RNAi screening of the tyrosine kinome identifies therapeutic targets in acute myeloid leukemia

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Despite vast improvements in our understanding of cancer genetics, a large percentage of cancer cases present without knowledge of the causative genetic events. Tyrosine kinases are frequently implicated in the pathogenesis of numerous types of cancer, but identification and validation of tyrosine kinase targets in cancer can be a time-consuming process. We report the establishment of an efficient, functional screening assay using RNAi technology to directly assess and compare the effect of individually targeting each member of the tyrosine kinase family. We demonstrate that siRNA screening can identify tyrosine kinase targets containing activating mutations in Janus kinase (JAK) 3 (A572V) in CMK cells and c-KIT (V560G) in HMC1.1 cells. In addition, this assay identifies targets that do not contain mutations, such as JAK1 and the focal adhesion kinases (FAK), that are crucial to the survival of the cancer cells. This technique, with additional development, might eventually offer the potential to match specific therapies with individual patients based on a functional assay. (Blood. 2008;111: 2238-2245) © 2008 by The American Society of Hematology

Introduction

The recent success of monoclonal antibodies and small-molecule inhibitors of tyrosine kinases in numerous malignancies have highlighted the potential of targeted therapy for the treatment of cancer.1-4 However, broad application of this strategy will require a more detailed understanding of the principal genetic targets involved in cancer pathogenesis in each individual patient. Tyrosine kinases constitute a gene family of 91 members that have an integral role in signal transduction of mammalian cells, including critical cellular processes as diverse as proliferation, apoptosis, differentiation, and cell motility. Aberrant regulation of any of these processes might contribute to oncogenesis, thus it is not surprising that dysregulation of tyrosine kinase activity has been observed in numerous types of malignancy.5

Acute myeloid leukemia (AML) represents one malignancy in which tyrosine kinases are abnormally regulated. Previous studies have shown that phosphorylation of signal transducer and activator of transcription 5 (STAT5) is present in blast cells from at least 70% of patients with AML.6-8 Because STAT5 phosphorylation is tightly controlled by tyrosine kinase signaling networks, this suggests the presence of constitutively active, mutated tyrosine kinases in these patients. To date, the only known activating mutations in tyrosine kinases in AML are point mutations in c-KIT (5%), mutations or internal tandem duplications in FLT3 (30%), and rare mutations observed in JAK2, JAK3, and PDGFR (Figure 1).9-12 These known abnormalities in tyrosine kinases give mechanistic insight into the genetics underlying approximately half of the cases of AML with phosho-STAT5. Of the remaining cases with unknown genetic etiology, the presence of phosphorylated STAT5 suggests that the tyrosine kinase family is one likely source of unknown oncogenic mutations (Figure 1).
construct functional profiles of AML cell lines, revealing targets critical for the proliferation and viability of these cells. These profiles revealed expected targets based on previously known activating mutations harbored by the CMK and HMC1.1 cell lines. In addition, they revealed unexpected targets that were informative about previously undefined signaling mechanisms of the primary targets. Taken together, this technique has the possibility of offering an efficient and cost-effective alternative to large-scale sequencing projects, and with further development it might provide a useful clinical service for molecular genetic diagnosis of the unique targeted therapy profile of malignancies.

Methods

Cell culture

K562 cells were obtained from American Type Culture Collection (Manassas, VA). CMK and HEL cells were obtained from the German Resource Centre for Biological Material (DSMZ; Braunschweig, Germany). HMC1.1 cells were provided by Dr Michael Heinrich. All cells were maintained in RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), L-glutamine, and penicillin/streptomycin (Invitrogen, Carlsbad, CA). For isolation of RNA, cells were serum starved overnight in RPMI supplemented with 0.1% bovine serum albumin, L-glutamine, and penicillin/streptomycin (Invitrogen; Promega, Madison, WI). For HMC1.1 cell stimulation, cells were serum starved overnight in RPMI supplemented with 0.1% bovine serum albumin, L-glutamine, and penicillin/streptomycin. The following day, cells were stimulated for 5 minutes with 100 ng/mL bovine serum albumin, L-glutamine, and penicillin/streptomycin (Invitrogen, Carlsbad, CA). For proliferation assays, cells were incubated for 72 hours in the presence of JAK Inhibitor I, JAK3 inhibitor II, PP2, or Src Kinase Inhibitor I (EMD Biosciences, San Diego, CA). For evaluation of gene knockdown, the above procedure was repeated at the optimal voltage using siRNA targeting GAPDH (Dharmacon, Lafayette, CO) at 500 or 1000 nM. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene silencing was determined at 48 hours after electroporation by immunoblot analysis for GAPDH and β-actin as a loading control.

