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How to cite

GREEN, Alexa S. et al. Pim kinases modulate resistance to FLT3 tyrosine kinase inhibitors in FLT3-ITD acute myeloid leukemia. In: Science advances, 2015, vol. 1, n° 8, p. e1500221. doi: 10.1126/sciadv.1500221

This publication URL: https://archive-ouverte.unige.ch/unige:161519

Publication DOI: 10.1126/sciadv.1500221

SIGNAL TRANSDUCTION

10.1126/sciadv.1500221

Pim kinases modulate resistance to FLT3 tyrosine kinase inhibitors in FLT3-ITD acute myeloid leukemia

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Fms-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) is frequently detected in acute myeloid leukemia (AML) patients and is associated with a dismal long-term prognosis. FLT3 tyrosine kinase inhibitors provide short-term disease control, but relapse invariably occurs within months. Pim protein kinases are oncogenic FLT3-ITD targets expressed in AML cells. We show that increased Pim kinase expression is found in relapse samples from AML patients treated with FLT3 inhibitors. Ectopic Pim-2 expression induces resistance to FLT3 inhibition in both FLT3-ITD–induced myeloproliferative neoplasm and AML models in mice. Strikingly, we found that Pim kinases govern FLT3-ITD signaling and that their pharmacological or genetic inhibition restores cell sensitivity to FLT3 inhibitors. Finally, dual inhibition of FLT3 and Pim kinases eradicates FLT3-ITD$^+$ cells including primary AML cells. Concomitant Pim and FLT3 inhibition represents a promising new avenue for AML therapy.

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive disease caused by the transformation of hematopoietic progenitor cells due to the acquisition of genetic alterations (*1*). Although heterogeneous, many of these lesions lead to constitutive activation of protein tyrosine kinases (PTKs) and growth factor signaling pathways, resulting in the proliferation and clonal expansion of hematopoietic progenitors (*2*), with significant prognostic implications (*3*).

Fms-like tyrosine kinase 3 (FLT3) is a member of the class III receptor tyrosine kinase family, which also includes the FMS, KIT, and platelet-derived growth factor (PDGF) receptors (*4*). The FLT3 internal tandem duplication (FLT3-ITD) mutation is found in blast cells of 20 to 30% of AML patients and confers a poor prognosis (*1*). These mutations occur in the juxtamembrane domain of FLT3 and lead to receptor dimerization and ligand-independent constitutive activation of downstream signal transduction pathways, including mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) and signal transducer and activator of transcription 5 (STAT5) (*5*).

Although the question of whether FLT3-ITD is a cooperating or initiating mutation in AML remains the subject of debate (*6*, *7*), FLT3-ITD may drive leukemogenesis from early hematopoietic progenitors (*8*). The high relapse rate observed after chemotherapy prompted the development of FLT3 inhibitors (FLT3i), which demonstrated clinical efficacy in FLT3-ITD–positive (FLT3-ITD$^+$) AML but failed to induce long-lasting remissions (*9*). Twenty percent of patients relapsing on FLT3i therapy have detectable FLT3-ITD tyrosine kinase domain mutations (TKD)—mostly at the D^{835} position—representing the most commonly described resistance mechanism to FLT3i (*6*, *9*). Elucidation of the molecular pathways involved in FLT3i resistance is therefore critical for the development of targeted therapies capable of inducing durable responses in affected patients.

Pim serine/threonine kinases are frequently overexpressed in human cancers (*10*). Named for their role as the provirus integration site for the Moloney murine leukemia virus, Pim kinases, including Pim-1, Pim-2, and Pim-3, have both overlapping and nonoverlapping functions and contribute to cell transformation in cooperation with oncogenes such as myc or deregulated PTKs, including *TEL/JAK2*, *BCR/ABL*, and *FLT3-ITD* (*11*–*15*). We previously reported increased Pim-2 protein expression in AML cells compared to normal CD34$^+$ hematopoietic progenitors (*16*).

Here, we aimed to explore the role of Pim kinases in FLT3i-resistant AML. We found that Pim kinase expression is increased in primary samples from AML patients relapsing on FLT3i therapy with sorafenib. Ectopic Pim kinase expression impairs the response to FLT3i in vitro and in models of FLT3-ITD–induced myeloproliferative neoplasm (MPN) and AML, using a *PIM2* allele, in mice. Pim kinase inhibition enhanced FLT3i activity across multiple FLT3-mutant cell lines and in FLT3-ITD$^+$ primary AML samples. Mechanistically, Pim kinases exert proximal control of FLT3-ITD signaling, and their inhibition facilitates the activity of FLT3i against FLT3-ITD receptors. Combined inhibition of Pim and FLT3 therefore warrants further evaluation in clinical trials in AML.

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RESULTS

Increased Pim kinase expression is found in sorafenib-resistant primary AML samples and confers resistance to FLT3 inhibition in vivo

Pim protein kinases are well-documented FLT3-ITD targets and therefore may have a potential role in FLT3-ITD–mediated cell transformation (*14*, *17*). We investigated the role of these oncogenic kinases in FLT3i resistance, which inevitably occurs after a short interval in FLT3-ITD$^+$ AML patients (*9*, *18*).

We compared Pim kinase protein expression in paired primary samples from FLT3-ITD$^+$ AML patients at the time of diagnosis (FLT3i-naïve) and after relapse on treatment with sorafenib (FLT3i-resistant) (Table 1). Whereas Pim kinase expression decreased in three of seven sorafenib-treated samples—which was expected considering the documented regulation of Pim kinases by FLT3-ITD (*14*, *17*)—Pim-2 expression unexpectedly increased in four of seven FLT3i-resistant compared to FLT3i-naïve paired samples (Fig. 1A). Pim-1 protein expression, which was low to absent in FLT3i-naïve samples, was increased in two FLT3i-resistant samples, decreased in one sample, and not detected in three samples (Fig. 1A). We found an *FLT3-ITD* kinase domain mutation by sequencing in four of seven sorafenib-resistant samples, which was not detected in sorafenib-naïve samples but without any correlation with the expression of Pim kinases (Fig. 1A, right, and Table 1). Thus, increased Pim kinase expression may occur in FLT3i-resistant primary AML cells.

We further used Pim-2 as a representative model of the Pim kinase family because Pim-2 is more frequently detected than Pim-1 in primary AML samples and AML cell lines (fig. S1A). We used a well-characterized experimental model of *FLT3-ITD*–induced MPN (*19*), in which mice received FLT3-ITD$^+$ transformed hematopoietic progenitor cells or cells expressing both *FLT3-ITD* and human *PIM2* (*FLT3-ITD/PIM2*). Compared to their syngeneic counterparts, recipients of FLT3-ITD$^+$ cells had decreased body weight (Fig. 1B), increased spleen weight and cellularity (Fig. 1, C and D), increased white blood cell (WBC) and monocyte counts, and decreased platelet counts (Fig. 1E). These parameters did not differ between mice receiving *FLT3-ITD*– or *FLT3-ITD/PIM2*–transduced cells (Fig. 1, B to E), suggesting that ectopic Pim-2 expression did not modify tumor burden. Engraftment of these cells was confirmed by immunohistochemistry of mouse spleen (Fig. 1F).

Treatment with the FLT3i AC220 commenced at disease onset, as defined by a WBC count of more than 10^4/ml. AC220 therapy resulted in a reduction in WBC and monocyte counts, diminished myeloid cells expansion (Fig. 1, G and H), and decreased spleen weight (Fig. 1I) in mice injected with *FLT3-ITD* but not *FLT3-ITD/PIM2* cells. Moreover, cellular proliferation in the splenic red pulp as measured by Ki-67$^+$ staining was reduced with AC220 treatment in *FLT3-ITD* but not in *FLT3-ITD/PIM2* recipients (Fig. 1J). Pim-2 expression in FLT3-ITD$^+$ hematopoietic cells is thus sufficient to induce FLT3i resistance in vivo.

Pim kinases are FLT3-ITD targets involved in resistance to FLT3 inhibition in AML

We used a doxycycline (Dox)–inducible short hairpin RNA (shRNA) to achieve targeted FLT3 knockdown in AML cell lines. FLT3 protein expression was efficiently suppressed in all cell lines tested (HL-60, OCI-AML3, MV4-11, and MOLM-14) but correlated with reduced Pim-1 and Pim-2 expression only in FLT3-ITD$^+$ cell lines (MV4-11 and MOLM-14, Fig. 2A). In MOLM-14 and MV4-11 cells, FLT3 knockdown increased annexin V binding, in contrast to the results observed in two *FLT3* wild-type AML cell lines, OCI-AML3 and HL-60 (fig. S2A), suggesting an addiction to FLT3-ITD signaling in these cell lines.

In the FLT3-ITD$^+$ MOLM-14 AML cell line, Pim-2 expression was constitutive and controlled by multiple signaling relays downstream of FLT3-ITD, in contrast to the observations made in the FLT3 wild-type

Table 1. Clinicopathologic characteristics of seven patients with FLT3-ITD$^+$ AML treated on sorafenib monotherapy trial. G, gender; A, age (in years); FAB, French-American-British classification; WCC, white cell count (10^9/liter); Blast, percentage of bone marrow blast cells; NPM1, nucleophosmin 1 mutation status; Karyo., karyotype; FLT3-TKD, FLT3 tyrosine kinase domain mutation status; Naïve, pre-sorafenib sample; Res., sample collected after disease evolution upon sorafenib treatment; NS, not specified; NA, not available; WT, wild type; Mut, mutated; 7 + 3, daunorubicin and cytarabine; HDAC, high-dose cytarabine; MTZ/VP16/AC, mitoxantrone, etoposide, and cytarabine; MACE-DEX, mitoxantrone, cytarabine, etoposide, and dexamethasone; ICE, idarubicin, cytarabine, etoposide; CLARA, clofarabine, cytarabine; MTZ/MDAC, mitoxantrone, medium-dose cytarabine; 5AZA, 5-azacytidine; Y and N, presence or absence of TKD mutations in sorafenib-naïve and sorafenib-resistant samples.

