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Activating alleles of *JAK3* in acute megakaryoblastic leukemia

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Summary

Tyrosine kinases are aberrantly activated in numerous malignancies, including acute myeloid leukemia (AML). To identify tyrosine kinases activated in AML, we developed a screening strategy that rapidly identifies tyrosine-phosphorylated proteins using mass spectrometry. This allowed the identification of an activating mutation (A572V) in the JAK3 pseudokinase domain in the acute megakaryoblastic leukemia (AMKL) cell line CMK. Subsequent analysis identified two additional *JAK3* alleles, V722I and P132T, in AMKL patients. JAK3^{A572V}, JAK3^{V722I}, and JAK3^{P132T} each transform Ba/F3 cells to factor-independent growth, and JAK3^{A572V} confers features of megakaryoblastic leukemia in a murine model. These findings illustrate the biological importance of gain-of-function *JAK3* mutations in leukemogenesis and demonstrate the utility of proteomic approaches to identifying clinically relevant mutations.

Introduction

Tyrosine kinases comprise a family of 90 enzymes involved in the regulation of various cellular processes, including proliferation, survival, differentiation, and motility (Krause and Van Etten, 2005). The activity of these kinases is normally tightly controlled. However, tyrosine kinases can become aberrantly activated by different mechanisms, including point mutation; fusion with unrelated genes that lead to constitutive dimerization and activation; and in the case of receptor tyrosine kinases, mutation in the juxtamembrane domain that results in constitutive kinase activation (Paul and Mukhopadhyay, 2004). Dysregulated tyrosine kinases have been shown to have a significant role in a variety of cancers, including leukemia. In the case of acute myeloid leukemia (AML), mutations in *FLT3* or *c-KIT* have been implicated as aberrations that confer a proliferative advantage to hematopoietic progenitors (Stirewalt and Radich, 2003; Tse et al., 2000; Yamamoto et al., 2001; Yokota et al., 1997).

Despite the importance of dysregulated tyrosine kinases in cancer, the identification of specific oncogenes within complex activation pathways is difficult in malignant cells. Recently, proteomic approaches focusing on the phosphotyrosine content of the cell have demonstrated that large numbers of tyrosine-phosphorylated proteins can be consistently identified in cancer cell lines. Indeed, Rush et al. (2005) and Walters et al. (2006) demonstrated that this methodology is capable of identifying activated tyrosine kinases even when the signaling pathways are unknown (Rush et al., 2005; Walters et al., 2006).

A particularly interesting disease in which to search for activated tyrosine kinases is AML. STAT5 has been found constitutively tyrosine-phosphorylated in the leukemic cells of approximately 70% of AML patients (Birkenkamp et al., 2001; Hayakawa et al., 1998). The presence of *FLT3* or *KIT* activating

SIGNIFICANCE

We used a mass spectrometry-based screen to identify activating alleles of *JAK3* in acute megakaryoblastic leukemia (AMKL). Activating alleles of *JAK3* had not previously been detected in human cancer. The JAK3^{A572V} mutation occurs at a conserved residue in the pseudokinase domain, providing mechanistic insights into the role of this domain in regulation of the JAK3 catalytic domain. Recent development of JAK3-selective small molecule inhibitors raises the possibility of therapeutic intervention in this subset of patients. This study thus provides insights into the molecular pathogenesis of AMKL, suggests that a more broadly based screen for *JAK3* mutations in cancer is warranted, and validates high-throughput mass spectrometry of phosphopeptides as a strategy for identification of therapeutic targets in cancer.

mutations can account for STAT5 phosphorylation in up to 35% of patients. However, the mechanism of constitutive STAT5 phosphorylation remains unclear in a significant percentage of patients lacking these mutations. As STAT5 tyrosine phosphorylation is a marker for tyrosine kinase activation, we focused our investigations on AML cell lines with constitutive STAT5 phosphorylation for analysis by phosphopeptide mass spectrometry.

Using this approach, we identified several activated tyrosine kinases in an acute megakaryoblastic leukemia (AMKL) cell line that lacked *FLT3* or *KIT* mutations yet possessed constitutive STAT5 phosphorylation. Subsequent siRNA-induced downregulation experiments validated the role of candidate kinases identified by LC-MS/MS in the activation of STAT5. These studies guided DNA sequence analysis of candidate genes and enabled rapid identification of an A572V mutation in the pseudokinase domain of JAK3. Correlation of the structural location of A572 with biochemical data implicates the structural integrity of the pseudokinase catalytic cleft region as potentially important for proper JAK signaling. These findings provided a rationale for DNA analysis of the entire coding sequence of *JAK3* in AMKL patients and resulted in identification of two additional *JAK3* mutations. Each of these mutant *JAK3* alleles resulted in constitutive activation of JAK3, phosphorylation of STAT5, and transformation of the hematopoietic cell line Ba/F3 to factor-independent growth. Furthermore, $JAK3^{A572V}$ recapitulated certain key features of megakaryoblastic leukemia in a murine model of disease.

Results

Constitutive STAT5 activation in CMK cells depends on JAK3

The baseline phosphorylation status of a spectrum of human AML cell lines was assessed, and STAT5 was constitutively phosphorylated in the AMKL cell line CMK (Figure 1A). CMK cells are a megakaryoblastic cell line derived from a patient with Down's syndrome and AMKL (Sato et al., 1989). Activating *FLT3* or *KIT* mutations are commonly responsible for constitutive activation of STAT5 in AML; however, denaturing-HPLC analysis revealed that the CMK cell line does not possess activating mutations in *FLT3* or *KIT*. To identify the upstream activator of STAT5, we used a phosphoproteomic approach in which CMK cell lysates were enzymatically digested with two different proteases to maximize the number of phosphopeptides identified after immunoprecipitation and subsequent analysis by LC-MS/MS mass spectrometry (Rush et al., 2005). Using this approach, we identified a total of 348 nonredundant phosphopeptides corresponding to 274 nonredundant phosphorylation sites in 210 proteins (Table S1 in the Supplemental Data available with this article online). Among these proteins, we detected numerous phosphorylated peptides corresponding to tyrosine kinases (Table 1), including JAK3, which was found to be phosphorylated in two sites, including Y980, within the activation loop—an indication of JAK3 activation in these cells. Other kinases, such as TYK2, JAK2, c-Kit, and c-Abl were also found to be tyrosine phosphorylated in the CMK cell line by mass spectrometry.

Activated JAK proteins are known to directly phosphorylate STAT proteins (Flores-Morales et al., 1998; Fujitani et al., 1997). Therefore, we next determined whether the pan-JAK inhibitor JAK Inhibitor I would affect the growth and viability of the CMK cell line. Treatment of CMK cells with the pan-JAK inhibitor resulted in a significant decrease in cell proliferation and viability (Figure 1B). However, treatment of CMK cells with imatinib, which inhibits both c-ABL and c-KIT, had little effect on CMK cell proliferation (Figure 1C). This indicated that JAK proteins, but not c-KIT or c-ABL, were essential for CMK growth and viability. Treatment of CMK cells with JAK Inhibitor I also impacted tyrosine phosphorylation of STAT5, p42/44 mitogen-activated protein (MAP) kinase, and STAT3 and was associated with an increase in the apoptotic death rate (Figures 1D and 1E). This effect could not be attributed to nonspecific toxicity of the inhibitor, as growth, viability, or STAT5 phosphorylation was not inhibited by the JAK Inhibitor I in K562 cells that express the BCR-ABL fusion protein.