siRNA knockdown using tyrosine kinase library

CMK or HMC1.1 cells (10^7) were washed one time in OptiMEM (Invitrogen) and resuspended in 4.2 mL of siPORT buffer (Ambion). Cells were aliquoted at 42 μL per well onto a 96-well electroporator (Ambion) and 2 μL of siRNA at 20 μM was added to each well. The tyrosine kinase library used in this study contains 4 siRNA targeting constructs per well (purchased from Dharmacon), and we manually added single and pooled nonspecific siRNA as well as siRNA pools (4 constructs per target) against ephrin type-A receptor (EPHA)5, EPHA6, src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation (SRMS), apoptosis-associated tyrosine kinase (AATK), lemur tyrosine kinase (LMTK)3, N-RAS, K-RAS (all from Dharmacon). These were added separately because they are not included in the standard tyrosine kinase library. Cells were electroporated at 2220 V (equivalent to a 96-well electroporator (Ambion) and 100 μsec, 2 pulses and 15 000 cells per well were replated using a Hydra 96-channel automated pipetter (Matrix Technologies, Hudson, NH) into triplicate plates containing 100 μL per well of standard culture media. For determination of cell viability and proliferation, cells were subjected to the CellTiter 96 AQueous One solution cell-proliferation assay (MTS; Promega). All values were normalized to the mean of the 2 nonspecific siRNA control wells.

Confirmation of RNAi silencing

For confirmation of efficient knockdown in HMC1.1 cells, siRNA targeting JAK1, JAK2, JAK3, EPHA4, PTK2, PTK2B, PTK6, PTK9, LTK, LYN, SRC, and c-KIT, was transfected at 300 V, 100 μsec, 2 pulses and whole cell lysates (see “Immunoblotting”) or total cellular RNA (Qiagen, Valencia, CA) were harvested after 48 hours using standard procedures. Total RNA was used to synthesize cDNA (Invitrogen SuperScript III) and quantitative polymerase chain reaction (PCR) against each respective gene (primers listed in Document S1, available on the Blood website; see the Supplemental Materials link at the top of the online article) as well as GAPDH was performed on each sample using SYBR Green qPCR.
SuperMix (Invitrogen) and a DNA Engine Opticon 2 system for real-time PCR (Bio-Rad). Quantitative PCR cycle values were converted to arbitrary qPCR units based on a standard curve for each gene or GAPDH, each value was normalized to its respective GAPDH value, and percent knockdown was calculated based on the following formula: (nonspecific – gene-specific)/nonspecific × 100. Whole cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using antibodies against JAK1 (BD Biosciences), JAK2, EPHA4, LYN, SRC (Millipore, Billerica, MA), JAK3, PTK6, LTK (Santa Cruz Biotechnology, Santa Cruz, CA), PTK2, PTK2B (Cell Signaling Technology, Danvers, MA), and β-actin (Millipore). Densitometry was performed and the value for each band was normalized to its respective β-actin loading control and the above formula was used to calculate percent knockdown.

### Immunoblotting

For direct immunoblots, cells were lysed in sample buffer (75 mM Tris pH 6.8, 3% SDS, 15% glycerol, 8% β-mercaptoethanol, 0.1% bromophenol blue). For immunoprecipitation, cells were lysed in 1 × cell lysis buffer (Cell Signaling Technology) supplemented with tyrosine phosphatase inhibitor cocktail, Aprotinin, and 4-(2-aminomethyl)benzene-sulfonyl fluoride hydrochloride (AEBSF; Sigma-Aldrich, St Louis, MO) and incubated overnight with antibodies specific for c-KIT (EMD Biosciences), JAK1 (BD Biosciences), or JAK3 (Santa Cruz Biotechnology). Immune complexes were precipitated with protein A-agarose beads (Amersham Biosciences, Piscataway, NJ), washed 3 times in lysis buffer, resuspended in sample buffer, and immunoprecipitations as well as whole cell lysates were separated by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) and subjected to immunoblot analysis with the above antibodies specific for c-KIT, JAK1, JAK3, as well as phospho-JAK1 (Cell Signaling Technology), GAPDH (Santa Cruz), or β-actin (EMD Biosciences).