Patient	G/A	FAB	WCC	Blast	NPM1	Karyo.	Previous treatments	FLT3-TKD*	
								Naïve	Res.
AML#1	M/54	NS	167	81	NA	Complex†	7 + 3, HDAC×2, MACE-DEX	N	N
AML#2	M/34	M6	2	6 (75)‡	WT	46, XY	7 + 3, ICE, CLARA, MTZ/MDAC, 5AZA	N	Y
AML#3	F/57	M1	19	54	WT	46, XX	7 + 3, MTZ/MDAC×3	N	N
AML#4§	F/47	NS	25	78	Mut.	46, XX	7 + 3, ICE×2	N	Y
AML#5	F/87	M1	260	94	NA	46, XX	Not eligible	N	Y
AML#6	F/43	NS	18	55	WT	Complex¶	7 + 3, HDAC×2, MTZ/VP16/AC	N	N
AML#7	F/67	M1	102	95	NA	46, XX	7 + 3, HDAC×2, 5AZA	N	Y

*D^{835} mutation of FLT3-TKD was examined as described (*18*) on peripheral blood cells.
†42~48,XY,del(5)(p13),+del(6)(q16),del(12)(p11.2),-14,-15,-17,t(17;19)(q21;p13),-20,+1~6mar[cp8]/46,XY[7].
‡The percentage of blast cells of bone marrow was 75% among the nucleated cells.
§The clinical information and the FLT3-TKD status of patient AML#7 have been reported (*18*).
¶46,XX,i(17)(q10)[6]/46,XX,del(5)(q34)[3]/46,XX,add(11)(p11.2)[2]/46,XX,del(11)(15.2)[2]/46,XX[20].

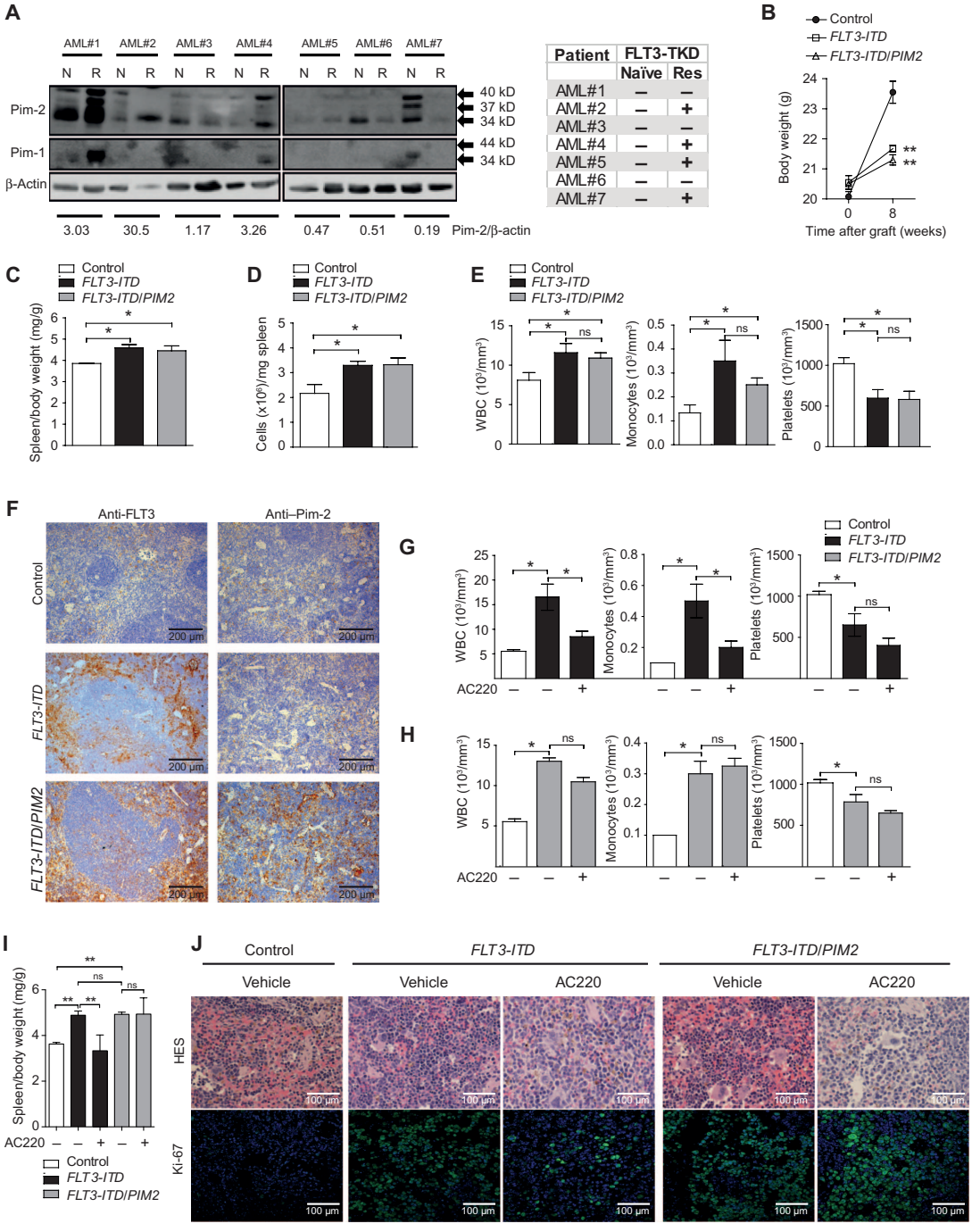

Fig. 1. Increased Pim kinase expression is found in sorafenib-resistant primary AML samples and confers resistance to FLT3 inhibition in vivo. (**A**) Left: Western blotting with Pim-1 and Pim-2 antibodies was performed on seven paired primary AML samples from patients before TKI treatment [naïve (N)] and after relapse (R) following sorafenib therapy. Right: FLT3-TKD sequencing (–, absence of mutation; +, detection of a D^{835} mutation) in naïve or resistant/relapsed (Res) AML samples. (**B** to **F**) Six- to 8-week-old B6 mice were transplanted with bone marrow (BM)–derived hematopoietic precursors transduced with *FLT3-ITD* (black), *FLT3-ITD* and human Pim-2 (*FLT3-ITD/PIM2*; gray), or control (white) constructs. Eight weeks after adoptive transfer, body weight (B), spleen weight (C), spleen cell counts (D), and peripheral blood counts (total leukocytes, monocytes, and platelets) (E) were determined, and immunohistochemical analysis of FLT3 and Pim-2 protein expression was performed on spleen sections (F) ($n = 4$ for each). (**G** and **H**) Peripheral blood counts (total leukocytes, monocytes, and platelets) from mice adoptively transferred with bone marrow precursors expressing *FLT3-ITD* (black bars) or *FLT3-ITD/PIM2* (gray bars), treated or not with AC220 for 1 week ($n = 4$ for each). (**I**) Spleen weight relative to body weight in mice transduced with *FLT3-ITD* (white bars) or *FLT3-ITD/PIM2* (black bars) and treated with vehicle or AC220. (**J**) Hemalun-Erythrosine-Safran (HES) and Ki-67 staining of spleen sections from (G) and (H). Results in the graphs are expressed as means ± SEM. *$P < 0.05$; **$P < 0.01$; ns, not significant. β-Actin was used as a loading control for all Western blots.