We next assessed whether JAK2, JAK3, or TYK2 contributed an essential role in growth and viability of CMK cells using a siRNA approach. Expression of JAK2, JAK3, or TYK2 was individually downregulated with specific siRNAs. Immunoblot analysis revealed that the expression of these proteins was specifically and significantly reduced by approximately 95% at 48 hr following transfection of the respective JAK family member siRNA into CMK cells (Figure 2A). We observed that downregulation of JAK3, but not JAK2 or TYK2, resulted in inhibition of CMK cell growth (Figure 2B). Moreover, treatment with JAK3 siRNA resulted in inhibition of STAT5 tyrosine phosphorylation and increased apoptosis of CMK cells, while downregulation of JAK2 or TYK2 had no effect (Figures 2A and 2C). In addition, we observed constitutively phosphorylated JAK3 in CMK cells following immunoprecipitation and phosphotyrosine immunoblot (Figure 2D). These findings indicated that activated JAK3 was essential for growth and survival of CMK cells.

JAK3 contains an activating mutation in CMK cells

We next analyzed *JAK3* for activating mutations. Constitutively phosphorylated JAK3 migrated at the expected molecular weight in CMK cells (Figure 2), indicating that point mutation rather than gene rearrangement was a likely mechanism for activation. Subsequent DNA sequence analysis identified a heterozygous C to T mutation at nucleotide position 1774 that is predicted to result in substitution of valine for alanine at amino acid position 572 in the JH2 domain of JAK3 (Figure 3A).

To assess the transforming ability of the $JAK3^{A572V}$ mutation, we transduced Ba/F3 cells with either *MSCV-IRES-EGFP* (*MIG*)-$JAK3^{WT}$ or *MIG*-$JAK3^{A572V}$. Twenty-four hours after transduction, GFP-expressing cells were selected by flow cytometry, plated in liquid culture in the absence of IL-3, and counted daily to assess IL-3-independent growth. As shown in Figure 3C, expression of $JAK3^{A572V}$ conferred IL-3-independent growth to Ba/F3 cells, whereas $JAK3^{WT}$-transduced cells retained dependence on IL-3 for proliferation. In addition, $JAK3^{A572V}$ also conferred IL-3-independent growth to the 32D myeloid cell line (data not shown). Biochemical analysis in Ba/F3 cells revealed that JAK3 as well as downstream targets STAT5, phosphoinositol-3-kinase-Akt, and MAP kinase (p42/44) were each constitutively phosphorylated in cells transduced with $JAK3^{A572V}$, but not in cells transduced with $JAK3^{WT}$ (Figure 3B). As a functional correlate to the observed increase in JAK3 phosphorylation, $JAK3^{A572V}$ exhibited increased kinase activity compared with $JAK3^{WT}$ in an in vitro immunoprecipitation kinase assay (Figure S1). Together, these results indicate that the *JAK3*^{A572V} mutation results in constitutive activation of the JAK3 tyrosine kinase and can transform cytokine-dependent hematopoietic cell lines in vitro.

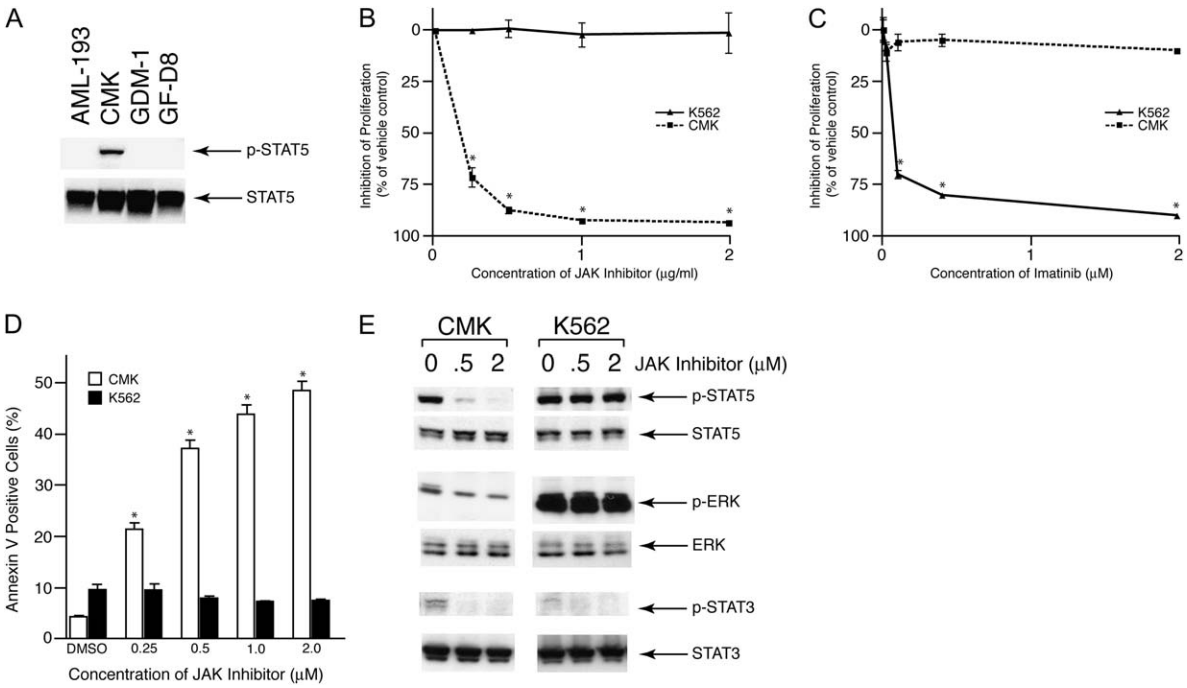

Figure 1. Constitutively activated STAT5 is caused by the JAK family in CMK cells

A: Cell lines were serum starved for 16 hr. Lysates were subjected to immunoblot analysis for STAT5 and phospho-STAT5.

B: CMK and K562 control cells were treated with increasing concentrations of the pan-JAK inhibitor JAK Inhibitor I. Seventy-two hours later, cells were subjected to an MTS assay for determination of total viable cells. Values represent mean ± SEM (n = 3).

C: CMK and K562 control cells were treated with increasing concentrations of imatinib. Seventy-two hours later, cells were subjected to an MTS assay for determination of total viable cells. Values represent mean ± SEM (n = 3).

D: CMK and K562 control cells were treated as in **B**. Seventy-two hours later, cells were stained with annexin V-FITC and analyzed by flow cytometry for determination of apoptotic cells. Values represent mean ± SEM (n = 3).

E: CMK and K562 control cells were treated as in **B**. Cell lysates were subjected to immunoblot analysis for total and phospho-STAT5, ERK, and STAT3. *$p < 0.05$.

AMKL patients have activating JAK3 mutations

These findings indicated that activating mutations in *JAK3* contribute to the pathogenesis of AMKL. To further address this possibility, we screened 19 AMKL patient samples, of which 3 were derived from Down's syndrome and 16 from non-Down's syndrome patients. While none of these patients had the $JAK3^{A572V}$ mutation, two had other *JAK3* mutations, one having a heterozygous V722I substitution in the JH2 pseudokinase domain and the second having a heterozygous P132T change in the JH6 domain of the receptor binding region (Figure 4A and Table S2). None of these 19 AMKL patient samples had the common myeloproliferative disorder mutation $JAK2^{V617F}$. Of note, as for the AMKL patient from whom the CMK cell line was derived, the patient with the $JAK3^{V722I}$ allele had Down's syndrome and harbored a loss-of-function mutation in the GATA-1 gene (Wechsler et al., 2002). Transduction of Ba/F3 cells with $MIG\text{-}JAK3^{WT}$, $MIG\text{-}JAK3^{V722I}$, or $MIG\text{-}JAK3^{P132T}$ confirmed that, similar to $JAK3^{A572V}$, the $JAK3^{V722I}$ and $JAK3^{P132T}$ alleles also conferred IL-3-independent growth to Ba/F3 cells as well as constitutive JAK3 and STAT5 phosphorylation (Figures 4B and 4C).

