MECHANISM OF TRANSFORMATION AND THERAPEUTIC TARGETS FOR HEMATOLOGICAL NEOPLASMS HARBORING ONCOGENIC KIT MUTATION

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MECHANISM OF TRANSFORMATION AND THERAPEUTIC TARGETS FOR HEMATOLOGICAL NEOPLASMS HARBORING ONCOGENIC KIT MUTATION

Gain-of-function mutations in the KIT receptor tyrosine kinase have been associated with highly malignant human neoplasms. In particular, an acquired somatic mutation at codon 816 in the second catalytic domain of KIT involving an aspartic acid to valine substitution is found in patients with systemic mastocytosis (SM) and acute myeloid leukemia (AML). The presence of this mutation in SM and AML is associated with poor prognosis and overall survival. This mutation changes the conformation of the KIT receptor resulting in altered substrate recognition and constitutive tyrosine autophosphorylation leading to constitutive ligand independent growth. As there are currently no efficacious therapeutic agents against this mutation, this study sought to define novel therapeutic targets that contribute to aberrant signaling downstream from KITD816V that promote transformation of primary hematopoietic stem/progenitor cells in diseases such as AML and SM. This study shows that oncogenic KITD814V (murine homolog) induced myeloproliferative neoplasms (MPN) occurs in the absence of ligand stimulation, and that intracellular tyrosines are important for KITD814V-induced MPN. Among the seven intracellular tyrosines examined, tyrosine 719 alone has a unique role in regulating KITD814V-induced proliferation and survival. Residue tyrosine 719 is vital for activation of the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), p85α, downstream from KITD814V. Downstream effectors of the PI3K signaling pathway, in

particular p21 activated kinase (Pak) and its upstream effectors including guanine exchange factors (GEF) Vav1 and the Rho family of GTPases (Rac) exhibit constitutive activation and contribute to gain-of-function mutant-mediated transformation. Treatment of leukemic cells bearing KITD814V with an allosteric inhibitor of Pak or its genetic inactivation results in growth repression due to enhanced apoptosis. To assess the role of Rac GEFs in KITD814V induced transformation, EHop-016, an inhibitor of Rac, was used to specifically target Vav1, and found to be a potent inhibitor of human and murine leukemic cell growth. *In vivo,* the inhibition of Vav or Rac or Pak delayed the onset of MPN and rescued the associated pathology in mice. These studies provide insight on mechanisms and potential novel therapeutic targets for hematological malignancies harboring an oncogenic KIT mutation.

Reuben Kapur, Ph.D., Chair

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
7AAD	7-amino-actinomycin D
AC	Acidic motif
AML	Acute Myeloid Leukemia
APC	Allophycocyanin (640 nm/660nm)
ATP	Adenosine triphosphate
ASM	Aggressive systemic mastocytosis
BM	Bone marrow
Bp	Base pair
CBF	Core Binding Factor
CH	Calponin homology domain
CHR	Chimeric receptor
CPM	Counts per minute
DH	Dbl Homology
DMEM	Dulbecco's Modified Eagle Medium
EGFP	Enhanced green fluorescent protein
ENU	Ethylnitrosourea
ETO	Eight-Twenty One
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate (488 nm/520 nm)

FL	Flt3 Ligand
FLT3	Fms-like tyrosine kinase 3
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GIST	Gastrointestinal Stromal Tumor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine triphosphate
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
HSC/P	Hematopoietic stem and progenitor cell
Ig	Immunoglobulin
IL-3	Interleukin 3
IL-6	Interleukin 6
IRES	Internal ribosome entry site
ISM	Indolent systemic mastocytosis
ITD	Internal tandem duplication
JM	Juxtamembrane domain
kDa	Kilodalton
LDMNC	Low density mononuclear cell
MC	Mast cells
MCL	Mast cell leukemia
MCS	Mast cell sarcoma

M-CSFR	Macrophage colony stimulating factor receptor
MSCV	Murine stem cell virus
MPN	Myeloproliferative neoplasms
NK	Natural killer
PAK	p21 activated kinase
PB	Peripheral blood
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptors
PE	Phycoerythrin
PH	Pleckstrin homology domain
PI3K	Phosphatidylinositol 3-Kinase
polyI:polyC	Polyinosinic:polycytidylic acid
RHD	Runt Homolog Domain
RTK	Receptor Tyrosine Kinase
RUNX	Runt-related Transcription Factor
SCF	Stem cell factor
SD	Standard deviation
SH2	Src homology domain 2
SH3	Src homology domain 3
SM	Systemic mastocytosis
STAT	Signal transducer and activator of transcription
SM-AHNMD	SM associated with a clonal hematopoietic non-MC disorder
TF	Transcription factor

TPO	Thrombopoietin
WBC	White blood cell counts
WT	Wild type

CHAPTER ONE

INTRODUCTION

Core Binding Factor-Acute Myeloid Leukemia

The core binding factor (CBF) is a heterodimeric transcription factor containing transcriptional subunits of enhancers and promoters that play an integral role in hematopoiesis and leukemogeneis (2). *RUNX1* was the first CBF gene to be isolated and is known also as *AML1*, *CBFα2*, and *PEBP2αB*. *RUNX1* is part of the Runt-related transcription factors (RUNXs), and plays a specific role in regulating early embryonic hematopoiesis and specifying hematopoietic stem cells (HSC) (3, 4). The importance of RUNX1 can be appreciated as *RUNX1* knockout mice do not develop fetal liver hematopoiesis, and are embryonic lethal by E11.5-E12.5 due to hemorrhaging in the central nervous system (5). Similar effects are also seen when another CBF subunit, CBFβ, is knocked out during development (6). Furthermore, RUNX1 plays not only a role in fetal hematopoiesis, but is critical for adult hematopoiesis and HSC self-renewal. RUNX1 forms a heterodimeric complex with CBFβ that binds to DNA at the Runt Homolog Domain (RHD) located in the N-terminus of RUNX1. Complexing with CBFβ facilitates RUNX1 binding to DNA and stabilizes the complex from proteolysis. The RUNX1/ CBFβ complex of transcription factors recruits additional cofactors like p900 and CREB-binding protein, all of which is controlled by RUNX1. These cofactors help in acetylating histones and unraveling condense DNA making promoter regions more accessible for transcription. The CBF complex binds the DNA consensus sequence of PyGPyGGTPy (Py is a pyrimidine base) (7). The CBF complex regulates transcription of

a number of crucial hematopoietic-specific genes, including interleukin 3 (IL-3), macrophage colony-stimulating factor receptor (M-CSFR), neutrophil elastase, myeloperoxidase and granzyme B (8-11).

Another transcription family of interest is the protein family Eight-Twenty One (ETO) that comprises of ETO (MTG8), ETO2 (MTG16 or MTGR2) and MTGR1 (12-14). The ETO family members suppress transcription by recruiting histone deacteylases and associated transcription repression complexes such as N-CoR, SMRT, or mSin3A. In 20% of acute myeloid leukemias (AML) a chromosome rearrangement of t(8;21) is found that results in a fusion of the gene RUNX1 on chromosome 21q22 with ETO on chromosome 8q22 (Figure 1.1) (15). As a consequence of this translocation, a fusion protein is generated which contains the N terminal and RHD of the RUNX1 protein and nearly the complete ETO protein (AML1-ETO) (16, 17). This chimeric fusion product, AML1-ETO, retains the ability to bind to CBFβ and to interact with the DNA consensus sequence (7). However, the fusion results in a protein in which ETO actively represses RUNX1-mediated transcription by recruitment of co-repressors N-CoR, SMRT, and mSin3A (18-20). The importance of the CBF complex is further demonstrated by the chromosomal rearrangement inv(16)(p13;q22) resulting in a fusion product CBFβ-MYH11. This fusion protein is present in about 12% of AMLs and 40% of M2-AMLs (21) (Figure 1.1). Like AML1-ETO, CBFβ-MYH11 leads to a disruption of transcription of definitive hematopoiesis and loss of differentiation.

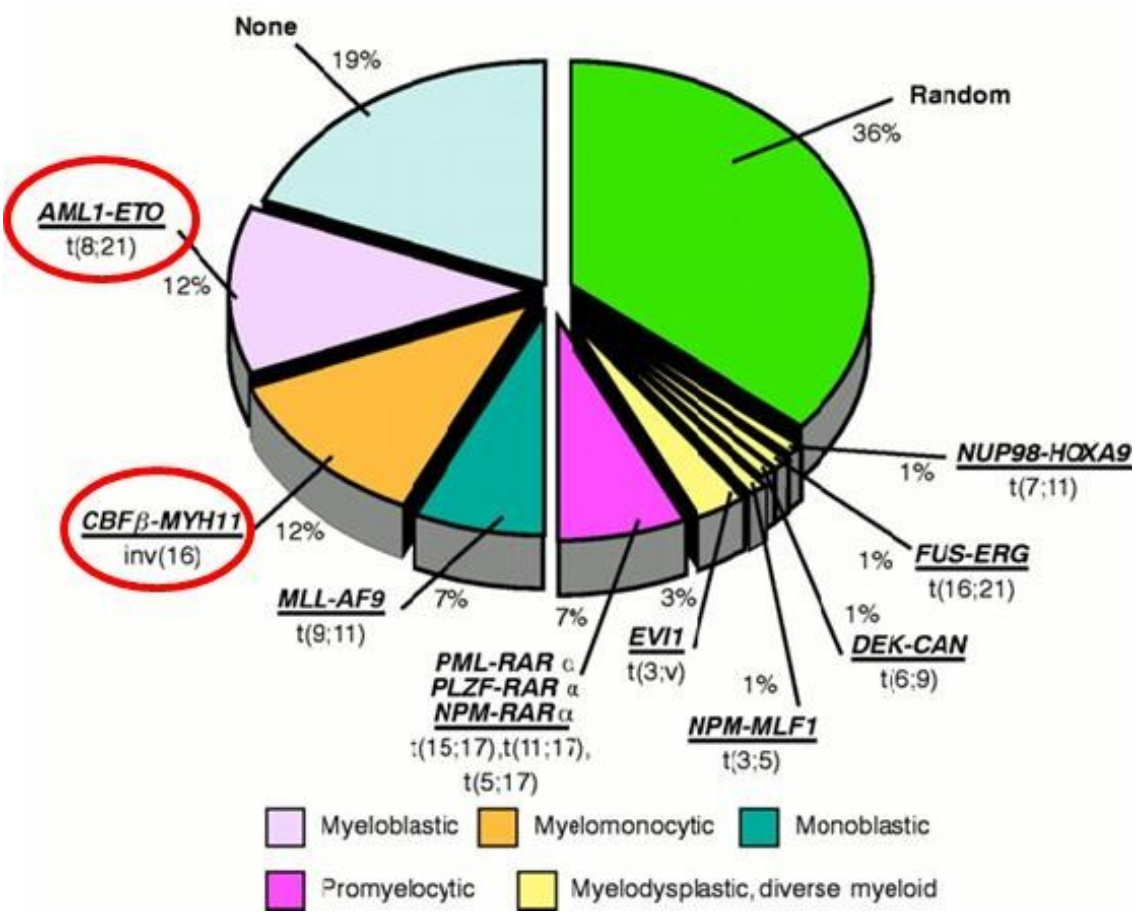


Figure 1.1. Prevalence of chromosomal rearrangements and their associated fusion products in AML patients.

Association of KIT with Core Binding Factor chromosome translocations

Many studies of adults and children show that *de novo* AML associated with t(8;21) or inv(16) alone have a favorable response to treatment with high complete response rates and overall survival (22-24). However, a cooperative model of AML has been substantiated in which more than one acquired mutation is present in most AMLs, a mutation that blocks differentiation and an additional mutation that drives proliferation (Table 1.1). Recent data and reviews support modifications to the Knudson's "two-hit"

hypothesis, in which involvement of genes regulating epigenetics are critical for leukemogeneis (25-28).

Table 1.1. Multiple hits associated with leukemogenesis.

A multiple hit model of leukemogenesis has been proposed to result from the cooperation between multiple classes of mutations. These mutation can be divided into classes that provide a proliferative advantage, can block differentiation potential of cells, and can affect epigenetic regulation.

Proliferation Advantage	Block Differentiation	Epigenetic Regulators
BCR-ABL	**CBFβ–MYH11**	TEL–AML1
N-Ras	**AML1–ETO**	ASXL1
K-Ras	TEL–AML1	DNMT3A
KIT (exon 8)	PML–RARα	EZH2
KIT (Asp 816)	NUP98–HOXA9	
Flt3 (ITD)	PU.1	
Flt3 (Asp 835)	C/CEPα	
PTPN11	AML1	
NF1	AML1–AMP19	

Clinical reports strongly suggest cooperation between activating KIT mutations and core binding factor TFs in a subset of AMLs. Approximately 12.8 to 46.1% of adults with core binding factor leukemias express activating KIT mutations (29-31). Acquired mutations in KIT have been associated with higher incidence of elevated white blood cell (WBC) counts, greater relapse incidence and worse overall survival in AML patients expressing t(8;21) or inv(16) (Figure 1.2). KIT mutations have been observed in CBF-

AMLs and have been observed in multiple clinical studies involving diverse ethnic groups and countries, including studies conducted in Japan, Italy and other parts of Europe and US (29, 31). Importantly, KIT mutations are not observed in many other subsets of AML (32, 33). These studies strongly support the notion of cooperativity between KIT mutations and CBF translocations in a subset of AML, and suggest that KIT mutations may be a poor prognostic indicator on outcome of CBF-AML in both adult and pediatric patients. However, these studies do not directly prove the notion that KIT mutations cooperate with CBF transcription factors (TFs) in CBF-AML. One of the specific aims of my thesis was to investigate the cooperation between KITD816V mutation and CBF TFs in AML.

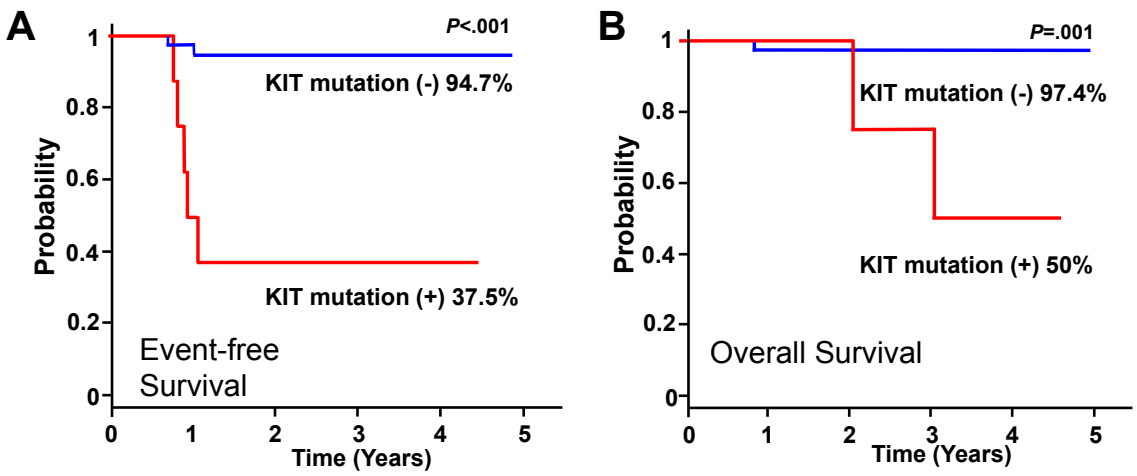


Figure 1.2. Kaplan-Meier survival analysis of AML (AML1-ETO) patients with or without KITD816V mutations.