### Sequencing analysis

JAK1 and JAK3 pseudokinase and activation loop domains were sequenced according to previously described protocols.17,41

### Statistical analyses

For tyrosine kinase siRNA library knockdown experiments, a Student t test was carried out for each well in comparison to both single and pooled nonspecific siRNA controls. The mean of the 2-tailed P value was determined for consideration of significance and data points with P value less than .05 and mean value less than 70% of nonspecific controls were considered significant. For cell proliferation assays, a Student t test was carried out for each dose point comparing HMC1.1 to K562 cell viability.

### Results

#### Optimization of electroporation conditions for siRNA transfection

We assessed the optimal conditions for delivery of siRNA molecules into CMK cells. As suspension cells are often recalcitrant to transfection by liposomal delivery methods, we focused instead on electroporation-based delivery. Initially, we incubated FITC-labeled siRNA molecules with CMK cells and delivered an increasing range of voltage to the cells in single pulses and at constant pulse duration. Two days after electroporation, cells were analyzed for viability by propidium iodide exclusion and incorporation, and we determined that optimal conditions for siRNA delivery into CMK cells was 300 V, 100 μsec, 2 pulses. For final confirmation of efficient siRNA delivery and target reduction, we used the above electroporation conditions to deliver 300 or 1000 nM GAPDH-targeting siRNA into CMK cells. We performed immunoblot analysis at 48 hours after electroporation for GAPDH and β-actin and demonstrated 93% knockdown of GAPDH in CMK cells using 1000 nM siRNA at these parameters (Figure 2C).

#### Functional profiling of CMK cells using a tyrosine kinase siRNA library

We previously determined that CMK cells harbor an activating mutation in JAK3 (A572V).17 To determine whether functional profiling with a tyrosine kinase siRNA library could be an effective tool for target identification in malignant cells, we tested CMK cells using this tyrosine kinase siRNA library with the expectation that knockdown of JAK3, as well as any other critical components in the JAK3 signaling cascade, would reduce the viability and proliferation of CMK cells. Using the above transfection conditions, we introduced siRNA individually targeting each member of the tyrosine kinase family as well as siRNA targeting the N-RAS and K-RAS oncogenes, and 2 nonspecific siRNA controls into CMK cells. Each siRNA well in this library is composed of a pool of 4 individual siRNA molecules that are each designed against a different region of the target transcript. We assessed cell viability and proliferation 4 days after electroporation (Figure 1). This time point was chosen as a result of numerous pilot studies over a time course from day 2 through day 6 showing peak functional effects at day 4 and a plateau thereafter (data not shown). As expected, siRNA directed against JAK3 significantly reduced the viability of CMK cells (Figure 3). This was consistent with our earlier findings that CMK cells depend on JAK3A572V function and activity for viability and proliferation. We also observed, however, that siRNA targeting JAK1 yielded an equally significant reduction in the  

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**Figure 2. Optimization of electroporation in CMK cells.** (A) CMK cells were incubated with a FITC-labeled siRNA molecule and left untreated or electroporated at 300 V, 100 μsec, 2 pulses. After 48 hours cells were analyzed for FITC incorporation by flow cytometry. (B) CMK cells were treated as in panel A and stained with propidium iodide. Viability as measured by PI exclusion was determined by flow cytometry on a Guava Technologies flow cytometer. (C) CMK cells were incubated with 0, 500, or 1000 nM siRNA targeting GAPDH and electroporated as in panel A. After 48 hours cell lysates were subjected to immunoblot analysis for GAPDH and β-actin.

