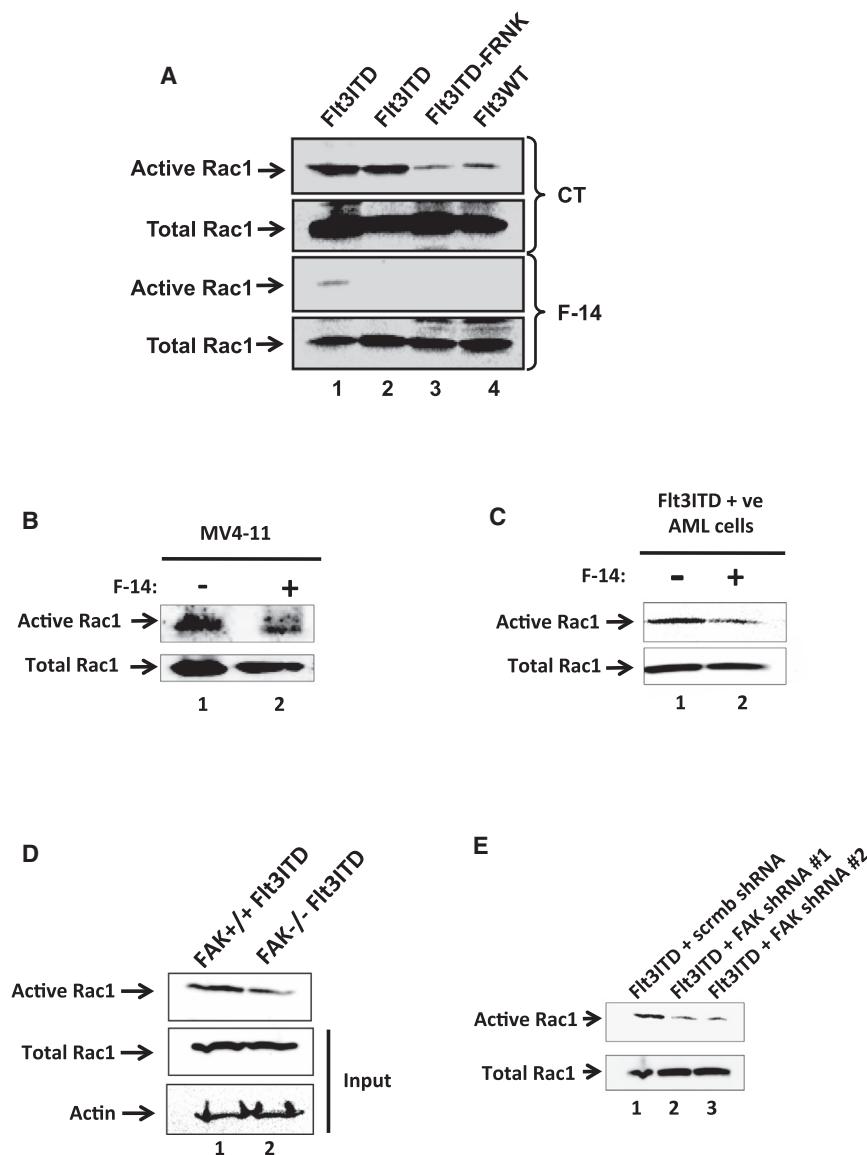
A

B

MV4-11

C

Flt3ITD + ve
AML cells

D

E

Figure 4. Rac1 Is a Downstream Effector of FAK in FLT3ITD-Bearing Oncogenic Pathway

(A) BaF3 (lane 1) or 32D (lane 2) cells expressing the FLT3WT receptor or FLT3ITD both alone or in combination with FRNK were starved and subjected to a Rac activation assay. These cells were either vehicle-treated alone (upper panel [CT]) or with F-14 (lower panel; n = 2).

(B and C) MV4-11 cells expressing endogenous FLT3ITD (B) and AML patient FLT3ITD+ cells (C) were subjected to Rac activation assay as described in (A).

(D) FLT3ITD-bearing BM cells in the setting of FAK deficiency were subjected to Rac activation assay as in (A) (n = 2).

(E) 32D FLT3ITD cells expressing shRNAs targeting FAK (lanes 2 and 3) and control shRNA (lane 1) were subjected to Rac1 activation assay as described in (A).

FLT3ITD-bearing cells expressing a dominant-negative version of Rac (RacN17; Figure S1F).

Active Stat5 is thought to play an essential role in regulating the transformation of FLT3 and KIT oncogene-bearing cells (Baumgartner et al., 2009; Brady et al., 2012). Although Stat5 is a transcription factor, mechanism(s) involved in the transport of this molecule in and out of the nucleus in oncogene-bearing cells remains poorly understood. We therefore performed cellular fractionation assays to determine the mechanism(s) behind active Stat5 translocation into the nucleus mediated by Rac1 and its upstream activator FAK in oncogene-bearing cells. We found active Stat5 in the nuclear fractions of FLT3ITD-bearing cells, which was associated with enhanced presence of Rac1, as compared with cells expressing

constitutive activation of Rac (Figure 4A; lane 3, CT panel). Furthermore, treatment of these cells with F-14 abolished Rac activation (lanes 1–4 upper CT panel versus lanes 1–4 lower [F-14] panel; Figure 4A). A similar reduction in Rac activation was also observed in FLT3ITD+ve human leukemic MV4-11 cells and FLT3ITD+ AML-patient-derived cells, respectively (Figures 4B and 4C) as well as in *FAK−/−*-deficient cells expressing FLT3ITD (Figure 4D). Small hairpin RNA (shRNA)-mediated downregulation of FAK in FLT3ITD-bearing cells also showed similar results (Figure 4E). These results suggest that FAK plays a role in the activation of Rac1 in FLT3ITD-bearing cells. Consistent with these observations, treatment of cells with a Rac1 inhibitor NSC23766 repressed the constitutive growth of FLT3ITD+ AML-patient-derived cells and of MV4-11 leukemic cells expressing the FLT3ITD receptor (Figures S1C and S1D). HL60 cells that express the FLT3WT receptor were used as a negative control (Figure S1E). Similar findings were observed in