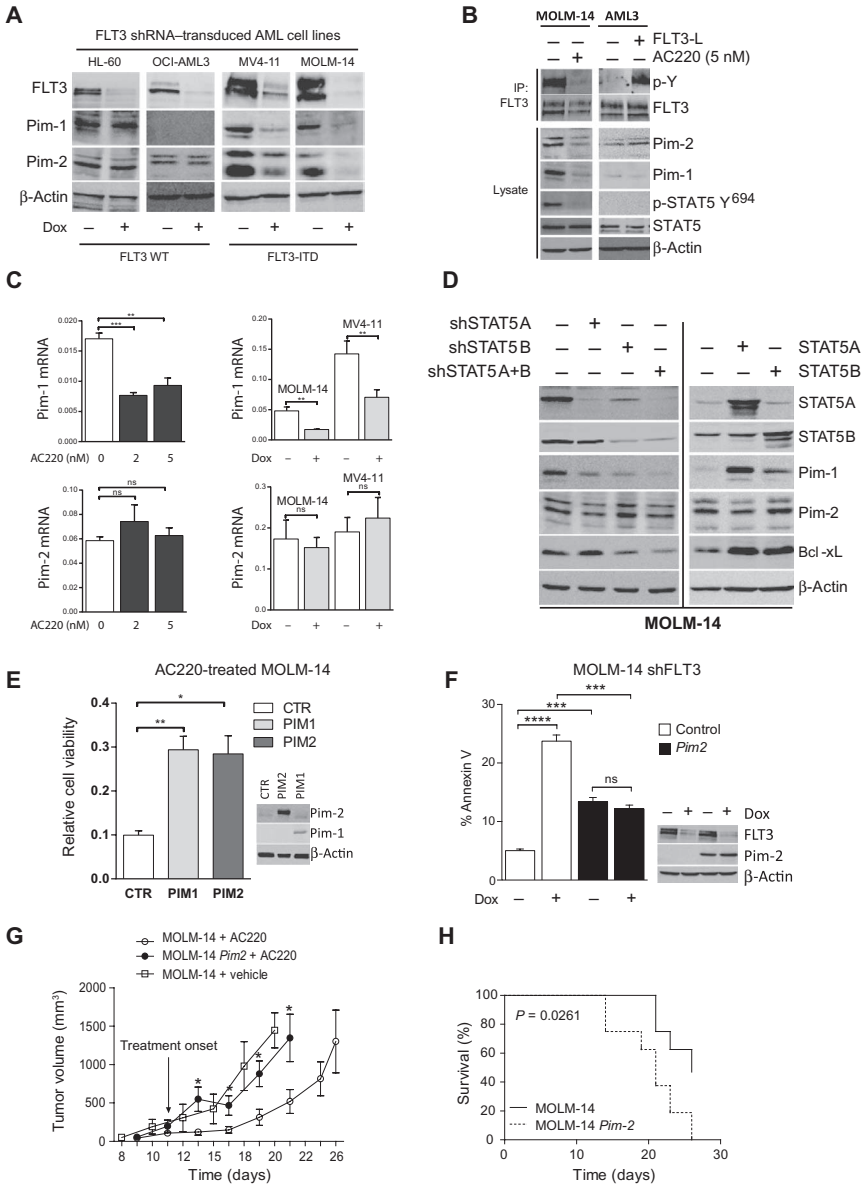

Fig. 2. Pim kinases are FLT3-ITD targets involved in resistance to FLT3 inhibition in AML. (**A**) AML cell lines (HL-60, OCI-AML3, MV4-11, and MOLM-14) were transduced via lentivirus with Dox-inducible anti-FLT3 shRNA vectors. shRNA induction was achieved with Dox (200 ng/ml). Western blots were performed using FLT3, Pim-1, and Pim-2 antibodies. WT, wild type. (**B**) MOLM-14 and OCI-AML3 cells were cultured with FLT3-L and/or 5 nM AC220. Tyrosine phosphorylation was evaluated in FLT3 immunoprecipitates. Pim-1, Pim-2, phospho-STAT5 (Y^{694}), and STAT5 levels were detected in whole-cell lysates by immunoblotting. (**C**) MOLM-14 cells were treated for 24 hours with 5 nM AC220 (left), and MOLM-14 and MV4-11 cells were transduced with inducible shFLT3 and treated with Dox (200 ng/ml) for 48 hours (right). Pim-1 and Pim-2 mRNA levels were quantified by real-time polymerase chain reaction. Gene expression was normalized to the HPRT (hypoxanthine-guanine phosphoribosyltransferase) levels ($n = 3$). (**D**) STAT5A/B gain (right) or loss (left) of function in MOLM-14 cells transduced with lentivirus. STAT5A, STAT5B, Pim-1, Pim-2, and Bcl-xL protein levels were evaluated by immunoblotting. (**E**) MOLM-14 cells were separately transduced with a *PIM1*- or *PIM2*-expressing vector or an empty vector. Ectopic expression of Pim-1 and Pim-2 was verified by immunoblotting (right). Cells were treated with vehicle or 1 nM AC220 for 48 hours, and cell viability was assessed by an UptiBlue assay (left) ($n = 3$). (**F**) MOLM-14 cells expressing an FLT3 shRNA in a Dox-inducible manner were transduced with a *Pim2* (murine Pim-2) allele or a control vector. After 48 hours of culture in the presence of Dox (200 ng/ml), FLT3 and murine Pim-2 expression was assessed by immunoblotting, and apoptosis was measured by annexin V staining. (**G** and **H**) MOLM-14 cells were transduced via lentivirus with a control vector (Dox-inducible anti–Pim-2 shRNA vector) or with *Pim2* lentivirus and xenografted into nude mice. (E) Tumor growth was assessed in mice treated with AC220 (1 mg/kg) (initiated once the tumor size reached 100 mm^3) with (black circle) or without (white circle) *Pim2* transduction. Growth of untreated xenotransplanted MOLM-14 cells is also provided (white square box). The means of the individual tumor sizes are plotted ($n = 8$). (F) Kaplan-Meier survival curve analysis of MOLM-14 cells transduced (dashed line) or not (solid line) with *Pim2*, transplanted into nude mice, and treated with AC220 ($n = 8$). Results are expressed as means ± SEM. β-Actin was used as a loading control for all Western blots. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

OCI-AML3 cell line (fig. S2, A and B). Pim-1 and Pim-2 protein expression also decreased with AC220 therapy in MOLM-14 cells (Fig. 2B). In OCI-AML3 cells, stimulation with FLT3 ligand (FLT3-L) enhanced FLT3 tyrosine phosphorylation but had no impact on Pim kinase expression (Fig. 2B). Collectively, these data suggest that Pim kinases are specific *FLT3-ITD* targets without implication of the wild-type *FLT3* allele in their regulation.

Previous work suggests that STAT5 activation by endoplasmic reticulum–anchored FLT3-ITD may transactivate Pim kinases along with other targets such as Bcl-xL (*20*, *21*). We quantified Pim-1 and Pim-2 mRNA upon FLT3 pharmacological or genetic inhibition. In MOLM-14 and MV4-11 FLT3-ITD$^+$ cells, FLT3 inhibition correlated to decreased Pim-1 transcript levels, whereas Pim-2 mRNA remained at baseline levels (Fig. 2C). Moreover, STAT5 knockdown in MOLM-14 cells induced Pim-1, but not Pim-2, depletion (Fig. 2D). In contrast, ectopic expression of STAT5A or STAT5B induced Pim-1 expression without Pim-2 modification (Fig. 2D). Pim-1 and Pim-2 are thus differentially regulated downstream of FLT3-ITD without implication of STAT5 in the case of Pim-2 in AML.

We expressed a *PIM1* or a *PIM2* allele separately in MOLM-14 cells (Fig. 2E). Upon treatment with AC220, cell viability was significantly preserved in MOLM-14 cells overexpressing Pim-1 or Pim-2 compared to MOLM-14 cells transduced with an empty vector (Fig. 2E). *Pim2* ectopic expression also protected MOLM-14 cells from apoptosis induced by genetic (Fig. 2F) or chemical (fig. S2D) FLT3 inhibition. Ectopic expression of *AKT* promoted annexin V staining similar to *Pim2* expression but did not protect MOLM-14 cells from AC220-induced cytotoxicity (fig. S2E). Pim kinase expression thus specifically prevented cytotoxicity upon FLT3 inhibition in AML.

We xenografted nude mice with MOLM-14 cells transduced with a control or *Pim2* allele. AC220 administration was initiated upon disease detection (Fig. 2G). FLT3i reduced tumor growth and delayed disease propagation in control animals, showing that MOLM-14 cells are sensitive to FLT3i in vivo, as previously reported (Fig. 2G) (*22*). In contrast, mice bearing xenografts ectopically expressing a *Pim2* allele were resistant to AC220 treatment and their survival was significantly shortened compared to controls (Fig. 2, G and H). Pim kinase expression thus drives FLT3i resistance in MOLM-14 cells in vitro and in vivo.

Pim kinases, with a predominant implication of Pim-2, regulate FLT3i-naïve and FLT3i-resistant AML cell survival

Pim-2 knockdown promoted apoptosis in two FLT3-ITD$^+$ AML cell lines (MOLM-14 and MV4-11; Fig. 3A). By contrast, it had no effect on annexin V binding in FLT3 wild-type AML cell lines (THP-1, OCI-AML3, and HL-60) or normal CD34$^+$ hematopoietic progenitor cells (Fig. 3, A and B). In contrast to other models (*10*) and to our observations in normal CD34$^+$ hematopoietic progenitor cells, no compensatory increase of other Pim kinase family members was seen after Pim-1 or Pim-2 knockdown in AML cells (fig. S3A). In MOLM-14 cells, Pim-1 knockdown did not induce annexin V binding (fig. S3, B and C) and reduced cell proliferation and viability, but to a lesser extent than did Pim-2 depletion (fig. S3D), suggesting the absence of functional compensation between these kinases in AML. Decreased cell viability induced by Pim-2 knockdown was rescued by ectopic murine Pim-2 expression but not by a mutant *Pim2* allele devoid of kinase activity [kinase-dead *Pim2* mutant (*Pim2KD*)] in MOLM-14 cells (Fig. 3C). These results suggest that Pim kinases, particularly Pim-2, are essential to the survival of FLT3-ITD$^+$ AML cells.