Modeling of JAK3 JH2 domain mutations

We next developed a structural model to assess the role of the *JAK3* mutations in constitutive kinase activation. The structure of the JAK3 JH1 catalytic domain, but not the JAK3 JH2 domain, has been solved (Boggon et al., 2005). Although the JAK3 kinase JH2 (pseudokinase) domain lacks several residues critical to phosphotransferase activity, this domain is expected to adopt the overall protein architecture characteristic of tyrosine and serine/threonine kinases. We used the Swiss-model automated comparative protein modeling server to construct a homology model of the JAK3 JH2 domain using the crystal structures of the epidermal growth factor receptor (Stamos et al., 2002), insulin receptor (Parang et al., 2001), Zap-70 (Jin et al., 2004), and Syk (Atwell et al., 2004) tyrosine kinases (Protein Data Bank accession codes 1M17, 1GAG, 1U59, and 1XBC, respectively) as a model. These tyrosine kinases have sequence identity to the JAK3 JH2 domain search sequence (Q501 to Q812) of 28%, 28%, 33%, and 33%, respectively. All four template crystal structures for the prediction of the JAK3 JH2 domain are active conformation tyrosine kinase domains. The model therefore assumes a conformation that is consistent with an active conformation kinase.

The predicted structure of the JAK3 JH2 domain highlights the expected locations of two residues discussed in this study. A572 is predicted to lie in the pseudokinase domain C helix (Figure 5). The model predicts that A572 is on the cleft side of the C helix located at the same position as the catalytic glutamic acid in active kinase domains. While the preferred conformation of the JAK3 pseudokinase domain activation loop is not known at this point, the model presented here is in an active kinase conformation. Correlation of the structural location of A572 with the data presented in this paper would suggest a role for the "catalytic cleft" region of the pseudokinase domain in autoregulation. Alternatively, the activation loop may fold in an autoinhibited-like conformation, potentially packing against A572 in a manner

Table 1. Tyrosine residues phosphorylated on kinases in CMK cells

Protein name	NCBI accession no.	NCBI site	Trypsin	Chymotrypsin
Protein kinase				
A6r	NP_009215	§309	•	
NM23	NP_000260	52		•
TIF1-β	NP_005753	458		•
Protein kinase, dual specificity				
DYRK1A	NP_001387	§145	•	
DYRK1A	NP_001387	319	•	
DYRK1A	NP_001387	§321	•	
Protein kinase, ser/thr (nonreceptor)				
Bcr	NP_004318	§644	•	
Cdc2	NP_001777	§15	•	•
Cdc2	NP_001777	§19	•	
CDK2	NP_001789	§15	•	
CDK2	NP_001789	§19	•	
CDK3	NP_001249	§15	•	
CDK3	NP_001249	§19	•	
CDK5	NP_004926	§15	•	
DNA-PK	NP_008835	779		•
ERK1	NP_002737	§204	•	
ERK2	NP_002736	§187		•
GSK3-β	NP_002084	§216	•	•
HIPK2	NP_689909	§352	•	•
HIPK3	NP_005725	§359	•	•
JNK1	NP_002741	§185	•	
p38-α	NP_001306	§182	•	•
p38-γ	NP_002960	185	•	
PKCD	NP_006245	§313	•	
PKCD	NP_006245	374	•	
PRP4	NP_003904	§849	•	•
Protein kinase, tyrosine (nonreceptor)				
Abl	NP_005148	§393	•	•
Ack	NP_005772	§518	•	•
Ack	NP_005772	§859	•	
Btk	NP_038510	§223	•	•
Btk	NP_038510	225	•	•
Btk	NP_038510	344	•	•
Btk	NP_038510	361	•	
Fer	NP_005237	§714	•	•
Fgr	NP_005239	§523		•
Fyn	NP_002028	§420	•	
Fyn	NP_002028	§531		•
Hck	NP_002101	§411	•	
Jak2	NP_004963	§570	•	
Jak3	NP_000206	§785	•	
Jak3	NP_000206	§980		•
Lck	NP_005347	§394	•	
Lyn	NP_002341	194	•	
Lyn	NP_002341	316	•	•
Lyn	NP_002341	473	•	
Lyn	NP_002341	§508	•	
Pyk2	NP_004094	§579	•	
Src	NP_005408	§530		•
Syk	NP_003168	§323	•	
Tyk2	NP_003322	§292	•	
ZAP70	NP_001070	§292	•	
ZAP70	NP_001070	§492	•	
ZAP70	NP_001070	§493	•	
Protein kinase, tyrosine (receptor)				
Axl	NP_001690	694	•	
Kit	NP_000213	§936	•	

Tyrosine-phosphorylated kinases identified by mass spectrometry in CMK cells. §, published sites.

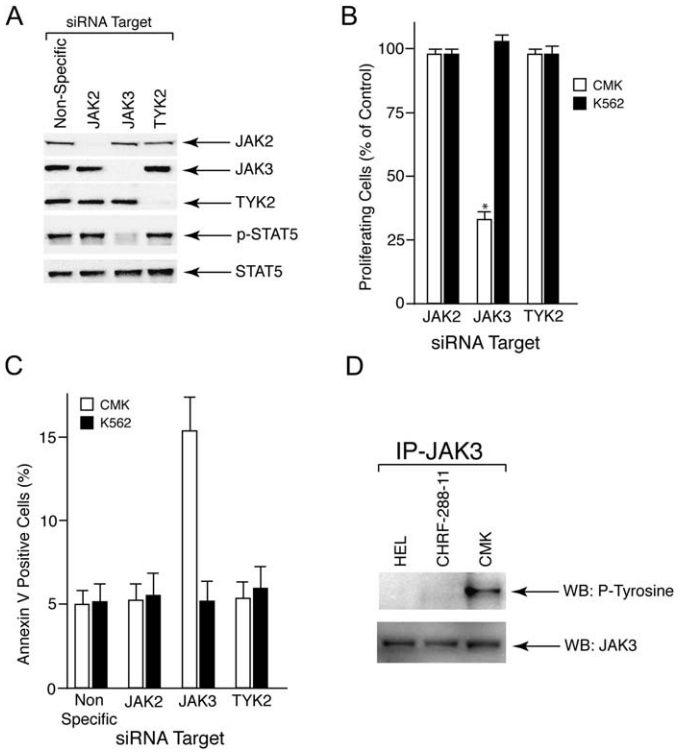

Figure 2. JAK3 is necessary for constitutive STAT5 activation, proliferation, and survival of CMK cells

A: CMK cells were transfected with nonspecific or JAK2-, JAK3-, or TYK2-specific siRNA. Forty-eight hours later, lysates were subjected to immunoblot analysis for JAK2, JAK3, TYK2, phospho-STAT5, and STAT5.

B: CMK and K562 control cells were transfected with siRNA as in **A**. Seventy-two hours later, cells were subjected to an MTS assay for determination of total viable cells. Values represent mean ± SEM (n = 7).

C: CMK and K562 control cells were transfected with siRNA as in **A**. Seventy-two hours later, cells were stained with annexin V-FITC for determination of apoptotic cells. Values represent mean ± SEM (n = 6).