(A) This study shows 4-year overall survival and (B) disease-free survival of AML1-ETO patients with KITD816V mutation (red line) or without a KITD816V mutation (blue line). The differences are statistically significant. Figure adapted from Shimada et al. (34).

Systemic mastocytosis

Mastocytosis is a heterogenous disorder characterized by an abnormal accumulation of mast cells (MC) in tissues. The disease was first described in 1869 as brown lesions that would welt following scratching (35). The disease was described in 1878 as urticaria pigmentosa and in the following year the mast cell was discovered by Paul Ehrlich (36). The association between an accumulation of MC in the dermis and urticaria pigmentosa was made by Unna in 1887 (37). Depending on the affected organs, mastocytosis can be divided into cutaneous mastocytosis, in which MC aggregates become deposited in the dermis, or systemic mastocytosis (SM) in which MCs become deposited in the bone marrow (BM) as well as extramedullary tissues like spleen, gastrointestinal tract, lymph node, and liver (38). The World Health Organization proposed major and minor criteria for the diagnosis and classification of mastocytosis (Table 1.2), and has described several subvariants: cutaneous mastocytosis, indolent systemic mastocytosis (ISM), aggressive SM (ASM), SM associated with a clonal hematopoietic non-MC disorder (SM-AHNMD), Mast cell leukemia (MCL), and MC sarcoma (MCS) (38-40).

Table 1.2. World Health Organization Diagnostic Criteria for Systemic Mastocytosis.

Criteria
Major criterion
1) Multifocal, dense infiltrates of MC (≥ 15 MC in aggregates) in bone marrow and/or other extracutaneous organ(s)
Minor criteria
1) ≥25% of MC in bone marrow or other extracutaneous organ(s) show an immature or abnormal morphology (typically spindle-shaped)
2) Activating KIT mutation at codon 816 in BM, blood, or other extracutaneous organ(s)
3) MC in BM express CD117 and CD2 and/or CD25 in addition to normal MC markers
4) Total serum trypase ≥20 ng/mL (unless patient has an associated clonal myeloid disorder)
• A diagnosis of systemic mastocytosis requires the fulfillment of the major criterion or at least 3 minor criteria

Although little is known about the pathogenic factors that contribute to mastocytosis, molecular determination of the mutation status of KIT has represented a major step in understanding the molecular mechanisms of this disease. Somatic point mutations in KIT at codon 816 (D816V > 95%; D816H, D816Y and others <5%) are detected in >90% of patients with SM (41-45). These findings suggest that the KIT mutation status is important for clinical diagnosis, and could represent a hallmark of pathogenesis in SM patients.

The KITD816V mutation is prevalent in both aggressive SM (SM-AHNMD, ASM, and MCL) and non-aggressive SM (ISM) (38, 39, 46, 47). The expression of KITD816V alone is insufficient to manifest all forms of mastocytosis *in vivo* (48). It is likely that additional factors may also contribute to aggressive subtypes of SM. To this

end, recent studies have identified genes that are frequently mutated in advanced SM including *TET2*, *DNMT3A*, *ASXL1*, *EZH2*, *RUNX1*, *CBL*, and *IDH1/IDH2* (49-51). TET2 is a tumor suppressor that functions in DNA demethylation (52, 53). Loss of function mutations of Tet2 have been documented in acute and chronic myeloid malignancies (50, 54). Furthermore, a significant growth advantage is observed in primary murine bone marrow-derived MCs expressing KITD816V that are Tet2 deficient relative to wild type cells (55). These findings suggest that additional genetic aberrations to KITD816V may contribute to a more aggressive form of SM.

Structure and function of receptor tyrosine kinase KIT

Receptor tyrosine kinases (RTKs) are widely expressed transmembrane proteins that have critical function in several developmental processes including regulation of cell survival, motility, growth and differentiation (56). Many of these functions are mediated in hematopoietic stem and progenitor cells (HSC/Ps) through the RTK KIT. The proto-oncogene KIT belongs to the RTK class III family, other members include platelet-derived growth factor receptor (PDGFR), macrophage colony stimulating factor receptor (M-CSFR), and Fms-like tyrosine kinase (FLT3) (57-59). The class III family members are membrane bound glycoproteins that are characterized by five immunoglobulin-like (Ig) domains on the extracellular region, a single transmembrane spanning domain and two intracellular kinase domains connected by a kinase insert domain (60). This family of RTKs has a pivotal role in normal hematopoiesis, and many of its family members have been associated with hematological malignancies.

RTK KIT signaling is important in erythropoiesis, lymphopoiesis, mast cell development, megakaryopoiesis, gametogenesis and melanogenesis. KIT activation is driven by binding of the ligand stem cell factor (SCF), which exists as a non-covalent dimer. In the murine system, SCF is encoded by the *steel* (*Sl*) locus while KIT is encoded by *dominant white spotting* (*W)* locus. Loss of function mutations of KIT in humans leads in piebaldism, a congenital hypopigmentation disease characterized by hypopigmented patches on the midforehead, chest, and extremities due to melanocyte defects (61, 62). In mice, the loss of function mutations in the loci of SCF or KIT affects development of hematopoietic cells, melanocytes, germ cells, and survival (63-65). Transgenic mice with knock out of the *KIT* gene or of its ligand, *SCF* gene, are embryonic lethal by day 16 of gestation due to severe anemia (66).

Activation of KIT is driven by SCF binding to the three distal Ig-like domains on the extracellular portion of the receptor. This binding of SCF brings two KIT monomers together and facilitates interactions of the fourth and fifth Ig-like domain causing the formation of a rigid KIT homodimer (67). The cytoplasmic domain of KIT involves a kinase insert that splits the kinase domain into an adenosine triphosphate (ATP)-binding domain as well as a phosphotyrosine activation domain. KIT also has a juxtamembrane domain (JM) that inhibits receptor dimerization and enzyme activity by maintaining control of the α-helix and activation loop, in the cytoplasmic domain. After dimerization, transphosphorylation of two tyrosine residues, tyrosine 568 and tyrosine 570, in the autoinhibitory domain causes a conformational change that keeps the JM domain from immobilizing the activation loop (68). The activation loops are then converted from a compact conformation to an open active conformation allowing for transphosphorylation

of the activation loop tyrosine 823, thus stabilizing activity for binding of the enzyme (69).

Upon non-covalent binding of SCF binding, the KIT homodimer undergoes tyrosine autophosphorylation at specific tyrosine residues in the cytoplasmic domain. The phosphorylated tyrosine residues act as high-affinity-binding site for protein substrates to dock. Recruitment of specific src homology 2 domains (SH2) containing proteins leads to KIT-mediated signal transduction. The activated KIT can then bind an array of intracellular signaling molecules including Src family kinases at tyrosine 567 and 569, Grb2 at tyrosine 702, p85α subunit of class IA of phosphatidylinositol 3-kinase (PI3K) at tyrosine 719, SHP2 phosphatase, phospholipase Cγ at tyrosine 728, Ras-GAP at tyrosine 745, and Grb7 at tyrosine 934, thereby initiating a signaling cascade resulting in various cellular changes (70-72). Although these interactions have been well characterized in WT KIT, little is known concerning individual contribution of these pathways in oncogenic KIT transformation. An additional specific aim of my project was to understand the individual contribution of these pathways on oncogenic KIT induced transformation.

KIT mutations in human neoplasms

A number of tyrosine kinase receptors that function in hematopoiesis have been found to have crucial roles in leukemogenesis. To this end, activating mutations of KIT have been reported in AML, gastrointestinal stromal tumors (GIST), melanomas, NK/T cell lymphomas, SM, and germ line cell tumors (41, 73). Two distinct types of mutations provoke oncogenic activation of KIT, one which involves the JM domain and the other involves the phosphotransferase domain in normal hematopoietic stem cells and cell lines

(Figure 1.3) (74, 75). Mutations of the JM domain lead to constitutive dimerization of KIT in the extracellular domain leading to constitutive activation (e.g. KITV560G, associated with **(**GISTs) in the absence of ligand stimulation. In contrast, KIT mutations of the activation loop (e.g. KITD816V, associated with AML and SM), do not result in dimerization, but are found rather to result in receptor self-association in the cytoplasmic domain (76). The mechanism of self-association in the absence of dimerization is not well understood, including whether the extracellular domain is necessary for KITD816V function, constitutive activation and capabilities for signal protein association, in a ligand independent manner (77). Transforming potential in the absence of the extracellular domain strongly suggests that the self-association observed in KITD816V is conferred upon the receptor via the D816V mutation in the cytoplasmic domain, rather than influences from the extracellular domain. Although a significant amount is known in our structural understanding of the KITD816V receptor, there is still relatively little understood about the oncogenic signaling pathways and regulation that contribute to the receptor's transforming ability.

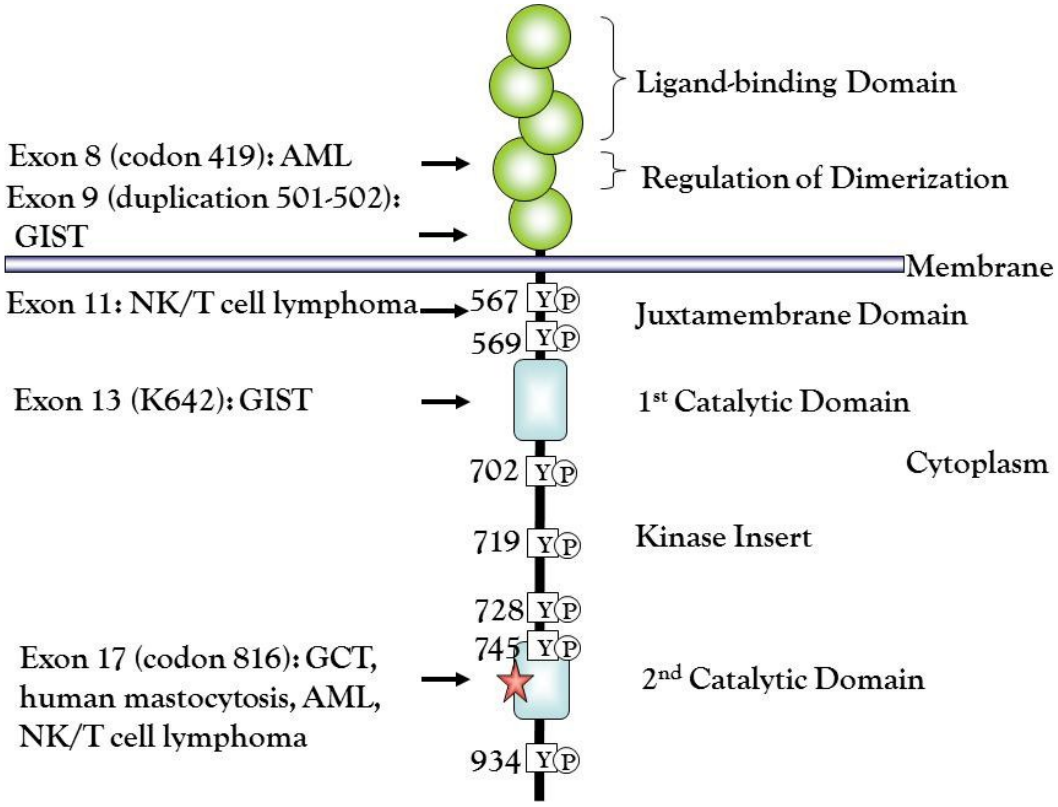


Figure 1.3. Schematic representation KIT and KIT mutations associated with AML, SM and other human malignancies.

Adapted from Heinrich et al. (1)

KIT-targeted therapies

Small molecule therapy has been shown to be beneficial for many neoplastic disorders including imatinib (STI-571, Gleevec), which is a clinically effective protein receptor tyrosine kinase inhibitor. Not only has imatinib been effective in treating chronic myelogenous leukemia with the t(9;22) (BCR-ABL) translocation, but this molecule has also been efficacious against activating mutations in KIT in the JM domain that have been associated with GISTs and melanomas (78). Imatinib functions by targeting the inactive conformation of KIT and binds sites in the ATP-binding domain thereby inhibiting its kinase activity (79, 80). These observations suggest that imatinib would be

beneficial for other neoplasms involving activating mutations of KIT like SM or AML. However, mutations within the activation domain of KIT, such as those found in the KITD816V version of the receptor, are highly resistant to such therapy (81). Modeling has demonstrated that mutations near sites of drug-protein interaction induce conformational changes in the activation loop that reduce the binding affinity of KIT for imatinib, thereby reducing drug efficacy (82). Imatinib is potentially promising for effective treatment against GISTs and melanomas with JM mutations or in ASM and MCL patients who are negative for KITD816V mutation. However, it is proven to be an ineffective treatment for neoplasms harboring the KITD816V mutation (1, 83).

Other small molecule inhibitors that have been found to be clinically active by US Food and Drug Administration include sunitinib for treatment against KIT in GISTs (84). Sunitinib inhibits KIT mutations and shows inhibitory effects against VEGF making it more effective than imatinib, likewise dasatinib is a Src/Abl kinase inhibitor that is 300-fold more potent than imatinib (85, 86). Although both of these inhibitors have significant activity against wild type KIT, they are both ineffective treatments against neoplasms bearing KITD816V, and have shown little to variable efficacy in clinic (87).

Several drugs aimed to target the KIT receptor have been largely ineffective at treating patients bearing the KITD816V mutation. Several other inhibitors of KITD816V are currently under clinical investigation that exhibit adequate efficacy and safety profiles to be considered for further development as therapeutic in combination with standard therapy for AML. For instance midostaurin (PKC412), a broad-spectrum inhibitor that targets FLT3, KIT, PDGFR, and c-FMS, is presently recruiting AML patients bearing a KITD816V mutation for a Phase I/II trial (88).

An approach to bypass KITD816V resistance to inhibitors targeting receptor tyrosine kinases would be to target downstream effectors of KITD816V signaling. Therefore, downstream effectors of oncogenic KIT need to be evaluated as potential novel therapeutic targets for patients with AML and SM bearing a KITD816V mutation. An additional specific aim of my thesis was to identify novel therapeutic targets that may be involved in the transforming potential of the KITD816V mutation downstream from oncogenic KIT.