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viability and proliferation of CMK cells (Figure 3). This was an intriguing result, given that JAK1 had not been detected by phospho-proteomic profiling and did not contain any activating mutations as assessed by sequencing (data not shown). The sequence of the predicted tryptic phosphopeptide containing the JAK1 activation loop phosphorylation site is EY*YTVK. This 6 amino acid peptide is almost certainly too hydrophilic to bind to the C18 resin we had previously used for peptide purification and analysis. Thus, we would not expect to have detected this phosphopeptide in our previous mass spectrophotometric analysis of the CMK cell lysates. However, our current siRNA profiling data suggested that the oncogenic signaling from JAK3A572V in CMK cells depends on JAK1, a finding that is consistent with the known association of JAK1 and JAK3 downstream of numerous cytokine receptors.42 While JAK3 and JAK1-specific siRNA reduced the growth and viability of CMK cells far more significantly than any other siRNA molecules, a few other targets did modulate CMK cell growth to a more subtle extent (a complete list of viability values and significance calculations can be found in Table S1).

Association of JAK1 and JAK3 in CMK cells

Because siRNA profiling indicated a reliance on JAK1 for the proliferation of CMK cells, we hypothesized that JAK1 is a signaling partner of JAK3 in these cells. To assess whether JAK1 was critical for JAK3 signaling in CMK cells we adopted 3 strategies. First, we looked for association of these 2 proteins by immunoprecipitation of JAK1 or JAK3, followed by immunoblotting for both JAK1 and JAK3. In both circumstances, we observed coimmunoprecipitation of JAK1 and JAK3, indicating that there is interaction between JAK1 and JAK3 in CMK cells (Figure 4A). Second, we used siRNA to individually knockdown each member of the JAK family and then performed immunoblot for JAK1 and JAK3 as well as their phosphorylated, active versions. Using siRNA targeting JAK1, we observed a reduction in total and phospho-JAK1 with no effect on the phosphorylation status of JAK3. Using siRNA targeting JAK3, however, we observed a reduction in total and phospho-JAK3 as well as a reduction in phospho-JAK1, indicating that JAK3 is a critical upstream activator of JAK1 (Figure 4B). Knockdown of JAK2 or tyrosine kinase (TYK)2 showed no effect on JAK1 or JAK3 phosphorylation (Figure 4B). Finally, we tested whether JAK1 was critical for downstream JAK3 signaling events. We previously published that JAK3, but not JAK2 or TYK2, can induce phosphorylation and activation of STAT5 in CMK cells.17 To test whether JAK1 is necessary for this signaling event, we targeted JAK1 using siRNA in CMK cells and assessed phosphorylation of STAT5. Knockdown of JAK1 resulted in a decrease in levels of phosphorylated STAT5, indicating that JAK1 is a necessary component of activated JAK3 signaling in CMK cells (Figure 4C).

Functional profiling of HMC1.1 cells using a tyrosine kinase siRNA library

We next tested our siRNA functional profiling approach using a cell line with a known mutation in a receptor tyrosine kinase to determine whether the breadth of targets and extent of functional knockdown is different from cells with an activating mutation in a cytosolic protein such as JAK3. For these experiments, we chose HMC1.1 cells, as they harbor an activating mutation in the stem

Figure 3. siRNA functional profiling in CMK cells. CMK cells (10^5) were suspended in siPORT buffer and incubated with 1 μM siRNA from an siRNA library individually targeting each member of the tyrosine kinase family as well as N-RAS, K-RAS, and single and pooled nonspecific siRNA controls. Cells were electroporated on a 96-well electroporation plate at 2220 V (equivalent of 300 V). 100 μsec, 2 pulses. Cells were replated into culture media and cell viability and proliferation was determined by an MTS assay at day 4 after electroporation. Values represent percent mean (normalized to nonspecific control wells) plus or minus SEM (n = 3).

