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Identification of Driver and Passenger Mutations of FLT3 by High-Throughput DNA Sequence Analysis and Functional Assessment of Candidate Alleles

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SUMMARY

Mutations in the juxtamembrane and kinase domains of FLT3 are common in AML, but it is not known whether alterations outside these regions contribute to leukemogenesis. We used a high-throughput platform to interrogate the entire *FLT3* coding sequence in AML patients without known FLT3 mutations and experimentally tested the consequences of each candidate leukemogenic allele. This approach identified gain-of-function mutations that activated downstream signaling and conferred sensitivity to FLT3 inhibition and alleles that were not associated with kinase activation, including mutations in the catalytic domain. These findings support the concept that acquired mutations in cancer may not contribute to malignant transformation and underscore the importance of functional studies to distinguish "driver" mutations underlying tumorigenesis from biologically neutral "passenger" alterations.

INTRODUCTION

The receptor tyrosine kinase (TK) FLT3 and its cognate ligand are important for the expansion of early hematopoietic progenitor cells and for the generation of mature natural killer cells and dendritic cells. Binding of FLT3 ligand to the extracellular (EC) domain of FLT3 induces receptor dimerization, which promotes phosphorylation of the

SIGNIFICANCE

High-throughput DNA sequencing has provided insights into the mutational profiles of human cancers and represents a promising strategy for the identification of therapeutic targets. However, recognizing the subset of functionally relevant mutations has proven difficult. We used a combined genetic and functional approach to evaluate a series of candidate mutations in the receptor tyrosine kinase FLT3 that were identified in patients with AML. This strategy enabled distinction between activating alleles that conferred sensitivity to a small-molecule inhibitor and bystander mutations that did not result in kinase activation. Remarkably, these latter alterations included mutations in key functional domains of FLT3. These results emphasize that complementary functional studies are critical for validation of suspected oncogenic alleles from large-scale genomic screens.

FLT3 kinase domain, thereby activating the receptor and several downstream signaling pathways, primarily the PI3K/AKT pathway and the RAS/RAF/MEK/ERK cascade (Parcells et al., 2006).

In patients with acute myeloid leukemia (AML), somatic mutations that result in constitutive activation of FLT3 have been identified in two functional domains of the receptor, the juxtamembrane (JM) domain and the kinase domain. The JM domain, which has been shown to be critical for kinase autoinhibition (Griffith et al., 2004), is disrupted by internal tandem duplications (ITDs) in 25%–30% of adult AML patients (Stirewalt and Radich, 2003), whereas JM domain point mutations have been described in approximately 1% of cases (Reindl et al., 2006; Stirewalt et al., 2004). FLT3 ITDs induce constitutive phosphorylation of several signal transduction intermediates, including STAT5, AKT, and ERK1/2 (Parcells et al., 2006) and cause myeloproliferative disease in various murine models (Kelly et al., 2002; Lee et al., 2005). JM domain point mutations also result in constitutive activation of STAT5 and AKT (Reindl et al., 2006), but their in vivo transforming potential has not been studied.

The activation loop (AL) in the carboxy-terminal lobe of the kinase domain is affected by point mutations, insertions, or deletions in approximately 7% of AML cases (Frohling et al., 2005). AL mutations share several signal transduction properties with FLT3 ITDs, for example, constitutive phosphorylation of AKT and ERK1/2 (Choudhary et al., 2005; Spiekermann et al., 2003). However, substantial differences between the two mutation types in the activation of STAT5 have been observed in some studies (Choudhary et al., 2005; Grundler et al., 2005; Rocnik et al., 2006), but not in others (Bagrintseva et al., 2004; Grundler et al., 2003; Spiekermann et al., 2003), and mice transplanted with BM expressing FLT3 AL mutations do not develop myeloid disease but rather, a T cell lymphoproliferative disorder with longer latency (Grundler et al., 2005). A mutation in the amino-terminal lobe of the kinase domain was found to induce constitutive activation of AKT, ERK1/2, and STAT5 (Schittenhelm et al., 2006).

From a clinical perspective, FLT3 mutations are important as a result of their prognostic relevance and because constitutively activated FLT3 is an attractive therapeutic target. FLT3 ITDs are associated with an increased risk of relapse and short survival (Yanada et al., 2005), and patients with low or absent levels of wild-type (WT) FLT3 appear to have a particularly dismal outcome (Thiede et al., 2002; Whitman et al., 2001). In contrast, the prognostic relevance of FLT3 AL mutations remains controversial despite several studies on large patient series (Yanada et al., 2005). Currently, there are four selective FLT3 inhibitors at various stages of clinical development, including PKC412 (midostaurin), CEP-701 (lestaurtinib), MLN518 (tandutinib), and SU11248 (sunitinib) (Knapper, 2007). These compounds are well tolerated at doses that achieve inhibition of FLT3 and have shown moderate activity in relapsed or refractory AML patients with activating FLT3 mutations. In addition, some patients without FLT3 ITDs or known AL mutations have responded to FLT3 inhibitors,

suggesting the possibility of other mutations that result in constitutive activation of FLT3. Ongoing clinical trials are evaluating the use of FLT3 inhibitors in combination with chemotherapy.

Previous studies have focused on the detection of mutations in the JM and kinase domains of FLT3, whereas the frequency and spectrum of sequence alterations outside these regions have not been systematically studied. We therefore performed high-throughput DNA sequencing of all coding exons of FLT3 in AML patients without FLT3 ITDs or known AL mutations. The primary aim of such a genomic screen is to identify "driver" mutations that are causally implicated in cancer development. However, recent large-scale sequencing studies in solid tumors that used statistical approaches to evaluate the impact of DNA sequence variants have demonstrated the difficulties in distinguishing driver mutations from "passenger" alterations that confer no clonal growth advantage (Forrest and Cavet, 2007; Getz et al., 2007; Greenman et al., 2007; Rubin and Green, 2007; Sjoblom et al., 2006; Wood et al., 2007). To address this problem, we experimentally tested the consequences of each candidate oncogenic allele identified in our mutation screen. This combined genetic and functional approach successfully identified gain-of-function mutations in the EC domain, the JM domain, and the AL of FLT3 and several passenger alterations that did not result in constitutive kinase activity. Furthermore, we investigated the structural implications, signal transduction properties, and sensitivity to FLT3 inhibition of the activating alleles.

RESULTS

High-Throughput DNA Sequence Analysis of *FLT3*
We performed bidirectional sequencing of all *FLT3* coding exons in pretreatment samples from 222 adult AML patients without known activating mutations of FLT3, KIT, and NRAS. Sequence data were evaluated for quality and coverage within the region of interest of each exon that included all coding bases and the five flanking intronic bases at the 5′ and 3′ ends. High-quality, bidirectional sequence reads were obtained for more than 90% of all samples analyzed (see Table S1 in the Supplemental Data available with this article online).