D: Cell lines were serum starved for 16 hr. Lysates were immunoprecipitated with a JAK3-specific antibody, and immunoprecipitates were subjected to immunoblot analysis for phosphotyrosine and JAK3. *$p < 0.05$.

that could be disrupted by an A572V mutation. The second residue that is located in the pseudokinase domain, V722, is predicted to be located C-terminal to the short kinase fold F helix (Figure 5). In the model, this residue is expected to be abutting the short D helix and partially surface exposed; however, a putative mechanism of activation for the V722I substitution is not readily apparent from the structural model. Hence, molecular modeling of the A572V mutation is consistent with functional data indicating an activating phenotype; however, numerous JAK3 pseudokinase domain mutations have been identified that induce loss-of-function phenotypes despite being constitutively phosphorylated (Chen et al., 2000; Notarangelo et al., 2001). As such, it will be important to assess the position of these residues in the context of a solved structure of the composite JAK3 JH1/JH2 complex.

JAK3^{A572V} induces a lethal hematologic malignancy in vivo

We used a murine model to further characterize the transforming potential of JAK3^{A572V}. C57BL/6 mice were transplanted with bone marrow (BM) cells that were retrovirally transduced with

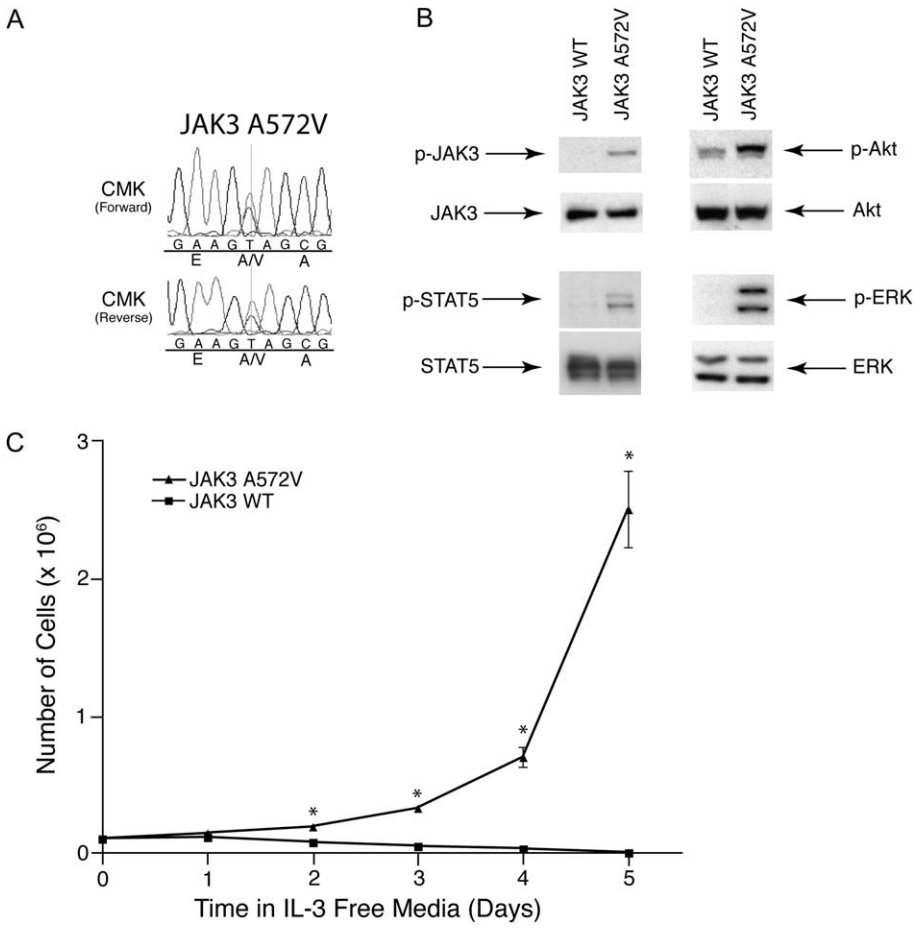

Figure 3. JAK3 mutation causes constitutive JAK3 activity in CMK cells

A: The *JAK3* JH2 domain was sequenced, revealing an alanine to valine substitution at amino acid position 572.

B: Ba/F3 cells were transduced with MIG-JAK3^{WT} or MIG-JAK3^{A572V}. GFP-expressing cells were selected by flow cytometry, cultured for 5–8 days in IL-3-containing media, and serum starved for 4 hr prior to biochemical analysis. Lysates were immunoprecipitated with anti-JAK3 followed by immunoblot analysis for phosphotyrosine or JAK3 or immunoprecipitated with anti-STAT5 followed by immunoblot analysis for phospho-STAT5 or STAT5. Immunoblot analyses for p42/44 and phosphorylated and total Akt were performed.

C: Ba/F3 cells were infected and sorted as in **B**. Cells were then incubated in media without IL-3 for 5 days and counted using trypan blue every day for determination of total viable cells. Values represent mean ± SEM (n = 3). *p < 0.05.

either *MIG-JAK3^{WT}* or *MIG-JAK3^{A572V}*. JAK3^{A572V} mice exhibited significantly decreased survival compared with JAK3^{WT} mice (Figure 6A) and showed several phenotypic characteristics of megakaryoblastic leukemia. There was splenomegaly in JAK3^{A572V} versus JAK3^{WT} animals (476 ± 94 mg versus 98 ± 6 mg, respectively). Multiparameter flow cytometry showed an approximate 8-fold expansion in the megakaryocyte-erythroid progenitors (MEP), defined as lineage−, c-KIT+, Sca1−, CD34low, and CD16/CD32low in spleens derived from JAK3^{A572V} compared with JAK3^{WT} animals. As a functional correlate of this finding, we observed an expansion in megakaryocyte colony formation in JAK3^{A572V} compared with JAK3^{WT} animals (Figure 6B). In addition, we detected abnormally high numbers of megakaryocytes infiltrated in the spleen and liver of JAK3^{A572V} transplanted mice as determined by staining for the megakaryocyte-specific marker von Willebrand factor (vWF) (Figure 6D and Figure S2B). To further characterize the effect of JAK3^{A572V} on megakaryocyte maturation, we assessed terminal differentiation by ex vivo culture of bone marrow cells from wild-type or mutant mice for 4 days in the presence of thrombopoietin (TPO) and stem cell factor (SCF). In both JAK3^{A572V} and JAK3^{WT}, mature proplatelet-forming megakaryocytes were observed in the cultures. Nevertheless, ploidy analysis on CD41+ cells showed a significant decrease in the median ploidy of megakaryocytes from JAK3^{A572V} compared to JAK3^{WT} mice, and an increase in megakaryocytes in S phase (Figure 6C). Despite the increased incidence of megakaryocytes in spleen and liver, JAK3^{A572V} mice exhibited normal platelet counts in peripheral blood (Figure S2C). Also, secondary transplantation of either bone marrow or spleen cells into sublethally irradiated mice did not result in visible disease in the recipient animals (data not shown). Taken together, these findings indicate that JAK3^{A572V} recapitulates several, but not all, of the phenotypic characteristics of AMKL, including splenomegaly, expansion of the megakaryocyte progenitors, and impaired megakaryocyte differentiation as assessed by analysis of polyploidization.

In addition to the megakaryocyte phenotype in JAK3^{A572V}-transduced animals, we observed marked leukocytosis that was comprised primarily of lymphocytes (Figure 6D). There was also lymphocytic infiltration of bone marrow, liver, spleen, lungs, and lymph nodes (Figure S2A and data not shown). Immunophenotypic analysis did reveal a marked expansion of CD8+ cells in the thymus, peripheral blood, bone marrow, and spleen in JAK3^{A572V} compared with JAK3^{WT} animals (Figure 6F and data not shown). These findings are consistent with a coexistent T cell lymphoproliferative disorder, in addition to the aberrancies in the megakaryocytic lineage induced by JAK3^{A572V}.