FLT3WT (Figure 5A, lane 1 versus 3). To determine whether nuclear translocation of Stat5 could be mimicked in FLT3WT-expressing cells upon cytokine stimulation, cells were treated with IL-3 and analyzed for Stat5 and Rac1 localization. As seen in Figure 5A (lane 2), addition of IL-3 resulted in activation of FLT3WT-mediated Stat5-Rac1 nuclear localization. Next, we assessed if the effect of FAK inhibition could be overcome after ligand stimulation. FLT3ITD-expressing cells were treated with FLT3 ligand, followed by treatment with F-14. As seen in Figure 5B, stimulation of FLT3ITD with its ligand FL resulted in a modest increase in nuclear localized active Stat5 and Rac1 (Figure 5B, lane 1 versus 3; Zheng et al., 2011), which was repressed in the presence of F-14 (Figure 5B, lane 4). A similar reduction in the activation of Stat5 and accumulation of Rac1, respectively, was noted in the nuclear fractions of primary FLT3ITD+ AML (Figure S2A), KITD816V+ mastocytosis (HMC1.2; Figure 5C), and AML-patient-derived cells (MV4-11)
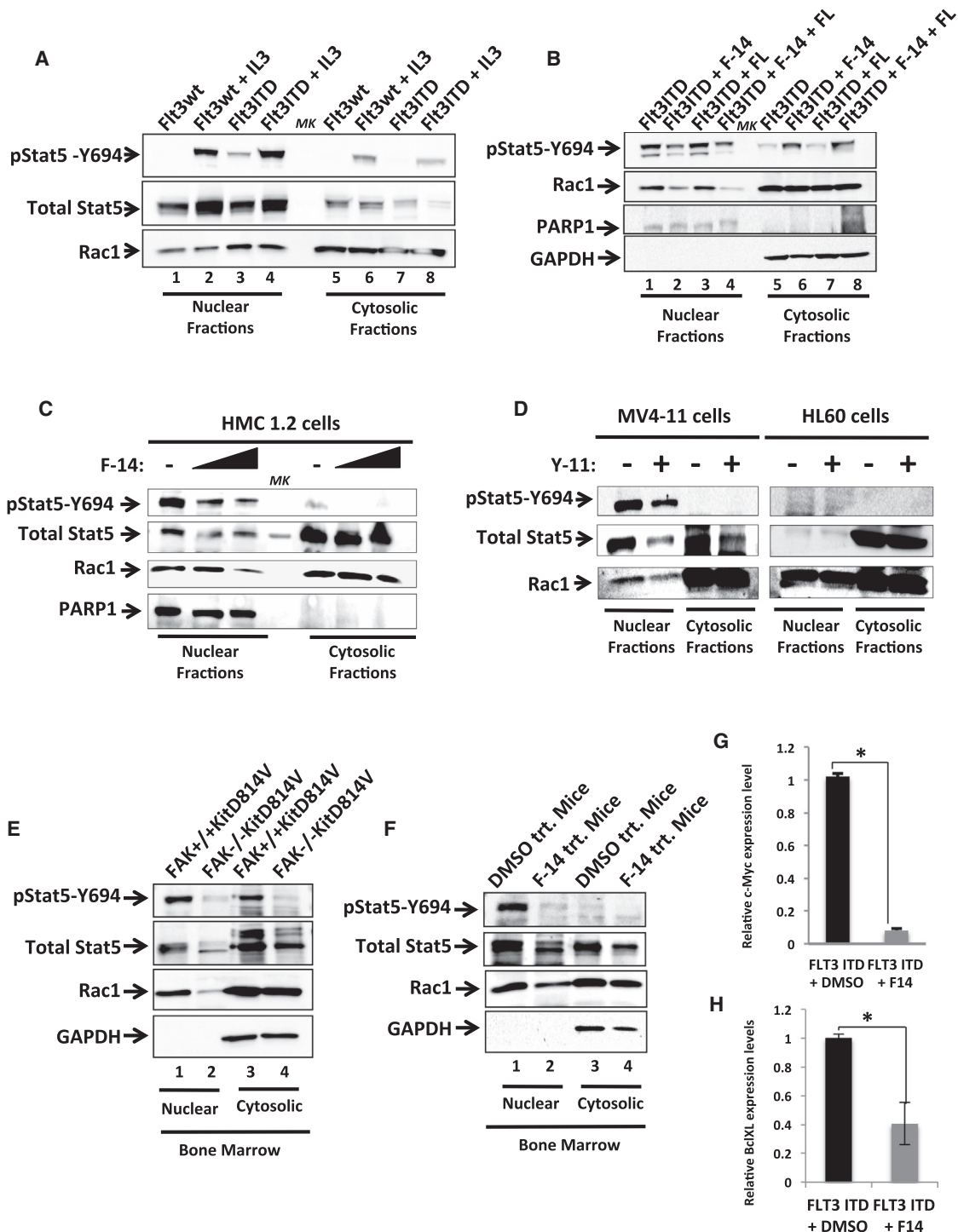
Figure 5. FAK Regulates the Translocation of Active Stat5 and Rac1 to the Nucleus in FLT3ITD- and KITD816V-Expressing Cells

(A) 32D cells expressing FLT3ITD or FLT3WT were serum starved and subjected to fractionation assays and nuclear and cytosolic fractions analyzed for levels of active Stat5 (pY694), total Stat5, and Rac1.

(B) 32D cells expressing FLT3ITD were subjected to fractionation assays after treatment with DMSO control, F-14, FL, or with F-14 followed by FL (B; n = 3).

(C) HMC1.2 cells bearing KITD816V+G560V mutations were subjected to fractionation assay as described in (A).

(D) MV4-11 and HL60 cells derived from leukemia patients harboring endogenous FLT3ITD and FLT3WT mutations were subjected to fractionation assay in presence of F-14 or Y-11 as in (A).

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treated with F-14 (Figure 5D). HL60 cells that express the FLT3WT receptor served as a negative control (Figure 5D, right panels). AC220-resistant driver mutations of FLT3 also demonstrated reduced nuclear accumulation of active Stat5 and total Rac1, respectively, when treated with F-14 (Figure S2B). Although we did not observe inhibition in the activation of MAPK or PI3K/Akt pathway in FLT3ITD-bearing cells lacking FAK or in which FAK was inhibited, we nonetheless directly examined the contribution of these pathways in the nuclear translocation of Stat5 and total Rac1. We performed a fractionation assay in the presence of MEK (PD98059), Raf (PLX4720), or Akt (124005) inhibitor and observed no significant inhibition of Rac1 or active Stat5 nuclear translocation (Figure S2C). Raf and MEK inhibitors also failed to repress the constitutive growth of FLT3ITD-bearing cells (Figures S2D and S2E).

A direct role for FAK in regulating the translocation of active Stat5 can be seen in Figure S2F. Expression of FLT3ITD failed to translocate active Stat5 and total Rac1 to the nucleus in *FAK*−/−-deficient BM cells (Figure S2F, lane 1 versus 2). The expression of FLT3 receptor in WT (*FAK*+/+) and *FAK*−/− BM cells is shown in Figure S8E. To further understand the mechanism behind FAK-regulated Stat5 translocation in FLT3 and KIT oncogene-bearing cells in an in vivo setting, we used cells derived directly from mice that were transplanted with BM from WT (*FAK*+/+) and FAK-deficient (*FAK*−/−) mice expressing KITD814V. Mice transplanted with WTFAK BM cells expressing KITD814V came down with disease significantly earlier than *FAK*−/− KITD814V mice (Figure 7G), at which point time-point-matched control mice (*FAK*−/− KITD814V) were euthanized and BM cells harvested from both groups of mice and subjected to cellular fractionation analysis. As seen in Figure 5E (lane 1 versus 2), and consistent with our in vitro findings (Figure S2F), genetic ablation of FAK in vivo inhibited the nuclear translocation of active Stat5 and Rac1 in KITD814V-bearing BM-derived leukemic cells. Furthermore, similar results were also observed after F-14 treatment of leukemic mice transplanted with BM cells expressing KITD814V. As seen in Figure 5F, BM cells derived from leukemic mice bearing the KITD814V mutation that were treated with F-14 also showed a reduction in Stat5 activation and nuclear accumulation of Rac1, as compared to cells derived from vehicle (DMSO)-treated mice (Figure 5F, lane 1 versus 2).

Active Stat5 downstream of FLT3ITD translocates to the nucleus to bind DNA and express Stat5-responsive genes like c-Myc and BclXL that play a crucial role in leukemogenesis (Li et al., 2007; Zhang et al., 2000). We next performed quantitative RT-PCR (qRT-PCR) analysis to determine the relative expression of c-Myc and BclXL genes in FLT3ITD cells treated with F-14. As seen in Figures 5G and 5H, a significant reduction in the expression of Stat5-responsive genes c-Myc and BclXL was observed

upon FAK inhibition. Similar results were observed in *FAK*−/− FLT3ITD cells (Figures S3A and S3B). To ascertain whether FAK also regulates the nuclear association between Rac1 and Stat5, besides directly activating Rac1, F-14-treated nuclear fractions were subjected to Rac1 immunoprecipitation assay. As seen in Figure 6A, the amount of active Stat5 that interacts with Rac1 was significantly reduced upon treatment of FLT3ITD cells with F-14 (Figure 6A, lane 1 versus 2). These results explain our initial observation demonstrating reduced levels of total Rac1 and active Stat5 in nuclear fractions of FLT3ITD-bearing cells in which FAK activation was repressed and is directly a consequence of FAK's role in the activation of Rac1 and more importantly its association and subsequent translocation with active Stat5 into the nucleus. To further assess whether FAK regulates the association between active Rac1 and active Stat5, we performed active Rac1 pull-down assay from WT and *FAK*−/− BM cells expressing FLT3ITD and analyzed for active Stat5 binding. As seen in Figure 6B, increased levels of active and total Stat5 protein were bound to activated Rac1 in WT (*FAK*+/+) BM cells (lane 1), which was reduced in FAK-deficient (*FAK*−/−) FLT3ITD-bearing cells (lane 2). Taken together, these findings suggest that first FAK regulates the formation of an active Rac1-active Stat5 complex and second it modulates the translocation of Rac1-Stat5 complex into the nuclear compartment.