FLT3 Sequence Variants in Patients with AML
We identified a total of 17 heterozygous nucleotide changes. Five of these changes corresponded to germline variants listed in single-nucleotide polymorphism (SNP) databases. Of the remaining 12 sequence alterations, 9 were predicted to change the amino acid sequence of FLT3 (nonsynonymous substitutions; Table 1 and Figure S1), whereas 3 were annotated as synonymous substitutions, resulting in a ratio of nonsynonymous to synonymous mutations of 3.

The nine nonsynonymous sequence variants that were not known SNPs have not been observed in 48 normal control samples in which the entire coding region of *FLT3* had previously been sequenced (Ley et al., 2003)

Table 1. Nonsynonymous Sequence Variants Identified by High-Throughput Sequencing of *FLT3* in 222 AML Patients

| DNA	Exon	Protein	Domain
581A > G	5	T167A	extracellular
662G > A	5	V194M	extracellular
1052G > A	8	D324N	extracellular
1172T > C	9	Y364H	extracellular
1434C > T	11	S451F	extracellular
1751G > A	13	V557I	transmembrane
1797A > G	14	Y572C	juxtamembrane
1857T > G	14	V592G	juxtamembrane
2293G > C	18	M737I	kinase
2574G > A	20	G831E	activation loop
2583G > A	20	R834Q	activation loop

Sequence numbering is according to Ensembl Transcript/Peptide ID ENST00000241453. D324N (refSNP ID rs35602083) and V557I (refSNP ID rs35958982) are known germline polymorphisms. Different mutations involving amino acids V592 and R834 (V592A, R834_D835 del, R834_D835insALG) are reported in the COSMIC database (version 30; http://www.sanger.ac.uk/genetics/CGP/cosmic/).

and were not among the germline variants identified in a mutation screen of all protein kinase genes in 210 human cancers (Greenman et al., 2007). These alterations were,

therefore, considered to be candidate leukemogenic mutations that warrant experimental validation. Paired diagnostic and remission material was available from a patient with an M737I substitution in the FLT3 kinase domain. This variant was present at diagnosis but not in the remission sample, demonstrating that it was somatically acquired (Figure S1). The three synonymous sequence variants that were not known SNPs were considered to be nonfunctional and were, therefore, not further investigated.

FLT3 S451F, Y572C, V592G, and R834Q Are Gain-of-Function Mutations that Induce Constitutive Kinase Activation

To determine the functional consequences of all nine candidate mutations, we tested their ability to transform murine hematopoietic BaF3 cells to cytokine-independent growth, a property conferred by a broad spectrum of oncogenic TK alleles. Cells expressing S451F in the EC domain, Y572C or V592G in the JM domain, and R834Q in the AL were able to grow in the absence of interleukin-3 (IL-3; Figure 1A). Immunoprecipitation of FLT3 followed by western blotting with a phosphotyrosine-specific antibody demonstrated that these mutant kinases induced constitutive phosphorylation of FLT3 (Figure 1B). These results indicate that the S451F, Y572C, V592G, and R834Q alleles are gain-of-function mutations that result in constitutive kinase activation. To perform a rapid and inexpensive secondary screen for these mutations, we designed allele-specific primer extension assays and performed genotype determination in an independent cohort
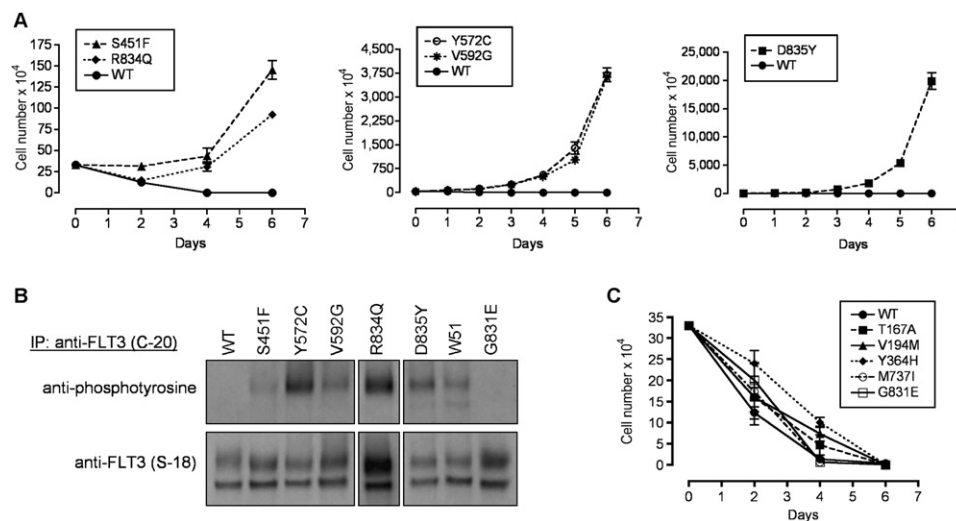
Figure 1. Constitutive Kinase Activation by FLT3 S451F, Y572C, V592G, and R834Q

(A) Expression of FLT3 S451F, Y572C, V592G, and R834Q resulted in IL-3-independent growth of BaF3 cells. Cells expressing the known AL mutation D835Y were used as positive control. Cells expressing WT FLT3 were used as negative control. Experiments were performed in triplicate. Values are represented as mean ± SEM.

(B) Expression of FLT3 S451F, Y572C, V592G, and R834Q in BaF3 cells resulted in constitutive FLT3 autophosphorylation. Cells were maintained in IL-3-free medium for 6 hr, FLT3 was immunoprecipitated from whole-cell lysates, and immunoprecipitates were analyzed by western blotting with an anti-phosphotyrosine antibody. Cells expressing a FLT3 ITD mutation (W51) or FLT3 D835Y were used as positive controls. Cells expressing WT FLT3 or the nontransforming G831E allele were used as negative controls.

(C) Expression of FLT3 T167A, V194M, Y364H, M737I, and G831E did not result in IL-3-independent growth of BaF3 cells. Experiments were performed in triplicate. Values are represented as mean ± SEM.

of 127 adult AML cases. This analysis identified one additional case positive for the S451F substitution and one additional case with the R834Q allele. Thus, the overall prevalence of activating FLT3 mutations in this study was 1.7% (6 of 349 cases).

Cells expressing any of the remaining five nonsynonymous sequence variants in the EC domain, the kinase domain, and the AL underwent apoptosis when cultured in the absence of exogenous cytokines, and constitutive phosphorylation of FLT3 was not observed (Figures 1B and 1C). These results indicate that a substantial proportion of the nonsynonymous sequence variants detected in FLT3 are likely to be passenger mutations. To increase confidence that the four presumed passenger mutations for which no matched normal DNA sample was available (T167A, V194M, Y364H, and G831E) did not represent germline polymorphisms or sequence artifacts, the following steps were taken. First, we sequenced the corresponding exons of *FLT3* in 102 normal DNA samples and detected no abnormalities. Second, we determined by mass spectrometry genotyping that the G831E allele in the FLT3 AL was not present in the panel of 270 normal individuals collected by the International HapMap Consortium (2003). Third, we introduced PCR-amplified genomic DNA from each case into a cloning vector and sequenced between 50 and 55 individual transformants. In two cases (T167A and G831E), the observed mutant-to-wild-type allelic ratio was substantially different from 1 (0.19 and 3.2, respectively), suggesting that these alleles did not represent heterozygous SNPs. In the remaining two cases (V194M and Y364H), the number of recombinant clones carrying the mutant allele was similar to that of clones harboring the WT sequence (24 versus 27 and 31 versus 21, respectively), a finding compatible with either a somatic mutation that was present in most of the cells from which the original DNA sample was derived or with a heterozygous germline variant.