Altered signaling via the KITD816V receptor

Mutation of aspartic acid to valine in exon 17 of the activation loop of KIT, KITD816V (human) and KITD814V (murine), leads to alterations in the recognition of downstream substrates (89). The KITD816V mutation results in constitutive activation of the receptor and leads to aberrant signaling regulation and promiscuous signaling. Alterations in signaling include the activation of signal transducer and activator of transcription 3 (STAT3) as well as degradation of KIT-signal inhibitory molecules, such as SHP-1 (90). Identifying molecular and regulatory changes in the activating KIT mutation that contributes to its oncogenic transformation may allow for alternative therapeutic targets. In the murine model, one strategic approach is to identify and directly target KITD814V effector molecules that contribute to the transformation ability of oncogenic KITD814V-bearing cells. Functional and pharmacologic studies using cell line models and an inhibitor of PI3K, wortmannin, have indicated that the lipid kinase PI3K may have a role in contributing to the oncogenic potential of KITD814V (91). Utilizing an *in vivo* model of myeloproliferative neoplasms (MPN) our lab has demonstrated that

deficiency of p85α regulatory subunit of PI3K rescues the transformation by KITD814V. Unfortunately, although useful in *in vitro* systems, the PI3K inhibitor wortmannin is less than ideal in an *in vivo* setting due to its broad spectrum, nonspecific inhibition, and extreme toxicity (92). PI3K is a key regulator in many different physiological events including cell proliferation, differentiation, apoptosis, cytoskeletal organization and membrane trafficking (93-95). Thus targeting specific downstream effectors of PI3K may provide a better therapeutic alternative. Lines of evidence indicate that PI3K activity is necessary for several Rac-regulated cellular functions, and that products of PI3K activate guanine exchange factors (GEFs) for Rac including Vav1 (Figure 1.4.A), which promote the conversion of inactive guanosine diphosphate (GDP)-bound Rac to active guanosine triphosphate (GTP)-bound form (Figure 1.4.B) (72, 96, 97). An additional specific aim of my thesis was to assess the role of Vav1 and Rac in KITD816V induced transformation and their potential as therapeutic targets.

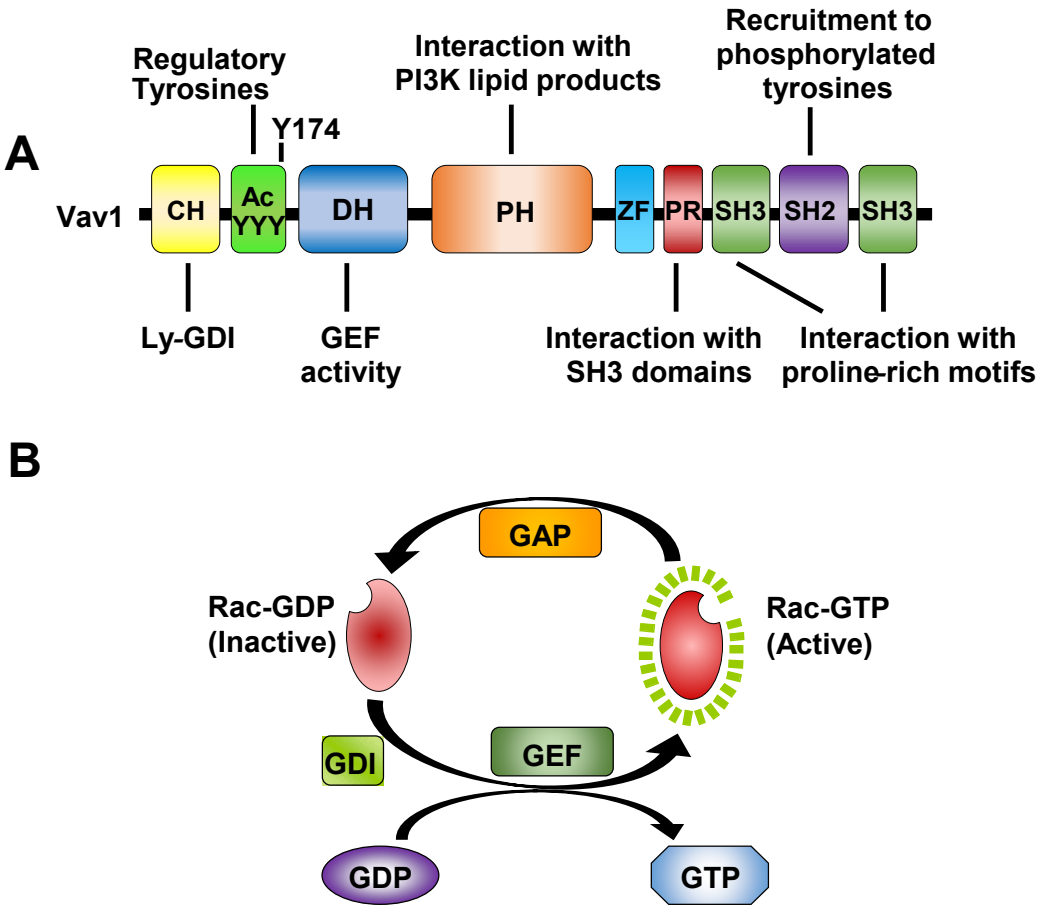


Figure 1.4. Schematic of (A) guanine nucleotide exchange factor (GEF) Vav1 and (B) Rac GTPase activation loop.

Regulation of guanine exchange factor Vav1

Dysregulation of Vav1 has been associated with transformation, in which mutations including substitution of tyrosine to phenylalanine at residue 174 of Vav1 or the amino terminal deletion of the calponin-homology (CH) domain and acidic (AC) motif of Vav1 induces transformation (Figure 1.4.A) (98, 99). Vav1 has a role in transformation, it is unclear what the biological effects of hyperactivation of Vav1 by oncogenic mutation in a receptor tyrosine kinase, such as KIT are. The effects in primary hematopoietic cells have yet to be addressed. Vav1, as well as family members Vav2 and

Vav3, consist of several conserved domains, including a dbl homology (DH) region, a pleckstrin homology (PH) domain, a SH2 and two Src-homology3 (SH3) domains, and exhibit GEF function that activate specific members of the Rho GTPase family (Figure 1.4.A) (100). Vav1's catalytic activity is tightly regulated by phosphorylation of tyrosine 174 in the AC domain, relieving auto-inhibitory intra-molecular interactions involving the N-terminal of Vav1 that block access to the DH domain, thereby activating GEF activity (101). The PH domain also regulates GEF activity through the binding of PI3K products (Figure 1.4.A). Of the known Rac GEFs, Vav proteins are the only ones known to have three Src-homology domains that are able to recruit adaptor molecules independent of its GEF activity.

Regulation of Rac GTPases

The GEF catalytic activity of Vav1 is relatively specific to the Rac subfamily of Rho GTPases (Rac1 and Rac2). The Rac subfamily of Rho GTPases have been implicated in a wide range of cellular functions, including structure of actin cytoskeleton, transcriptional and translational activity, cell survival, cell cycle entry as well as being implemented in transformation (102, 103). Dysregulation of the Rac subfamily has been associated with a number of human cancers including, leukemias, lymphomas, gastric tumors, breast cancer, and head and neck squamous cell carcinomas (104, 105). These proteins act as molecular switches that cycle between an inactive GDP-bound conformation and an active GTP-bound conformation (Figure 1.4.B). The Rac GTPase subfamily includes three structurally related proteins, Rac1 and Rac3 are ubiquitously expressed, while the third protein Rac2 is restricted to the hematopoietic compartment.

Studies involving Rac1 and/or Rac2 deficient mice have shown that the Rac proteins are essential in multiple hematopoietic lineages; the deficiency of both Rac1 and Rac2 demonstrates the most profound effects (106).

Summary and Significance

It has been hypothesized that KIT activation loop mutants, including KITD816V (human) and its murine homologue KITD814V, alter the specificity of KIT substrate recognition and utilization (89). As a result signals emanating from the oncogenic KIT (KITD816V) can be promiscuous in nature and induce aberrant signals not normally regulated by wild type KIT, including the activation of STAT3 and the degradation of KIT-signaling inhibitory molecules, such as SHP-1 (89, 90, 107). Given the fact imatinib and other second generation tyrosine kinase inhibitors, such as sunitnib and dasatinib, have shown poor efficacy against this receptor in clinical trials, an alternative therapeutic approach to directly targeting KIT could involve targeting KIT effector molecules that contribute to the transformation of KITD816V-bearing cells. Previous studies by our lab and others have shown that the lipid kinase, PI3K, maybe necessary and sufficient in the transforming ability of D816V (murine D814V) (91, 97, 108). Although the use of wortmannin as a PI3K inhibitor is useful in experimental *in vitro* systems, this inhibitor is non-specific, and is associated with high cytotoxicity *in vivo* (92). Multiple lines of evidence demonstrate that several Rac-regulated cellular functions depend on PI3K activity, and that products of PI3K activate GEFs for Rac including Vav, which promote the conversion of inactive Rac-GDP to active Rac-GTP (96, 109). Importantly among the various Vav and Rac family members Vav1 and Rac2 are expressed specifically in

hematopoietic cells and deficiency of these molecules perturbs specialized functions in mature leukocytes without significantly affecting the growth of hematopoietic progenitors. We hypothesized that the Vav/Rac pathway may be a useful pathway to target disease involving KITD814V mutation.

The main objective of this study was to understand the molecular mechanisms contributing to KITD816V-induced transformation in order to determine molecular targets and potential molecular-based therapeutic options to treat KITD816V-positive AML and SM patients. Our studies specifically address the following aims:

SPECIFIC AIM 1: We hypothesized that endogenous ligand binding is not necessary for KITD814V-induced MPN. We utilized a chimeric receptor lacking the extracellular region of the KIT receptor to determine if the KITD814V mutation can induce ligand-independent growth *in vitro* and MPN *in vivo*.

SPECIFIC AIM 2: We hypothesized that the tyrosine residues within the JM and/or the kinase insert region of KITD814V play an essential role in ligand independent growth *in vitro* and transformation *in vivo*. We dissected the role of tyrosines in the JM and kinase insert region of KITD814V in transformation and activation and binding of downstream effectors such as PI3K, AKT, ERK, and STAT5.

SPECIFIC AIM 3: We hypothesized that the hematopoietic specific guanine exchange factor Vav1 contributes to transformation by KITD814V. We determined the extent to which deficiency of Vav1 in hematopoietic stem/progenitor cells contributes to the MPN phenotype by KITD814V using a genetic approach.

SPECIFIC AIM 4: We hypothesized that the Rho family GTPases Rac1 and Rac2, downstream effectors of Vav1, contribute to KITD814V induced transformation.

We determined the extent to which Rac1 and/or Rac2 GTPases contribute to KITD814V induced transformation *in vitro* and MPN *in vivo.*

SPECIFIC AIM 5: We hypothesized that AML1-ETO and KITD814V cooperate *in vitro.* We determined the contribution of co-expressing AML1-ETO and KITD814V *in vitro* on growth and activation of downstream effectors.

SPECIFIC AIM 6: We established murine models to study primary human AML and SM patient samples or patient-derived cells to study AML and SML *in vivo* both locally and systemically as models for therapeutic regimes.

Our overall proposed molecular mechanism for KITD814V activation is generalized by the schematic diagram in Figure 1.5.

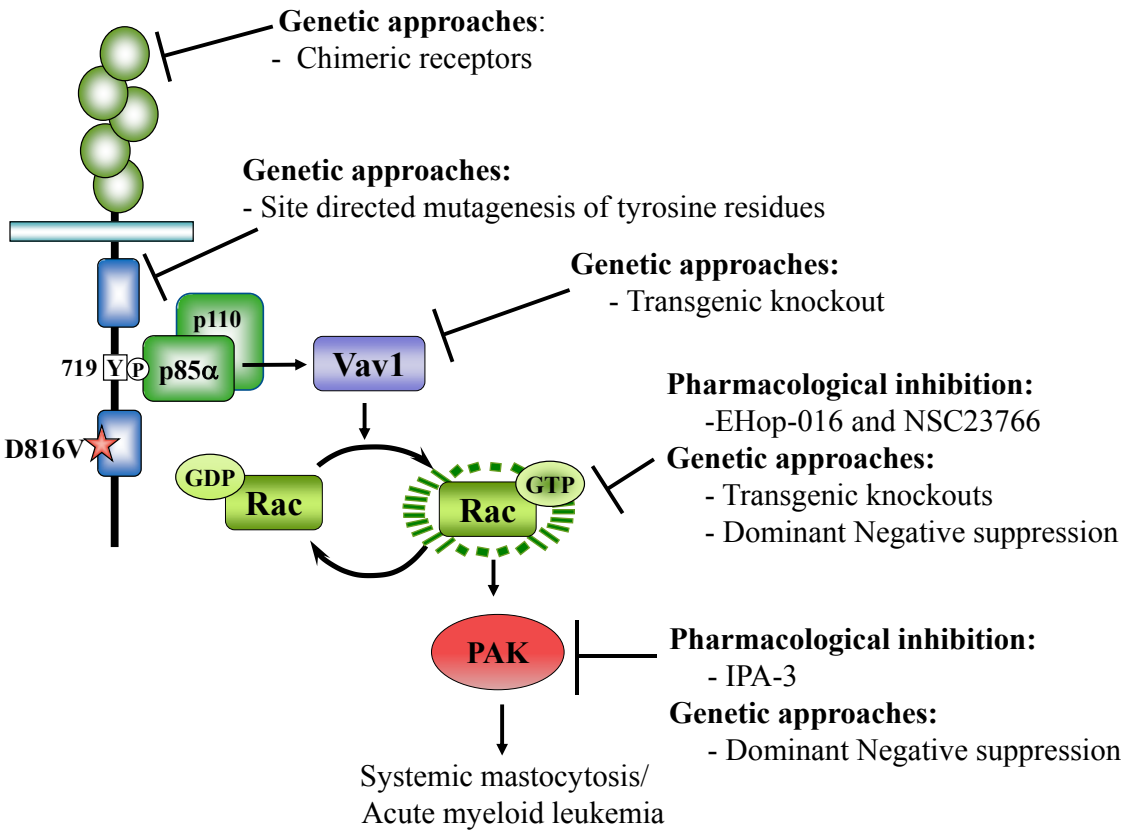


Figure 1.5. Schematic of proposed KITD814V molecular mechanisms described and identified in these studies.

CHAPTER TWO

MATERIALS AND METHODS

A. Materials

1. Plasmids

cDNA for WT KIT, KITD814V, AML1-ETO, RACN17, and CHR receptor constructs were expressed into an improved murine stem cell virus (MSCV)-based bicistronic retroviral vector, MIEG3, upstream of the internal ribosome entry site (IRES) and the enhanced green fluorescent protein (EGFP) or human CD4 (hCD4) gene.

2. Primers

Standard polymerase chain reaction (PCR) reactions for genotyping transgenic animals were performed using oligonucleotides synthesized by Invitrogen.