Discussion

Using a screening strategy involving phosphopeptide immunoprecipitation followed by LC-MS/MS mass spectrometry, we have identified a JAK3 mutation, *JAK3^{A572V}*, associated with human cancer in the megakaryoblastic cell line CMK. These findings prompted DNA sequence analysis of primary cells

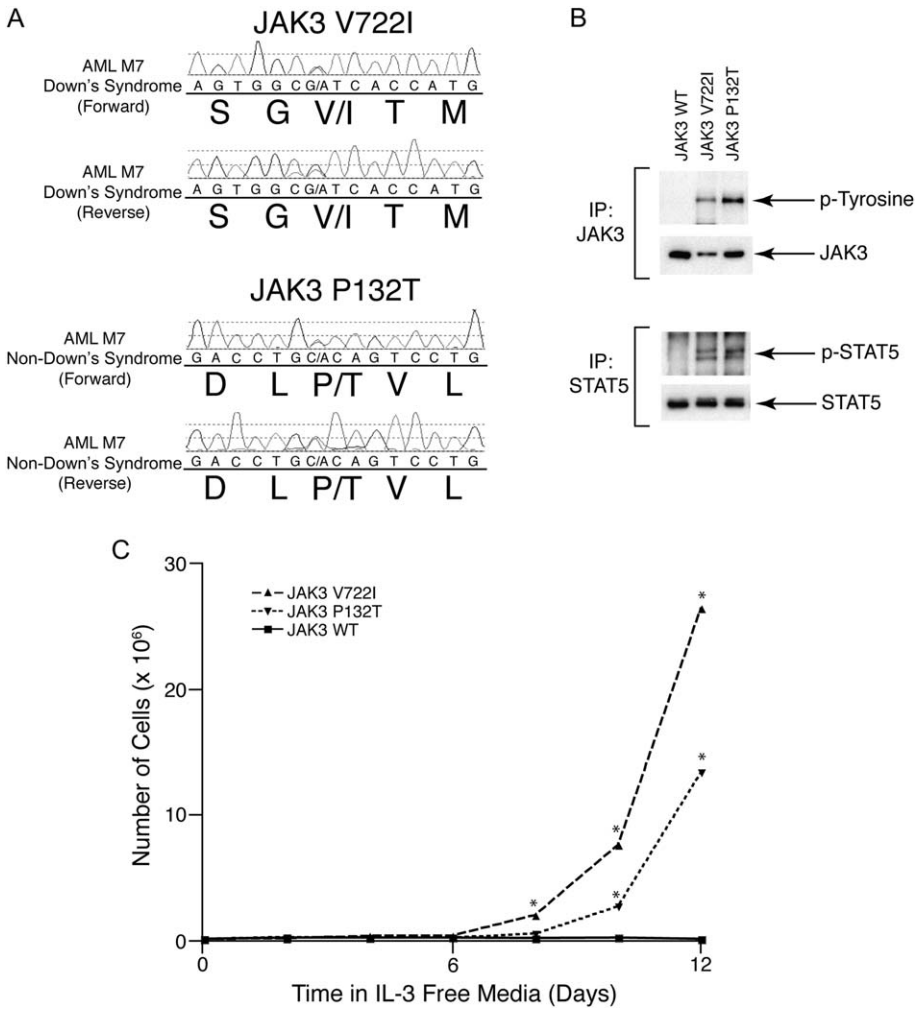

Figure 4. Two JAK3 mutations from AMKL patients transform BaF3 cells

A: JAK3 was sequenced in 19 megakaryoblastic AML patients (3 with Down's syndrome, 16 non-Down's syndrome), and two mutations, V722I and P132T, were identified.

B: Ba/F3 cells were transduced and sorted as in described in Figure 3, and biochemical analysis of Ba/F3 cells infected with $JAK3^{WT}$, $JAK3^{V722I}$, and $JAK3^{P132T}$ was performed. Cells were serum starved for 4 hr, and lysates were immunoprecipitated with anti-JAK3 and subjected to immunoblot analysis for phosphotyrosine or JAK3 or immunoprecipitated with anti-STAT5 and subjected to immunoblot analysis for phospho-STAT5 or STAT5.

C: Sorted cells were incubated in media without IL-3 for 12 days and subjected to an MTS assay every 2 days for determination of total viable cells. Values represent mean ± SEM (n = 3). *$p < 0.05$.

derived from patients with AMKL and identified two additional activating alleles of *JAK3*: $JAK3^{V722I}$ and $JAK3^{P132T}$. Expression of these mutations transforms Ba/F3 cells to factor-independent growth that is associated with constitutive activation of the respective *JAK3* allele in the absence of cytokine, and activation of known downstream effectors of JAK signaling, including STAT5, ERK, and AKT.

While this report demonstrates an activating mutation of *JAK3* associated with leukemia, several studies have previously suggested an association of *JAK3* with cancer phenotypes in the absence of known mutations. Initially, JAK3 expression was found preferentially in hematopoietic cells, including samples of B cell lymphomas, supporting a possible involvement of JAK3 in transformation of hematopoietic cells (Tortolani et al., 1995). Also, JAK3 splice variants were discovered in cancer cells from hematopoietic and epithelial origin, and JAK3 was found to be important for activation of the proto-oncogenes c-*fos*, c-*myc*, and bcl-2 (Kawahara et al., 1995; Lai et al., 1995). In addition, peripheral T lymphocytes infected and transformed with HTLV-1, a major cause of adult T cell leukemia, were subsequently shown to exhibit constitutively phosphorylated JAK3, and this event was shown to correlate with IL-2-independent growth (Migone et al., 1995; Xu et al., 1995). Finally, oligomerization of JAK3 induced by fusion with TEL, although an artificial construct not found in patients so far, has been shown to constitutively activate JAK3, resulting in factor-independent growth of BaF3 cells as well as activation of downstream signaling partners, including STAT1, -3, and -5 (Lacronique et al., 2000). In this manuscript, we identified activating, mutated alleles of *JAK3* in patients with leukemia and demonstrated that constitutively activated JAK3 can induce a lethal hematopoietic malignancy in vivo.

Several inferences on the mechanism of activation of JAK3 can be drawn for the two mutations residing in the JAK3 JH2 domain, A572V and V722I. Deletion of the JH2 domain in experimental systems results in constitutive activation of JH1 (Saharinen and Silvennoinen, 2002). These observations have led to the hypothesis that JH2, which is nearly identical in amino acid sequence to JH1, but lacks catalytic activity, serves an autoinhibitory function. In support of this hypothesis, there are other examples of activating alleles in the JH2 domain of JAK family members that include the E695K *Hopscotch* allele in the single *Drosophila* JAK, and the $JAK2^{V617F}$ allele observed in the majority of cases of polycythemia vera, myeloid metaplasia with myelofibrosis, and essential thrombocythemia (Baxter et al., 2005; James et al., 2005; Jones et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). The E695K *Hopscotch* allele is predicted to disrupt a salt bridge and result in loss of structure, and thereby loss of autoinhibitory activity of the JH2 domain. A predicted structure of the JAK2 JH1 and JH2 domains suggests