For comparison with our functional analyses, we also predicted the effects of all nine candidate mutations in silico using the SIFT (http://blocks.fhcrc.org/sift/SIFT.html) and PMut (http://mmb2.pcb.ub.es:8080/PMut) software programs (Table S2). Three sequence variants (S451F, G831E, and R834Q) were predicted to affect the function of FLT3, whereas four changes (T167A, V194M, Y364H, and M737I) were predicted to be tolerated. The remaining two mutations (Y572C and V592G) had conflicting results between the SIFT and PMut algorithms. These findings illustrate that bioinformatics approaches and functional assays provide complementary information as to the potential impact of mutations identified in large-scale genomic screens.

Ortholog alignments for all nonsynonymous sequence variants identified in this study, as well as relevant sequence alignments of all five class III receptor TKs (PDGFRA, PDGFRB, KIT, CSF1R, and FLT3) and other TKs with a known role in cancer (EGFR, ERBB2, RET, MET, and ABL1), are shown in Figure S2. Clinical characteristics of the patients with activating FLT3 mutations are given in Tables S3 and S4.
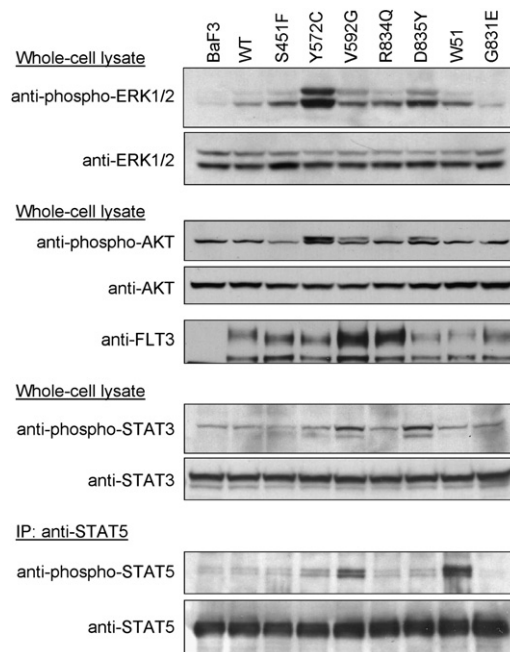

Figure 2. Differential Activation of Signal Transduction Pathways by FLT3 S451F, Y572C, V592G, and R834Q

Expression of FLT3 S451F, Y572C, V592G, and R834Q in BaF3 cells resulted in constitutive phosphorylation of varying signaling proteins. Cells were maintained in IL-3-free medium for 6 hr, and whole-cell lysates or immunoprecipitates from whole-cell lysates were analyzed by western blotting as indicated. The signaling characteristics of the four mutations were compared with those of FLT3 D835Y and the FLT3 ITD mutation W51. Cells expressing WT FLT3 or the nontransforming G831E allele were used as negative controls. Expression of FLT3 in the different stable cell lines was confirmed by reprobing the blot demonstrating constitutive phosphorylation of AKT with an anti-FLT3 antibody.

FLT3 S451F, Y572C, V592G, and R834Q Differentially Activate Downstream Signaling Pathways

To examine the signal transduction properties of the four activating mutations, BaF3 cells expressing FLT3 S451F, Y572C, V592G, or R834Q were deprived of IL-3 and then analyzed by western blotting for phosphorylation of signaling proteins (Figure 2 and Table 2).

Expression of the JM domain mutations Y572C and V592G resulted in constitutive phosphorylation of ERK1/2, AKT, STAT3, and STAT5. We recently identified Y589 and Y591 as sites in the JM domain that are necessary for aberrant activation of STAT5 in cells expressing a FLT3 ITD mutation (Rocnik et al., 2006). To investigate whether these residues are also required for signal transduction mediated by JM domain point mutations, we generated Y589/591F double substitutions in the cDNAs encoding FLT3 Y572C and V592G. Coexpression of Y589/591F delayed the induction of IL-3-independent growth in BaF3 cells (Figure S3A), decreased the proliferation rate of BaF3 cells in the absence of IL-3 (Figure S3B), and altered the signaling properties of the two alleles (Figure S3C). The

Table 2. Constitutive Phosphorylation of Signaling Molecules in BaF3 Cells Expressing Activating FLT3 Mutations

Signaling molecule	FLT3 Mutation					
	S451F	Y572C	V592G	R834Q	D835Y	W51
FLT3	+	+	+	+	+	+
STAT5	−	+	+	−	−	+
ERK1/2	+	+	+	+	+	+
AKT	−	+	+	−	+	−
STAT3	−	+	+	−	+	−

S451F, Y572C, V592G, and R834Q were identified in this study; D835Y and W51 are known activating FLT3 mutations that have been described previously. +, constitutive phosphorylation; −, no constitutive phosphorylation.

Y589/591F mutations strongly reduced constitutive phosphorylation of ERK1/2, STAT3, and STAT5 and attenuated aberrant activation of AKT. These results, along with our analysis of the FLT3 structure (see below), support the hypothesis that point mutations in the JM domain, like FLT3 ITD mutations, can alter the conformation of the JM domain, potentially resulting in exposure of occult docking sites such as Y589 and Y591, in turn enabling recruitment and activation of specific downstream signaling proteins.

The signaling properties of the R834Q substitution were compared to those of the most frequent AL mutation, D835Y. Consistent with previous reports (Choudhary et al., 2005; Grundler et al., 2005; Rocnik et al., 2006), BaF3 cells expressing D835Y showed constitutive activation of ERK1/2 and AKT, but not STAT5. Furthermore, we found that expression of D835Y was associated with constitutive phosphorylation of STAT3. The R834Q mutation, on the other hand, resulted in phosphorylation of ERK1/2 but not AKT, STAT3, or STAT5. This signaling pattern was associated with increased sensitivity to growth inhibition

by the MEK inhibitor PD98059 as compared to cells harboring the D835Y mutation (Figures 3A and 3B), indicating a higher relative contribution of ERK signaling to the transforming activity of the R834Q allele. Similar to R834Q-positive cells, cells expressing the S451F mutation also showed constitutive phosphorylation of ERK1/2, but no induction of AKT, STAT3, or STAT5. These results suggest that activation of the MAPK pathway in the absence of PI3K/AKT, STAT3, or STAT5 signaling is sufficient for transformation of hematopoietic cells by specific FLT3 alleles and that cells carrying different FLT3 mutations are differentially dependent on signaling mechanisms that involve the MEK/ERK cascade.