Table 2.1. Genotyping primers

Gene	Forward (5′-3′)	Reverse (5′-3′)	Null (5’-3’)
Rac1 flox	5’-TCC AAT CTG TGC TGC CCA TC-3’	5’-CAG AGC TCG AAT CCA GAA ACT AGT A- 3’	5’-GAT GCT TCT AGG GGT GAG CC-3’
Rac2 null	5′-GAC GCA TGC TCC ACC CCC T-3’	5′-CAC ACA CTT GAT GGC CTG CAT-3’	5′-TGC CAA GTT CTA ATT CCA TCA GAA GC-3’
Mx-Cre	5′-CAA CCT CAG TAC CAA GCC AAG-3′	5′-CTT CCC AGG GAG AAT CAA AGC-3′	5′-CGC ATA ACC AGT GAA ACA GCA T- 3′
KITD814V	5′-GAA AGA GCG GCA GAC AAG AG-3’	5′-TGA GGT CTC TCA GCT CAG GTG-3’	5′-AGA GGC CAC TTG TGT AGC GC-3’

Table 2.2. Sequencing Primers for Confirmation of Mutagenesis of Internal Tyrosine Residues

CHR primers	Sequence	Region
KIT 2270	5'-GGA TGA TTT GCT GAG CTT CTC- 3'	Covers region with codon 814 of KIT
KIT 1	5′-CAT TGA GTG CAG AAG GTT C-3'	Covers region with codon 814 of KIT
KIT 2	5′-AAG AAG AGC AGG CAG AAG C-3′	Covers region with codon 814 of KIT
CSFR-1	5′-TTC ACC ATC CAC AGG GCC AAG-3'	Covers region with codons 567 & 569 of KIT
CSFR-2	5'-TTT TAA CTG GAC CTA CCT GG-3'	Covers region with codons 567 & 569 of KIT
CSFR-3	5'-TGG GAT GAC TTT CTG CAC TTT C-3'	Covers region with codons 567 & 569 of KIT
Y934F	5'-GCA CCA AGC ACA TTT TTT CCA ACT TGG CAA AC-3'	Covers region with codon 934 of KIT
Y934R	5'-GTT TGC CAA GTT GGA AAA AAT GTG CTT GGT GC-3'	Covers region with codon 934 of KIT

3. Patient Samples

AML- and SM- derived patient peripheral blood, or bone marrow aspirate samples were obtained with prior patient consent. Using ficoll gradient, low density mononuclear cells were isolated and provided to us by Drs. Ramon Tiu and Valeria Visconte at Cleveland Clinic (Cleveland, OH) and Dr. Scott Boswell (IUSM).

4. Mice

All mice were maintained under specific pathogen-free conditions at the Indiana University Laboratory Animal Research Center (Indianapolis, IN, USA), and were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine study numbers 3137 and 10311.

a. C57BL/6

C57Bl/6 mice were purchased from Jackson Laboratory (Bay Harbor, ME, USA). All mice used in this study were between 6 to 12 weeks of age.

b. Rac1$^{flox/flox}$

Rac1 deficient mice are embryonic lethal at day 8 (110). Using a Rac1$^{flox/flox}$ conditional mice were crossed with Mx1-Cre animals. These mice possess a flox allele that contains *loxP* sites on either side of exon 1 of the targeted Rac1 gene (111). To delete the flox sequences a Mx1-Cre promoter was used, in which Cre was driven by the interferonγ-inducible Mx1 promoter following three intraperitoneal injections of 200μg polyinosinic:polycytidylic acid (polyI:polyC) given at 48 hour intervals.

c. Rac2$^{-/-}$

Rac2 null mice are a transgenic knockout mouse in C57BL/6 background strain (112).

d. Rac1$^{flox/flox}$;Rac2$^{-/-}$

To generate this strain Mx1-Cre;Rac1$^{flox/flox}$ mice were crossed with Rac2$^{-/-}$ transgenic knockout mice. Double heterozygote mice with Mx1-Cre were crossed to generate Mx1-Cre;Rac1$^{flox/flox}$;Rac2$^{-/-}$ mice. The Mx1-Cre double knockout mice strain was generated in which Rac1 transgene was under the control of Mx1-Cre promoter, with Rac2 being a complete transgenic knockout.

e. Vav1$^{-/-}$

Guanine exchange factor, Vav1, knockout mice were in C57BL/6 background (113).

f. C3H/HeJ

C3H/HeJ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA).

g. NOD/SCID (NOD.CB17-*Prkd^{scid}/SzJ)*

The NOD.CB17-*Prkd^{scid}/SzJ* mice, commonly known as NOD/SCID, do not express the *Prkdc* gene. These mice lack functional T and B lymphoid cells. NOD genetic background results in reduced NK cell activity. These mice accept allogeneic and xenogeneic grafts effectively and have been used successfully to transplant a variety of normal and malignant human cells and tissues, including hematopoietic neoplasms (114). Mice were purchased from the In Vivo Therapeutics Core at Indiana University School of Medicine.

h. NOD/SCID/IL2Rnull (NSG) (NOD.CB17-*Prkd^{scid}Il2rh^{tmWjl}*/SzJ)

The NOD.CB17-*Prkdc^{scid}Il2rh^{tmWjl}*/SzJ mice, commonly known as NOD/SCID gamma (NSG), do not express the *Prkdc* gene nor the X-linked *Il2rg* gene. Histologically these mice have the absence of mature B and T lymphoid cells and lack functional natural killer cells. NSG mice lack some cystic structures in the thymus, have an absence of follicles in the spleen and a markedly diminished cellularity of the lymph nodes. These mutant mice readily support engraftment of human CD34+ hematopoietic stem cells and represent a superior, long-lived model suitable for studying localized and systemic xenotransplantation (115). Mice were purchased from the In Vivo Therapeutics Core at Indiana University School of Medicine.

i. NSG 3 Cytokine (CMV-IL3, CSF2, KITLG) (NOD.CB17-*Prkdc*^{scid}*Il2rh*^{tmWjl}*(CMV-IL3 CSF2, KITLG)*1Eav.MloySzJ)

The NOD.CB17-*Prkdc*^{scid}*Il2rh*^{tmWjl}*(CMV-IL3 CSF2, KITLG)*1Eav.MloySzJ mice were developed at Cincinnati Children's Hospital Medical Center. This NOD scid gamma (NSG) strain does not express the *Prkdc* gene nor the X-linked *Il2rg* gene, while allowing constitutive transgenic expression of three human cytokines stem cell factor (SCF), granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3). These mice harbor the same physiological properties as the NSG mice, while expressing three human cytokines leading to improvements in expansion of normal human myeloid cells and xenotransplantation of AML samples (116). Mice were purchased from the In Vivo Therapeutics Core at Indiana University School of Medicine.

5. Antibodies

Table 2.3. Antibodies for Immunoblotting

Primary Antibodies	**Company**	**Molecular Weight**
KIT (C-19)	Santa Cruz	~145 kDa
Rac1, clone 23A8	Millipore	~21 kDa
Rac2	Millipore	~21 kDa
Vav (C-14)	Santa Cruz	95 kDa
β-actin	Santa Cruz	40 kDa
Bcl-Xs/l (L-19)	Santa Cruz	27 kDa
Phospho-PAK	Cell Signaling	68 kDa(Pak1) and 61kDa (Pak2)
PAK	Cell Signaling	68 kDa(Pak1) and 61kDa (Pak2)
Phospho-Tyrosine	Cell Signaling	multiple
Erk	Cell Signaling	42 and 44 kDa
Phospho-Erk	Cell Signaling	42 and 44 kDa
Akt	Santa Cruz	60 kDa
Jak2	Santa Cruz	125 kDa

Stat5	Santa Cruz	90 kDa
Phospho-Stat5	Cell Signaling	90 kDa
Bcl-2	Santa Cruz	28 kDa
P85α	Santa Cruz	85 kDa
P-Akt	Cell Signaling	60 kDa
Bax	Santa Cruz	20 kDa
Rac1/2/3	Millipore	21 kDa
Cdc42	Millipore	21 kDa

Table 2.4. Horseradish Peroxidase Secondary Antibodies

Secondary Antibody	Vendor
Goat Anti-Rabbit IgG horseradish peroxidase (HRP)	Santa Cruz
Goat Anti-Mouse IgG HRP	Santa Cruz

6. Commercially purchased kits

Table 2.5. Commercially purchased kits

Kit	Vendor	Catalog Number
Plasmid Maxi-Prep	Invitrogen	K210007
SuperSignal West Dura Extended Duration Substrate	Thermo Scientific	34076
Mini-prep	Qiagen	27106
Profection Mammalian Transfection System	Promega	E1200
Rac1 Activation Assay	Millipore	17-283
Annexin V Apoptosis	BD Pharmingen	556547

QuikChange Site-Directed Mutagenesis	Agilent Technologies	200519

B. Methods

1. Cell Culture

a. 32D Cells

The 32D cell line is an immortalized interleukin-3 (IL-3) dependent myeloblast-like cell line derived from murine bone marrow that was purchased from American Type Culture Collection (ATCC). These cells were cultured in RPMI-1640 (Invitrogen), 10% heat-inactivated fetal bovine serum (FBS) (Hyclone; Thermo Fisher Scientific), 2% penicillin/streptomycin (Invitrogen), and 10ng/ml murine IL-3 (PeproTech) (117). Cells were grown at 37˚C and 5% CO_2.

b. P815

The murine P815 lympho-blast like mastocytoma cell line was maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% FBS (Hyclone; Thermo Fisher Scientific), 2% penicillin/streptomycin (Gibco) (118). Cells were grown at 37˚C and 5% CO_2.

c. HMC1.2

The HMC1.2 cell line was established from the peripheral blood of a patient with mast cell leukemia (119). This cell line harbors a juxtamembrane domain mutation Gly560Val and an additional Asp816Val tyrosine kinase domain mutation in KIT (120-122). HMC-1.2 cells were cultured in RPMI 1640 (Invitrogen), supplemented with 15% heat-inactivated FBS (Hyclone; Thermo Fisher Scientific) and 1%

Penicillin/streptomycin at 37˚C and 5% CO_2. The HMC1.2 cell line was a kind gift from Dr. Clifford Takemoto (John Hopkins Children's Center, Baltimore, Maryland).

d. Kasumi-1

The Kasumi-1 cell line was derived from peripheral blood of a 7-year-old Japanese boy with AML in relapse after bone marrow transplantation. Kasumi-1 leukemic cells line has an 8;21 chromosome translocation that juxtaposes AML1 with ETO (or MTG8) gene, giving rise to the fusion gene AML1-ETO, hence the cells produce a chimeric AML1-ETO protein (123). The cell line also harbors an additional Asp822Lys ligand-independent KIT activating mutation. The cells are positive for myeloperoxidase showing a morphology of myeloid maturation (124). The cell line was cultured in in RPMI 1640 (Invitrogen), supplemented with 15% heat-inactivated FBS (Hyclone; Thermo Fisher Scientific) and 1% Penicillin/streptomycin at 37˚C and 5% CO_2. The Kasumi-1 cell line was a gift from Dr. Christopher Klug (University of Alabama at Birmingham, Birmingham, Alabama).

e. Primary Low Density Mononuclear Cells

Primary murine low density mononuclear cells (LDMNCs) were cultured in RPMI 1640 (Invitrogen), 20% FBS (Hyclone; Thermo Fisher Scientific), 2% Penicillin/streptomycin (Invitrogen), 100 ng/mL Stem Cell Factor (SCF), 100ng/mL Thrombopoietin (TPO), 50ng/mL Flt-3 Ligand (FL), and 4ng/mL murine interleukin-6 (IL-6) all purchased from Peprotech (Rocky Hill, NJ). LDMNCs were maintained at 37˚C and 5% CO_2.

f. Phoenix Packaging Cells

Phoenix-GP is a second generation retrovirus producer cell line for the generation of ecotropic retroviruses. This cells line is highly transfectable with calcium phosphate mediated transfection and expresses only gag-pol but may be further pseudotyped with retroviral virions with envelope, this cell line was developed by Garry P Nolan at Stanford University. These cells were cultured in DMEM (Invitrogen), supplemented with 10% FBS (Hyclone; Thermo Fisher Scientific), 1% L-glutamine, and 2% penicillin/streptomycin on gelatin-coated, tissue culture-treated 10cm plates at 37˚C and 5% CO_2.

g. Patient-derived leukemic blasts

Cells from the bone marrow and peripheral blood (PB) of patients with systemic mastocytosis (SM) were cultures in 100ng/mL of human recombinant SCF in IMDM supplemented with 20% heat-inactivated FBS and 2% penicillin/streptomycin in tissue culture treated 6-well plates at 37˚C and 5% CO_2.

h. MV4;11

MV4;11 cell line was established from the blast cells of a 10-year-old male with biphenotypic B-myelomonocytic leukemia harboring a translocation t(4;11). These cells express a homozygous 30-base pair (bp) FLT3-ITD mutation. Cultures were maintained in RPMI-1640 (Invitrogen), supplemented with 10% heat-inactivated FBS (Hyclone; Thermo Fisher Scientific) and 1% Penicillin/streptomycin at 37˚C and 5% CO_2.

2. Thymidine Incorporation Assay

Proliferation was assessed by conducting a thymidine incorporation assay. Cells were washed and starved in IMDM containing 0.2% BSA without serum or growth factors for 6 hours. Cells (5 x 10^4) were plated in replicates of four in a 96-well plate in 200 μL complete medium (IMDM, 10% FBS, 2% Penicillin-Streptomycin) either in the presence or absence of indicated growth factors with or without inhibitors. Cells were cultured for 48 hours and subsequently pulsed with 1.0 μCi (0.037 MBq) [^{3}H] thymidine (Perkin Elmer, Shelton, CT) for 6 hours. Cells were harvested using an automated 96-well cell harvester (Brandel, Gaithersburg, MD) and thymidine incorporation was determined as counts per minute (CPM).

3. Subcloning

cDNA encoding wild type murine KIT or KITD814V were subcloned from a murine stem cell virus (MSCV)-based bicistronic retroviral vector, MIEG3, with an enhanced green fluorescent protein (EGFP) tag to a bicistronic retroviral vector MIEG3 with a hCD4 tag (125). The EcoR1-NotI fragment containing the KITD814V mutation was verified by sequencing and ligated into the EcoR1-NotI sites of the MIEG3-hCD4 retroviral vector. pCMV6 expressing a Myc-tagged kinase-dead (K299R) Pak was a gift from Jeffrey Field (University of Pennsylvania, Philadelphia, Pennsylvania, USA) (126). Plasmids encoding RacN17 have been previously described (104). The cDNAs were subcloned into a bicistronic retroviral vector, MIEG3, upstream of the internal ribosome entry site (IRES) and the EGFP gene (125) or human CD4 gene. The pLNCX2-

PAK1T423E-IRES2- EGFP plasmid has been described (127). Plasmids encoding MIEG3-RacN17 and MSCV-puro-RacV12 have been previously described (128).

4. Retroviral Supernatant Production

Retroviral supernatants for the transduction of 32D cells and primary BM LDMNCs were generated with a Phoenix ecotropic packaging cell line transfected with retroviral vector plasmids using a calcium phosphatase transfection kit (ProFection® Mammalian Transfection System, Promega, Madison, WI). Supernatants were collected 48 hours after transfection and filtered through 0.45-μm membranes. Supernatants were used fresh or following storage at -80°C.

5. Retroviral Infection

a. Primary murine bone marrow low density mononuclear cells

BM LDMNCs were suspended in non-tissue culture plates along with prestimulation media (IMDM containing 20% FBS and 2% penicillin-streptomycin supplemented with 100ng/ml TPO, SCF, 50ng/mL Flt3 Ligand, and 4ng/mL IL-6) for 2 days prior to two rounds of retroviral infection using 2ml of retroviral particles encoding pMIEG3-WT KIT, pMIEG3-KITD814V, pLNCX2-PAK1T423E, or MSCVpuro-RacV12 plasmids on fibronectin fragments (Retronectin; Takara). Forty-eight hours after the last infections, cells expressing WT KIT or KITD814V were sorted to homogeneity based on EGFP expressing. MSCV puro cells were selected with 1μg/ml puromycin (Sigma-Aldrich) for an additional 48 hours.

b. 32D cells

32D cells were infected with 2 ml of high-titer virus supernatant in the presence of 8 μg/ml polybrene in complete media (RPMI 1640 containing 10% FBS and 2% penicillin-streptomycin supplemented with 10 ng/ml murine IL-3), and plated into 6-well plates. The plates were centrifuged for one hour at 1,200 rpm to ensure the viral particles and cells were in close proximity prior to incubation at 37˚C for 5 hours. Cells were collected and incubated in fresh complete media and IL-3 overnight at 37 ˚C. Infection was repeated the following day before cells were sorted and expanded. EGFP-positive cells or human CD4-positive (hCD4-positive) cells were sorted to homogeneity and used in the experiments.