Because Rac1 contains a functional nuclear localization signal and also forms a complex with active Stat5 in the nucleus, we next investigated the role of Rac1 in nuclear translocation of active Stat5 in FLT3ITD-bearing cells. Cells were treated with or without Rac inhibitor NSC23766 and subjected to a fractionation assay. Levels of active Stat5, along with total Rac1, were reduced in FLT3ITD cells treated with NSC23766 (Figure S3C). Likewise, cells coexpressing FLT3ITD along with a dominant-negative form of Rac1 (Rac1N17) showed reduced levels of active Stat5 in the nuclear fractions (Figure S3D). Equal expression levels of GFP-Rac1N17 in FLT3ITD and WT cells can be seen in Figures S3E and S3F. As seen in Figure 6C (lane 1 versus 2), robust levels of active Stat5 were present in nuclear fractions of FLT3ITD-expressing *Rac1*+/+ BM cells, whereas a significant reduction in the levels of nuclear localized active Stat5 was observed in *Rac1*−/− BM cells, with no detectable levels of Stat5 activation in FLT3WT-bearing cells. These results demonstrate an essential role for Rac1 in the translocation of active Stat5 into the nucleus.

RacGEF Tiam1 Is Essential for FLT3ITD-Induced Leukemic Development in Mice

To identify Rac guanine nucleotide exchange factors (GEFs) involved downstream of FAK in activating Rac1 in FLT3ITD-bearing cells, we analyzed the role of Tiam1. We ascertained whether Tiam1 is active in cells bearing FLT3ITD. As seen in Figure 6D, increased levels of active Tiam1 were observed in

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(E) Fractionation assays were performed in BM cells harvested from primary transplanted mice cohorts transplanted with KITD814V in a wild-type FAK (*FAK*+/+) or FAK-deficient (*FAK*−/−) background (n = 2).

(F) Fractionation assay from BM cells harvested from F-14- or DMSO (vehicle)-treated primary transplant mice cohorts. The level of Stat5 phosphorylation/expression and Rac1 expression in the nuclear and cytosolic fractions is indicated. Expression of GAPDH was used as an indicator of cytosolic marker and loading control. MK denotes lane with protein ladder.

(G and H) qRT-PCR analysis of relative mRNA expression levels of Stat5 responsive genes c-Myc (G) and BclXL (H) in FLT3ITD cells treated with F-14 or vehicle (DMSO; n = 2); *p < 0.05.
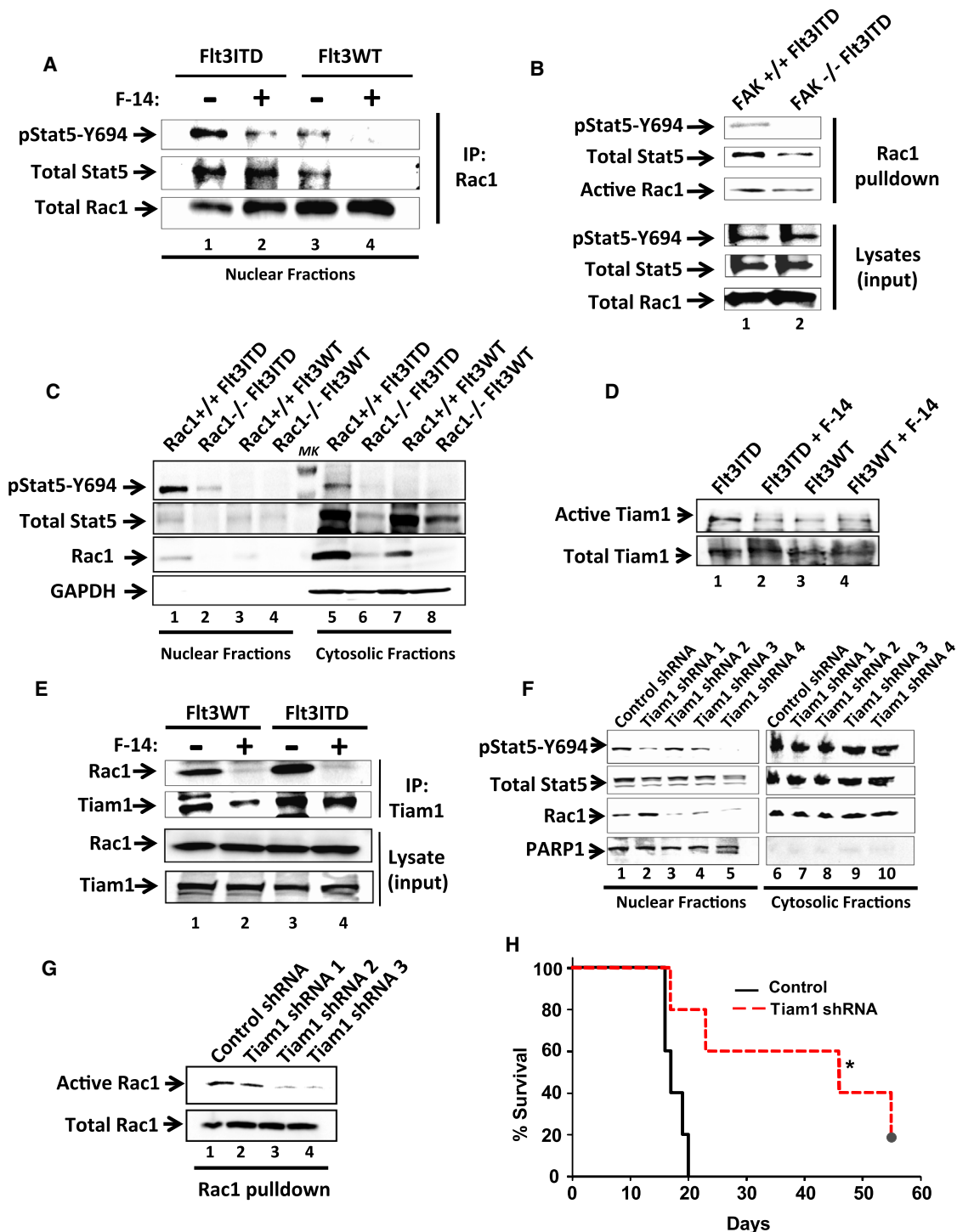
Figure 6. Downstream of FAK, Tiam1 Regulates the Activation of Rac1 and Subsequent Translocation of Active Stat5 to the Nuclear Compartment to Develop Leukemia in Mice

(A) Nuclear fractions from F-14-treated FLT3ITD- and FLT3WT-bearing cells were subjected to Rac1 immunoprecipitation assay to assess the level of Rac1 binding to active Stat5. Level of active Stat5 (pY694) and total Stat5 and Rac1 were analyzed.

(B) Active Rac1 fractions from WT and *FAK*−/−-deficient BM cells expressing FLT3ITD were determined, along with levels of active and total Stat5. Total Rac1 levels are shown in the lowermost panel (n = 2).

(C) Fractionation assay was performed using WT and *Rac1*−/− BM cells expressing FLT3ITD or FLT3WT receptors. Nuclear and cytosolic fractions were analyzed as described above. GAPDH was used as a loading control and cytosolic marker (n = 3). MK denotes lane with protein ladder.