FLT3 S451F, Y572C, V592G, and R834Q Confer Varying Sensitivity to the TK Inhibitor PKC412

To determine the sensitivity of the four activating mutations to FLT3 inhibition, BaF3 cells expressing FLT3 S451F, Y572C, V592G, or R834Q were treated with increasing concentrations of the small-molecule TK inhibitor PKC412. FLT3 Y572C, V592G, and R834Q conferred high sensitivity to PKC412 (inhibitory concentration of 50% [IC_{50}], 2.5 nM, 5 nM, and 2.3 nM, respectively), whereas a considerably higher IC_{50} (48 nM) was observed for FLT3 S451F (Figure 4A). Western blot analysis showed that treatment with PKC412 led to dose-dependent decreases in FLT3 autophosphorylation and in phosphorylation of ERK1/2 that correlated with inhibition of cell growth (Figure 4B). WT FLT3-expressing BaF3 cells grown in the presence of IL-3 were used as a control for toxicity unrelated to FLT3 inhibition, and no antiproliferative effect was observed for concentrations of PKC412 up to 50 nM (Figure 4A). These data show that the four activating FLT3 mutations identified in our mutation screen can be inhibited by PKC412 and that Y572C, V592G, and R834Q are considerably more sensitive to this compound than S451F.
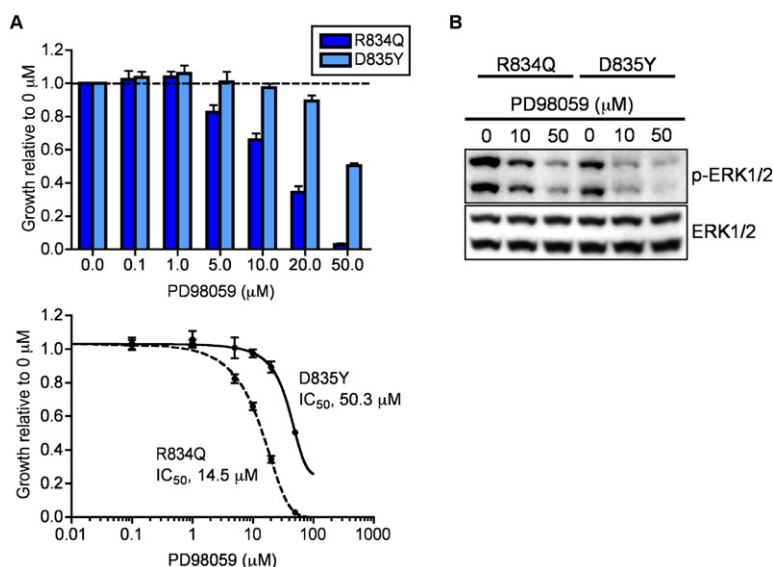

Figure 3. Varying Sensitivity of Different FLT3 AL Mutations to MEK Inhibition

(A) Expression of FLT3 R834Q was associated with a higher sensitivity to MEK inhibition as compared to FLT3 D835Y. BaF3 cells stably expressing FLT3 R834Q or D835Y and growing in the absence of IL-3 were treated with PD98059 as indicated. Cell viability was measured after 48 hr, and the proportion of viable cells relative to the control (no inhibitor) was plotted. IC_{50} values are indicated. Experiments were performed in triplicate. Values are represented as mean ± SEM.

(B) PD98059 treatment of BaF3 cells expressing FLT3 R834Q or D835Y resulted in dose-dependent inhibition of ERK1/2 phosphorylation. Cells were incubated with varying drug concentrations for 15 min, and whole-cell lysates were analyzed by western blotting as indicated.
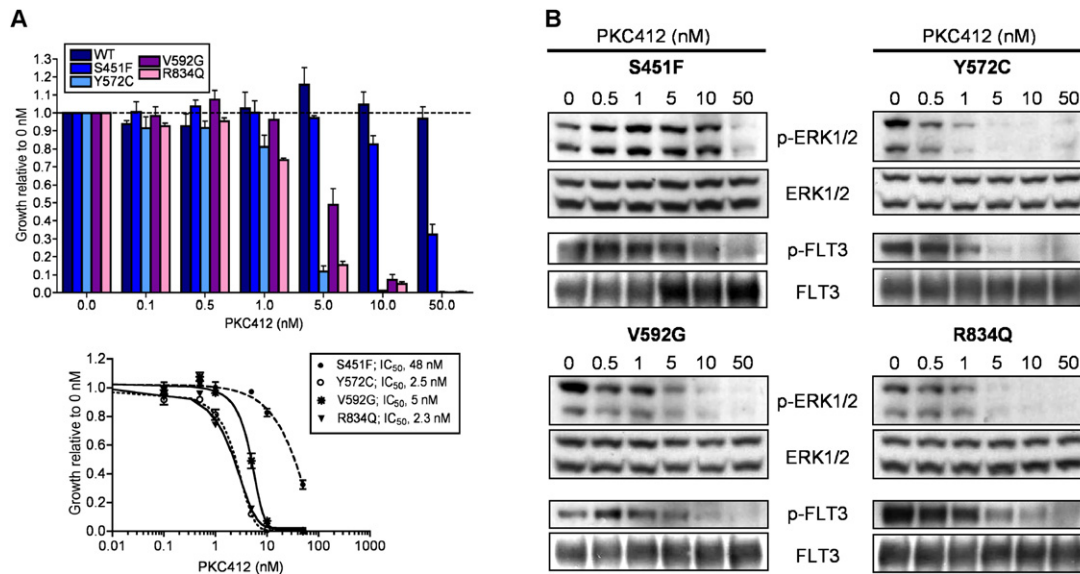
Figure 4. Varying Sensitivity of FLT3 S451F, Y572C, V592G, and R834Q to Kinase Inhibition by PKC412

(A) Expression of FLT3 Y572C, V592G, or R834Q was associated with higher sensitivity to FLT3 inhibition as compared to FLT3 S451F. BaF3 cells growing in the absence of IL-3 were treated with PKC412 as indicated. Cell viability was measured after 48 hr, and the proportion of viable cells relative to the control (no inhibitor) was plotted. IC_{50} values are indicated. Experiments were performed in triplicate. Values are represented as mean ± SEM. (B) PKC412 treatment of BaF3 cells expressing FLT3 S451F, Y572C, V592G, and R834Q resulted in dose-dependent decreases in FLT3 and ERK1/2 phosphorylation. Cells were incubated with varying drug concentrations for 2 hr, and whole-cell lysates were analyzed by western blotting as indicated.