6. Cell Sorting

Retroviral transduced cells were collected and sorted using fluorescence-activated cell sorting (FACS) analysis, collecting either enhanced green fluorescent protein positive (EGFP+) or hCD4 positive expressing cells.

7. Immunoprecipitation and Western Blotting

0.5-1mg total cell lysates were immunoprecipitated with anti-p85α, anti-Vav1, or anti- KIT antibody and protein-A or protein-G sepharose beads (GE Healthcare) in Cell Lysis Buffer (consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM Na_2EDTA, 1% Triton, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 0.1 mM leupeptin) supplemented with 10 μl/ml Protease Inhibitor Cocktail (Sigma-Aldrich) overnight while rotating at 4˚C. The following day, beads were

centrifuged and washed several times with cold Cell Lysis Buffer, and immunoprecipitated proteins resuspended in sample buffer. Immunoprecipitated products were boiled before loaded and run on SDS-PAGE gels. The gels were transferred to nitrocellulose which was blocked in 5% nonfat milk in TBS, following blocking the membranes were incubated with the indicated primary antibody, followed by incubation with secondary HRP-linked antibodies (Cell Signaling, Beverely, MA). Detection of protein bands was performed using Super Signal West Dura Extended Duration Chemiluminescence (Thermo Scientific, Rockford, IL). Chemiluminenesce signals were detected with a BioRad BioImager and analyzed using ImageLab software.

8. Apoptosis Assay

Apoptosis was assessed following starvation of cells and subsequently plating them in 24-well plates (2 x 10^5 cells) in 2 mL of complete media (IMDM, 10% FBS, 2% Penicillin/Streptomycin) in the absence of growth factors. Apoptosis was determined following staining with an Annexin V Apoptosis Detection kit (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol and analyzed by flow cytometry.

9. Cell Cycle Assay

For cell cycle assay, cells were deprived of serum and growth factors and plated in 24-well plates (2x10^5 cells/ well) in 2 mL of complete media in the absence of growth factors for 48 hours at 37°C. Cells were collected into individual 5 mL polystyrene tubes (FACS tubes), washed with PBS and the cells were stained with propidium iodide (PI)

staining solution (0.1 μg/ml PI, 0.6% NP-40 , and 100μg/ml DNase-free RNase A) in PBS for 10 min at room temperature. Stained cells were analyzed on a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) and data was analyzed using the CellQuest software or ModFit LT for Mac (Verity Software House, Topsham, ME).

10. Transplantation Assay

a. Syngeneic Transplantation Model

Transplantation into C3H/HeJ mice was carried out by administering a single i.v. injection of 2×10^6 32D cells bearing WT KIT or KITD814V with or without RacN17 or PakK299R, or 1×10^6 KITD814V-bearing 32D cells cultured overnight with DMSO (vehicle), 25 μM NSC23766, or 2.5 μM EHop-016. Mice were harvested at the time of moribundity, and PB, femurs, spleen, lungs, and liver were collected for histopathological and flow cytometric analysis.

b. Primary Murine Transplantation Model

WT or *Vav1*$^{-/-}$, *Rac2*$^{-/-}$, and Mx1-cre;*Rac1*$^{flox/flox}$;*Rac2*$^{-/-}$ C57BL/6 mice that were 6-to-8 weeks of age were given a single i.p. injection of 150 mg/kg of 5-fluorouracil (5-FU) (APP Pharmaceuticals). BM cells were collected 72 hours after injection from the tibia, femur, and iliac crest, and LDMNCs were prestimulated for 2 days in prestimulation media, as described above prior to transduction with retrovirus encoding WT KIT or KITD814V. After two rounds of infection as described above, cells were sorted to homogeneity, and 1×10^6 cells were counted and mixed with 1×10^5 supporting fresh splenocytes.

11. Flow Cytometry

Cells expressing either WT KIT or KITD814V were incubated for 30 minutes at 4°C in 10% rat serum before staining with a combination of the indicated antibodies. Cells were washed two times with 0.2% BSA in PBS (Sigma-Aldrich) and analyzed by FACS (FACSCaliber, and LSR; BD).

12. Rac Activation Assay

Rac Activation Assays were performed using the manufactures directions (Millipore, catalog number 17-218). Cells were collected on ice and washed with PBS, and lysed with 500 µl of Mg^{2+} Lysis Buffer for every 10^7cells. Lysates were centrifuged to remove cellular debris and the supernatants were quantified using BCA assay buffer. Lysates were set aside as input and lysates were immunoprecipitated with 20-30µg of the GTPase Protein Binding Domain/agarose slurry and incubated for 45 minutes at 4°C with gentle agitation. The agarose beads were pelleted by centrifugation at 14,000 rpm for 10 seconds. The supernatant was discarded and the beads were washed with MLB three times. The beads were resuspended in 40 µl of 2X Laemmli reducing sample buffer and boiled for 5 minutes to liberated the bound protein and run by immunoblot analysis, probing with anti-Rac1, Rac2, Rac1/2/3 and cdc42 antibodies (EMD Millipore)

13. Site-directed mutagenesis of Chimeric KIT receptors

Chimeric KIT receptors (CHR) were previously generated in our laboratory as described by Tan et al.(128). Utilizing CHR receptors as templates we introduced a

KITD814V mutation by mutagenesis. We generated the mutant CHRD814V and CHRD814V with none (CHRD814V-F7) or single intracellular tyrosine add-back mutants using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the following primer pair (forward: 5'GGGCTAGCCAGAGTCATCAGGAAT GATTCG3'; reverse: 5'-CGA ATCATTCCTGATGACTCTGGCTAGCCC-3'). Generation of plasmids containing the KITD814V mutation was verified by sequencing described in Table 2.2.

14. Statistical Analysis

All graphical data were evaluated by paired Student's *t*-test and results were considered significantly different with p-value <0.05. Data are expressed as mean values± standard deviations (SD) unless otherwise stated. Sigma Plot 12.0 software (Systat Software Inc, San Jose, CA) was used for statistical analysis. All data are represented as mean values ± SD. Survival of transplanted mice cohorts were compared using a Kaplan–Meier survival analysis using a log rank test in which statistical significance was determined as p-values <0.05 by log-rank test.

15. Study Approval

Mice were maintained under specific pathogen-free conditions at the Indiana University Laboratory Animal Research Center, and this study was approved by the IACUC of the Indiana University School of Medicine. Samples from AML and SM patients were collected after obtaining written informed consent as approved by the Institutional Review Board of the Cleveland Clinic/Case Comprehensive Cancer Center

and the Institutional Review Board of the Indiana University School of Medicine (Study# 1468). AML and SM patient samples were obtained in accordance with the Declaration of Helsinki.

CHAPTER THREE

ROLE OF INTRACELLULAR TYROSINE RESIDUES IN ONCOGENIC KIT-INDUCED TRANSFORMATION

3.A ABSTRACT

Gain-of-function mutations in KIT receptor in humans are associated with gastrointestinal stromal tumors (GIST), systemic mastocytosis (SM), and acute myelogenous leukemia (AML). The intracellular signals that contribute to oncogenic KIT induced myeloproliferative disease (MPN) are poorly understood. Here, we show that oncogenic KITD814V induced MPN occurs in the absence of ligand stimulation. The intracellular tyrosine residues are important for KITD814V induced MPN, albeit to varying degrees. Among the seven intracellular tyrosines examined, tyrosine 719 alone plays a unique role in regulating KITD814V induced proliferation and survival *in vitro*, and MPN *in vivo.* Importantly, the extent to which AKT, ERK and Stat5 signaling pathways are activated via the seven intracellular tyrosines in KITD814V impacts the latency of MPN and severity of the disease. Our results identify critical signaling molecules involved in regulating KITD814V induced MPN, which might be useful for developing novel therapeutic targets for hematologic malignancies involving this mutation.

3.B INTRODUCTION

The proto-oncogene KIT belongs to the receptor tyrosine kinase class III family, which includes the M-CSFR, the PDGFR, as well as FLT3 (68). The KIT receptor plays a crucial role in cell proliferation, differentiation, survival and migration through activation of diverse signaling pathways (56). Binding of its ligand, stem cell factor (SCF), induces KIT receptor dimerization and autophosphorylation on intracellular tyrosine residues leading to the recruitment and docking of SH2 containing signaling molecules to the seven critical intracellular tyrosines (129). Although the individual and combined importance of these tyrosines in normal KIT receptor signaling is beginning to emerge; the role of these tyrosines in oncogenic KIT induced MPN is poorly understood. Furthermore, it is unclear whether these tyrosines play a unique, redundant or overlapping function in inducing MPN.

Activating mutations in KIT receptor have been shown to be involved in various human diseases including GIST, SM, and AML (45, 130-132). An activating KIT mutation within the tyrosine kinase domain, such as KITD814V in mice or KITD816V in humans, results in ligand independent tyrosine kinase activity leading to constitutive autophosphorylation and activation of downstream signaling pathways (76, 89). As a result, KITD814V bearing cells demonstrate ligand independent proliferation *in vitro* and MPN *in vivo* (75, 76, 89, 133, 134). However, the intracellular mechanisms that contribute to KITD814V induced MPN are not known. In addition, primary hematopoietic stem and progenitors (HSC/Ps) bearing KITD814V show a further increase in proliferation in the presence of KIT ligand, SCF, relative to cells grown in the absence of SCF (133), which suggests that endogenous ligand stimulation may contribute

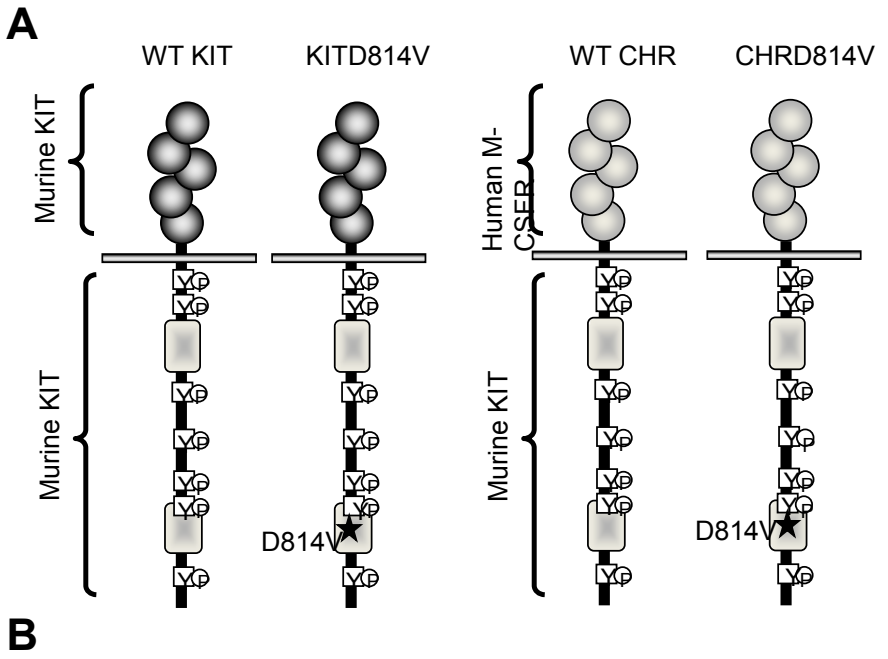
to oncogenic KIT induced transformation *in vivo*. Therefore, it is still unclear whether ligand independent growth observed *in vitro* via KITD814V is sufficient to induce MPN *in vivo* or whether presence of SCF is necessary to drive MPN.

Although KIT mutations within the juxtamembrane domain found in GIST are highly sensitive to inhibition by imatinib (i.e. Gleevec), KIT mutations within tyrosine kinase domain involved in SM and AML, including KITD816V, are resistant to imatinib treatment (83, 135, 136). Currently, there are no therapies available for human diseases involving KITD816V mutation. Thus, it is important to identify signaling pathways that are involved in KITD814V induced MPN to develop novel therapeutic targets for diseases involving this mutation. Utilizing biochemical and genetic approaches, we demonstrate that endogenous ligand (i.e. SCF) binding is dispensable for KITD814V induced MPN. Furthermore, the intracellular tyrosine residues are important for KITD814V induced MPN, albeit to varying degrees. Among the seven intracellular tyrosines examined, tyrosine 719 alone plays a unique role in regulating KITD814V induced proliferation *in vitro*, and MPN *in vivo.* Importantly, tyrosine 719 is vital for the activation of PI3K and Stat5 downstream from KITD814V. Our results identify critical signaling molecules downstream from KITD814V, which might be useful for developing therapeutic targets for hematologic malignancies involving oncogenic forms of KIT.

3.C RESULTS

Construction of wild-type and mutant KIT chimeric receptors

We and others have previously shown that KITD814V is sufficient to induce ligand independent growth *in vitro* as well as myeloproliferative disease (MPN) *in vivo* (75, 76, 89, 97, 133). It is however unclear whether KITD814V induced ligand independent growth observed *in vitro* is sufficient to cause MPN *in vivo* or whether presence of endogenous SCF induced signals are essential for the development of MPN. To determine the contribution of ligand independent growth in KITD814V induced MPN *in vivo*, we generated a chimeric KIT receptor (CHR) in which the extracellular domain of KIT was replaced with the extracellular domain of human macrophage colony stimulating factor receptor (h-MCSFR) to inhibit the endogenous binding of murine SCF, but to maintain the transmembrane and intracellular domains of the murine KIT receptor (69, 72) (Figure 3.1.A). This receptor allows studying the ligand independent functions of KIT receptor *in vivo* as it maintains the intracellular functions of KIT receptor intact without endogenous binding of murine SCF or M-CSF, but is specifically activated by human M-CSF (69, 72). The wild-type chimeric receptor (WT CHR) is functionally and biochemically similar to the wild-type endogenous KIT receptor as previously reported (69, 72). In addition, we constructed a mutant chimeric receptor (CHRD814V) that contains an oncogenic mutation of aspartic acid to valine at residue 814 of the WT CHR (Figure 3.1.A), this mutant was verified by sequencing (Figure 3.1.B). Parental and chimeric KIT receptors with or without D814V mutation were cloned into a bicistronic retroviral vector, MIEG3, which expresses EGFP through an internal ribosome entry site as previously described (69, 72).



GENE ID: 16590 Kit | kit oncogene [Mus musculus] (Over 100 PubMed links)

Score = 449 bits (1154), Expect(2) = 8e-125
Identities = 239/263 (90%), Positives = 241/263 (91%), Gaps = 3/263 (1%)
Frame = +2

D814V

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Figure 3.1 Construction of chimeric CHRD814V receptor.