We subcutaneously injected nude mice with MOLM-14 cells expressing in a Dox-inducible manner either a Pim-2 shRNA or a scrambled shRNA and followed leukemia propagation. Administration of Dox impaired tumor growth in Pim-2 shRNA–expressing tumors relative to scrambled shRNA–expressing as well as vehicle-treated tumors (Fig. 3, D and E). Corroborating these findings, Pim-2 knockdown increased mouse survival (Fig. 3, F and G). Immunohistochemical analysis of tumor sections confirmed the efficiency and specificity of Pim-2 knockdown (Fig. 3H). Pim-2 knockdown also correlated with inhibition of 4E-BP1 phosphorylation, suggesting an efficient inhibition of Pim-2–dependent signaling pathways in vivo (Fig. 3H) (*16*). Finally, tumors with knockdown of Pim-2 exhibited increased cell death as measured by TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) staining (Fig. 3H).

We used AC220-treated MOLM-14 xenografts that became resistant to therapy (Fig. 2E). Ex vivo cultures of these cells (referred to as MOLM-14-R) devoid of FLT3 tyrosine kinase domain mutation (FLT3-TKD; data not shown) confirmed our in vivo results (Fig. 2, E and F), suggesting that these cells acquired AC220 resistance compared to parental FLT3i-naïve MOLM-14 cells (Fig. 3I). Augmentation of AC220 proapoptotic activity was observed in parental MOLM-14 cells upon Pim-2 knockdown (Fig. 3I). Strikingly, Pim-2 knockdown induced by Dox sensitized MOLM-14-R cells to AC220-induced apoptosis, suggesting that AC220 resistance is associated with Pim-2 expression in this model (Fig. 3I). Together, these data show that Pim-2 is involved in FLT3i resistance in vitro and in vivo.

Pim kinase inhibition directly facilitates FLT3-ITD receptor blockade by AC220

We measured intracellular calcium flow as a dynamic marker of FLT3-dependent signaling pathway activation. Calcium mobilization was promptly induced in Ba/F3-ITD cells—used as a minimal model of oncogene addiction to FLT3-ITD signaling (*23*)—after FLT3-L stimulation, consistent with the sustained sensitivity of FLT3-ITD receptors to FLT3-L in AML patients (Fig. 4A) (*24*). Ectopic expression of *Pim2*, but not *Pim2KD*, allele abrogated FLT3-L–induced signaling, whereas induction of calcium flux by thapsigargin was similar regardless of transfectant (Fig. 4A). Co-incubation of Pim-2 and FLT3 recombinant proteins decreased FLT3 tyrosine phosphorylation in vitro compared to the level of FLT3 autophosphorylation (fig. S4A), and MOLM-14 cells transfected with *Pim2* exhibited reduced FLT3 and STAT5 tyrosine phosphorylation, in contrast to *Pim2KD* transfectants (Fig. 4B). These results suggest that Pim-2 activity inhibits FLT3-ITD signaling.

We used Ba/F3 cells transduced with *FLT3-ITD* alleles with wild-type or D835Y mutated kinase domains. The *FLT3-ITD-D835Y* allele modeled here conferred mutational resistance to FLT3i (*6*). We treated both FLT3-ITD and FLT3-ITD-D835Y cells with the pan-Pim kinase inhibitor LGB321 (*25*). FLT3-L stimulation increased calcium flux in both FLT3-ITD and FLT3-ITD-D835Y cells treated with LGB321 compared to vehicle-treated cells (Fig. 4C). In support of these results, incubation of a mix of Pim-2 and FLT3 recombinant proteins with LGB321 in vitro increased FLT3 tyrosine phosphorylation (fig. S4B). These results suggest that Pim-2 directly controls FLT3 tyrosine phosphorylation and FLT3-ITD–dependent signaling pathway activation. We co-incubated FLT3, Pim-2, and STAT5 recombinant proteins in vitro without or with AC220 or LGB321 and then analyzed STAT5 tyrosine phosphorylation by immunoblotting using STAT5 as a direct FLT3 substrate in vitro (*26*). Whereas LGB321 intrinsically had no

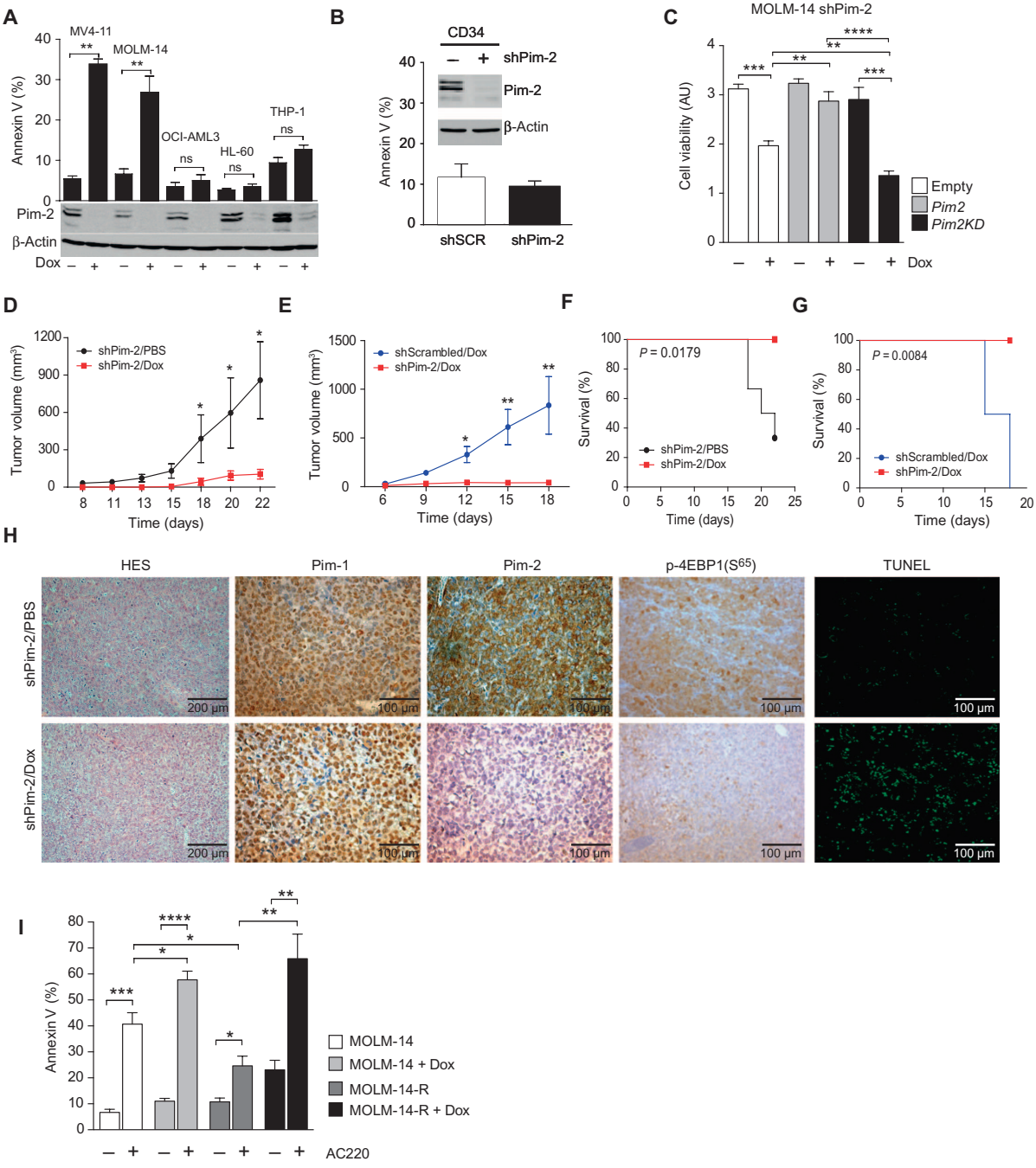

Fig. 3. Pim kinases, with a predominant implication of Pim-2, regulate FLT3i-naïve and FLT3i-resistant AML cell survival. (**A**) AML cell lines (MOLM-14, MV4-11, HL-60, OCI-AML3, and THP-1) were transduced via lentivirus with a vector promoting the expression of an anti–Pim-2 shRNA after induction with Dox (200 ng/ml). Apoptosis was quantified by annexin V staining after 4 days of shRNA induction ($n = 3$). The extent of Pim-2 knockdown in each cell line was determined by Western blot (bottom). (**B**) Western blot for Pim-2 expression (top) and annexin V staining in normal $CD34^+$ hematopoietic progenitor cells lentivirally transduced with scrambled (–) or Pim-2 (+) shRNA ($n = 3$). (**C**) Pim-2 shRNA–transduced MOLM-14 cells were cotransfected with *Pim2* or *Pim2KD* as indicated. Cell viability after Dox treatment was assessed by staining with the UptiBlue fluorescent reagent ($n = 6$). (**D** to **G**) Tumor growth and survival (Kaplan-Meier curve) of MOLM-14 cells transfected with scrambled or Pim-2 shRNA and xenografted into nude mice. Animals were treated with vehicle [phosphate-buffered saline (PBS), black line, $n = 8$] or Dox (200 μg/ml) (Pim-2 shRNA in red, scrambled shRNA in blue; $n = 8$ for each). (**H**) Tumor sections stained by HES and TUNEL or labeled with Pim-1, Pim-2, and phospho-4E-BP1 (S^{65}) antibodies. Representative images from three separate experiments are shown. (**I**) MOLM-14 cells were harvested from AC220-treated xenografted mice as depicted in Fig. 2, E and F. Pim-2 knockdown was induced with Dox (200 ng/ml) for 24 hours, and 2 nM AC220 was then added to the culture for an additional 24 hours. Apoptosis was quantified by annexin V staining. Results are expressed as means ± SEM. β-Actin was used as a loading control for all Western blots. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