Figure 4. JAK3 signals through JAK1 in CMK cells. (A) CMK cell lysates were immunoprecipitated with antibodies specific for JAK1 and JAK3 and subjected to immunoblot analysis for JAK1 and JAK3. (B) CMK cells were incubated with siRNA targeting JAK1, JAK2, JAK3, and TYK2 and electroporated as in (1A). After 48 hours, cell lysates were analyzed by immunoblot using antibodies specific for total and phospho-JAK1 and JAK3. (C) CMK cells were incubated with siRNA targeting JAK1 and treated as in Figure 1A. After 48 hours, cell lysates were subjected to immunoblot analysis for JAK1 and total and phospho-STAT5.
cell factor (SCF) receptor, c-KIT (V560G). Immunoblot analysis confirmed that c-KIT expression could be effectively reduced in HMC1.1 cells using our electroporation parameters (Figure S1). As expected, we observed significant reduction in viability and proliferation of HMC1.1 cells after siRNA targeting of c-KIT, although the extent of the reduction in viability was not as great as that observed with JAK1 or JAK3 in CMK cells (Figure 5). In addition to reduction of HMC1.1 viability after c-KIT knockdown, we also observed equivalent reductions in the viability of cells after targeting of 10 other genes: EPHA4, JAK1, JAK3, leukocyte tyrosine kinase (LTK), LYN, protein tyrosine kinase (PTK)2 (FAK), PTK2B (FAK2), PTK6 (BRK), PTK9, and SRC (a complete list of viability values and significance calculations can be found in Table S2). To confirm expression and efficient target reduction with our siRNA library, we performed quantitative PCR and immunoblot analysis after transfection of each of these 10 siRNAs into HMC1.1 cells. Significant target reduction at both the mRNA and protein levels was seen with each of these siRNAs (Figure S1). With the exception of EPHA4 and LTK, these proteins are all cytosolic tyrosine kinases that are widely implicated in various signaling pathways. In particular, SRC and its family member LYN have been extensively documented as downstream signaling partners of c-KIT. The rest of these secondary targets, however, have not been previously documented as critical components of c-KIT signaling.

Confirmation of secondary targets using small-molecule kinase inhibitors

Small-molecule kinase inhibitors exist for several of the secondary targets observed in HMC1.1 cells, notably SRC, LYN, JAK1, and JAK3. To determine whether these targets are important for HMC1.1 growth and viability, as implied by the siRNA library data, we performed proliferation assays with HMC1.1 cells treated with a gradient of concentrations of PP2, SRC kinase inhibitor I, JAK inhibitor I, AG-490, and JAK3 inhibitor III. PP2 and SRC kinase inhibitor I are both small-molecules that exhibit broad activity against the SRC family of tyrosine kinases, with little specificity to any individual SRC family kinase. JAK inhibitor I potently inhibits all members of the JAK family, while JAK3 inhibitor III specifically inhibits JAK3. AG-490 has specificity for JAK2 at concentrations below 50 μM, however, it has been shown to inhibit JAK3 at 50 μM and above. As a control cell line with a different primary oncogenic lesion, we used K562 cells that are transformed by the BCR-ABL fusion oncogene. As has been previously reported, K562 cells show some sensitivity to SRC inhibition by PP2 and other SRC family inhibitors. Our findings were consistent with these previous observations showing an IC50 of 5 μM for K562 cell proliferation after 3 days in culture. HMC1.1 cells treated under the same conditions exhibited even greater sensitivity to PP2, with an IC50 of 1.5 μM (Figure 6A). Parallel studies with SRC inhibitor I showed equivalent findings with HMC1.1 cells exhibiting greater sensitivity to the inhibitor than K562 cells with an IC50 of 5 μM (Figure S2A). To assess whether JAK1 and JAK3 were viable targets in HMC1.1 cells, we tested the effect of JAK inhibitor I on these cells as well as K562 cells. As we had seen previously, K562 cells are resistant to JAK inhibitor I at concentrations up to and including 10 μM; however, HMC1.1 cells exhibited sensitivity to this pan-JAK inhibitor, reaching an IC50 at 5 μM (Figure 6B). Because this inhibitor also has activity against JAK2 and TYK2, we additionally tested the JAK3-specific, JAK3 inhibitor III, on HMC1.1 cells and saw an IC50 of 8.5 μM (predicted IC50 of 11 μM) while K562 cells did not reach an IC50 by 20 μM (Figure S2B). As an additional control, we treated HMC1.1 and HEL cells with the JAK2 inhibitor, AG-490. HEL cells depend on a mutated allele of JAK2 (V617F) for viability and, thus, exhibited sensitivity to AG-490 with an IC50 of 45 μM (predicted IC50 of 11 μM) while K562 cells did not reach an IC50 by 20 μM (Figure S2B).