(A) Schematic of WT CHR and CHRD814V receptors. Wild-type (WT) and oncogenic chimeric (CHR) KIT receptors were constructed by replacing the extracellular ligand binding domain of murine WT KIT or KITD814V with the ligand binding domain of human M-CSF receptor. (B) Mutagenesis of an aspartic acid (D) to valine (V) at indicated position 814 (indicated by arrow) by site-directed mutagenesis using WT CHR as a template.

Ligand independent growth is sufficient to induce KITD814V induced MPN in vivo

We first verified whether our constructed chimeric KIT receptors function similar to their wild-type and KITD814V counterparts. Viral supernatants expressing these receptors were used to infect an immortalized, murine, interleukin-3 (IL-3) dependent myeloid cell line (32D) which is absent of intrinsic KIT expression; transduced cells were sorted to homogeneity based on EGFP expression and used to perform functional and biochemical studies. 32D cells are particularly useful because they lack endogenous wild-type KIT expression. Transduced cells were observed to have comparable levels of protein expression of the wild-type and chimeric KIT receptors. To verify that our constructed chimeric KIT receptors function similar to their wild-type and KITD814V counterparts, we performed proliferation assay in 32D myeloid cells bearing parental or chimeric KIT receptors with or without D814V mutation by assessing thymidine incorporation. We starved these cells of serum and growth factors for six hours and performed a thymidine incorporation assay ($2x10^4$ cells/200 µl) in the absence of growth factors and incubated the cells for forty-eight hours at 37°C. The cells were pulsed with $1\mu Ci$ tritiated thymidine and incubated for 6 hours at 37°C, prior to analysis using an automated 96-well cell harvester (Brandel, Gaitgersburg, MD). As expected, cells bearing KITD814V or CHRD814V showed similar levels of ligand independent growth (Figure 3.2.A). In contrast, cells bearing WT KIT or WT CHR showed minimal thymidine incorporation in the absence of growth factors (Figure 3.2.A). In addition, cells bearing WT CHR showed increased growth in the presence of human M-CSF, but not murine M-CSF (Figure 3.2.B). These findings demonstrate that chimeric receptors (WT CHR and CHRD814V) function in a manner similar to their parental counterparts with respect to

ligand independent growth and specifically respond to human M-CSF, but not murine M-CSF. Therefore, these chimeric receptors could be used to determine the impact of ligand independent growth in KITD814V induced MPN *in vivo*.

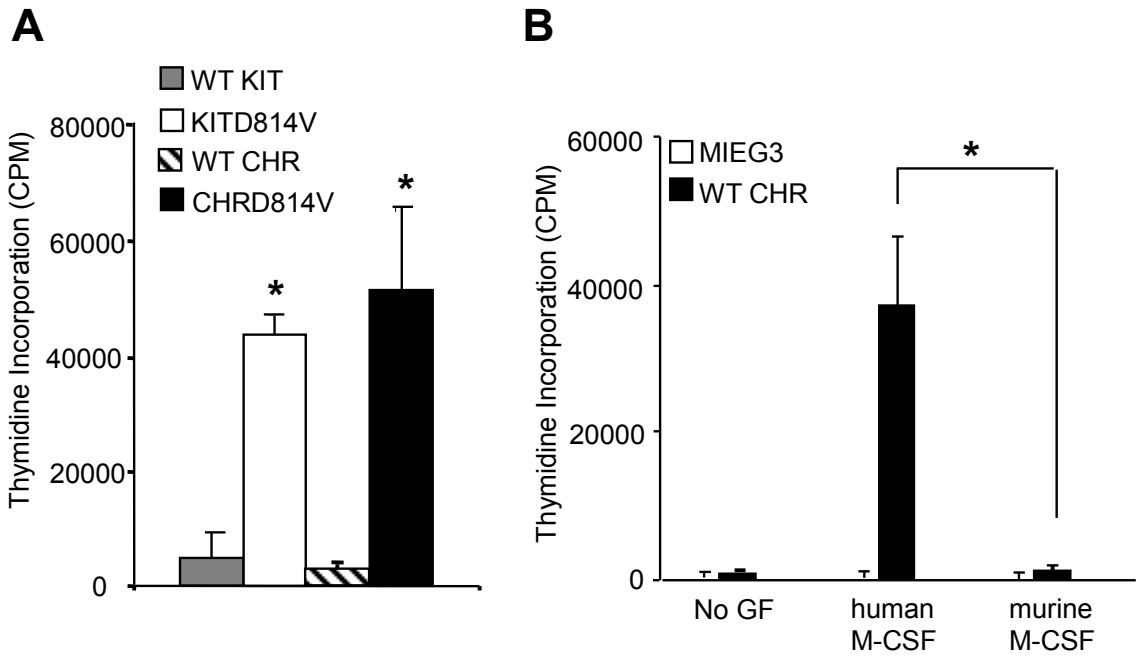

Figure 3.2. Intracellular tyrosine residues of KIT receptor are essential for KITD814V-induced ligand-independent growth in vitro.

(A) Cells bearing KITD814V and CHRD814V exhibit similar ligand independent growth. Cells expressing WT KIT, KITD814V, WT CHR or CHRD814V receptors were starved and subjected to proliferation assay by thymidine incorporation in the absence of growth factors as described in methods. Bars denote the mean thymidine incorporation (CPM ± SD) from one of three independent experiments performed in quadruplicates. *p-value<0.05, KITD814V or CHRD814V vs. WT KIT or WT CHR. (B) Chimeric KIT receptor responds specifically to human M-CSF, but not to murine M-CSF. Cells expressing MIEG3 vector or WT CHR were starved in serum- and growth factor-(GF) free media for 6 hours and cultured in the presence or absence of human or murine M-CSF for 48 hours and analyzed for proliferation by thymidine incorporation. Bars denote the mean thymidine incorporation (CPM ± SD) from one of at least three independent experiments performed in quadruplicate. *p-value<0.05.

To determine the role of ligand independent growth in KITD814V induced MPN *in vivo*, we used a murine transplantation model that we have previously described (97). LDMNC from 5-FU-treated C57BL/6 mice were transduced with WT CHR, WT KIT, KITD814V or CHRD814V, and sorted cells were transplanted into lethally irradiated recipient mice. Transplanted mice were monitored for MPN development and survival. Consistent with our previous results, all mice transplanted with WT CHR cells appeared normal and healthy past 180 days post transplantation (data not shown). In contrast, all recipient mice transplanted with CHRD814V bearing cells died within 81 days of transplantation (Figure 3.3.A). No significant difference in the survival of mice transplanted with cells bearing KITD814V or CHRD814V was observed suggesting that endogenous SCF mediated stimulation of KITD814V receptor is not necessary for KITD814V induced MPN *in vivo*. Mice transplanted with cells expressing CHRD814V developed a series of fatal diseases, including MPN, as seen by a significant increase in white blood cell counts compared to WT CHR controls (Figure 3.3.B). In addition, mice transplanted with CHRD814V-bearing cells showed splenomegaly (Figure 3.3.C). Figure 3.4.A shows representative pictures of spleen and liver from mice transplanted with cells bearing either WT CHR or CHRD814V.

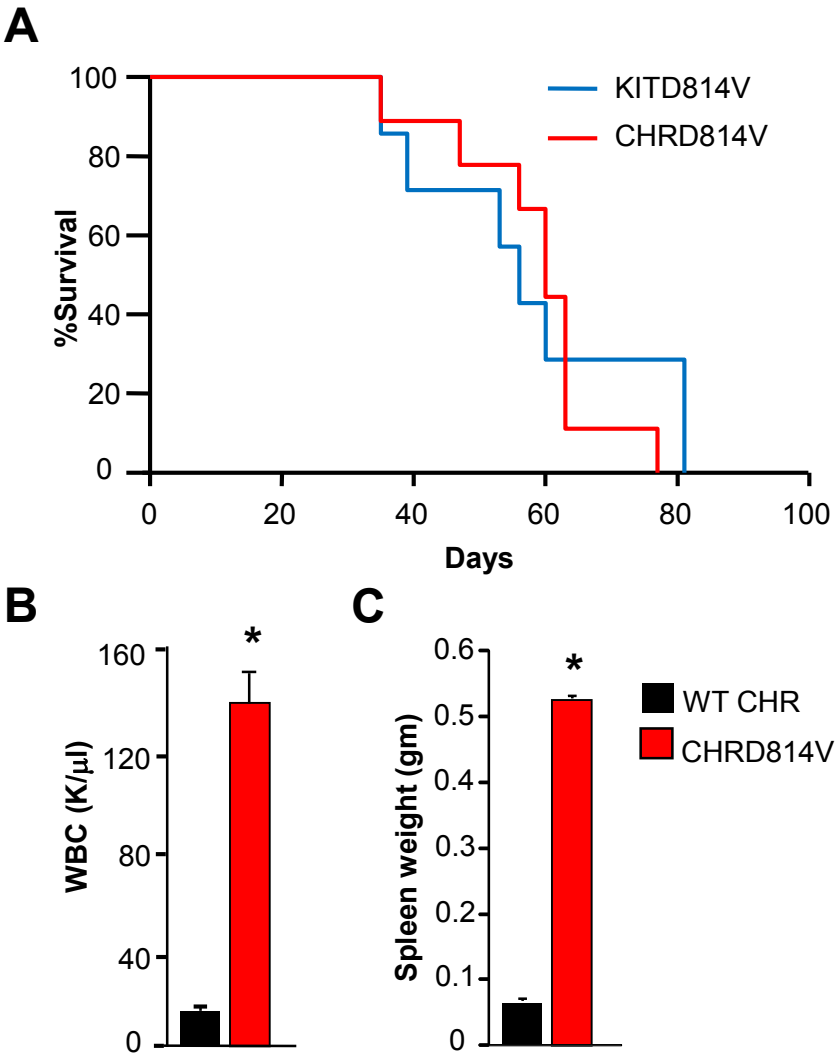

Figure 3.3. KITD814V induced MPN is not dependent on endogenous SCF.

(A) Kaplan–Meier analysis of mice transplanted with 5-fluorouracil (5-FU) derived primary hematopoietic stem and progenitor cells bearing WT KIT, KITD814V or CHRD814V. The percentage of surviving mice (*y*-axis) is plotted with respect to post-transplantation time in days (*x*-axis). Mice transplanted with WT KIT (n=8), KITD814V (n=7) or CHRD814V (n=9) expressing cells succumbed to a fatal MPN within 81 days of transplantation. No significant difference between the survival of mice bearing KITD814V or CHRD814V was observed. (B) & (C) enhanced peripheral blood (PB) cell counts and splenomegaly in mice transplanted with cells bearing CHRD814V (n=9) compared to WT CHR (n=4 to 6). *p-value<0.05.

Histologic analysis of bone marrow (BM), spleen, liver and lungs from mice transplanted with CHRD814V bearing cells showed signs of MPN including infiltration of immature cells in BM and disruption of the normal architecture of red and white pulp in spleen (Figure 3.4.B). Furthermore, flow cytometric analysis of peripheral blood, spleen and BM from mice transplanted with cells bearing CHRD814V showed increase in the presence of Gr-1 and Mac-1-positive cells compared to mice transplanted with cells bearing WT CHR (Figure 3.4.C). Some mice transplanted with cells bearing CHRD814V also demonstrated erythroleukemia, B-cell and T-cell lymphomas in addition to MPN, similar to other published models of oncogene-induced MPN (data not shown) (137). Taken together, these results indicate that KITD814V mutation does not require a direct engagement with its ligand SCF to induce MPN *in vivo*.

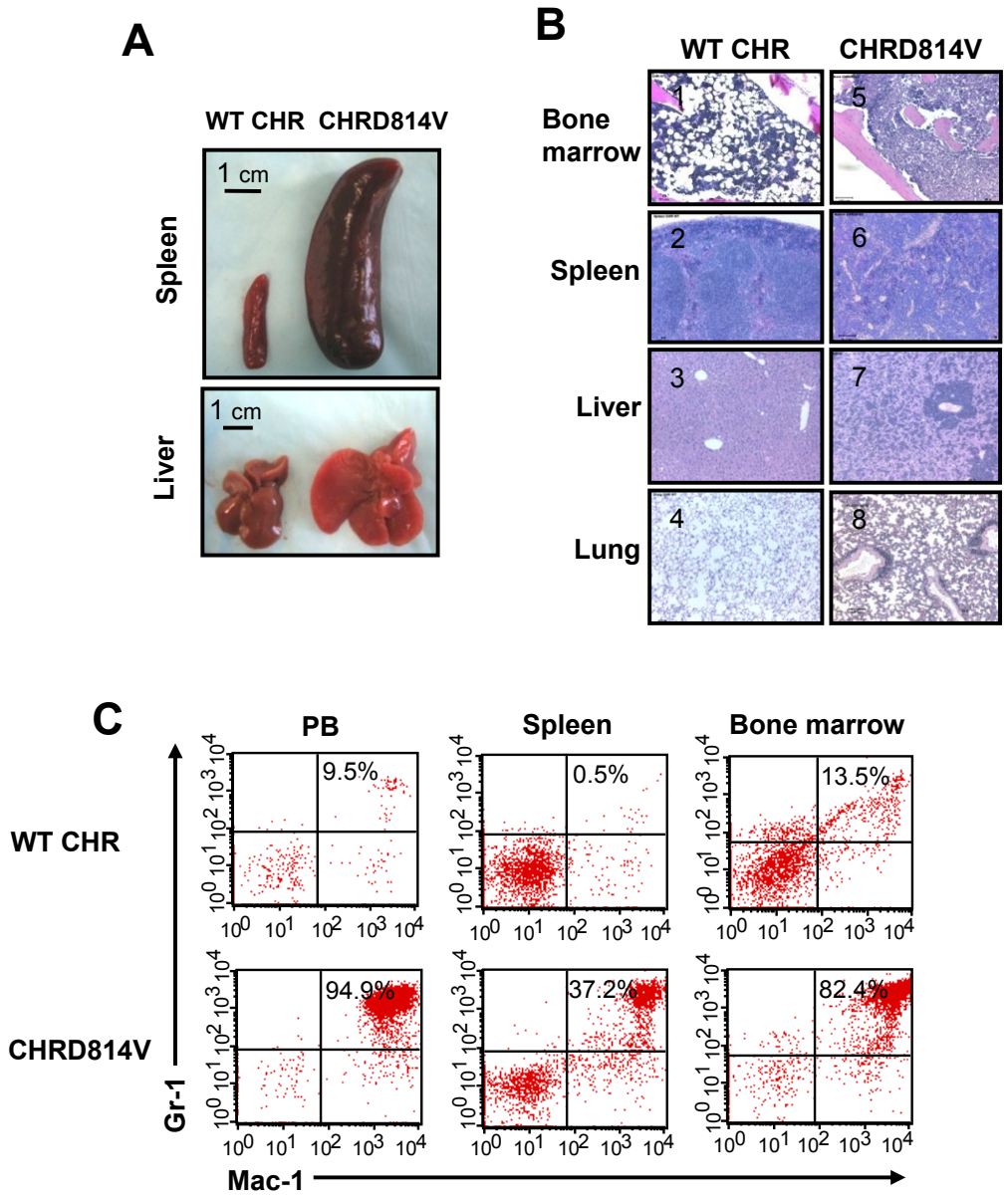

Figure 3.4. CHRD814V induced elevated WBC counts and splenomegaly.