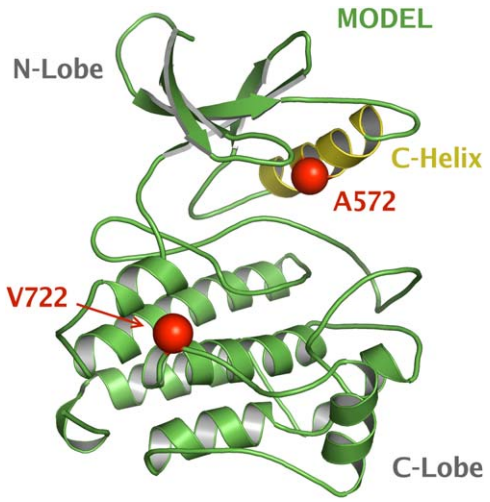

Figure 5. Homology model of the JH2 pseudokinase domain of human JAK3
The model was constructed using the Swiss-Model automated protein modeling server using the JAK3 pseudokinase domain and the crystal structures of epidermal growth factor receptor, insulin receptor, Zap-70, and Syk tyrosine kinases (Protein Data Bank accession codes 1M17, 1GAG, 1U59, and 1XBC, respectively) as a model. Although several residues critical for kinase activity are not conserved, the pseudokinase domain is expected to retain the essential features of the protein kinase fold, including the smaller β sheet-rich N lobe and the larger, mostly helical C lobe. The locations of A572 and V722 are indicated with red spheres. A572 is expected to lie within the kinase fold C helix, and V722 is expected to be C-terminal to the kinase fold F helix. The figure was made using the program PYMOL (http://www.pymol.org/).

that the JH1 and JH2 regions may interact to prevent kinase activation (Lindauer et al., 2001) and predicts that residues V617–E621 of JAK2 are important for autoinhibition. However, V617 is conserved between JAK1, JAK2, and TYK2, but not JAK3. Indeed, substitution of the homologous M592 residue with phenylalanine in the context of JAK3 does not result in kinase activation (Staerk et al., 2005). Our structural modeling suggests that the A572V substitution is on the cleft side of the C helix at the same position as the catalytic glutamic acid residue in active kinase domains and that there may be a role for the catalytic cleft region of the pseudokinase domain in autoregulation of kinase activity. However, the crystallographic structure of the respective JH1–JH2 domains of JAK family members will be required to formally test these hypotheses.

Expression of JAK3^{A572V} in a murine bone marrow transplant model induces several features of AMKL that include splenomegaly, increase in megakaryocyte-erythroid progenitors, and impaired megakaryocyte differentation. However, other features of AMKL, including bone marrow fibrosis and serially transplantable disease, were not observed, suggesting that full AMKL transformation may require other cooperating mutations. Consistent with this hypothesis, two of three *JAK3* mutations were found in samples from patients with Down's syndrome and AMKL that also harbored an additional chromosome 21 and a *GATA-1* mutation resulting in expression of a truncated protein. Recent reports indicate that *GATA-1* mutations alone do not cause AMKL in a murine knockin model but result in abnormal transient proliferation of megakaryocyte progenitors during embryonic development (Li et al., 2005). Taken together, our findings indicate that *JAK3* mutations likely cooperate with other mutations in the pathogenesis of AMKL, including *GATA-1* mutant alleles or oncogenic events phenocopying trisomy 21.

Of note, mice transplanted with JAK3^{A572V} also develop a T cell lymphoproliferative disorder. As demonstrated by studies on *JAK3*-deficient mice and genotypic analyses of severe combined immunodeficiency disorders, JAK3 signaling plays an important role in T cell development, proliferation, and function by virtue of association with the common gamma chain of a spectrum of T cell-specific cytokine receptors that include IL-2, -4, -7, -9, -15, and -21 (Brown et al., 1999; Johnston et al., 1994; Miyazaki et al., 1994; Noguchi et al., 1993; Nosaka et al., 1995; Pesu et al., 2005; Russell et al., 1994; Sohn et al., 1998; Thomis et al., 1995; Witthuhn et al., 1994). The murine bone marrow transplantation assay enables retroviral transduction of the full spectrum of myeloid and lymphoid hematopoietic progenitors. It is of interest that the *JAK3*^{A572V} allele induces a T cell lymphoproliferative disorder in this murine BMT model, whereas the *JAK2*^{V617F} allele associated with myeloproliferative disorders does not (Wernig et al., 2006). This observation suggests that JAK3 mutations may also be relevant in the pathogenesis of T cell lymphoproliferative disorders.

Previously identified *JAK3* mutations have been loss-of-function mutations associated with a severe combined immunodeficiency. These observations indicate that inhibition of JAK3 would be predicted to inhibit T cell function and have spawned an effort to develop JAK3 inhibitors as immunomodulatory agents in autoimmune disorders that include asthma and allergies (Malaviya et al., 1999), type I diabetes (Cetkovic-Cvrlje et al., 2003; Tian et al., 1998), allograft rejection (Hall, 1991; Stepkowski et al., 2002), and amyotrophic lateral sclerosis (Cetkovic-Cvrlje and Tibbles, 2004). This study suggests that JAK3 inhibitors that are currently in development for treatment of autoimmune disease (Borie et al., 2004; Changelian et al., 2003; O'Shea et al., 2004) may also have clinical activity in AMKL patients with mutant activating alleles of JAK3, and potentially in T cell lymphoma/leukemia. Our murine model should provide a useful tool for assessing these potential therapeutic activities.

Another important aspect of this study was the use of a mass spectrometry-based strategy to identify novel therapeutic targets in cancer. Recent data indicate that using high-throughput DNA sequence analysis of the tyrosine and serine-threonine kinome may yield few cancer alleles (Davies et al., 2005). Mass spectrometric assessment of phosphopeptides that denote constitutive activation of signal transduction pathways may provide a useful surrogate screen for functional activation of cancer disease alleles that are, in turn, potential therapeutic targets.

These findings demonstrate a role for activating alleles of *JAK3* in human cancer and indicate that JAK3 contributes to the pathogenesis of AMKL and can engender a T cell lymphoproliferative phenotype in vivo. As such, screening for mutations in *JAK3* may be warranted, as this study prompts a thorough screening of both types of leukemias for activating *JAK3* mutations.

Experimental procedures

Cell lines and reagents

AML-193, GDM-1, CMK, K562, HEL, CHRF-288-11, and Ba/F3 cells were obtained from the German National Resource Centre for Biological Material (DSMZ). GF-D8 cells were generously provided by Dr. Orietta Spinelli, Laboratorio Paolo Belli, Bergamo, Italy. All cell lines were maintained in RPMI 1640