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FLT3ITD-bearing cells compared to FLT3WT-expressing cells (lane 1 versus 3), which was attenuated upon treating these cells with F-14 (lane 1 versus 2). Further, F-14 treatment perturbed the interaction between Rac1 and Tiam1 (Figure 6E). Having observed the presence of Tiam1 in active Rac1 complex in FLT3ITD-bearing cells and the perturbation of this association upon FAK inhibition, we next examined the functional significance of Tiam1 in FLT3ITD-induced transformation. We knocked down Tiam1 using shRNA in cells bearing FLT3ITD (Figure S3G). Cells bearing FLT3ITD and Tiam1 shRNA showed significant reduction in constitutive growth in comparison to cells bearing scrambled shRNA (Figure S3H). Moreover, when FLT3ITD and Tiam1shRNA coexpressing cells were subjected to a fractionation assay, the levels of active Stat5 and total Rac1 in nuclear fractions were significantly reduced compared to scrambled shRNA-expressing cells (Figure 6F, lane 1 versus 2–5). As shown in Figure 6G (lane 1 versus 2–4), knockdown of Tiam1 significantly inhibited the activation of Rac1 compared to cells coexpressing FLT3ITD and scrambled shRNA. These results indicate that Tiam1 plays an important role in the activation of Rac1 in FLT3ITD-expressing cells, which in turn regulates the nuclear translocation of the Rac1/Stat5 complex. To further investigate the role of Tiam1 in FLT3ITD-induced leukemogenesis, we performed transplantation experiments. Figure 6H shows that mice transplanted with FLT3ITD and Tiam1 shRNA survived significantly longer compared to mice transplanted with FLT3ITD and scrambled vector (*p < 0.01).

Inhibition of FAK Delays the Onset of FLT3ITD- and KITD814V-Induced MPN Development and Prolongs the Survival of Mice

We next examined the in vivo impact of FAK inhibition on FLT3ITD- and KITD814V-induced leukemogenesis. Although mice bearing FLT3ITD cells treated with DMSO died within 30 days posttransplant, mice treated with F-14 showed significantly prolonged survival (Figure 7A). F-14-treated mice showed significantly reduced spleen weight (Figures 7B and 7C) and demonstrated absence of lesions in lungs compared to DMSO-treated mice (Figures S4A and S4B). Histopathologic analysis showed leukemic infiltration of myeloid cells and destruction of alveolar architecture in lungs and of normal architecture in spleens of DMSO-treated mice but significant improvement in the F-14-treated mice (Figures S4C and S4D). Moreover, F-14-treated mice also showed reduced percentage of leukemic cells in tissues (peripheral blood and spleen) as determined by the presence of GFP-positive cells (Figures S4E and S4F), relative to vehicle-treated mice. Likewise, F-14 treatment of mice transplanted with cells expressing KITD814V survived significantly longer than vehicle-treated mice (Figure S5A; *p < 0.01). We next assessed these findings in mice transplanted with primary

BM cells expressing KITD814V. One cohort of mice was treated with F-14 and the other with vehicle (DMSO). As seen in Figure 7D, white blood cell (WBC) counts remained constant over the entire duration of F-14 treatment in KITD814V-bearing mice (6 weeks), whereas KITD814V-bearing mice treated with vehicle demonstrated a steady rise in WBC counts over time, a hallmark of MPN development and progression. At the end of 6 weeks, all mice were euthanized and analyzed. As seen in Figures 7E and 7F, vehicle-treated mice demonstrated significant enlargement of spleen compared to F-14-treated mice. Collectively, these data support the observation that targeting FAK rescues the development of FLT3ITD- and KITD814V-induced MPN in vivo. To further investigate the role of FAK in FLT3ITD-induced MPN, we knocked down FAK expression using shRNA in cells bearing FLT3ITD and transplanted into mice as described in Figure 7A. Knockdown of FAK not only repressed the constitutive growth of FLT3ITD-bearing cells (Figure S5B), but more importantly, mice transplanted with cells coinfected with FLT3ITD and FAK shRNA survived significantly longer compared to mice transplanted with FLT3ITD and scrambled vector (Figure S5C; *p < 0.025). To rule out nonspecific effects of F-14 and diminished, but not absolute, effects of shRNA-mediated knockdown of FAK, we performed transplantation studies using primary BM cells from WT (*FAK+/+*) and *FAK−/−*-deficient mice expressing the oncogenic KITD814V receptor. As seen in Figure 7G, genetic ablation of FAK significantly prolonged the survival of leukemic mice (*FAK−/−* KITD814V) compared to controls (*FAK+/+* KITD814V). Leukemic mice harboring the KITD814V oncogene in the *FAK−/−* background demonstrated reduced spleen size (Figures 7H and 7I) and WBC counts relative to controls (Figure S5D). To further ascertain whether targeting FAK in the context of KITD814V-induced MPN inhibits the growth of cells that give rise to leukemia, we performed secondary transplants using BM cells derived from the primary cohorts. As seen in Figure 7J, mice transplanted with BM cells from primary donor harboring KITD814V in a FAK-deficient background (*FAK−/−* KITD814V) survived significantly longer than mice transplanted with BM from WT background (*FAK+/+* KITD814V). The prolonged survival of these mice correlated with reduced splenomegaly (Figures 7K and 7L). Importantly, loss of FAK in hematopoietic stem cells did not impair the engraftment or the self-renewal of these cells (Lu et al., 2012).

Targeting PAK1 Inhibits the Nuclear Translocation of Active Stat5

To determine the functional role of p21-activated kinase (PAK), a downstream effector of Rac1, in Stat5 regulation and leukemogenesis, we utilized a recently described allosteric PAK inhibitor, IPA-3 (Deacon et al., 2008). As seen in Figure 8A (lane 5 versus 7),

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(D) 32D FLT3ITD and FLT3WT cells were treated with DMSO or with F-14 and subjected to Tiam1 activation assay (n = 2).

(E) 32D FLT3ITD and FLT3WT cells were subjected to Tiam1 IP in presence or absence of F-14. Samples were analyzed for amount of Rac1-binding Tiam1 (IP:Tiam1 panels). Lower two panels (lysate [input]) depict the total protein levels of Rac1 and Tiam1 (n = 2).

(F) 32D cells coexpressing FLT3ITD and Tiam1 shRNA or scrambled shRNA were subjected to cellular fractionation assay, and nuclear and cytosolic fractions were analyzed for the levels of active Stat5 (pY694), total Stat5 and Rac1, and nuclear marker/loading control PARP-1.

(G) 32D cells coexpressing FLT3ITD and Tiam1 shRNAs (lanes 2–4) or scrambled shRNA (lane 1) were subjected to active Rac1 pull-down assay. The amount of active and total Rac1 are shown in the upper and lower panels, respectively.