Mutation of FLT3 V592 Promotes Aberrant Signal Transduction and Proliferation of Human AML Cells

To investigate the role of mutant FLT3 in human AML cells, we analyzed the MONO-MAC-6 cell line that has been reported to contain a V592A substitution in the FLT3 JM domain (Spiekermann et al., 2003). DNA sequence analysis confirmed the presence of a homozygous V592A mutation in this cell line (data not shown). Lentiviral transduction of MONO-MAC-6 cells with two short hairpin RNA (shRNA) constructs targeting different regions of the *FLT3* transcript resulted in stable downregulation of *FLT3* mRNA (Figure 5B) and reduced levels of FLT3 protein (Figure 5C). Knockdown of FLT3 was associated with a substantial reduction in cell viability as compared to cells transduced with a nontargeting control shRNA (Figure 5A). Western blot analysis showed that suppression of FLT3 resulted in loss of STAT5 phosphorylation (Figure 5C), indicating that STAT5 is a downstream target of mutations involving FLT3 V592, consistent with our signaling studies in BaF3 cells. These results suggest that endogenous point mutations of FLT3 V592 promote the growth and survival of human AML cells and that this effect is mediated through aberrant STAT5 activation.

Structural Analysis of FLT3 Mutations

Based on the crystal structure of the intracellular domains of FLT3 (Protein Data Bank accession code 1RJB; Figure 6A), we assessed the potential mechanism by which point mutations in the JM domain and in the AL result in constitutive FLT3 kinase activity. Residues Y572 and R834 seem to be involved in maintaining the energetic favorability of the autoinhibited conformation of FLT3. Loss of the stabilizing effects of these residues should favor the active conformation. Y572, which has thus far not been found mutated in AML, is described as the first amino-terminal residue visible in the electron density of the crystal structure (Griffith et al., 2004). It is a key "hook" for the JM domain and is critical to maintain the autoinhibited conformation of FLT3 (Figure 6B). The Y572C substitution is expected to result in a substantial loss of hydrogen bonding networks and extensive hydrophobic interactions, with a consequent reduction in the energetic favorability of this conformation. Likewise, loss of hydrogen bonding networks that would result from the R834Q and D835Y mutations is expected to destabilize the autoinhibited kinase conformation (Figures 6C and 6D).

Conversely, structural analysis suggests that the nontransforming G831E substitution may not deleteriously affect the stability of the autoinhibited conformation. The molecular surface of autoinhibited FLT3 shows that there is a cavity directly adjacent to G831. This cavity is filled with water molecules and falls between the autoinhibited-conformation AL and JM domain (Figure 6E). Even though mutation of this glycine residue to glutamic acid is a substantial change, the glutamic acid residue should be able to be accommodated in the autoinhibited conformation by displacement of a number of water molecules. G831 lies within the invariant protein kinase DFG motif required for correct $Mg^{2+} \bullet ATP$ coordination (Karlsson et al., 1993) and is important for conformational movements of the DFG motif between active and inactive states
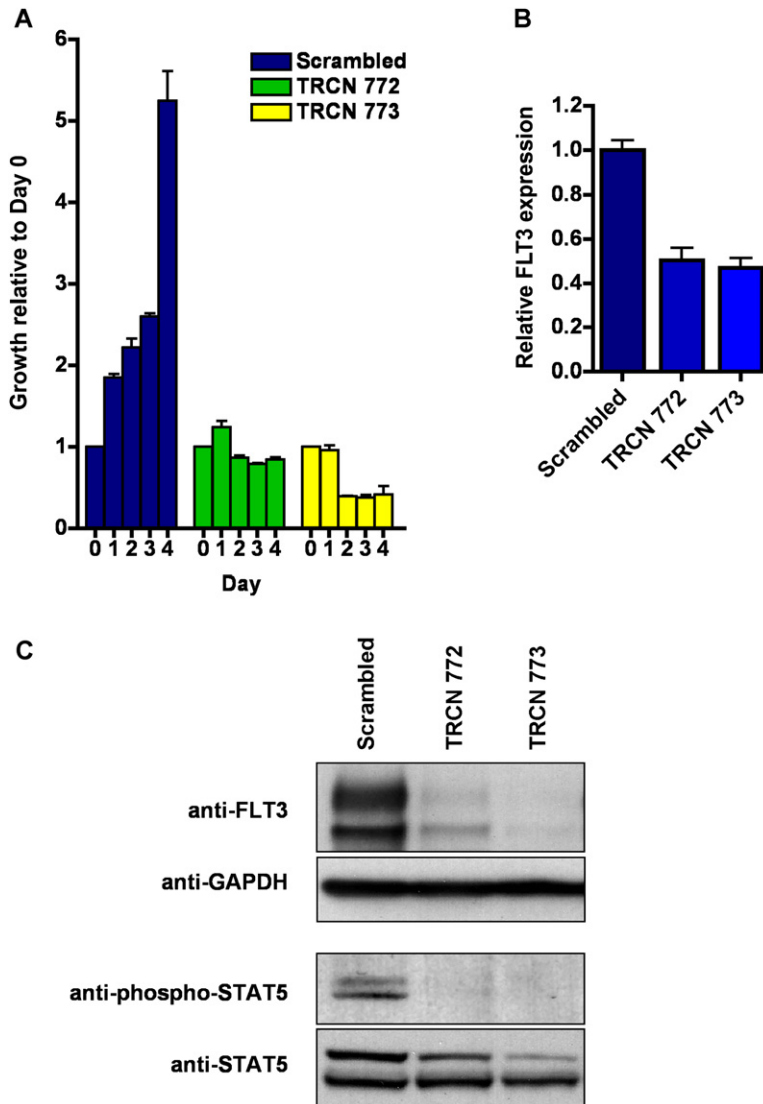
Figure 5. Effects of FLT3 Knockdown in Human AML Cells

(A) Downregulation of FLT3 using shRNA constructs TRCN 772 and TRCN 773 inhibited the growth of a human AML cell line, MONO-MAC-6, that harbors a homozyogus V592A mutation in the FLT3 JM domain. Experiments were performed in triplicate. Values are represented as mean ± SEM.

(B) Transduction of MONO-MAC-6 cells with shRNA constructs TRCN 772 and TRCN 773 resulted in decreased *FLT3* mRNA levels as compared to a nontargeting control construct. Experiments were performed in duplicate. Values are represented as mean ± SEM.

(C) Suppression of *FLT3* mRNA was associated with a reduction in FLT3 protein (upper panels) and with loss of STAT5 phosphorylation (lower panels).

([Levinson et al., 2006](#)). Consequently, the G831E mutation may stabilize FLT3 in the autoinhibited conformation and result in reduced kinase activity, consistent with our data showing a lack of FLT3 autophosphorylation in cells expressing FLT3 G831E.

DISCUSSION

We have used high-throughput DNA sequence analysis to determine the frequency and spectrum of mutations in the *FLT3* gene in adult AML patients without known FLT3 ITDs or AL mutations. Sequencing of all *FLT3* coding exons identified nine candidate leukemogenic alleles in six exons corresponding to different domains of the FLT3 receptor.