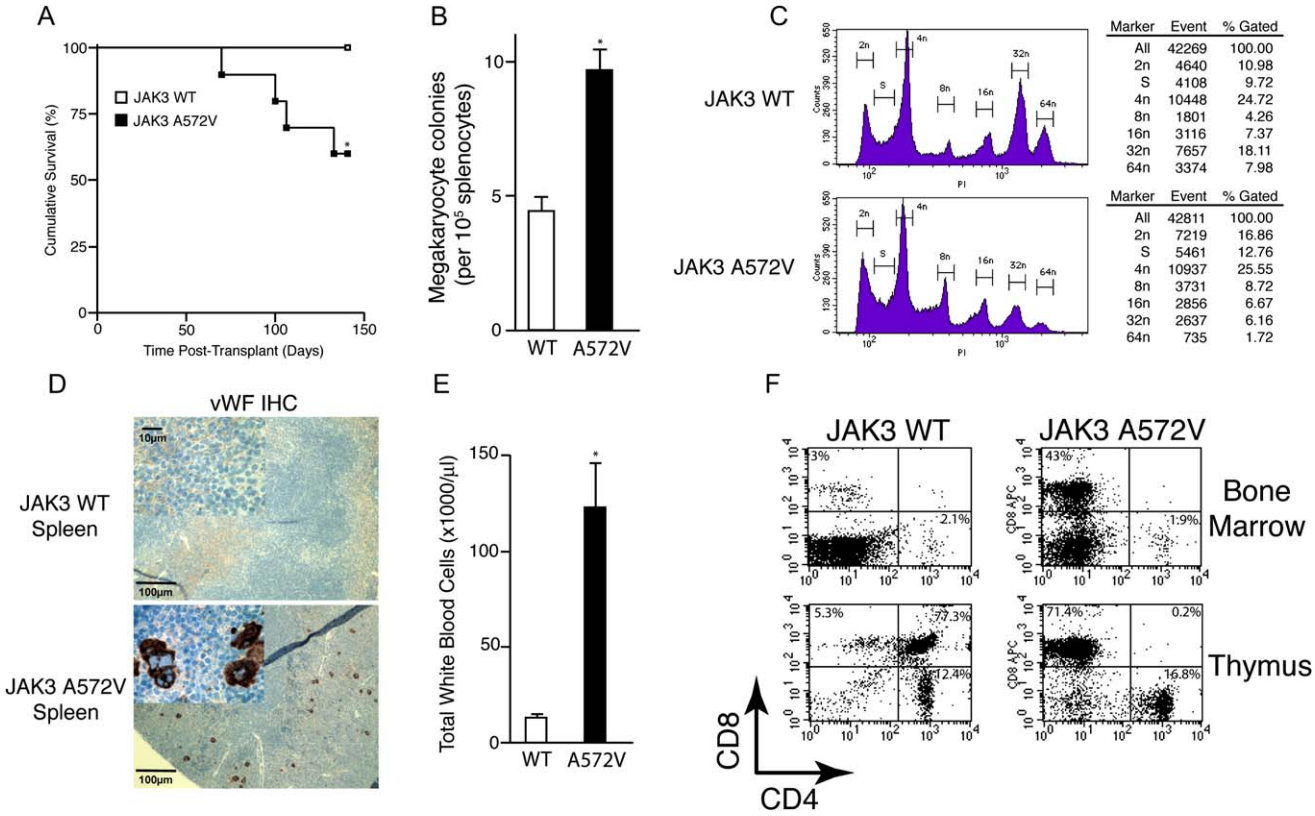

Figure 6. In vivo expression of JAK3^{A572V} causes a lethal hematopoietic malignancy with megakaryoblastic features

A: MSCV retroviruses expressing either JAK3$^{WT\text{-}GFP}$ or JAK3$^{A572V\text{-}GFP}$ were used to transduce bone marrow cells from wild-type C57BL/6 mice. Transduced bone marrow cells were then transplanted into lethally irradiated C57BL/6 recipient mice, and survival was monitored (n = 10).

B: Splenocytes from transplanted JAK3^{WT} and JAK3^{A572V} were plated onto collagen-based culture containing Tpo, IL-11, IL-3, and IL-6. Colonies were stained for acetylcholinesterase activity and counted after 7 days. Values represent mean ± SEM (n = 3).

C: Bone marrow cells from JAK3^{WT} or JAK3^{A572V} transplanted mice were cultured in the presence of Tpo and SCF for 4 days. They were subsequently stained with anti-CD41 antibody and propidium iodide (PI) and analyzed by flow cytometry. Shown are PI histograms of CD41-positive cells.

D: Spleens were harvested from JAK3^{WT} or JAK3^{A572V} transplanted mice at 120 days posttransplantation. Splenic sections were subjected to immunohistochemistry for von Willebrand factor (vWF) and analyzed by light microscopy.

E: Total white blood cells count at 120 days posttransplantation from C57BL/6 animals transduced with JAK3$^{WT\text{-}GFP}$ or JAK3$^{A572V\text{-}GFP}$. Values represent mean ± SEM (n = 5).

F: Flow cytometric analysis of CD4 and CD8 expression on bone marrow and thymus cells from transplanted animals. A representative analysis is shown. *$p < 0.05$.

supplemented with 10% FCS, 1 unit/ml penicillin G, and 1 mg/ml streptomycin at 37°C and 5% CO_2. The pan-JAK inhibitor JAK Inhibitor I was purchased from Calbiochem.

KIT and *FLT3* mutational analysis

Mutational analysis was performed as previously described (Goemans et al., 2005; O'Farrell et al., 2003).

Phosphopeptide immunoprecipitation

Phosphopeptides were prepared using PhosphoScan Kit (Cell Signaling Technology).

Analysis by LC-MS/MS mass spectrometry

Peptides in the immunoprecipitation eluate (40 µl) were concentrated and separated from eluted antibody using Stop and Go extraction tips (StageTips) (Rappsilber et al., 2003). Peptides were eluted from the microcolumns with 1 µl of 60% MeCN, 0.1% TFA into 7.6 µl of 0.4% acetic acid/0.005% heptafluorobutyric acid (HFBA). The sample was loaded onto a 10 cm × 75 µm PicoFrit capillary column (New Objective) packed with Magic C18 AQ reversed-phase resin (Michrom Bioresources) using a Famos autosampler with an inert sample injection valve (Dionex). The column was developed with a 45 min linear gradient of acetonitrile in 0.4% acetic acid, 0.005% HFBA delivered at 280 nl/min (Ultimate, Dionex). Tandem mass spectra were collected in a data-dependent manner with an LTQ ion trap mass spectrometer (ThermoFinnigan), using a top-ten method, a dynamic exclusion repeat count of 1, and a repeat duration of 10 s. TurboSequest (ThermoFinnigan) searches were done against the NCBI human database released on August 24, 2004 (containing 27,175 proteins), allowing for tyrosine phosphorylation (Y + 80) and oxidized methionine (M + 16) as dynamic modifications. Sequence assignments were accepted based on the score-filtering criteria described previously (Rush et al., 2005), with the exception that assignments having score characteristics of false-positive assignments, determined by searching against a database of reversed protein sequences (Peng et al., 2003), were not accepted.

Patient sample collection, sequencing, and mutational analysis

Primers for the kinase domain and JH2 pseudokinase domain of JAK3 were as described (Levine et al., 2005). Primers for *JAK3* sequencing of AMKL patient samples were as follows: exon 4 forward: 5′-GTAAAACGACGGCCAGTGGGGTCAGCCCAGGATTG-3′, reverse: 5′-CAGGAAACAGCTATGACCGCCCTGGGTCATAGGAACAC-3′; exon 16 forward: 5′-GTAAAACGACGGCCAGTACCCCCACTTTGACAGAAGG-3′, Reverse: 5′-CAGGAAACAGCTATGACCCAACCTCACCAGACACACAGG-3′. Bidirectional sequence traces were assembled and analyzed for mutations using Mutation Surveyor version 2.5 (SoftGenetics, State College, PA). All clinical samples were obtained with

informed consent with approval by the University of Chicago or Brigham and Women's Hospital Institutional Review Board.

siRNA and siRNA transfection

JAK2, JAK3, and TYK2 *SMART*pool siRNA duplexes were purchased from Dharmacon Research, Inc. A nonspecific *SMART*pool siRNA was used as a control. Cells were transfected with the various siRNAs via electroporation. Briefly, cells were pulsed once (CMK: 20 ms, 250 V; K562: 20 ms, 285 V) using a square-wave electroporator (BTX Genetronics), incubated at room temperature for 30 min, and transferred to 96-well, 24-well, and/or 6-well plates (Becton Dickinson).

Flow cytometry and immunoblotting

Detection of apoptosis was performed as previously described (Walters et al., 2005). Detection of total and phosphorylated protein targets was performed using standard immunoprecipitation and immunoblotting procedures. Antibodies used were anti-phospho-STAT5, anti-phospho-Akt, anti-Akt, anti-phospho-p42/44, and anti-p42/44 (Cell Signaling Technology, Beverly, MA); anti-STAT5 and anti-TYK2 (BD Transduction Laboratories, BD Pharmingen, San Diego, CA); anti-JAK3 and anti-phospho-JAK3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and anti-phosphotyrosine 4G10 (Upstate Biotechnologies). Megakaryocyte/erythrocyte progenitor (MEP) analysis was performed using a previously described protocol (Na Nakorn et al., 2002) modified to accommodate the presence of GFP in sorted cells. Briefly, spleen cells were collected from $JAK3^{WT}$ and $JAK3^{A572V}$ animals. Red blood cells were lysed using RBC Lysis buffer (Gentra).