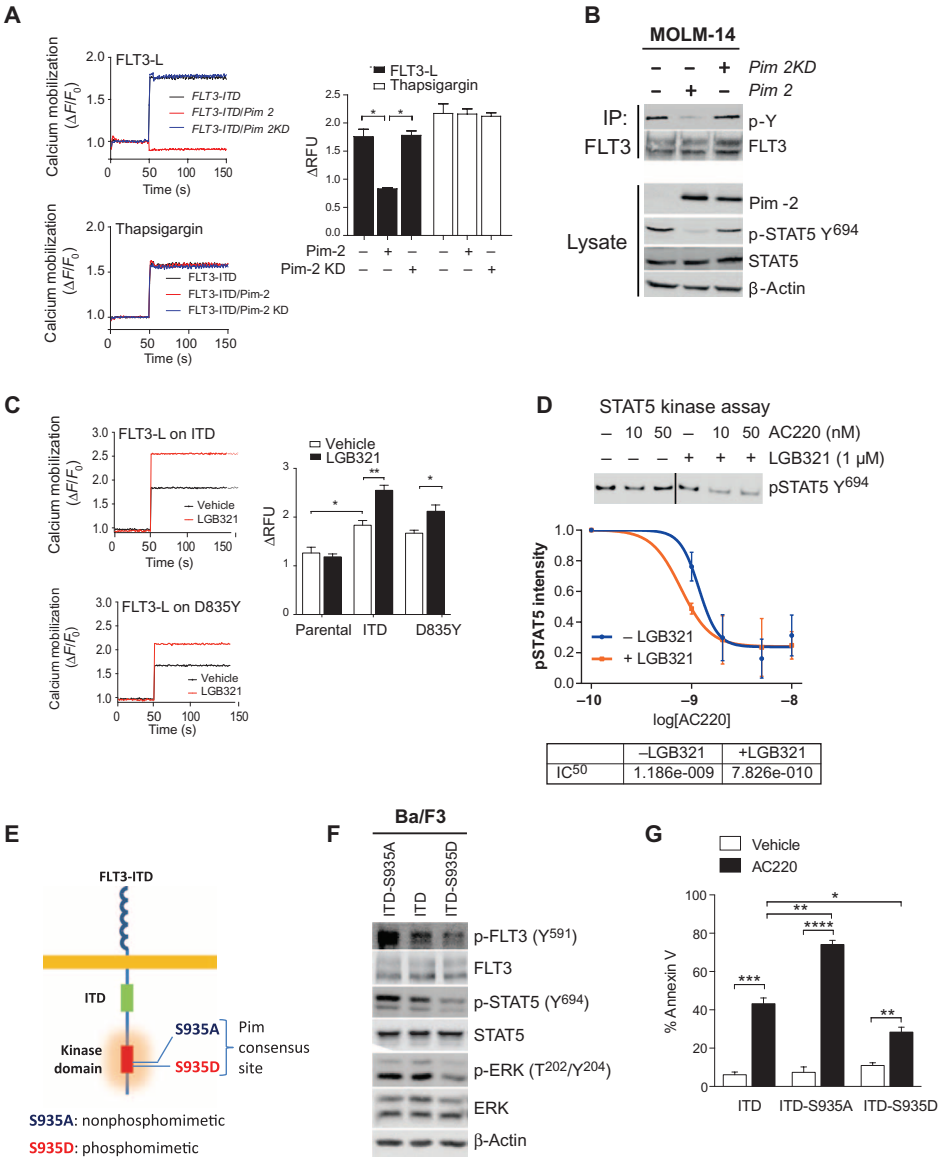

Fig. 4. Pim kinase inhibition directly facilitates FLT3-ITD receptor blockade by AC220. (**A**) Ba/F3 cells expressing *FLT3-ITD*, *Pim2*, and *Pim2KD* alleles as single or combined transfectants as indicated were stained with Fluo-4 AM and treated or not with FLT3-L (30 ng/ml). Variations in intracellular calcium concentrations ($[Ca^{2+}]_i$) were evaluated. Fluorescence (485-nm excitation/516-nm emission) was acquired over time to evaluate the kinetics of response. Variations are expressed as differences between the baseline and experimental $[Ca^{2+}]_i$ elevations ($\Delta F/F_0$) (left), and $[Ca^{2+}]_i$ elevations ($\Delta F/F_0$) are expressed as the mean area under the curves [change in relative fluorescence units (ΔRFU)] (right). Results are expressed as the mean of at least four independent samples. Thapsigargin (10 μM) was used as a control for calcium mobilization. (**B**) Protein extracts from MOLM-14 cells expressing a control vector, *Pim2*, or *Pim2KD* were assessed for FLT3 tyrosine phosphorylation (after FLT3 immunoprecipitation) as well as STAT5 (Y^{694}) phosphorylation and Pim-2 and STAT5 expression by immunoblotting. (**C**) Parental Ba/F3 cells and Ba/F3 cells expressing *FLT3-ITD* or *FLT3-ITD-D835Y* alleles were treated with vehicle or 1 μM LGB321 for 1 hour, and calcium flux was measured as detailed in (A). (**D**) STAT5, Pim-2, and FLT3 recombinant proteins were mixed together in a kinase buffer without or with 1, 2, 5, 10, or 50 nM AC220 and without or with 1 μM LGB321 for 1 hour. Then, 200 μM ATP was added for 30 min, and proteins were solubilized in Laemmli buffer and analyzed by immunoblotting with a phospho-STAT5 Y^{694} antibody. A representative Western blot is provided (top). Signal intensity was quantified using MultiGauge software (Fujifilm), and results are presented with AC220 concentrations given in log scale and using the log(inhibitor) versus response variable slope (four parameters) function of GraphPad v6 software. Results of IC_{50} for STAT5 phosphorylation without (–) or with (+) LGB321 are provided (bottom) (n = 3). (**E**) Schematic representation of FLT3-ITD receptors and of Pim kinase consensus S^{935} site with either nonphosphomimetic or phosphomimetic amino acid substitutions. (**F** and **G**) Ba/F3 cells were transduced with FLT3-ITD receptors either unmodified (ITD) or harboring nonphosphomimetic (ITD-S935A) or phosphomimetic (ITD-S935D) amino acid substitutions. (F) Western blotting with phospho-FLT3 (Y^{591}), phospho-ERK (T^{202}/Y^{204}), phospho-STAT5 (Y^{694}), FLT3, STAT5, and ERK antibodies. (G) Ba/F3 cells were cultured for 48 hours with vehicle or 5 nM AC220. Apoptosis was measured by annexin V binding. Results are expressed as means ± SEM. β-Actin was used as a loading control for all Western blotting experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

impact on STAT5 phosphorylation (Fig. 4D and fig. S4C), addition of this compound decreased the AC220 median inhibitory concentration (IC_{50}) for STAT5 phosphorylation (Fig. 4D), suggesting that Pim-2 inhibition facilitated AC220 competition with adenosine triphosphate (ATP) for FLT3 receptors, facilitating AC220-induced FLT3 activity blockade in vitro.

FLT3 harbors a consensus Pim phosphorylation motif (RKRPSF) surrounding a serine residue at position 935 (S^{935}), which is conserved among species (fig. S4D). Phosphorylation of S^{935} by Pim-1 has recently been reported to regulate FLT3 stability (*27*). We generated both phosphomimetic (*FLT3-ITD-S935D*) and nonphosphomimetic (*FLT3-ITD-S935A*) mutant alleles and expressed them in Ba/F3 cells (Fig. 4E). Compared to Ba/F3-ITD cells, Ba/F3 cells expressing the *FLT3-ITD-S935D* phosphomimetic mutant had reduced FLT3 Y^{591} phosphorylation as well as decreased FLT3-dependent signaling as measured by ERK (T^{202}/Y^{204}) and STAT5 (Y^{694}) phosphorylation (Fig. 4F). In contrast, the *FLT3-ITD-S935A* nonphosphomimetic allele showed increased Y^{591} phosphorylation along with enhanced ERK and STAT5 phosphorylation (Fig. 4F). Furthermore, introduction of the S935A mutation augmented the proapoptotic effects of AC220, whereas the S935D mutation abrogated AC220-induced annexin V binding (Fig. 4G). Together, these results suggest that a FLT3-ITD consensus site for Pim kinase phosphorylation regulates FLT3-dependent signaling and that abrogation of S^{935} phosphorylation restores FLT3-ITD sensitivity to FLT3i.

Combined inhibition of Pim and FLT3 eradicates FLT3-mutated cells

The F691L and D835Y FLT3 kinase domain mutants (Fig. 5A) are frequently correlated to FLT3i resistance in AML patients (*6*). *FLT3-ITD* alleles harboring these mutations were expressed in Ba/F3 cells via lentivirus. Relative to Ba/F3-ITD cells, expression of these mutants conferred resistance to AC220-induced cytotoxicity but had no impact on sensitivity to LGB321 (fig. S2, A and B). Coadministration of LGB321 sensitized FLT3-ITD as well as FLT3-ITD-F691L and FLT3-ITD-D835Y cells to AC220, as measured by the reduction of IC_{50} for AC220 cytotoxicity (Fig. 5B and fig. S5C).