Large-scale mutational profiling studies in cancer typically result in extensive lists of validated nonsynonymous sequence variants. A major challenge of these studies is to distinguish driver mutations that are responsible for malignant transformation from nonfunctional passenger alterations that arise in the malignant clone by chance and

accumulate during repeated rounds of cell division. To identify genetic changes with a role in tumorigenesis, statistical models have been developed that incorporate mutational frequency and distribution, and it has been inferred from such approaches that nonsynonymous passenger mutations are present at a higher frequency than previously anticipated ([Greenman et al., 2007; Sjoblom et al., 2006; Wood et al., 2007](#)). We provide functional documentation of this concept by using a different strategy that combined both high-throughput DNA sequence analysis of *FLT3* in primary AML samples with experimental validation of all potential driver mutations identified. Cytokine independence assays and biochemical analysis revealed that of the 9 candidate leukemogenic alleles, 4 are gain-of-function mutations that result in constitutive kinase activation and stimulation of downstream signaling pathways, properties that are relevant to leukemogenesis and can be exploited therapeutically, whereas the remaining 5 alleles were not associated with increased kinase activity and aberrant signal transduction.
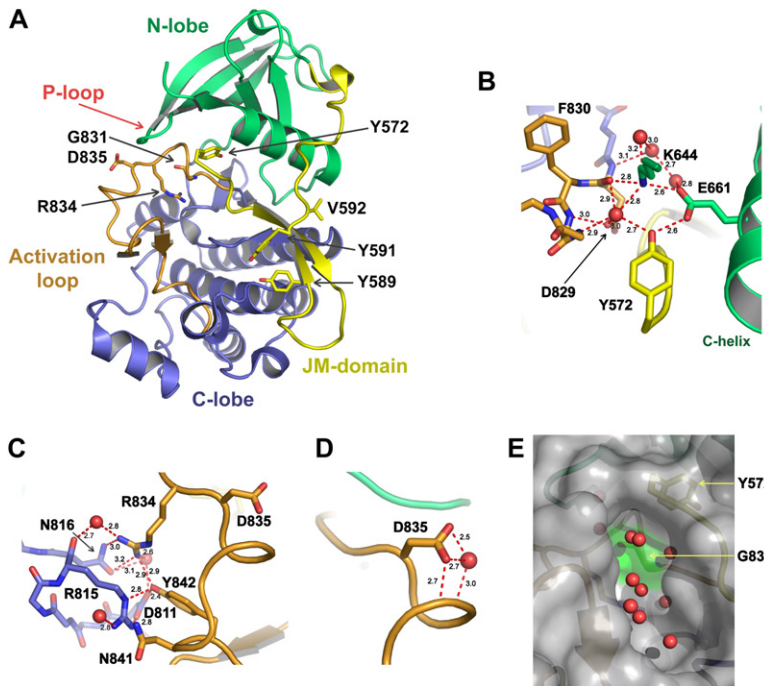
Figure 6. Structural Analysis of FLT3 Mutations

(A) Ribbon representation of the crystal structure of the FLT3 kinase domain (Protein Data Bank, accession code 1RJB). The structure is a typical kinase fold crystallized in the autoinhibited conformation with the JM domain packing closely to the kinase domain and locking the protein in this conformation.

(B) Hydrogen bonding network formed by FLT3 Y572. Y572 inserts into the catalytic cleft of FLT3, where the phenolic hydroxyl oxygen forms hydrogen bonds to the catalytic glutamic acid, E661, and an ordered water molecule. The aromatic ring also makes extensive hydrophobic contacts. The Y572C mutation will disrupt this "hook" that locks the JM domain in the autoinhibited conformation.

(C) Hydrogen bonding network formed by FLT3 R834. R834 is critical to the formation of a hydrogen bonding network that incorporates interactions with the side chains of residues N816, R815, Y842, D811, the backbone carbonyl oxygen of R815, and two water molecules. This network is directly adjacent to a hydrogen bonding network in which N841 plays a critical role.

(D) Hydrogen bonding network formed by FLT3 D835. D835 is an integral part of a hydrogen bonding network that seems to stabilize the AL backbone in the autoinhibited conformation. There is a hydrogen bond directly between the carboxyl group and the backbone amide of residue S836. There is also an indirect hydrogen bond between the caryboxyl group and the backbone amide of M837 mediated by a water molecule.

(E) Surface representation of the region proximal to G831. The molecular surface of FLT3 protein atoms is shown as a transparent gray surface. The surface is colored green where it contacts G831, and water molecules are shown as red spheres. The G831E mutation may not disrupt this conformation due to the proximity of a water-filled cavity adjacent to G831. In this figure, amino acid residues are shown in stick representation with nitrogen atoms in blue, oxygen in red, and carbon atoms the same color as the corresponding domain. Water molecules are shown as red spheres and hydrogen bonds as dashed red lines with their lengths labeled in Å. This figure was made using the program PyMOL (www.pymol.org).

These results also indicate that functional analyses can provide information beyond that derived from statistical methods for identifying driver mutations. First, we show that rare sequence variants occurring at frequencies that would not allow them to be distinguished from unselected passenger changes can be drivers. Second, we report that alleles in the kinase domain and in the AL—highly conserved and functionally relevant domains that, based on mathematical approaches, are likely to harbor driver mutations—may not be associated with a detectable gain of function. Third, statistical techniques result in identification of candidate cancer genes, but do not predict the ability of individual alleles to contribute to transformation. Our analysis of a series of nonsynonymous sequence variants in *FLT3*, a validated cancer gene (Futreal et al., 2004), indicates that functional studies are needed to complement the bioinformatic approaches that have been described to date.

In addition to mutational frequency and distribution, statistical methods for assessing the functional relevance of DNA sequence variants in cancer rely on discerning acquired mutations from inherited polymorphisms. While the analysis of germline DNA is essential to assess the origin of cancer-associated genomic alterations and to determine whether a mutation has been selected for during

tumorigenesis, previous findings in solid tumors indicate that the presence of a kinase mutation in constitutional DNA does not exclude the possibility that it has a potential role in malignant transformation (Bell et al., 2005; Jeffers et al., 1997; Mulloy et al., 2007; Plaza-Menacho et al., 2006). These data further support the conclusion that the interpretation of high-throughput sequencing studies can be improved through functional assessment of candidate oncogenic alleles.

The four activating mutations that were identified in our screen are located in the EC domain (S451F), the JM domain (Y572C and V592G), and the AL (R834Q) of FLT3. Sequence variants in the EC region (D324N and N520Y) have been described previously in patients with AML (Ley et al., 2003; Syampurnawati et al., 2007). However, D324N is also present in normal individuals and in nonhematopoietic tissues from AML patients and does not confer cytokine-independent growth to BaF3 cells (Schnittger et al., 2006), whereas the functional consequences of N520Y have not been assessed. Our results thus document that FLT3, like EGFR and KIT (Gari et al., 1999; Lee et al., 2006), can be activated by mutations in the EC domain. To determine the mechanism by which the S451F mutation results in constitutive FLT3 kinase activity, structural analysis of

a larger FLT3 molecule that includes the EC domain will be required.