(H) Kaplan-Meier survival curve of mice transplanted with 32D cells coexpressing FLT3ITD and Tiam1 shRNA (n = 5) or scrambled shRNA (n = 5); *p < 0.01.
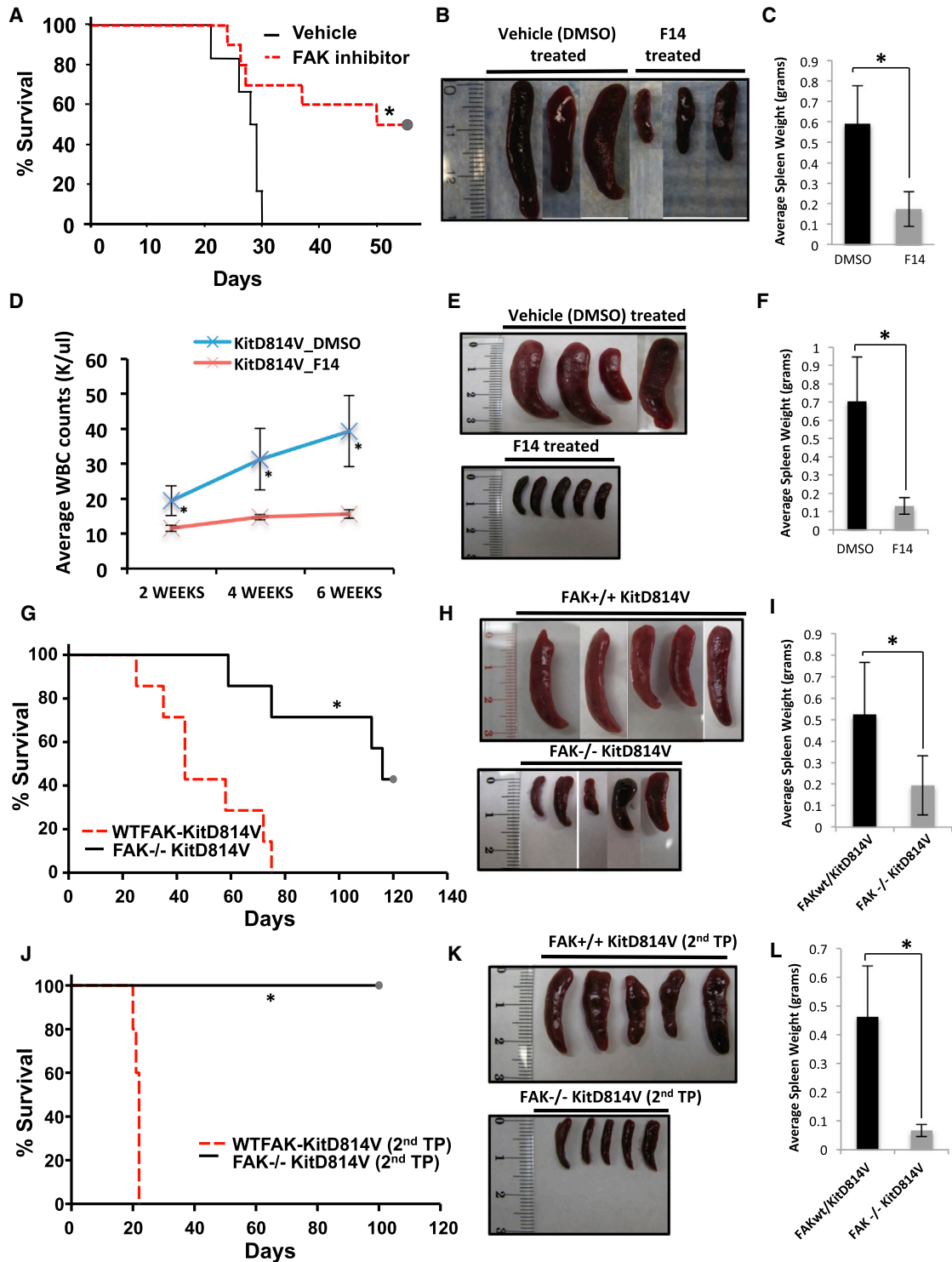
Figure 7. In Vivo Inhibition of FAK Delays the Development of MPN in Mice Transplanted with FLT3ITD- and KITD814V-Bearing Cells

(A) C3H/HeJ mice were transplanted with 32D cells bearing FLT3ITD and treated with 20 mg/kg body weight F-14 for 28 days.

(B and C) Kaplan-Meier survival analysis of vehicle- (n = 14) versus F-14 (n = 15)-treated mice showed significant increase in overall survival (*p < 0.02) and significant reduction of spleen size and weight in F-14-treated mice as compared to vehicle (DMSO) control-treated mice.

(D) BoyJ mice were irradiated and transplanted with 5′ fluorouracil (5-FU)-treated BM cells expressing KITD814V. Mice were randomly divided into two groups and treated with vehicle (DMSO; n = 7) or F-14 (n = 7) after 3 weeks posttransplantation, for 6 weeks. Peripheral blood from mice was analyzed at intervals of 2, 4, and 6 weeks.

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IPA-3 treatment significantly inhibited the presence of activated nuclear Stat5 in FLT3ITD-expressing cells. As seen in Figure 8A (lane 1 versus 2), addition of ligand also resulted in activation of the Stat5 oncogenic pathway in FLT3WT cells, which was also inhibited by IPA-3 (Figure 8A, lane 4). Next, we used another PAK inhibitor PF-3758309 (PF) (Murray et al., 2010). As seen in Figure 8B (lane 1 versus 2), similar results were observed upon treating FLT3ITD cells with PF-3758309, resulting in repression of nuclear translocation of active Stat5. Consistent with these results, expression of a dominant-negative form of PAK1 (K299R) in FLT3ITD-expressing cells (Figure S5F) demonstrated reduced tyrosine-phosphorylated Stat5 (Figure S5E). These results suggest that, downstream from Rac1, PAK contributes to the translocation of active Stat5 in the nucleus in FLT3ITD-bearing cells. To determine whether PAK is active in FLT3ITD and KITD814V oncogene-bearing cells, whole-cell lysates were analyzed for PAK1 activity. As seen in Figure S6A, cells expressing KITD814V showed increased levels of constitutive PAK1 activation (pPAK1) compared to KITWT cells (lane 1 versus 5), which was readily inhibited upon treatment with F-14 (lane 2 versus 6). Similar results were seen in FLT3ITD-expressing cells treated with F-14 (Figure S6B, lane 1 versus 2). Inhibition of active PAK1 levels was also observed in FLT3ITD-expressing cells treated with PAK inhibitors IPA-3 and PF-3758309 (Figure S6C, lane 1 versus 2 and 3). These results suggest that PAK1 is hyperactive downstream of a FAK/Rac1-signaling pathway in FLT3ITD and KITD814V oncogene-bearing cells.

Group I family of PAKs consist of three members including PAK1, PAK2, and PAK3. Whereas PAK1 and PAK2 are ubiquitously expressed, PAK3's expression is predominantly restricted to the brain (Ye and Field, 2012). We assessed the role of PAK1 and PAK2 in oncogene-induced transformation by examining Stat5 activation in FLT3ITD cells in which the expression of these two isoforms was knocked down (Figure S6E). As seen in Figure S6D, knockdown of PAK1 (lane 1 versus 2 and 3), but not PAK2 (lane 1 versus 4–6), significantly reduced the nuclear accumulation of active Stat5 in FLT3ITD-expressing cells. Consistent with these findings, expression of FLT3ITD in PAK1-deficient BM (*PAK1*−/−) cells also impaired the nuclear translocation of active Stat5 (Figure S6I, lane 1 versus 2). Furthermore, loss of active Stat5 nuclear import in *PAK1*−/− FLT3ITD cells resulted in reduced expression of Stat5 target genes including Bcl-xL and c-Myc compared to controls (Figure S6F) and also reduced relative mRNA levels of c-Myc and BclXL in FLT3ITD cells treated with PAK inhibitor PF-3758309 (Figures 8C and 8D).

Inhibition of PAK1 Delays the Onset of FLT3ITD- and KITD814V-Induced MPN and Prolongs the Survival of Mice

To determine the role of PAK isoforms in FLT3ITD- and KITD814V-mediated MPN development, BM transplant studies were performed. Transplantation studies utilizing myeloid cells bearing FLT3ITD in the context of PAK2 knockdown did not prolong the survival of leukemic mice compared to controls (Figure S6G). In contrast, PAK1 knockdown in the context of FLT3ITD expression significantly enhanced the lifespan of leukemic mice (Figure S6H). To further confirm PAK1's involvement in KITD814V-mediated leukemogenesis, we transplanted mice with KITD814V-bearing *PAK1*−/− BM cells or WT (*PAK1*+/+) controls. Mice transplanted with WTPAK1 BM cells expressing KITD814V came down with disease significantly earlier than *PAK1*−/− KITD814V mice (Figure 8G), at which juncture time-point-matched control mice (*PAK1*−/− KITD814V, *PAK1*+/+ KITWT, and *PAK1*−/− KITWT) were euthanized and BM (Figure 8E) and spleen (Figure 8F) cells harvested and subjected to cellular fractionation analysis. As seen in Figures 8E (lane 1 versus 2) and 8F (lane 1 versus 2), similar to our data with cell lines and PAK inhibitors, genetic ablation of PAK1 in vivo abrogated the nuclear translocation of active Stat5 in KITD814V-bearing BM-derived cells. As seen in Figure 8G, genetic ablation of PAK1 significantly prolonged the survival of leukemic mice (*PAK1*−/− KITD814V) compared to controls (*PAK1*+/+ KITD814V) and modulated the development of MPN in mice as shown by reduced splenomegaly and WBC counts (Figures 8H, S6J, and S6K). Mice transplanted with WTKIT did not demonstrate any signs of MPN and showed normal survival and spleen size (Figures 8H and S6J). To further ascertain whether targeting PAK1 in the context of KITD814V-induced MPN selectively impacts the leukemia initiating cell "LIC" population, we performed secondary transplants using BM cells from primary recipients. As seen in Figure 8I, mice transplanted with BM cells from primary donor harboring KITD814V in a PAK1-deficient background (*PAK1*−/− KITD814V) survived significantly longer than mice transplanted with BM from WT background (*PAK1*+/+ KITD814V). The survival of mice correlated with correction in spleen size (Figures 8I, 8J, and S6L), similar to primary transplants described above.