AC220 monotherapy induced annexin V binding in Ba/F3-ITD and moderately in Ba/F3-ITD-D835Y, but not in Ba/F3-ITD-F691L cells (fig. S5D). LGB321 enhanced the proapoptotic effect of AC220 in FLT3-ITD and FLT3-ITD-D835Y cells and triggered apoptosis in FLT3-ITD-F691L cells (fig. S5D). In FLT3-ITD-D835Y cells, the combination of LGB321 and AC220—but not AC220 monotherapy—inhibited ERK and STAT5 phosphorylation (Fig. 5C). In FLT3-ITD cells, coadministration of LGB321 amplified AC220-induced inhibition of STAT5 and ERK phosphorylation (fig. S5E). Similar results were obtained with the dual Pim/FLT3 inhibitor SGI-1776 (fig. S5F) (*28*). These results indicate that LGB321 restores sensitivity to AC220 in FLT3-ITD cells with kinase domain mutation–induced resistance to FLT3i.

We compared the effects of four clinically used FLT3i agents in Ba/F3 cells expressing FLT3-ITD (Ba/F3-ITD, sensitive to FLT3i) or FLT3-ITD with the F691L mutation (Ba/F3-F691L, resistant to most FLT3i) (*29*). All four FLT3i agents induced annexin V binding, and cotreatment with LGB321 significantly enhanced their efficacy in Ba/F3 FLT3-ITD (Fig. 5D). In contrast, F691L cells were resistant to type II tyrosine kinase inhibitors (TKIs) (AC220 and sorafenib) and were only sensitive to high doses of type I TKIs (PKC412 and crenolanib) (Fig. 5D) (*2*, *30*). Strikingly, cotreatment with LGB321 sensitized these cells to both type I and type II TKIs (Fig. 5D).

We expressed *FLT3-ITD* or *FLT3-ITD-D835Y* alleles in MOLM-14 cells, and Pim-2 expression was still observed despite FLT3 inhibition by AC220 (Fig. 5E). Whereas MOLM-14-ITD cells were sensitive to AC220, expression of the D835Y allele blunted AC220-induced apoptosis (Fig. 5F), suggesting that this transgene exerts dominant effects on the endogenous FLT3-ITD receptor. In MOLM-14-D835Y cells, coadministration of LGB321 enhanced AC220-induced apoptosis (Fig. 5F). AC220 induced annexin V binding in FLT3-ITD^+ cell lines (MOLM-14 and MV4-11) and LGB321 enhanced this effect, contrary to the observations in FLT3 wild-type cells (OCI-AML2, OCI-AML3, and THP-1) (Fig. 5G). AC220 and LGB321 demonstrated synergistic activity in those cell lines (fig. S5G). In primary FLT3-ITD^+ AML samples (table S1), AC220 monotherapy induced minimal apoptosis in vitro, which is a frequent finding even in the absence of previous exposure to FLT3i (*31*, *32*), but coadministration with LGB321 significantly induced annexin V binding ($n = 7$; Fig. 5H). On the other hand, these agents had no impact on annexin V binding in FLT3 wild-type primary AML samples ($n = 3$; Fig. 5H). These results suggest that Pim kinase inhibition enhances the efficacy of FLT3i in the context of various FLT3 mutations.

DISCUSSION

FLT3-ITD is a frequent genetic event in AML and confers a poor prognosis due to an increased relapse rate despite intensive chemotherapy (*33*). In phase 1/2 clinical trials, ATP-competitive FLT3i have shown promising results with acceptable toxicity (*4*, *34*). However, responses are transient, and FLT3i resistance is virtually inevitable (*34*). The rapid emergence of TKD-mutated subclones in a subset of FLT3i-treated patients pinpointed FLT3 signaling as a critical molecular event for disease propagation (*6*). However, most relapsing patients do not manifest new FLT3 mutations, suggesting the existence of alternative pathways involved in FLT3i resistance (*9*). Compensatory activation of alternative signaling pathways (*18*, *21*, *35*), leukemia-permissive interactions with the bone marrow microenvironment (*20*, *36*), and hyperactivation of spleen tyrosine kinase (SYK) (*37*) have been proposed as explanatory models.

We and others have previously reported an important role for Pim kinases in AML biology (*16*, *38*, *39*) and suggested that combining FLT3 and Pim kinase inhibitors may be relevant in FLT3-ITD^+ AML (*40*, *41*). Here, we built on these studies by showing that Pim kinases (particularly Pim-2) are overexpressed in FLT3-ITD^+ AML patients refractory to FLT3i, regardless of their FLT3-TKD mutational status. Whereas FLT3-ITD^+ cells are addicted to both Pim-1 and Pim-2 expression, the impact of Pim-2 on AML cell survival appears higher without significant functional compensation between these kinases. In addition, a STAT5-independent regulation of Pim-2 downstream of FLT3-ITD receptors—in contrast to Pim-1 in our model—led us to consider these kinases separately and to focus on Pim-2 in AML. In murine FLT3-ITD–induced MPN and AML models, ectopic Pim-2 expression decreases FLT3i efficacy, and in AML xenografts resistant to AC220, Pim-2 knockdown restores FLT3i sensitivity. Augmented Pim kinase activity thus drives FLT3i resistance in FLT3-ITD^+ AML.

Previous studies in minimal cellular models suggest that Pim kinase overexpression reduces the efficacy of FLT3i (*11*, *17*). However, the molecular mechanisms, cellular consequences, and clinical application of this observation have yet to be fully characterized.