Interestingly, the reduction of viability and proliferation of HMC1.1 cells induced by JAK inhibitor I did not exceed much beyond 50% despite increasing concentrations of the drug. This suggests that alternate signaling pathways might be able to rescue these cells in the absence of functional JAK1 and JAK3 signaling. Because these data were consistent with our siRNA library findings, where no single well exhibited less than 50% reduction in viability, we tested whether combining a SRC inhibitor with this JAK kinase inhibitor could achieve an additive effect on reduction of HMC1.1 cell growth and viability. As such, we incubated
molecule inhibitors targeting these 2 proteins significantly reduced the viability of HMC1.1 cells. Sequence analysis of JAK1 and JAK3 did not reveal genetic abnormalities (data not shown), implicating JAK1 and JAK3 rather than JAK2 in c-KIT signal transduction in this setting. To test this hypothesis, we immunoprecipitated c-KIT, JAK1, and JAK3 from HMC1.1 cells after stimulation with SCF. Immunoblotting for c-KIT, JAK1, and JAK3 indicated that all 3 of these proteins were interacting in HMC1.1 cells both with and without stimulation with SCF, a finding that is consistent with the constitutive kinase activity of the V560G mutation in c-KIT (Figure 7). After stimulation with SCF, we did see additional evidence of JAK1 signaling downstream of c-KIT as we observed increases in the level of phospho-JAK1 in these SCF-stimulated HMC1.1 cells. We did observe coimmunoprecipitation of c-KIT and JAK2 as well as increased phospho-JAK2 after SCF stimulation in HMC1.1 cells; however, our siRNA library findings indicate that this signaling event is not crucial for cell viability. These data indicate that in this setting of a mast cell leukemia line, signaling of c-KIT through JAK1 and JAK3 is crucial for cell growth and viability identifying JAK1 and JAK3 as viable therapeutic targets. In contrast, JAK2 does become activated by SCF-c-KIT signaling in HMC1.1 cells, but it is not necessary for maintenance of cell viability.

Discussion

We demonstrate that high-throughput functional screening with an siRNA library targeting all tyrosine kinases is an efficient and useful approach toward identifying therapeutic targets in malignant cells. With this approach, we could rapidly identify the primary, mutated targets, JAK3 and c-KIT, in 2 AML cell lines. In addition to these 2 genes we were also able to identify numerous secondary targets, some of which were not observed by phospho-proteomic profiling, and all of which would have remained undisclosed by conventional sequencing approaches. These secondary targets serve 2 useful purposes. First, they offer insight into signaling pathways originating from the mutated oncoprotein, and second, they offer alternative targets for therapeutic intervention in patients.

One cell line that was functionally profiled had an activating mutation in a cytosolic tyrosine kinase and the other had an activating mutation in a receptor tyrosine kinase. The viability profile of these 2 cell lines is suggestive of different levels of redundancy emanating from mutations in these 2 different classes of tyrosine kinases. CMK cells, with an activating mutation in the cytosolic protein, JAK3, exhibit sensitivity to siRNA targeting JAK3 or its critical signaling partner, JAK1. While there are a few other genes whose targeting yields subtle effects on cell growth, there are no other genes whose contribution to growth and viability of CMK cells seems nearly as crucial as these 2 genes. Alternatively, HMC1.1 cells, with an activating mutation in the receptor

HMC1.1 cells over the same drug concentrations of JAK inhibitor I, but in combination with an IC50 (1.5 μM) of PP2. The combination yielded lower viability readings at all dose points compared with the JAK inhibitor alone (Figure 6B). These data indicate that HMC1.1 cells depend on JAK and SRC family members for growth and viability and that these 2 signaling pathways might be partially redundant. To further clarify which specific JAK and SRC family members were functioning in this redundant fashion, we transfected HMC1.1 cells with all possible combinations of siRNA targeting JAK1, JAK3, LYN, and SRC. Unexpectedly, we were not able to observe any further decrease in cell viability with any of these combinations than with individual knockdown of these genes (data not shown). This indicates that the additive effect observed with JAK and SRC family inhibition might rely either on off-target effects of these inhibitors or on the functions of other gene family members in addition to JAK1, JAK3, LYN, and SRC.