Inhibition of PAK Inhibits the Constitutive Growth of FLT3ITD+ AML Cells and KITD816V (+) SM-Patient-Derived Cells

To assess the functional consequence(s) of PAK1 repression on the growth and transforming ability of FLT3ITD- and KITD814V-bearing cells, we performed a proliferation assay using cells expressing FLT3 and KIT receptors treated with or without PF-3758309. A dose-dependent reduction in the growth of FLT3ITD- and KITD814V-bearing cells was observed, but not that of FLT3WT- and KITWT-bearing cells (Figures S7A, S7B, S7C, and S7D, respectively). Similar results were observed for FLT3ITD cells treated with the PAK inhibitor IPA-3 (Figure S7E). To further validate these observations, we performed similar studies in FLT3ITD-bearing cells coexpressing a

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(E and F) After 6 weeks, mice were harvested to determine spleen size (E) and weight (F).

(G) 5-FU-treated BM cells from WTFAK or *FAK*−/− mice expressing KITD814V were transplanted into lethally irradiated C57BL/6 mice.

(H and I) Kaplan-Meier survival analysis of *FAK*+/+ KITD814V (n = 9) versus *FAK*−/− KITD814V (n = 9) mice; spleen size (H) and weight (I) is shown (*p < 0.002).

(J) Secondary transplants were performed using BM from *FAK*+/+ KITD814V and *FAK*−/− KITD814V primary recipients.

(K and L) Kaplan-Meier survival analysis of *FAK*+/+ KITD814V (n = 5) versus *FAK*−/− KITD814V (n = 5) is shown (*p < 0.003) and spleen size (K) and weight (L; *p < 0.05).
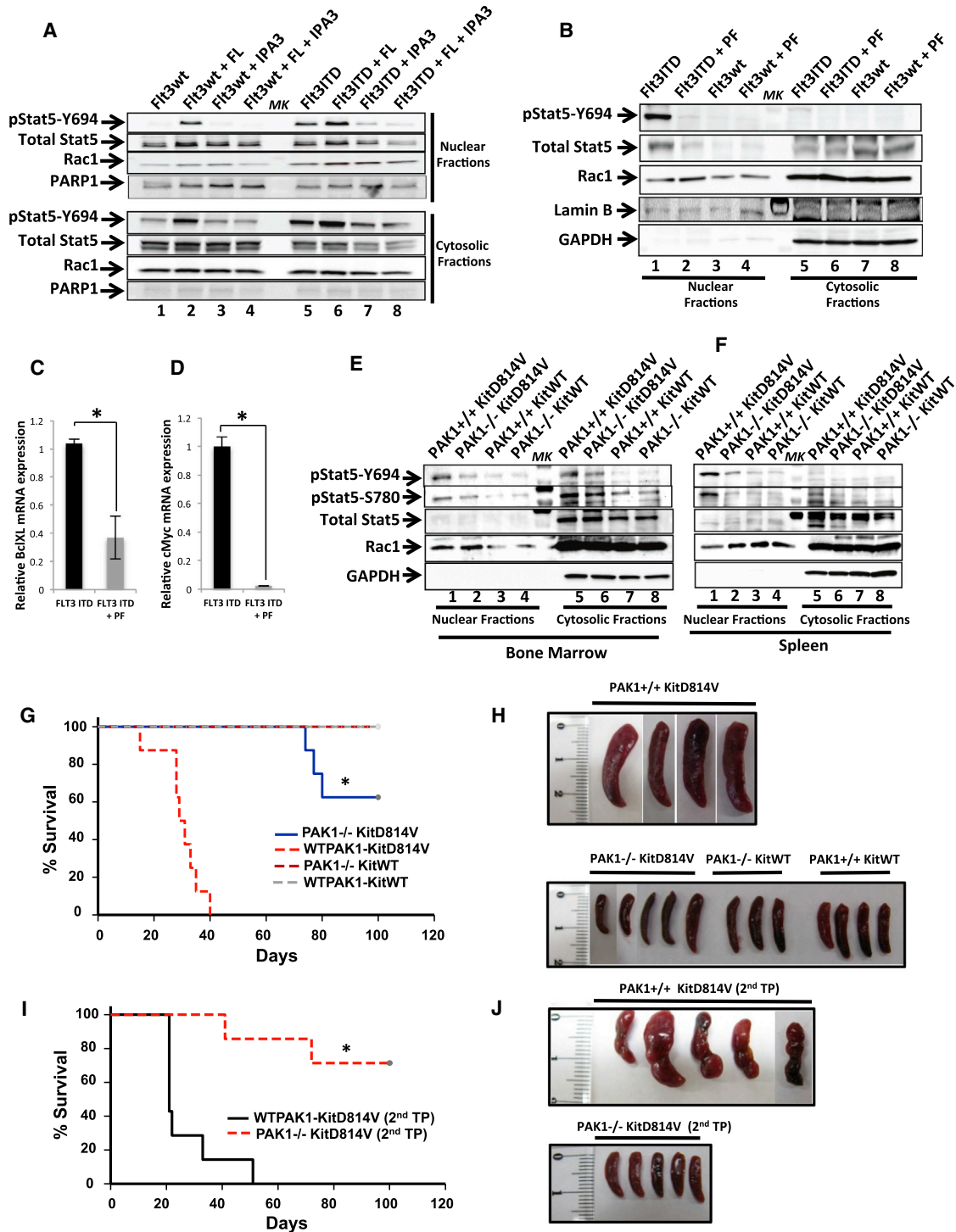
Figure 8. In Vivo Inhibition of PAK1 Delays the Onset of MPN in Mice Transplanted with KITD814V-Bearing Cells

(A) 32D cells expressing FLT3ITD or FLT3WT were starved of serum and treated with the PAK inhibitor IPA-3 (lanes 3 and 7) alone, with FL (lanes 2 and 6), or with IPA-3 followed by FL (lanes 4 and 8) as indicated and subjected to cellular fractionation assay. Nuclear and cytosolic fractions were quantitated, and equal lysates were loaded on a gel and probed with the indicated antibodies. Arrows indicate the activation/expression of the labeled molecules in nuclear as well as in cytosolic fractions of FLT3ITD- and FLT3WT-bearing cells. Expression of PARP-1 was used as an indicator of nuclear loading (n = 2).

(B) 32D FLT3ITD and FLT3WT were serum starved and treated with the PAK inhibitor PF-3758309 (PF) and analyzed as described in (A).

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dominant-negative version of PAK1 (K299R; Figure S7F) as well as in BM cells expressing FLT3ITD in the setting of PAK1 deficiency (Figure S7G). A significant inhibition in the growth of FLT3ITD-bearing cells was noted in the background of dominant-negative or genetic ablation of PAK1. A similar inhibition in the growth of AML patient cells (Figure S7I) and KITD816V (+) SM-patient-derived cells (Figure S7K) was observed in the presence of IPA-3. Lastly, we assessed the role of PAK1 overexpression on the rescue of ligand-independent growth of BM cells expressing KITD814V in PAK1-deficient (PAK1−/−) background. An activated version of PAK1 (PAK1T423E; Schürmann et al., 2000) was coexpressed with KITD814V in BM cells derived from PAK1−/− mice. As seen in Figure S7H, we observed rescue of ligand-independent growth of cells overexpressing PAK1T423E, as compared to vector control.