Point mutations in the region between amino acids 579 and 594 in the FLT3 JM domain have been detected in a small proportion of AML cases (Reindl et al., 2006; Stirewalt et al., 2004). We have found a valine to glycine substitution involving residue 592 and a mutation involving Y572. Y572 is the first amino acid of the JM domain, and analysis of the crystal structure of the FLT3 cytoplasmic domain has suggested a key role for this residue in kinase autoinhibition (Griffith et al., 2004). Our structural analysis suggests that mutation of Y572 would result in disruption of its extensive interactions with the surrounding FLT3 subdomains and consequent destabilization of the inactive kinase conformation. In support of this hypothesis, the Y572C substitution that was identified in our mutation screen results in constitutive FLT3 kinase activity. These data provide genetic evidence for the importance of Y572 in maintaining the autoinhibited conformation of FLT3 and suggest that any mutation that interferes with the stability of the JM domain in this conformation is likely to be transforming.

The signaling properties of the two JM domain point mutations are similar to those of FLT3 ITDs (Parcells et al., 2006). In particular, aberrant activation of STAT5 by JM domain point mutations appears to involve two tyrosine residues, Y589 and Y591, that are essential for FLT3 ITD-mediated induction of STAT5 and leukemic transformation in vivo (Rocnik et al., 2006). Analysis of the role of Y589 and Y591 also provided insights into the deregulation of other signaling pathways by mutant FLT3. For example, the majority of primary AML samples are characterized by activated MAPK and PI3K/AKT signaling; however, the fact that dysregulation of various upstream effectors can result in aberrant phosphorylation of ERK1/2 and AKT indicates that the MAPK and PI3K/AKT cascades may be activated through a variety of different mechanisms (Martelli et al., 2006; Platanias, 2003). In support of the hypothesis that mutant TKs activate downstream signaling pathways by distinct mechanisms, phosphorylation of ERK1/2 and AKT by FLT3 JM domain point mutations also requires Y589 and Y591, and similar observations were made for constitutive activation of STAT3. These results suggest that the different types of JM domain mutations (ITDs and point mutations) contribute in similar ways to myeloid leukemogenesis and demonstrate that Y589 and Y591 are critical for the induction of several signaling pathways that are activated in leukemias with FLT3 JM domain mutations. Furthermore, these findings illustrate the potential of point mutations to alter the substrate specificity of protein kinases. Whether FLT3 Y589/591-mediated signal transduction in AML involves direct engagement of downstream effectors by these residues or requires additional adaptor molecules is currently unknown.

Previously described mutations in the FLT3 AL include substitutions, insertions, or deletions within a region between amino acids 834 and 842, with D835 being the most commonly affected residue (Frohling et al., 2005). We identified nonsynonymous substitutions at codons 831 and 834. Analysis of the crystal structure of the FLT3 kinase domain suggests that mutation of residue R834 would interfere with the stability of the autoinhibited conformation, and in agreement with this hypothesis, FLT3 R834Q has constitutive kinase activity and confers IL-3-independent growth to BaF3 cells. In contrast, mutation of the conserved DFG motif G831 to glutamic acid may deleteriously affect the structural requirements for enzymatic activity, is expected to be compatible with the autoinhibited conformation, and does not result in transformation of hematopoietic cells. These results illustrate the value of structural analysis for understanding the atomic-level mechanisms by which TK mutations associated with human leukemias function. In addition, these data indicate that the autoinhibited conformation of FLT3 is critically dependent on the stabilizing contacts of a limited number of specific amino acids, whereas mutation of other residues within the AL does not alter the structure and function of the FLT3 kinase.

Analysis of the signaling properties of FLT3 R834Q demonstrated that this mutation, unlike the more common D835Y allele, results in activation of ERK1/2, but not AKT, STAT3, or STAT5. Consistent with this observation, the R834Q mutation is associated with enhanced sensitivity to MEK inhibition, indicating that cells expressing R834Q are more reliant on MAPK signaling than are D835Y-expressing cells that constitutively phosphorylate multiple signaling proteins. These results suggest that there are not only differences in signal transduction between various FLT3 mutation classes, for example, ITDs and AL mutations (Choudhary et al., 2005; Grundler et al., 2005), but also between distinct alleles within a given mutation class and that cells carrying different FLT3 mutations are differentially dependent on certain signaling pathways.

To determine the functional consequences of candidate mutations, we used an in vitro assay that is based on exogenous expression of mutated alleles in murine hematopoietic cells. This assay has proved to be of value for the assessment of increased TK activity and perturbed signal transduction and has been validated for numerous TK alleles identified in human hematologic malignancies, including FLT3 ITDs and AL mutations (Clark et al., 2004; Weisberg et al., 2002), as well as for TK alleles associated with solid tumors, such as mutations in the EGFR kinase domain and the recently discovered EML4-ALK fusion in patients with non-small-cell lung cancer (Jiang et al., 2005; Soda et al., 2007). On the other hand, conversion of BaF3 cells to cytokine independence may not faithfully assess the functional implications of certain mutations, either because they require expression of additional proteins (Lu et al., 2005) or because cooperativity between two or more mutations is required for signaling pathway activation. Lastly, this assay may fail to detect a gain of function conferred by kinase mutations that do not result in increased enzymatic activity. For instance, Wan et al. (2004) described three mutants of the serine-threonine kinase BRAF with impaired catalytic activity that are capable of stimulating downstream signaling through transactivation of CRAF. Although we observed no

activation of ERK1/2, AKT, STAT3, or STAT5 in cells expressing FLT3 mutations that read out as nonfunctional in the BaF3 assay, it is possible that some of these alleles stimulate other effector molecules that were not analyzed in our study. We recognize that it is impossible to definitively exclude any conceivable function of a given mutation. Nonetheless, the nontransforming mutations identified in our screen do not meet conventional criteria for effects on kinase activation.

The activating mutations identified in our study conferred varying degrees of sensitivity to the small-molecule TK inhibitor PKC412. Cells expressing Y572C, V592G, or R834Q were highly sensitive to this compound, as has been reported for other JM domain (Reindl et al., 2006) and AL mutations (Choudhary et al., 2005; Grundler et al., 2003; Weisberg et al., 2002). In contrast, cytotoxic responses in S451F-expressing cells required substantially higher drug concentrations. The determinants of response to pharmacologic inhibition of FLT3 are diverse and remain only partly understood. In general, the presence of mutant FLT3 is associated with increased drug sensitivity (DeAngelo et al., 2006; Fiedler et al., 2005), yet there appears to be substantial variation among the different mutation types (Choudhary et al., 2005; Grundler et al., 2003; Weisberg et al., 2002) as well as between individual alleles within the same mutation class (Clark et al., 2004; Grundler et al., 2003). In addition, it has been observed that patients without FLT3 ITDs or any of the known AL mutations may also derive benefit from FLT3 inhibitor therapy, which might be related to the presence of previously unrecognized mutations (Schittenhelm et al., 2006), and in vitro studies in primary AML samples have shown that there is substantial interpatient variation in the degree of dependency on FLT3 signaling (Knapper et al., 2006). Our findings further illustrate the complex relationship between FLT3 mutation status and response to pharmacologic FLT3 inhibition.