Association of c-KIT with JAK1 and JAK3 in HMC1.1 cells

Numerous studies have implicated JAK2 in signaling downstream of c-KIT; however, HMC1.1 cell viability and proliferation was normal after treatment with siRNA targeting JAK2 (Table S2). Instead, siRNA targeting JAK1 and JAK3 as well as small
tyrosine kinase, c-KIT, exhibit 11 genes that all appear equivalent in their contribution to viability of HMC1.1 cells. In addition, the decrease in viability observed with all of these 11 genes in HMC1.1 cells is less dramatic than that observed in CMK cells with siRNA targeting JAK1 or JAK3. This discrepancy could be explained in several ways. From a technical standpoint, it is possible that the extent of target reduction by siRNA is less efficient in HMC1.1 cells than in CMK cells. Indeed, quantitative densitometry of GAPDH immunoblots after siRNA targeting in these 2 cell lines revealed greater than 90% target reduction in CMK cells compared with approximately 70% reduction in HMC1.1 cells (data not shown). A second possibility is that oncogenic signals originating from receptor tyrosine kinases might have more redundancy in downstream signaling cascades making ablation of any one of these targets less detrimental for overall cell growth and viability.

Evidence for this concept comes from the fact that combination of inhibitors targeting the SRC and JAK families showed an additive effect in reduction of growth and viability of HMC1.1 cells (Figure 6B). Finally, the possibility remains that HMC1.1 cells could harbor a second activating mutation in another tyrosine kinase or in an oncogene that lies outside the tyrosine kinase family. While no specific evidence exists for this hypothesis, it might warrant further exploration.

These studies offer the potential of functionally analyzing signaling pathways in a larger scale than has previously been conducted. Previous studies have indicated that c-KIT interacts with JAK2. Indeed, stimulation of numerous cell types with SCF results in phosphorylation and kinase activation of JAK2, including in HMC1.1 cells (Figure S3). Our data, however, indicate JAK2 is dispensable for c-KIT signaling in HMC1.1 cells as cell viability was unaffected despite significant knockdown by JAK2 siRNA (Figures 4, S1). This conclusion is bolstered by the relative resistance of HMC1.1 cells to inhibition of JAK2 by AG-490 in comparison to the JAK2-dependent cell line, HEL (Figure S2C). In contrast, siRNA functional profiling clearly indicates that JAK1 and JAK3, previously not known to be associated with c-KIT signaling, are crucial signaling mediators of this pathway in HMC1.1 cells. Using this technology, it might be possible to explore numerous signaling pathways from a new perspective.

Here we have applied siRNA functional profiling to cells with constitutively active pathways; however, this analysis could also be performed by treating cells with the growth factor or other ligand of interest after siRNA targeting. Using this approach, we might expect to find data that complement our current biochemical understanding of signaling pathways as we found in CMK cells, or we might uncover new insights into pathways as we found with c-KIT signaling through JAK1 and JAK3 in HMC1.1 cells.

Finally, these data demonstrate the utility of siRNA functional profiling in target identification in malignant cells. Perhaps the most important facet of these findings is the ability of functional profiling to identify a wide range of targets, which is not limited to mutated targets, in malignant cells. Indeed, the ability of c-KIT to signal through JAK1 and JAK3 might prompt an investigation as to the efficacy of new JAK3 inhibitors in c-KIT derived cancer, especially in cases with imatinib-resistant mutations in c-KIT.57 Along similar lines, this assay might also prove useful for the determination of sensitivity profiles of other kinases with drug-resistant mutations, such as BCR-ABL or epidermal growth factor receptor (EGFR). Additionally, siRNA targeting of the focal adhesion kinases, PTK2 and PTK2B, in both CMK and HMC1.1 cells yielded reduction in cell growth and viability. This might reveal a novel target for therapeutic intervention in malignancies that depend on c-KIT, JAK1, or JAK3 signaling. Thus, siRNA functional profiling might provide an efficient diagnostic tool for identification of molecular targets for therapeutic intervention in malignant cells, such that patients might be matched with a cocktail of small-molecule kinase inhibitors that are individually tailored to their cancer.

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Authorship

Contribution: J.W.T. designed and performed research, analyzed data, and wrote the paper; D.K.W., S.W., M.L., J.O., H.E., and A.S.C. performed research; T.O. and M.L. designed research; and M.C.H., M.W.D., and B.J.D. contributed vital reagents, designed research, and revised the paper.

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RNAi screening of the tyrosine kinome identifies therapeutic targets in acute myeloid leukemia

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