DISCUSSION

Presence of active Stat5 in the nucleus and the subsequent expression of Stat5-dependent prosurvival and antiapoptotic genes plays a key role in the transformation of cells bearing oncogenic forms of FLT3 and KIT (Benekli et al., 2003; Choudhary et al., 2007; Tse et al., 2000). However, the mechanism(s) involved in regulating the active form of nuclear Stat5 remain poorly understood. To this end, a role for Rac1 GTPase/MgcRacGAP complex in the translocation of active Stat5 into the nucleus has been suggested; however, the upstream and downstream signaling proteins from Rac1 involved in this process have not been identified and the extent to which these proteins contribute to leukemogenesis is unknown (Sallmyr et al., 2008). Using pharmacologic, biochemical, and genetic approaches, we demonstrate that the FAK/Rac1-Tiam1/PAK1 axis plays a crucial role in the transformation induced by oncogenic forms of FLT3 (FLT3ITD) and KIT (KITD816V). Targeting FAK, Tiam1, and PAK1 in oncogene-bearing cells in vitro or in vivo inhibits the presence of active Stat5 in the nuclear compartment, which profoundly delays the onset of leukemia by repressing the expression of Stat5-responsive genes. These findings were validated in both murine and human models of AML and MPN and suggest that the signaling axis we have identified is highly conserved across species. More importantly, we show that this axis is active in leukemia-initiating cells as well as in leukemic cells that acquire AC220-resistant mutations of FLT3.

We and others have shown that FAK may contribute to Rac activation in hematopoietic cells as well as in other heterologous cell systems (Chang et al., 2007; Elias et al., 2010; Vemula et al., 2010) and that FLT3ITD can activate Rac1 and regulate the production of reactive oxygen species (ROS) via its association with Stat5 (Sallmyr et al., 2008). Furthermore, active Rac1 can also induce the activation and nuclear translocation of Stat3 (Simon et al., 2000). FLT3ITD can phosphorylate Stat5, independent of Jak kinase family members (Choudhary et al., 2007), whereas Rac1-mediated ROS production can induce the activation of Jak kinases and Stats downstream of G-protein-coupled receptors (Pelletier et al., 2003). Although significant work has been done in identifying the above described linkages, how these molecules connect and what is their relationship in regulating transformation via oncogenic forms of KIT and FLT3 has never been described. Our findings provide insight into how FAK-Rac1-Tiam1 and PAK1 axis contributes to leukemic transformation in part by regulating active nuclear Stat5.

The role of GEFs such as Vav1 and Vav2 is largely considered promiscuous, as they regulate the activity of all three members of the Rho GTPase family including Rac, Rho, and Cdc42 (Schmidt and Hall, 2002). In contrast, GEF Tiam1 is highly specific for Rac1 in vivo and has been implicated in activating Rac1 to mediate Stat3 activation and its subsequent nuclear localization in COS-1 cells (Simon et al., 2000). We demonstrate that FAK activates Rac1 via Rac GEF Tiam1 in FLT3ITD-bearing cells and targeting FAK and Tiam1 results in inhibition of Rac1. We also show that shRNA-mediated knockdown of Tiam1 prolongs the survival of FLT3ITD-bearing leukemic mice and genetic and pharmacologic inhibition of FAK and Tiam1 results in failure of active Stat5 to be expressed in the nucleus, along with Rac1. Whereas Tiam1 regulates epithelial cancers such as carcinomas of breast and colon (Bourguignon et al., 2000; Buongiorno et al., 2008), we show its role in regulating hematologic malignancies.

Although evidence in this study and reported earlier (Sallmyr et al., 2008) suggests a role of Rac1 in translocating Stat5 into the nucleus, the relationship between Stat5 and Rac GTPases in the context of FLT3 and KIT oncogenic mutations is unclear. An indirect role of PAK1 in nuclear shuttling of Stat5 has been suggested, where PAK1 plays a role in "switching" in occupancy of the same promoter region between BCL6 and Stat5. In colorectal cancer, PAK1, activated via Rac1, translocates into the nucleus and phosphorylates chromatin-bound BCL6, leading to its dissociation from the promoter, thereby allowing active Stat5 that is already present in the nucleus via a Rac1/MgcRacGAP-dependent mechanism to bind to the same promoter regions (Barros et al., 2012). In line with reported findings by Barros

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(C and D) qRT-PCR analysis of relative mRNA expression levels of Stat5-responsive genes BclXL (C) and c-Myc (D) in FLT3ITD cells treated with PAK inhibitor PF-3758309 (n = 2); *p < 0.05.

(E and F) Fractionation assays were performed in BM cells (E) and splenocytes (F). Mice cohorts transplanted with KITD814V in a WT PAK1 (PAK1+/+ KIT814V) or PAK1-deficient (PAK1−/− KITD814V) cells (n = 2). The level of phospho-Stat5 and total Stat5 and Rac1 in the nuclear and cytosolic fractions is indicated. Expression of GAPDH was used as an indicator of cytosolic marker and loading control. MK denotes lane with protein ladder.

(G and H) Primary transplants were carried out using 5-FU-treated BM cells from WTPAK1 or PAK1−/− mice transduced with KITD814V or KITWT and transplanted into lethally irradiated C57BL/6 mice. Four groups of mice were used: WTPAK1 KITD814V (n = 8); WTPAK1 KITWT (n = 5); PAK1−/− KITD814V (n = 8); and PAK1−/− KITWT (n = 5). Kaplan-Meier survival analysis of PAK1+/+ KITD814V versus PAK1−/− KITD814V, WTPAK1 KITWT, and PAK1−/− KITWT mice showed significant overall survival (*p < 0.0003; Hom-Sidak method) and significant reduction of spleen size (H).

(I and J) Secondary transplants were performed with BM cells from PAK1+/+ KITD814V and PAK1−/− KITD814V mice and transplanted into irradiated C57BL/6 mice. Kaplan-Meier survival analysis of PAK1+/+ KITD814V (n = 5) versus PAK1−/− KITD814V (n = 5) mice showed significant overall survival (*p < 0.001) (I) and significant reduction of spleen size (J).

et al., we have observed a reduction in BCL6 activation and its mRNA expression levels upon treatment of FLT3ITD cells with PAK1 inhibitor (Figures S8A and S8B). These data demonstrate that, in the FLT3 and KIT oncogenic pathway, the FAK/Tiam1/ Rac1-signaling axis activates PAK1, which in turn inhibits the transcriptional repressor BCL6, while correspondingly activating Stat5 to mediate leukemic transformation.