Cells were stained in five steps: (1) rat anti-mouse CD34 antibody; (2) PE Cy5.5-conjugated goat anti-rat antibody (Caltag); (3) biotin-conjugated Sca1 antibody; (4) APC Cy7-conjugated Streptavidin (Caltag), PE-conjugated FCgRII/III antibody, APC-conjugated cKit antibody (eBioscience), and PE Cy7-conjugated lineage markers antibodies (CD3, CD4, CD8, B220, CD19, Ter119, and Gr1); (5) propidium iodide. Cells were blocked with rat immunoglobulin between steps 2 and 3 and were washed with PBS (4°C) between each step. Lineage-negative cells (2×10^4) were analyzed. MEPs were defined as lineage−, c-KIT+, Sca1−, CD34low, and CD16/CD32low. Acquisition of the data was performed on a FACSAria (BD Biosciences) and analyzed with Flowjo software.

Generation of $JAK3^{A572V}$, $JAK3^{V722I}$, and $JAK3^{P132T}$ mutants and MTS assay

A cDNA encoding the S isoform of human *JAK3* was prepared from a purchased clone encoding the M isoform of human *JAK3* (Origene, Rockville, MD) (Lai et al., 1995). The required C-terminal region for the S isoform of *JAK3* was amplified by RT-PCR using cDNA from the CMK cell line, then directionally inserted as a 1.0 kb SrfI-XbaI fragment. Following transfer of the resulting *JAK3* (S isoform) cDNA to pRC/CMV as a 4.7 kb NotI fragment, the A572V, V722I, and P132T mutations were introduced using the GeneTailor site-directed mutagenesis system (Invitrogen, Carlsbad, CA) and then transferred into the MSCV-IRES-EGFP (MIG) vector. Full-length sequencing confirmed the identities of the respective cDNAs. Primer sequences are as follows:

RT-PCR, forward: 5′-CGCTAGGCGACAATACAGGT-3′, reverse: 5′-ATAA TTCTAGACACATATGCCCATCTG-3′, mutagenesis ($JAK3^{A572V}$) forward: 5′-GCATGGAGTCATTCCTGGAAGTAGCGAGCTTG-3′, ($JAK3^{A572V}$) reverse: 5′-CTTCCAGGAATGACTCCATGCAGTTCTTGTG-3′, ($JAK3^{P132T}$) forward: 5′-GTGCTATCCTTGACCTGACAGTCCTGGAGCACCTC-3′, ($JAK3^{P132T}$) reverse: 5′-GAGGTGCTCCAGGACTGTCAGGTCAAGGATAGCAC-3′, ($JAK3^{V722I}$) forward: 5′-GGGAAGTGTTTAGTGGCATCACCATGCCCATCAGT-3′, ($JAK3^{V722I}$) reverse: 5′-ACTGATGGGCATGGTGATGCCACTAAACACTTCCC-3′. Ba/F3 cells were transduced with viral supernatants from 293T cells transfected with either MIG-$JAK3^{WT}$, MIG-$JAK3^{A572V}$, MIG-$JAK3^{V722I}$, or MIG-$JAK3^{P132T}$. Cells were selected for expression of GFP 24 hr after infection by cell sorting, and GFP-positive cells were incubated in the absence of IL-3 for 5–12 days. Viable cell number was determined every 1–2 days by counting with trypan blue. The MTS assays using JAK Inhibitor I and siRNA were performed 72 hr after treatment.

In vitro kinase assay

$JAK3^{WT}$ and $JAK3^{A572V}$ were immunoprecipitated using standard procedures from Ba/F3 cells described above using a polyclonal JAK3 antibody (Santa Cruz). Immunoprecipitates were incubated in kinase assay buffer (Cell Signaling) with a JAK3-specific substrate (Upstate) according to manufacturer protocols.

Bone marrow transplant and animal analysis

Viral supernatants were obtained as previously described (Schwaller et al., 1998). Five days prior to bone marrow procurement, 8- to 10-week-old C57BL/6 (obtained from Taconic) mice were injected intraperitoneally with 150 mg/kg of 5-FU. On day 0, primary bone marrow cells were collected from femurs and tibiae and cultured overnight in RPMI 1640 supplemented with 10% FBS and IL-3, IL-6, and SCF. Cells were transduced with identical titer viral supernatants on day 1 and day 2. Each time, cells were centrifuged for 90 min at 2500 rpm at 30°C in the presence of virus, returned to the incubator for 3–4 hr, and resuspended into fresh media containing cytokines. After the second centrifugation, cells were resuspended in 1× Hank's balanced buffer, and 1×10^6 cells were injected in the tail veins of lethally irradiated C57BL/6 mice. Eye-bleeds were obtained using EDTA-coated capillary tubes, and blood counts were performed within 30 min on a HemaVet HV950FS (Drew Scientific). For histology, tibial bones, spleens, livers, peripheral blood, and lungs were harvested from transplanted mice, sectioned, and stained with hematoxylin and eosin. Histopathology was analyzed by light microscopy. Spleen and liver sections were stained by standard immunohistochemistry procedures with an antibody specific for von Willebrand factor (Dako Cytomation). Approval for the use of animals in this study was granted by the Children's Hospital Boston Animal Care and Use Committee under the protocol number A04-03-029.

Megakaryocyte colony-forming assay and ploidy analysis

For megakaryocyte colony-forming assays, primary spleen cells were collected from transplanted animals and subsequently treated with red blood cell lysis buffer (Puregene, Gentra systems). Cells (1×10^5) were mixed with MegaCult-C (StemCell Technologies), containing Tpo, IL-11, IL-3, and IL-6 and plated onto two double chamber culture slides, and colonies were stained for acetylcholinesterase and enumerated after 8 days. For ploidy analysis, bone marrow cells from transplanted animals were cultured 4 days in RPMI 1640 supplemented with 10% FBS, 10 ng/ml mouse SCF, and 10 ng/ml mouse Tpo prior to analysis. Cells were first stained with CD41 antibody, followed by staining with APC-conjugated secondary antibody. Cells were then incubated 30 min in a 0.1% sodium citrate solution containing 50 μg/ml of RNase A and 50 μg/ml of propidium iodide. Analysis of ploidy was performed on CD41+ cells.

Model of JAK3 JH2 domain structure

The Swiss-Model automated comparative protein modeling server (http://swissmodel.expasy.org/) was used to create an energy-minimized model of the JH2 domain of JAK3 (Schwede et al., 2003). The sequence for residues Q501 to Q812 of JAK3 was submitted to Swiss-Model for a first approach mode search using the Protein Data Bank (http://www.pdb.org/) deposited structures for epidermal growth factor receptor (Stamos et al., 2002), insulin receptor (Parang et al., 2001), Zap-70 (Jin et al., 2004), and Syk (Atwell et al., 2004) tyrosine kinases (Protein Data Bank accession codes 1M17, 1GAG, 1U59, and 1XBC, respectively) as three-dimensional templates (sequence identity 28%, 28%, 33%, and 33%, respectively). An energy-minimized model of the JH2 domain of JAK3 was determined and manually checked for global reasonableness. The locations of A572 and V722 in this model correspond well with the crystallographic coordinates of the template models for the equivalent residues. Further modeling runs using autoinhibited tyrosine kinase domain crystal structures as templates confirmed the location of these residues (data not shown).

Statistical analysis

Survival curves were analyzed for significance by the log-rank test. All other statistical analyses were performed using paired Student's t test. Values were considered statistically significant when $p < 0.05$.

Supplemental data

The Supplemental Data include two supplemental figures and two supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/1/65/DC1/.

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