(A) Representative pictures of spleen and liver from mice transplanted with cells bearing WT CHR (n=4) and CHRD814V (n=9). (B) Histopathologic analysis demonstrating MPN phenotype in mice transplanted with cells bearing CHRD814V. Bone marrow, spleen, liver and lungs from the mice transplanted with cells bearing WT CHR or CHRD814V were harvested, fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Shown are representative tissue sections from WT CHR and CHRD814V transplanted mice. (C) Flow cytometric analysis on cells derived from indicated tissues from mice bearing WT CHR or CHRD814V.

Intracellular tyrosine residues in KITD814V are essential for ligand independent growth in vitro

We next focused our studies to determine the importance of intracellular tyrosine residues in KITD814V induced ligand independent growth. To address this, we constructed a CHRD814V mutant receptor (CHRD814V-F7) in which all the intracellular tyrosine residues were converted to phenylalanine by site directed mutagenesis as shown in Figure 3.5.A. 32D cells were transduced with WT CHR, CHRD814V or CHRD814V-F7, sorted to homogeneity based on EGFP expression and used to perform proliferation assay. Consistent with our earlier result (Figure 3.2.A), cells bearing CHRD814V, but not WT CHR, showed ligand independent growth (Figure 3.6.A). However, conversion of all the seven intracellular tyrosine residues in CHRD814V to phenylalanine (CHRD814V-F7) resulted in loss of ligand independent growth (Figure 3.6.A). These results suggest that intracellular tyrosine residues in KITD814V are essential for ligand independent growth.

To identify which of the tyrosine residue plays a critical role in KITD814V induced ligand independent growth *in vitro* and transformation *in vivo*, we generated 7 different single tyrosine add-back mutants at residues 567, 569, 702, 719, 728, 745 and 934 in the KIT intracellular domain using CHRD814V-F7 as the template (Figure 3.5.A). These CHRD814V mutant receptors were designated CHRD814V-Y567, CHRD814V-Y569, CHRD814V-Y702, CHRD814V-Y719, CHRD814V-Y728, CHRD814V-Y745 and CHRD814V-Y934. 32D cells, which lack endogenous KIT receptor, were infected with these mutant CHRD814V receptors and sorted to homogeneity. A similar level of expression of these receptors was observed by western blot analysis (Figure 3.5.B). As

seen in Figure 3.6.B, 32D cells bearing only the chimeric receptor harboring tyrosine residue at position 719 (CHRD814V-Y719) was sufficient to induce ligand independent growth to a level similar to cells bearing the CHRD814V receptor. The other tyrosine add-back CHRD814V receptors induced modest to no ligand independent proliferation (Figure 3.6.B). Similar results were observed in transduced primary HSC/Ps from $Kit^{W\text{-}sh/W\text{-}sh}$ mice lacking endogenous KIT (Data not shown). In addition, cells bearing CHRD814V showed significantly increased survival compared to WT CHR bearing cells in the absence of growth factors and loss of intracellular tyrosine residues in CHRD814V (CHRD814V-F7) had significant decreased in ligand independent survival (Figure 3.7.A). Among all the single tyrosine add-back CHRD814V receptors, CHRD814V-Y719 was the only receptor whose expression maintained survival at a level similar to that of CHRD814V receptor (Figure 3.7.A). There was no significant difference in the cycling status of cells bearing various mutant CHRD814V receptors, including CHRD814V and CHRD814V-Y719, when grown in the absence of growth factors (Figure 3.7.B). These results demonstrate that intracellular tyrosine residues in KITD814V receptor are essential for ligand independent growth. Among these tyrosine residues, tyrosine at residue 719, which is the binding site for class I_A PI3K regulatory subunit p85α, is sufficient to rescue ligand independent proliferation *in vitro* to CHRD814V levels.

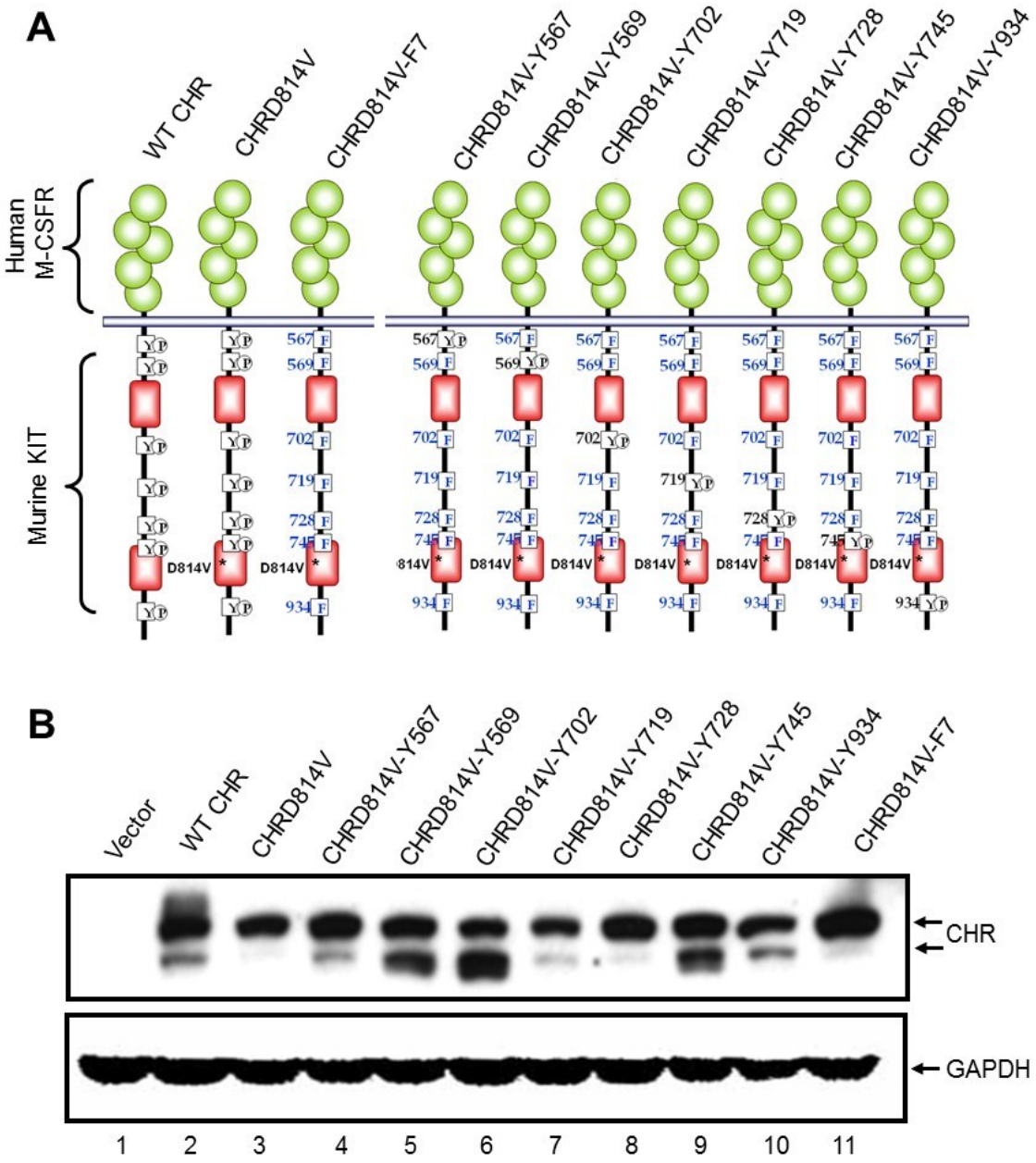

Figure 3.5. Schematic of mutant CHRD814V receptors and expression in 32D cells.

(A) Schematic of mutant CHRD814V receptors. In the single tyrosine add-back mutant CHRD814V receptors, phenylalanine at indicated positions in the CHRD814V-F7 receptor were restored to tyrosine on an individual basis. (B) Equal amounts of protein lysates from cells bearing MIEG3 vector or indicated CHRs were subjected to Western Blot analysis using an anti-KIT antibody.

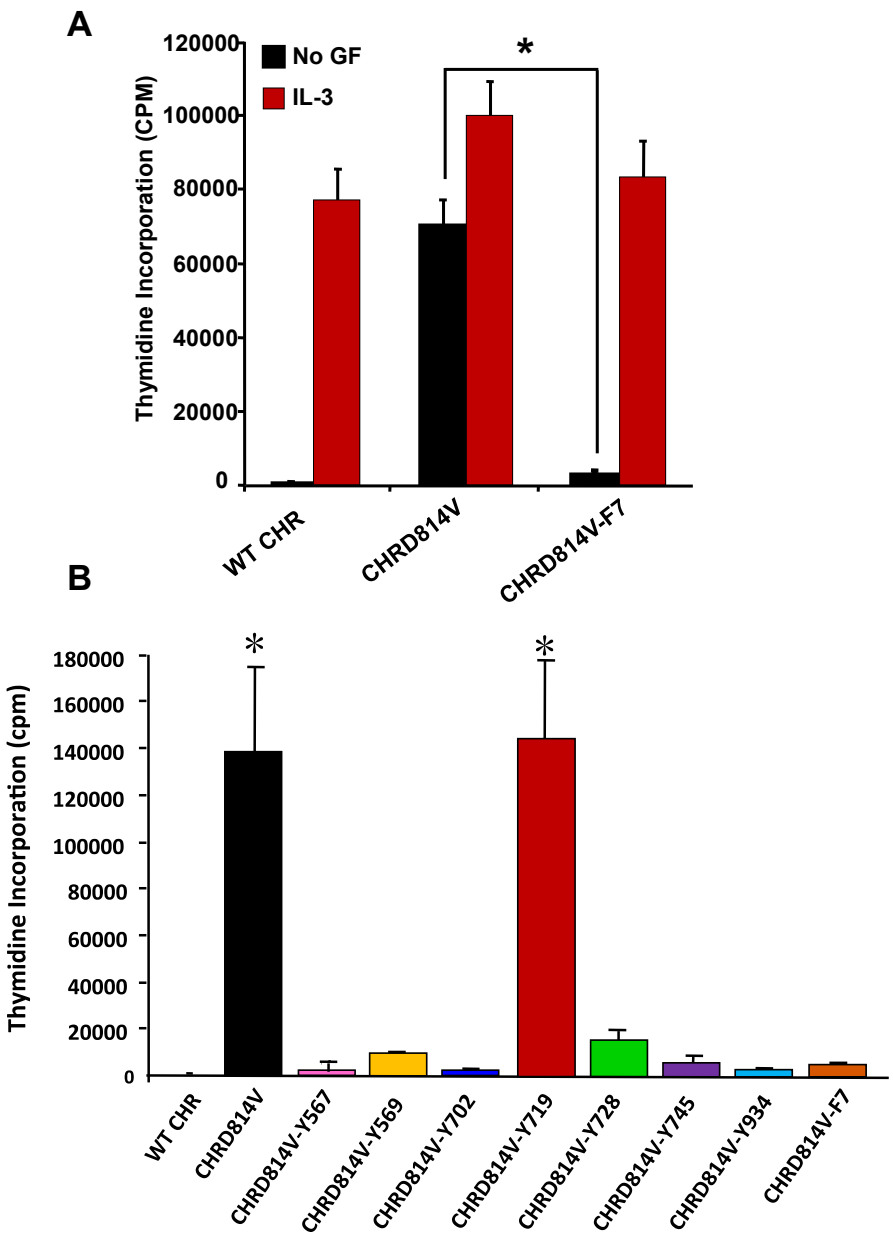

Figure 3.6. Differential contributions of intracellular tyrosine residues in KIT receptor to KITD814V-induced ligand-independent growth *in vitro*.

(A) 32D cells bearing the WT CHR, naked CHRD814V-F7, or CHRD814V were starved of serum and growth factors for 6 hours and subjected to thymidine incorporation assay in the presence or absence of 5 ng IL-3. (B) Cells bearing the indicated CHRD814V add-backs were grown in the absence of growth factors. Bars denote the mean thymidine incorporation (cpm ± sd) *p-value<0.05.

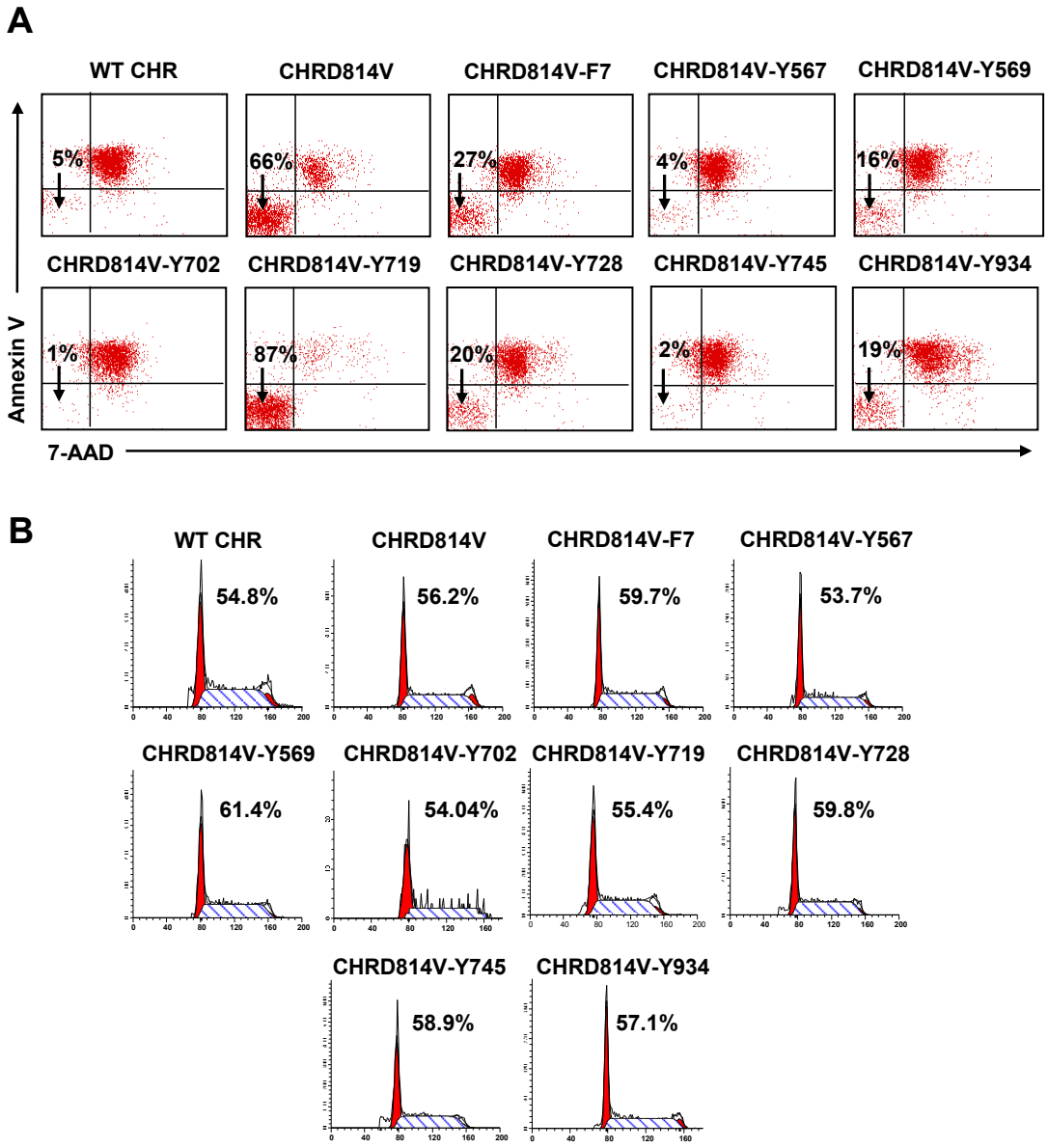

Figure 3.7. Reduced survival, but normal cycling of cells bearing various chimeric KIT receptors.