In BCR-ABL-induced CML, majority of Stat5 is persistently active and retained in the cytoplasmic compartment, primarily via an association of active Stat5 with Gab2 and PI3K/Akt, subsequently leading to leukemogenesis (Nyga et al., 2005). FLT3ITD also interacts with Gab2 and results in the activation of PI3K/ Akt-; Stat5- and Gab2-mediated recruitment of Src kinases can also result in the activation of Stat5. Thus, whereas FLT3ITD can directly activate Stat5 (Choudhary et al., 2007), other tyrosine kinases present in complexes with FLT3ITD may also be involved in regulating Stat5 activation including Src kinases, as mutating Src-kinase-binding sites Y589 and Y591 in FLT3 receptor inhibits Stat5 activation (Hayakawa et al., 2000; Rocnik et al., 2006). Our data using FLT3ITD specific inhibitor AC220 not only show inhibition in the phosphorylation of FLT3ITD on Y589/591 but also downstream inhibition of activating residue Y397 on FAK, indicating that oncogenic FLT3 mediates direct activation of FAK. In breast cancer cells, prolactin (PRL)-induced activation of FAK and Stat5 is mediated via Src family kinases. Upon phosphorylation by Src, phosphorylated FAK recruits Grb2/Gab2 to mediate activation of Ras/MAPK-signaling pathway. The involvement of the PI3K/Rac/Pak pathway in PRL-induced activation of Erk has also been suggested. These results demonstrate a complex crosstalk between various signaling pathways involved in breast cancer metastasis (Aksamitiene et al., 2011). Future studies will determine whether Gab2 or other Src kinases play a role in activating FAK downstream of FLT3ITD and KITD814V receptors, which results in oncogenic transformation mediated by the subsequent nuclear translocation of active Stat5.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Phycoerythrin-conjugated annexin V antibody and 7-amino actinomycin D were purchased from BD Biosciences PharMingen. Rabbit anti-phospho-PAK1, anti-PAK1, anti-phospho-Stat5 (Y694), anti-Stat5 antibodies, rabbit anti-phospho-FAK (Y397), and anti-FAK antibodies were purchased from Cell Signaling Technology. Anti-rabbit immunoglobulin G DyLight 649 from Biolegend, Anti-actin and GAPDH antibodies were purchased from Sigma. Tiam1 activation kit was purchased from Cell BioLabs. Anti-mouse-horseradish peroxidase (HRP), anti-rabbit-HRP, anti-goat-HRP, anti-phospho-Stat5 (S780), anti-Tiam1, PARP-1, LaminB, BclXL, and cMyc antibodies were purchased from Santa Cruz Biotechnology. FAK inhibitors F-14 and Y-11, PAK inhibitors IPA-3 and PF-3758309, and Rac inhibitor NSC23766 were purchased from R&D Systems. Lumina Forte Western HRP Substrate, Chemiluminescent Blocker (bløk-CH), anti-phospho-FAK (Y397) rabbit polyclonal, anti-Rac1 (23A8), and Rac1 activation kit were purchased from Millipore. Recombinant murine and human IL-3, Flt3, granulocyte macrophage-colony stimulating factor, stem cell factor, IL-6, and Tpo were purchased from Peprotech. Retronectin was obtained from Takara. Iscove's modified Dulbecco's medium was purchased from Invitrogen. Monothioglycerol was purchased from Sigma. [³H]thymidine was purchased from PerkinElmer. Protein A-Sepharose beads were purchased from Amersham Biosciences. MKK/MEK inhibitor PD98059 was purchased from Cell Signaling Technology, Raf inhibitor PLX4720 from Selleckchem, and Akt inhibitor 124005 from Millipore.

Mice

C57BL/6 and C3H/HeJ mice were purchased from Jackson Laboratory. FAK-, Rac1-, and PAK1-deficient mice have been previously described (Martin et al., 2013; McDaniel et al., 2008; Vemula et al., 2010). All mice used in this study were between 6 and 12 weeks of age and were maintained under specific pathogen-free conditions at the Indiana University Laboratory Animal Research Center. The studies were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

Patient Samples

Peripheral blood mononuclear cells from patients with AML were obtained at the time of diagnostic testing after informed consent. Approval was obtained from the institutional review boards of Indiana University School of Medicine. The buoyant fraction was isolated over Ficoll-Hypaque and then washed with PBS before processing as described previously (Hartman et al., 2006). KITD816V-positive or negative SM-patient-derived cells were obtained as described (Traina et al., 2012).

Cells

Primary low-density mononuclear cells were harvested as described earlier and used in the study (Mali et al., 2011). The murine IL-3-dependent myeloid cell line 32D cells bearing FLT3, FLT3ITD (N51), MIEG3 vector, KIT, or KITD814V have been described (Mali et al., 2011). Puromycin-resistant BaF3 cells bearing the AC220-resistant mutants (Flt3ITD+TKD_D835Y/F and F691L) have been described (Smith et al., 2012). The human mast cell leukemia line, bearing the KITV560G as well as KITD816V mutations, HMC1.2 and AML cell line, bearing the FLT3ITD mutation, MV4-11 have been described (Butterfield et al., 1988; Lange et al., 1987).

Expression of WT and Oncogenic Receptors

Transduction of 32D and primary BM-derived hematopoietic stem and progenitor cells was performed as described previously (Mali et al., 2011).

shRNA Silencing of FAK, Tiam1, and PAK

FAK-, Tiam1-, or PAK-specific shRNA expression plasmids were purchased from OriGene Technologies. Purified and sequence-verified plasmid containing a noneffective 29 mer shGFP cassette (Scrambled vector) was used as a negative control. Cells were transduced with scrambled vector or shRNA plasmid and grown in the presence of puromycin (10 ng/ml) to select for the transduced cells.

Proliferation and Apoptosis Assays

Proliferation assays were performed as previously described (Mali et al., 2011).

Cytoplasm and Nuclear Extraction

To extract nuclear and cytosolic fractions, the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) was used as per the manufacturer's instructions.

IP and WB

Immunoprecipitation (IP) and western blot analysis (WB) was performed as described previously (Mali et al., 2011).

qRT-PCR

Total RNA was isolated from 5×10^6 cells using RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was generated using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed using FastStart Universal SYBR Green master mix (Roche) and a Applied Biosystem 7500 Real Time PCR system. β-actin amplification was used to normalize sample RNA content.

Mouse Leukemia Induction and In Vivo Drug Treatment

Mouse leukemia induction and in vivo drug treatments were performed as described previously (Mali et al., 2011). We injected 1×10^6 32D cells bearing FLT3ITD in 200 μl PBS into C3H/HeJ mice intravenously. After 48 hr of transplantation, mice were treated with vehicle (PBS/DMSO) or FAK inhibitor F-14 (25 mg/kg body weight) by intraperitoneal injection at 24 hr interval for

21 days. Mice were closely monitored for MPN development and harvested at moribund. For F-14 treatment in a primary transplant model, irradiated BoyJ mice were transplanted with 2.0×10^6 GFP-KITD814V cells and 0.1×10^6 supporting BM cells. Three weeks posttransplant mice were treated with 10 mg/kg body weight F-14 for 5 days a week for 6 weeks. All mice were harvested, and peripheral blood (PB) counts were monitored using a Hemavet 950 (Drew Scientific). PB, BM, and spleens were analyzed for GFP expression. BM, spleen, and lungs were also fixed in 10% buffered formalin to perform histopathologic analysis by hematoxylin and eosin staining.

Primary and Secondary Bone Marrow Transplants

Eight- to ten-week-old C57BL/6, *PAK1*−/−, or *FAK*−/− mice were injected with 5-flurouracil (150 mg/kg body weight), and after 5 days, the mice were killed and BM was harvested. Cells were prestimulated overnight in Iscove modified Dulbecco medium supplemented with 20% fetal bovine serum with mouse SCF (100 ng/ml), mouse TPO (100 ng/ml), mouse Flt3 (50 ng/ml), and mouse IL-6 (10 ng/ml). Cells were plated on Retronectin-coated plates (Takara), and retroviral transduction was performed using viral supernatants as described before (Mali et al., 2011). GFP+ve cells were sorted using FACS Aria (BD Biosciences). We injected 1×10^6 GFP-positive cells along with 0.1×10^6 supporting splenocytes into the tail veins of lethally irradiated (11 Gy) female C57BL/6 recipient mice. Peripheral blood counts were monitored on a regular interval after transplantation on a Hemavet 950 (Drew Scientific) and by fluorescence-activated cell sorting analysis for GFP expression. All moribund mice were then euthanized, and peripheral blood, BM, and spleen cells were analyzed for GFP expression. At day 120, all remaining mice were euthanized and analyzed.

For secondary transplant, leukemic WT mice and time-point-matched *PAK1*−/− or *FAK*−/− mice, both bearing KitD814V-expressing cells, were harvested and 1×10^6 cells were injected into the tail veins of lethally irradiated (11 Gy) female C57BL6 recipients.

Statistics

All graphical data were evaluated by paired Student's t test (two-tailed), and results were considered significantly different with p value < 0.05. All data are represented as mean values ± SD. Survival probability of transplanted mice groups was compared using a Kaplan-Meier survival analysis in which statistical significance was determined as p values < 0.05 by log rank test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.039.

AUTHOR CONTRIBUTIONS

ACKNOWLEDGMENTS

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