In summary, our results demonstrate that previously unrecognized activating FLT3 mutations in the EC domain, the JM domain, and the AL occur in patients with AML, albeit at a low frequency. Since mutations lying outside the usually studied mutational hotspots may confer sensitivity to small-molecule TK inhibitors, eligibility for clinical trials of FLT3-targeted therapies should not be based on diagnostic screens that are limited to the detection of only a fraction of the FLT3 mutations associated with AML. The development of sensitive, allele-specific assays, such as mass spectrometry-based genotyping (Thomas et al., 2007), will allow all patients enrolled on trials of FLT3 inhibitors to be genotyped for all known FLT3 alleles, and it is hoped that similar strategies will be used in trials of molecularly targeted therapies for patients with different hematopoietic malignancies and solid tumors.

FLT3 is a highly annotated and well-studied cancer gene known to play a key role in myeloid leukemogenesis. The finding that only a fraction of the nonsynonymous FLT3 mutations associated with AML result in constitutive kinase activity illustrates the challenge in distinguishing between sequence variants that contribute to malignant transformation and nonfunctional passenger mutations. Given that the increasing throughput of DNA sequencing technologies will allow improved characterization of somatic cancer genomes, these data demonstrate that a combination of genetic and functional approaches will be crucial in order to identify the mutations that truly drive the development of cancer and to validate the potential of suspected oncogenic alleles as therapeutic targets.

EXPERIMENTAL PROCEDURES

Patient Samples
This study included 349 adult patients with AML, de novo or secondary after treatment for a primary malignancy or following myelodysplasia, as defined by French-American-British Cooperative Group criteria or the World Health Organization classification (Bennett et al., 1985; Harris et al., 1999). Diagnostic BM and peripheral blood samples were collected after obtaining informed consent according to the Declaration of Helsinki and with institutional review board approval of the relevant institutions. All samples were enriched for mononuclear cells by density centrifugation before banking. Only samples with more than 50% blasts were included for DNA sequence analysis, and more than 80% of the samples had at least 80% blasts. All specimens that were analyzed by DNA sequencing were negative for FLT3 ITDs, as assessed by PCR amplification of exons 14 and 15; for FLT3 AL mutations involving codons 835 and 836, as assessed by PCR amplification of exon 20 followed by EcoRV digestion; for activating KIT mutations, as assessed by sequencing of exons 8 and 17; and for mutations involving NRAS codons 12, 13, and 61, as assessed by sequencing of exons 1 and 2.

FLT3 Mutational Analysis and Selection of Candidate Mutations
Extraction of genomic DNA, amplification of FLT3 exons, bidirectional sequencing, and sequence detection were performed using a high-throughput resequencing approach as previously described (Levine et al., 2005). External gene-specific primers and internal M13-appended primers are listed in Table S5. Analysis of sequence traces was performed using Mutation Surveyor version 2.28 (SoftGenetics, State College, PA). Five steps were used to identify mutations of interest. First, any synonymous substitutions were not analyzed further. Second, known SNPs were excluded by comparison to the dbSNP database (release 127; http://www.ncbi.nlm.nih.gov/projects/SNP). Third, published data sets (Greenman et al., 2007; Ley et al., 2003) were queried to exclude any nonsynonymous substitutions in the FLT3 coding sequence that have been detected in DNA samples derived from normal tissues. Fourth, sequence chromatograms were visually inspected to remove false positive calls in the automated analysis. Fifth, candidate mutations were reamplified and sequenced from the original DNA sample for independent verification. Leukemic cells at diagnosis and a matched remission sample were analyzed in one case with a presumptive leukemogenic mutation in the FLT3 kinase domain, M737I.

Genotyping
Mass spectrometry genotyping was performed as previously described (Levine et al., 2005). Primers and primer extension probes for detection of the different FLT3 alleles are listed in Table S6.

DNA Constructs and Retrovirus Production
Sequence variants were introduced into the full-length FLT3 cDNA using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All mutations were confirmed by sequencing of the entire FLT3 open reading frame. The mutant cDNAs were cloned into the MSCV-PGK-neo retroviral vector, and full-length protein expression was documented by western blotting. Generation of retroviral

supernatants and infection of BaF3 cells were performed as described previously (Rocnik et al., 2006). BaF3 cells transduced with MSCV-PGK-neo constructs were selected with G418 in the presence of IL-3 for 14 days.

Cell Culture

BaF3 cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum and 10% WEHI-conditioned medium as a source of IL-3. For cytokine independence assays, cells transduced with each of the MSCV-PGK-neo retroviral contructs were seeded at a density of 0.33×10^6/ml in IL-3-free medium, and the number of viable cells was determined daily by trypan blue exclusion. For growth inhibition assays, cells were seeded at a density of 1×10^5/ml in IL-3-free medium with various concentrations of PKC412 (Novartis, Basel, Switzerland) or PD98059 (Calbiochem, San Diego, CA), and the number of viable cells was determined after 48 hr using the CellTiter 96AQ$_{ueous}$ One Solution Proliferation Assay (Promega, Madison, WI).

Immunoprecipitation and Western Blotting

Immunoprecipitation and western blotting were performed as described previously (Rocnik et al., 2006) using the following antibodies: anti-FLT3, anti-STAT5, anti-phospho-ERK1/2 (T202/Y204) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-STAT3, anti-phospho-STAT3 (Y705), anti-phospho-STAT5 (Y694), anti-ERK1/2, anti-AKT, anti-phospho-AKT (S473) (Cell Signaling Technology, Beverly, MA); and anti-phosphotyrosine (4G10) (Upstate, Lake Placid, NY).

Knockdown of FLT3 Expression

Two pLKO.1-based lentiviral vectors encoding shRNAs targeted to the 3′ untranslated region (TRCN 772) or the coding sequence (TRCN 773) of the human *FLT3* mRNA, and a pLKO.1 construct containing a non-targeting shRNA sequence were obtained from the MISSION TRC-Hs 1.0 (Human) shRNA library (Root et al., 2006). The oligonucleotide sequences of the shRNAs were as follows (21-nucleotide stem sequences matching the target transcript underlined; noncomplementary 6-nucleotide loop sequences italicized): TRCN 772, 5′-CCG G<u>CG TCT GCG TTT ACT CTT GTT T</u>*CT CGA G*<u>AA ACA AGA GTA AAC GCA GAC G</u>TT TTT-3′; TRCN 773, 5′-CCG G<u>GC TAA CTT CTA CAA ACT GAT T</u>*CT CGA G*<u>AA TCA GTT TGT AGA AGT TAG C</u>TT TTT-3′. Generation of lentiviral supernatants and infection of human AML cells were performed as described previously (Scholl et al., 2007).

Analysis of the FLT3 Structure

The crystal structure of the kinase domain of FLT3 was downloaded from the Protein Data Bank (http://www.pdb.org; accession code 1RJB) and analyzed in O (Jones et al., 1991).

Supplemental Data

The Supplemental Data include three supplemental figures and six supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/6/501/DC1/.

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