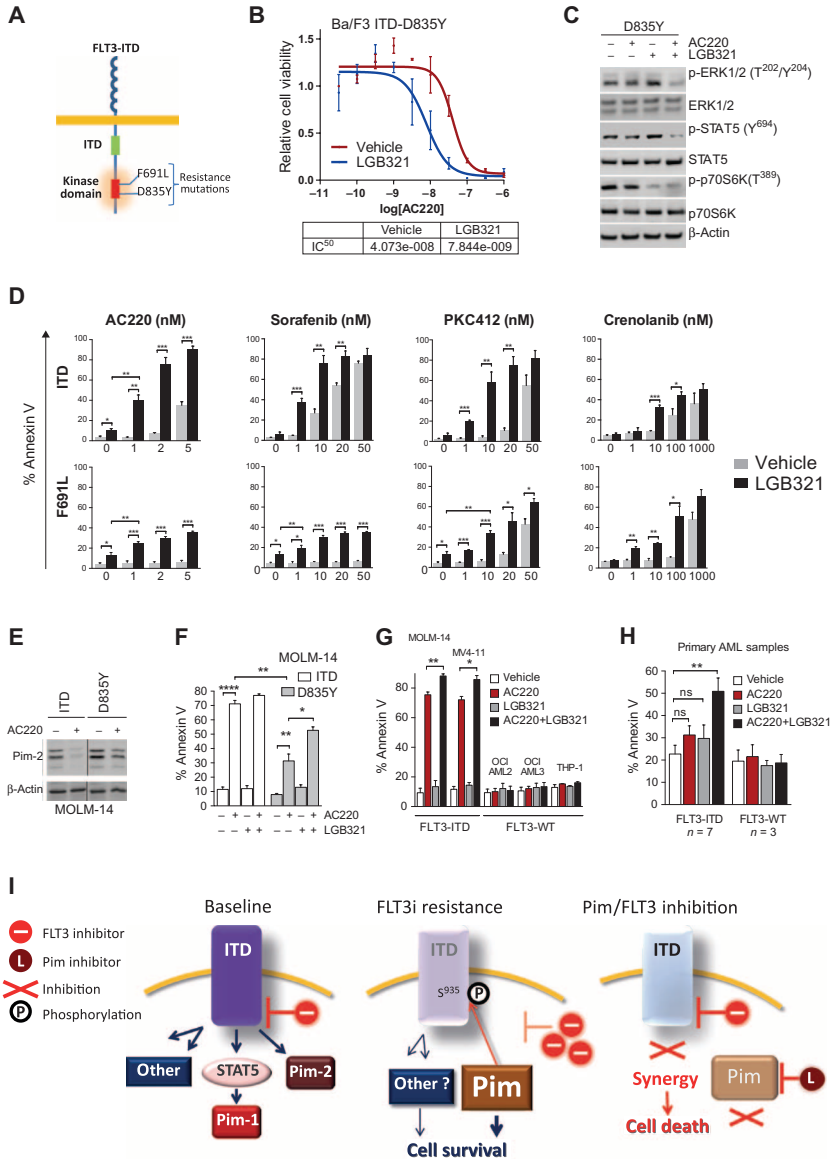

Fig. 5. Combined inhibition of Pim and FLT3 eradicates FLT3-mutated cells. (**A**) Schematic representation of FLT3i resistance-conferring mutations within the FLT3-ITD kinase domain. (**B**) Cell viability measured by a CellTiter-Glo assay in FLT3-ITD-D835Y–transduced Ba/F3 cells treated with log dilutions of AC220 without or with 3.2 nM LGB321. Relative luminescence (RLU) was normalized to vehicle-treated cells for each condition (normalized RLU). Results are presented using the log(inhibitor) versus response variable slope (four parameters) function of GraphPad v6 software. The IC_{50} values for this assay are provided (bottom) ($n = 3$). (**C**) Ba/F3 ITD-D835Y cells were cultured without or with 5 nM AC220 and/or 1 μM LGB321 for 4 hours. Western blots were done with phospho-STAT5 (Y^{694}), phospho-ERK (T^{202}/Y^{204}), phospho-P70S6K (T^{389}), STAT5, ERK, and P70S6K antibodies. (**D**) Ba/F3 cells expressing *FLT3-ITD* or *FLT3-ITD-F691L* alleles were cultured for 48 hours with 1 μM LGB321 and increasing doses of TKIs (AC220, sorafenib, PKC412, and crenolanib), as indicated. Apoptosis was determined by annexin V staining ($n = 3$ for each). (**E** and **F**) Ectopic expression of *FLT3-ITD* or *FLT3-ITD-D835Y* alleles with a lentiviral vector in MOLM-14 cells. (E) Western blotting with a Pim-2 antibody, using protein extracts from MOLM-14-ITD or MOLM-14-ITD-D835Y cells treated with vehicle or 5 nM AC220 for 24 hours. (F) Cells were treated with vehicle, 1 μM LGB321, 5 nM AC220, or the combination of LGB321 and AC220 for 48 hours, and apoptosis was quantified by annexin V staining. (**G** and **H**) AML cell lines (MOLM-14, MV4-11, OCI-AML2, OCI-AML3, and THP-1) (G) and primary FLT3-ITD$^+$ ($n = 7$) or FLT3-WT ($n = 3$) AML samples (H) were cultured with vehicle, 1 μM LGB321, 5 nM AC220, or the combination of LGB321 and AC220 for 48 hours, and annexin V staining was measured. (**I**) Explanatory model. Baseline: FLT3-ITD receptors (ITD) constitutively activate prosurvival signaling pathways including Pim-1 (through STAT5 activation) and Pim-2 (through an unknown STAT5-independent mechanism). FLT3i inhibit FLT3 activity and induce cytotoxicity. FLT3i resistance: Increased Pim kinase expression is found in FLT3i-resistant AML cells. Pim kinases have intrinsic oncogenic properties in favor of AML cell survival. They also induce direct FLT3-ITD receptor modifications (including S^{935} phosphorylation) contributing to FLT3i resistance. Pim/FLT3 inhibition: Genetic or pharmacological Pim kinase inhibition restores FLT3i activity against FLT3-ITD receptors, leading to synergistic cytotoxicity. β-Actin was used as a loading control for all Western blotting experiments. Results are expressed as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Pim kinases exert oncogenic effects by modulating multiple targets involved in cellular proliferation and apoptosis (*10*). Accordingly, Pim kinase inhibition reduces AML cell viability and Pim-2 knockdown blocks disease propagation in FLT3-ITD$^+$ AML xenografts. Addiction to Pim kinases may thus intrinsically contribute to FLT3i resistance. In addition, we unexpectedly found that Pim kinases directly modulate FLT3-ITD receptor activity and response to ATP-competitive inhibitors.

The regulation of FLT3-ITD by tyrosine phosphorylation is well established (*42*). Puissant and colleagues (*37*) reported that FLT3 tyrosine phosphorylation by SYK is critical for both FLT3-ITD–induced transformation and FLT3i resistance. In contrast, the role of FLT3 serine phosphorylation has only recently emerged. Natarajan and colleagues (*27*) showed that Pim kinases directly phosphorylate and stabilize FLT3 in vitro. Although in our model the impact of Pim kinase–induced FLT3 glycosylation on resistance to FLT3i seems limited, we suggest that the consensus Pim kinase residue S^{935} is involved in the affinity of FLT3-ITD receptors for FLT3i because a nonphosphomimetic mutant (S935A)—which mimics the impact of Pim kinase inhibition on this residue—augments AC220-induced cytotoxicity. These results provide an explanatory model to understand the synergy between FLT3 and Pim kinase targeting in AML (Fig. 5I).

We further hypothesize that FLT3i resistance mediated by Pim kinases may involve steric modification of the FLT3 kinase domain. Sensitivity to FLT3i was elicited by Pim kinase inhibition across multiple FLT3-ITD kinase domain–mutant cell lines, including those expressing the FLT3-F691L allele. The F691L amino acid substitution involves a kinase domain "gatekeeper" position, similar to the T315I substitution in ABL1 kinase, which confers broad-spectrum resistance to TKIs in BCR-ABL malignancies (*29*). In F691L-mutated cells, resistance to FLT3i is abrogated by Pim kinase inhibition—regardless of the use of type I or type II FLT3i—supporting our model of steric FLT3 kinase domain changes induced by Pim kinases. Because FLT3-ITD subcellular localization regulates its activity (*43*), we hypothesize that Pim kinases may have an impact on FLT3-ITD trafficking with potential implications in receptor conformation and affinity for FLT3i, and this point should be investigated for each FLT3-ITD kinase domain mutant allele in future studies.

Drug resistance invariably occurs in FLT3i-treated AML patients, underscoring the importance of understanding the landscape of resistance mechanisms to FLT3i as we design therapeutic modalities that are more effective for this disease (*9*). Our results suggest that deregulation of Pim kinase activity is a recurrent resistance mechanism to FLT3i and that dual inhibition of Pim and FLT3 kinase efficiently targets FLT3-ITD$^+$ malignancies, as shown in primary samples from AML patients. Whereas the pharmacological profile of Pim and FLT3 dual targeting may appear favorable, taking into account that multidrug resistance effectors such as the ABCG2 drug transporter are directly activated by Pim kinases (*44*), careful pharmacokinetic studies should be conducted before administration in AML patients. Ideally, dual-activity molecules rather than combination therapies should be developed in this setting, even if the first-in-class dual Pim/FLT3 inhibitor SGI-1776 was withdrawn from clinical development because of unanticipated cardiac toxicity (*45*). Given the recent development of new FLT3i-targeting TKD mutant receptors such as crenolanib (*29*, *32*, *46*), the design of highly active and safe dual Pim/FLT3 inhibitors represents a fascinating challenge to make possible new therapeutic strategies in this particularly aggressive subtype of AML.

METHODS

Primary cells

Patients with FLT3-ITD$^+$ AML who had relapsed or had been refractory after two courses of standard chemotherapy or who were considered unfit for conventional treatment were recruited into a phase 2 clinical trial of sorafenib monotherapy as approved by the local institutional review board (IRB) of Hong Kong West Cluster/The University of Hong Kong (IRB UW 10-393). Briefly, patients were treated with a continuous course of sorafenib at 200 to 400 mg twice daily until allogeneic bone marrow transplantation or leukemia progression. Bone marrow or peripheral blood samples were obtained before sorafenib treatment (naïve) or during subsequent progression (resistant). We also collected blast cells from the bone marrow or blood of FLT3-ITD$^+$ AML patients treated in Cochin Hospital after obtaining approval from the hospital's ethics committee. Normal CD34$^+$ hematopoietic cells were obtained from healthy donors. Informed consent was obtained from each patient and healthy donor in accordance with the Declaration of Helsinki.

Cell lines and reagents

The MOLM-14, MV4-11, OCI-AML2, OCI-AML3, THP-1, and HL-60 human AML cell lines were cultured in α-modified Eagle's medium (αMEM) supplemented with 10% fetal calf serum (FCS) (*47*). We evaluated these cell lines for FLT3-ITD, FLT3-TKD, NPM1, *n*-ras, IDH1, IDH2, DNMT3A, and TP53 gene mutations (table S2). Ba/F3 cells transfected or not with ITD-mutated FLT3 with or without a TKD mutation (F691L, D835Y, S935A, or S935D) were grown in RPMI with 10% FCS, supplemented with 10% WEHI-3–conditioned medium as a source of interleukin-3 (IL-3) in the case of parental Ba/F3 cells (*48*). Dox (Sigma-Aldrich) was used in vitro at a concentration of 200 ng/ml in cell culture experiments (*49*–*51*) and at 200 μg/ml in mouse experiments. The endoplasmic reticulum calcium ATPase inhibitor thapsigargin (Sigma-Aldrich) was used at 10 μM as a control for calcium mobilization. We also used AC220, PKC412, sorafenib, and crenolanib (all from Selleck Chemical) at various concentrations as indicated. AC220 was also used at 1 mg/kg in mouse experiments. Murine IL-3 and FLT3-L were purchased from PeproTech. The pan-Pim kinase inhibitor LGB321 was from Novartis Pharmaceutical (*25*). The construction of mammalian expression plasmids for *PIM1*, *PIM2*, *Pim2*, *Pim2* kinase domain K61A mutant (kinase-dead or KD), and various *FLT3-ITD* mutants is described in the Supplementary Materials.

Western blotting

Whole-cell protein extraction and Western blotting were performed as previously described (*16*, *41*, *52*). The antibodies used in this study are described in table S2.

Retroviral infection of bone marrow cells and transplantation

Bone marrow cells were harvested from the femurs and tibias of female C57BL/6 mice, immediately infected with *FLT3-ITD*–containing retroviruses, and cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS, IL-3 (10 ng/ml), IL-6 (10 ng/ml), and stem cell factor (100 ng/ml). Cells were transduced with *PIM2* or control lentiviruses and cultured for 5 days under puromycin selection. Transduced bone marrow cells (2×10^5) were transplanted via retroorbital intravenous injection into sublethally irradiated recipient mice (250 cGy, $n = 4$ for each group). Body weights were monitored

weekly. Peripheral blood was collected at 4, 8, and 12 weeks after transplantation in EDTA-coated tubes, and complete blood counts were measured on an MS9-5 Blood Analyzer (Melet Schloesing Laboratoires). After sacrifice, spleens were measured, weighed, and either fixed in 4% formalin or subjected to cell dispersion by passaging through a 70-μm cell strainer in PBS containing 1% bovine serum albumin. Cells were electronically counted with a Scepter automated cell counter (Millipore).

RNA interference

The Tet-pLKO-puro plasmid (*49*) (Addgene plasmid 21915) was used to express hairpins targeting Pim-2 or FLT3 in a Dox-dependent manner after lentiviral delivery. The sequences were 5′-GATGAACCCTACACT-GACTTT-3′ (Pim-2) and 5′-GCATCCCAGTCAATCAGCTTT-3′ (FLT3). Scrambled and Pim-2 hairpins expressed in a noninducible pLKO vector were purchased from Thermo Scientific.

Flow cytometry

Apoptosis was measured by annexin V–phycoerythrin (Becton Dickinson) staining according to the manufacturer's instructions.

AML xenografts in nude mice

MOLM-14 cells stably expressing the shPim-2 pLKO-Tet-On vector were subcutaneously injected into nude mice. Detailed procedures for follow-up of tumor growth, survival, immunohistochemistry, and TUNEL assays are provided in the Supplementary Materials. All experiments were conducted in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International and after approval of the local ethics committee.

Cell viability assays

UptiBlue. AML cells were plated in triplicate at 2.5×10^4 in 100 μl of 5% FCS–supplemented MEM for 48 hours. The UptiBlue viable cell counting reagent (Interchim) was then added for 3 hours according to the manufacturer's instructions, and fluorescence was measured with a Typhoon 8600 scanner (GE Healthcare Bio-Sciences).

CellTiter-Glo. Cells were plated at a concentration of 4×10^4/ml in 50 μl of 10% FCS–supplemented MEM (2×10^3 cells per well) and cultured in the presence of drugs for 48 hours at 37°C. Viability was quantified by the luminescence-based CellTiter-Glo assay (Promega) following the manufacturer's instructions. Luminescence was measured with the EnVision plate reader (Perkin Elmer). Luminescence values were normalized to dimethyl sulfoxide–treated controls for each cell line. IC_{50} values were calculated with GraphPad Prism v6 (GraphPad) (*53*).

Measurement of free intracellular calcium

Ba/F3 or Ba/F3 FLT3-ITD cells were transduced with control, *Pim2* (murine Pim-2), or *Pim2KD* lentiviruses. Free intracellular calcium content was measured using the Fluo-4 Direct Calcium Assay Kit (Invitrogen) as indicated by the manufacturer. Changes in cytosolic free calcium were quantified as RFU using a microplate reader (Tecan Infinite M200 and Magellan software, Tecan). Detailed procedures are provided in the Supplementary Materials.

In vitro kinase assays

STAT5 (reference no. SRP5142), Pim-2 (reference no. K3518), and FLT3 571-993 (reference no. F6432) recombinant proteins were from Sigma-Aldrich and were used at a concentration of 200 ng in a final volume of 100 μl. Proteins were mixed in a kinase reaction buffer (Cell Signaling Technology, reference no. 9802) with 0, 1, 2, 5, 10, or 50 nM AC220 and 0 or 1 μM LGB321 and incubated for 1 hour at 37°C. Then, 200 μM ATP (Cell Signaling Technology, reference no. 9804) was added, and the mixture was further incubated for 30 min at 37°C. The reactions were stopped by the addition of boiling Laemmli buffer.

Statistics

Differences between the mean values obtained for the experimental groups were analyzed using two-tailed Student's *t* test. Statistical analyses were performed using Prism software (GraphPad).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/1/8/e1500221/DC1

Fig. S1. Pim kinase expression in AML.
Fig. S2. Pim-2 regulation by downstream FLT3-ITD receptors.
Fig. S3. Pim-1 and Pim-2 regulation by FLT3-ITD receptors.
Fig. S4. Direct phosphorylation of FLT3 receptors by Pim-2.
Fig. S5. Dual inhibition of FLT3 and Pim kinases produces synergistic cytotoxicity in AML.
Table S1. Genotyping of AML cell lines used in the current study.
Table S2. References of the antibodies used in the current study.
Materials and Methods

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Acknowledgments: We are indebted to P. Dubreuil [Cancerology Research Centre of Marseille (CRCM), U1068, Marseille, France] for the gift of FLT3-ITD and pMX vectors; E. Campeau (University of Massachusetts Medical School, Worcester, MA) for providing Addgene plasmid 19068 [pLenti PGK Puro DEST (w529-2)]; D. Wiederschain and K. Mostov for sharing the Tet-pLKO-puro and pLKO.1-blast plasmids, respectively, through Addgene; I. Dusanter (Institut Cochin, Paris, France) for providing STAT5A/B expression vectors and shRNA constructs (*54*); and N. Azar (Hopital de la Pitié-Salpetrière, Paris, France) for contributing normal $CD34^+$ cell samples. We are also grateful to W. Shuo-Chieh (Dana-Farber Cancer Institute, Boston, MA) for help with the analysis of in vitro synergy experiments. Finally, we thank O. Thibaudeau (Institut Claude Bernard/IFR2—Faculté de Médécine Xavier Bichat, Paris), F. Watier and N. Gadessaud (Plateforme d'Histologie et de Morphologie—Fondation Imagine, Paris) for their assistance with histologic sample processing, and D. Damotte (Hotel-Dieu Hospital, Paris, France) for her assistance with histologic analysis. **Funding:** We thank the French INCa (Institut National du Cancer) organization for financial support. This work was supported by grants from the Ligue Nationale Contre le Cancer (LNCC comité de Paris, laboratoire associé), the Institut National du Cancer, cancéropôle d'Ile de France (INCa), the Fondation de France (comité leucémies), the Association Laurette Fugain, Association de la recherche contre le cancer (ARC), and the Agence Nationale de la Recherche (grants ANR-10-JCJC-1108, ANR-12-BSV1-0039, ANR-11-LABX-0051, IM, OH). The Imagine Institute and the Laboratory of Excellence GR-Ex are funded by "Investissements d'avenir" program of the French National Research Agency (ANR-10-IAHU-01 and ANR-11-IDEX-0005-02, respectively). M.-A.H. and S.P. are recipients of an INCa grant. T.T.M. received grant support from INSERM (poste vert). E.P. received grant support from the Fondation pour la Recherche Medicale (FRM). I.M. was a recipient of an INSERM contrat d'interface fellowship. Free sorafenib was provided by Bayer Health Ltd. and the S.K. Yee Medical Foundation. The work on sorafenib-naïve and sorafenib-resistant AML samples was supported by Hong Kong Blood Cancer Foundation. A.Y.L. is the Li Shu Fan Medical Foundation Professor in Haematology and received funding from its endowment. **Author contributions:** A.S.G., T.T.M., and M.-A.H. designed and performed all experiments, analyzed the data, and helped write the manuscript. C.Y. performed Western blotting and FLT3 gene sequencing of primary AML samples from the Hong Kong study. F.M. constructed Pim-1 expression vector and contributed to cytotoxicity assays. E.C.T. performed cell viability analysis. S.P. contributed to molecular biology and signaling experiments. M.L. supervised and/or performed molecular biology experiments. E.P. contributed to mouse experiments and provided helpful discussions. A.J. contributed to apoptosis experiments and provided helpful advises for the design of inducible shRNA experiments. F.Z. and J.D. contributed to mouse experiments. L.P., P.S., N.J., and K.A. contributed to Western blotting and molecular biology experiments. J.C.C.S. was involved in the sorafenib clinical trial. O.K. performed all FLT3 sequencing experiments. P.A., O.H., D.M.W., C.L., and P.M. provided helpful discussions and critical analysis of the data and helped write the manuscript. G.J.V. was in charge of the material transfer agreement for LGB321 and gave helpful advice on the design of LGB321 experiments. A.Y.L. was responsible for the sorafenib clinical trial in Hong Kong, provided care to the patients, and supervised Western blotting and sequencing experiments on samples from this cohort. I.C.M. and D.B. assisted with study design, data analysis, and manuscript writing. J.T. designed the study, supervised the overall project, performed experiments, analyzed the data, and wrote the manuscript. **Competing interests:** G.J.V. is a co-author of this manuscript and an employee of Novartis Institutes for BioMedical Research (Cambridge, MA) involved in the development of the pan-Pim kinase inhibitor LGB321. **Data and materials availability:** All necessary data is included in this paper.

Submitted 23 February 2015
Accepted 30 June 2015
Published 18 September 2015
10.1126/sciadv.1500221

Citation: A. S. Green, T. T. Maciel, M.-A. Hospital, C. Yin, F. Mazed, E. C. Townsend, S. Pilorge, M. Lambert, E. Paubelle, A. Jacquel, F. Zylbersztejn, J. Decroocq, L. Poulain, P. Sujobert, N. Jacque, K. Adam, J. C. C. So, O. Kosmider, P. Auberger, O. Hermine, D. M. Weinstock, C. Lacombe, P. Mayeux, G. J. Vanasse, A. Y. Leung, I. C. Moura, D. Bouscary, J. Tamburini, Pim kinases modulate resistance to FLT3 tyrosine kinase inhibitors in FLT3-ITD acute myeloid leukemia. *Sci. Adv.* **1**, e1500221 (2015).

ScienceAdvances

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Sci. Adv., 1 (8), e1500221. • DOI: 10.1126/sciadv.1500221