Cells bearing the indicated chimeric receptors were starved of serum and growth factors and cultured for 48 hours in the absence of growth factors. (A) Cells were harvested and stained with anti-Annexin V and 7-Amino-Actinomycin D (7-AAD) antibody followed by flow cytometric analysis. Double negative cells in the lower left quadrant are indicated as surviving cells. Representative dot blots are shown (n=2, *$p<0.05$). (B) Cells were stained with propidium iodide followed by flow cytometric analysis. Percentage of cells in S-phase is indicated. No significant difference in the cycling of cells bearing different chimeric KIT receptors was observed (n=2).

Intracellular tyrosine residues in KITD814V contribute to MPN in vivo, albeit to varying extent

To determine the physiologic role of the intracellular tyrosine residues in KITD814V induced MPN *in vivo*, we transduced primary HSC/Ps from 5-FU-treated C57BL/6 mice with WT CHR, CHRD814V or CHRD814V with none or single tyrosine add-back mutants. Transduced cells showing similar transduction efficiencies were sorted to homogeneity and transplanted into lethally irradiated recipient mice. Mice were monitored for MPN development and survival. While mice transplanted with cells bearing WT CHR showed no signs of disease and survived throughout the study, mice transplanted with cells bearing CHRD814V (red line) succumbed to death within 80 days of transplantation and developed a fatal MPN (Figure 3.3.A and Figure 3.8). In addition, mice transplanted with cells bearing CHRD814V-F7 (black line), which lack all the seven intracellular tyrosine residues, significantly delayed MPN development and prolonged overall survival (Figure 3.8. Panel 1). In the CHRD814V-F7 group, only 70% of the mice died within 180 days of transplantation and remaining 30% showed no signs of disease and appeared normal. These data suggest that the intracellular tyrosine residues in KITD814V are critical for efficient transformation *in vivo*.

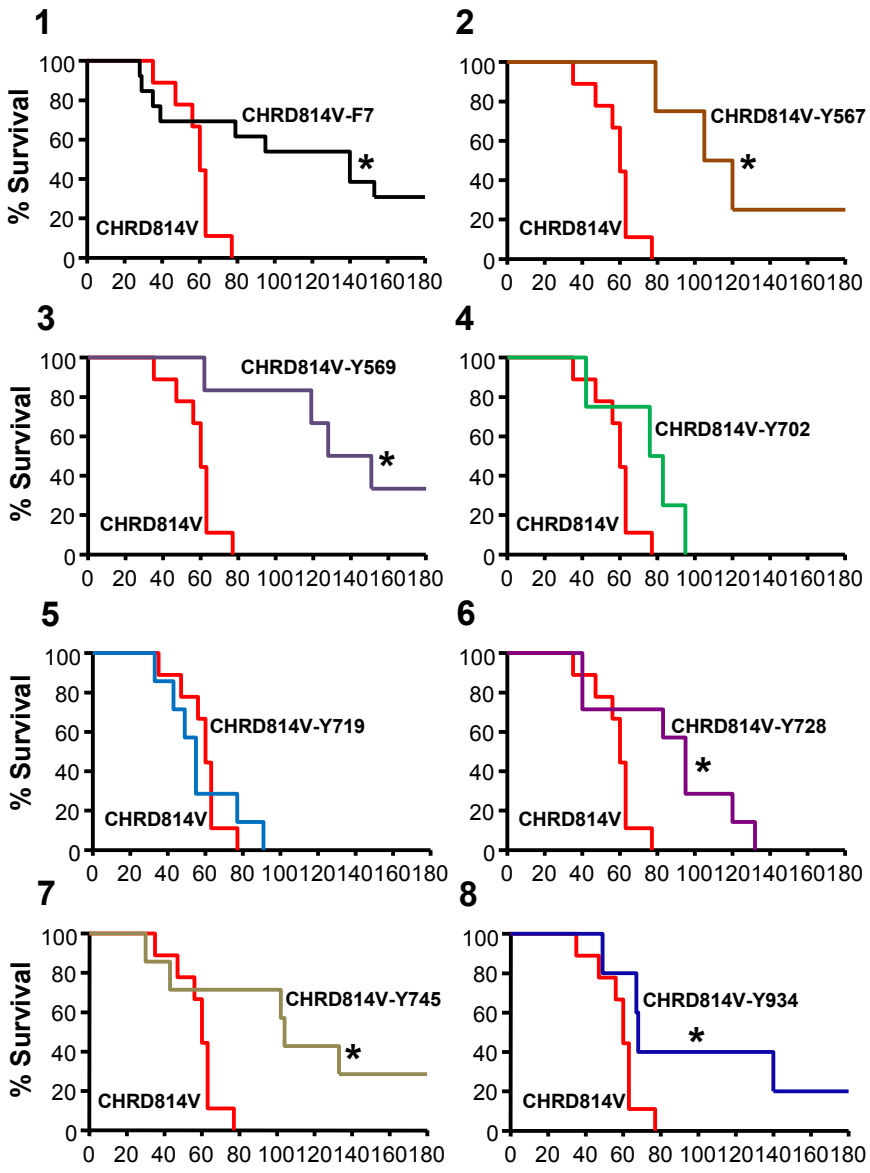

Figure 3.8. Intracellular tyrosine residues in KIT receptor are essential for KITD814V-induced MPN *in vivo*.

Kaplan-Meier survival analysis of mice transplanted with cells bearing indicated single tyrosine add-back mutant CHRD814V receptors (n=4-13 per group). Results show that loss of seven tyrosine residues in CHRD814V significantly delayed MPN development and prolonged overall survival (140 days median survival, n=13, *p-value<0.05. Presence of Y719 alone is sufficient for transformation *in vivo* (55 days median survival, n=7, *p-value<0.05. Compared with CHRD814V, restoration of Y567, Y569, Y728, and Y934 demonstrated a significant delay in disease onset in transplanted mice (95-128 days median survival, n= 4-13, *p-value<0.05). There is a modest delay in the survival of the recipient mice bearing CHRD814V-Y702 compared with CHRD814V-bearing mice (76 days median survival, n=4, p=0.077).

Consistent with *in vitro* proliferation, among all the mice transplanted with cells bearing various CHRD814V mutant receptors, only recipient mice expressing CHRD814V-Y719 (blue line) showed similar MPN progression and survival as the CHRD814V bearing mice (Figure 3.8. Panel 5). The median time of survival in these two groups was 60 days for CHRD814V vs 55 days for CHRD814V-Y719. In addition, recipient mice with cells bearing CHRD814V-Y567, CHRD814V-Y569, CHRD814V-Y728CHRD814V-Y745 and CHRD814V-Y934 showed a significant delay in MPN development and survival compared to CHRD814V bearing mice (Median time of survival 105, 128,95,104 and 68 days, respectively, *p-value<0.05). Restoration of Y702 demonstrated a modest but non-significant delay in the disease onset compared to KITD814V bearing mice (Median time of survival 76 days, p=0.077). Consistent with the survival data, histological analysis of bone marrow, spleen, liver and lungs from the recipient mice transplanted with cells bearing various single tyrosine add-back CHRD814V mutants showed variable degree of infiltration of immature cells relative to CHRD814V bearing mice or CHRD814V-Y719 bearing mice (Figure 3.4.B and Figure 3.9). These results suggest that among the seven intracellular tyrosine residues in KITD814V, tyrosine residue at 719 is sufficient to induce fully penetrant MPN *in vivo*. Other tyrosine residues at 567, 569, 702, 719, 728, 745 and 934 do contribute to KITD814V induced MPN, however to a lesser extent, resulting in delayed disease onset and reduced severity.

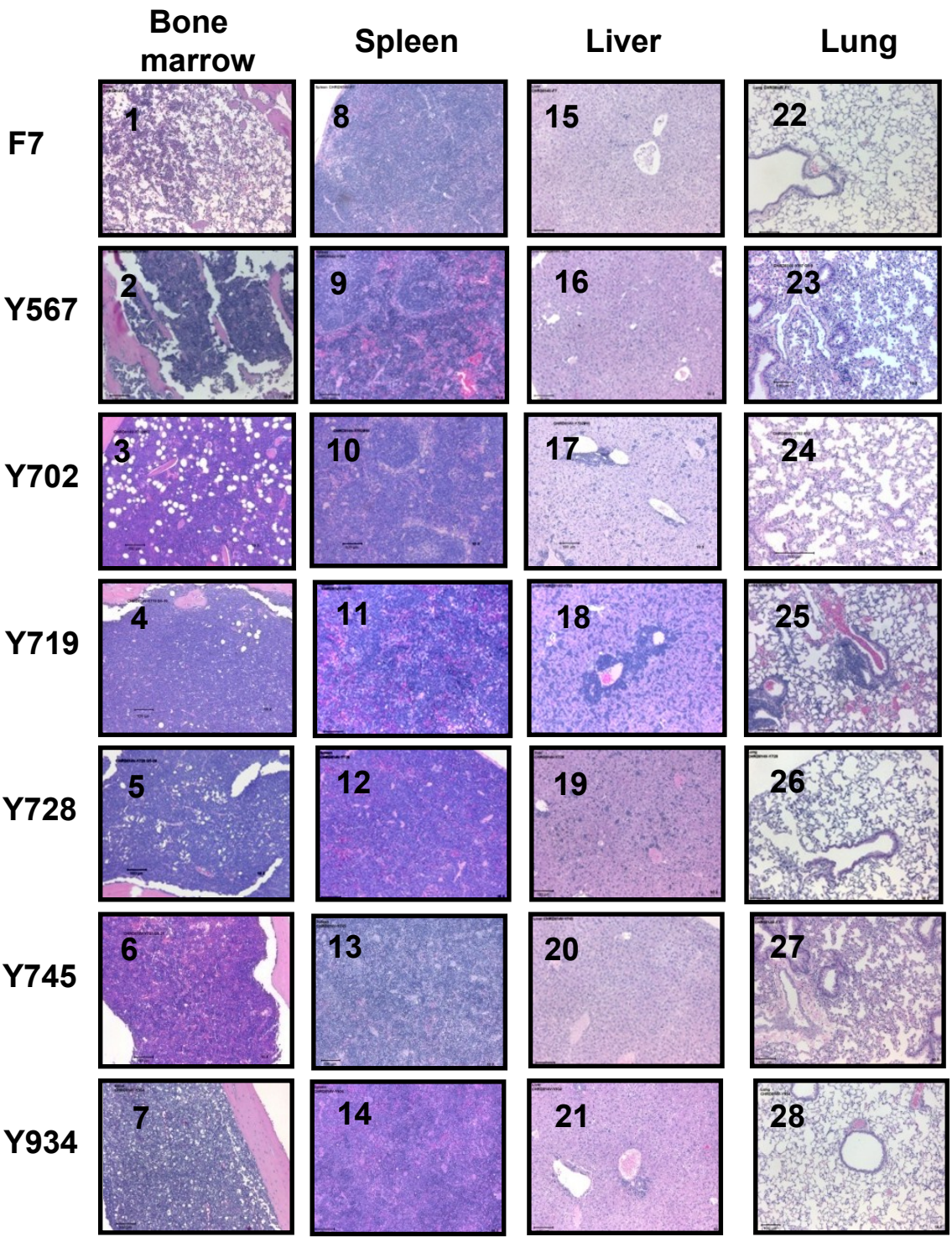

Figure 3.9. Immunohistopathologic analysis of BM, spleen, liver and lung from mice transplanted with cells bearing various single tyrosine add-back mutant CHRD814V receptors.

Samples were harvested, fixed in 10% buffered formalin, sectioned and stained with hematoxylin and eosin. Shown are representative tissue sections from mice transplanted with cells bearing various single tyrosine add-back mutant CHRD814V. Normal erythroid and myeloid components in BM, spleen, liver and lungs were replaced b sheets of immature tumor cells to various degrees in all the representative animals, but predominately in CHRD814V-Y719 (panel 4) followed by CHRD814V-Y745 (panel 6) and CHRD814V-Y728 (panel 5), respectively (first row).

Differential activation of PI3K, Stat5, and ERK mitogen-activated protein kinase in cells bearing various single tyrosine add-back CHRD814V mutant receptors

To assess the biochemical basis for the differential role of single tyrosine add-back CHRD814V mutants in ligand independent growth and MPN, activation of AKT, Stat5 and ERK, which are known to be critical for KITD814V induced MPN, was analyzed (97, 133, 138, 139). As seen in Figure 3.10.A, constitutive phosphorylation of AKT, Stat5, and ERK was observed in cells bearing CHRD814V, but not in WT CHR bearing cells. Interestingly, loss of seven intracellular tyrosine residues in CHRD814V resulted in complete abrogation of the constitutive activation of AKT, Stat5 and ERK (Figure 3.10.A, lane 11). Importantly, among the single tyrosine add-back CHRD814V mutants, only restoring tyrosine 719 completely rescued the robust constitutive phosphorylation of AKT, Stat5, and ERK observed in CHRD814V expressing cells (Figure 3.10.A, lane 7). Cells bearing CHRD814V-Y702 and CHRD814V-Y728 showed robust activation of AKT and ERK, but not Stat5 (Figure 3.10.A, lanes 6 and 8, respectively). Furthermore, a modest constitutive activation of AKT and ERK was observed in cells bearing CHRD814V-Y569 (Figure 3.10.A, lane 5). In contrast, no activation of AKT, Stat5 and ERK was observed in cells bearing CHRD814V-Y567, CHRD814V-Y745 or CHRD814V-Y934 (Figure 3.10.A, lanes 4, 9 and 10, respectively).

These results suggest that differential activation of AKT, Stat5 and ERK in cells bearing various single tyrosine add-back CHRD814V mutants likely contributes to variable level of disease progression and survival of transplanted mice.

Enhanced activation of Stat5, AKT, and ERK by CHRD814V-Y719

Previous studies of KITD814V have implicated a role in Stat5 and PI3K in aberrant signaling in activating mutations of KIT (97). To determine whether promiscuous signaling observed in cells bearing KITD814V utilized specific tyrosines for SH2 binding molecules we cultured 32D cells expressing the chimeric constructs in the absence of growth factor and examined the expression of activated Stat5. As shown in Figure 3.10.A, Stat5 was constitutively activated only in cells bearing CHRD814V and CHRD814V-Y719 (*lane 3 & 7*). There was also robust expression of activated AKT and ERK in the presence of CHRD814V, CHRD814V-Y719, and CHRD814V-Y702, while other single tyrosine add-backs, CHRD814V-Y569 and CHRD814V-Y728, showed modest constitutive activation of these molecules. Anti-apoptotic proteins, Bcl-xL and Bcl-2 had comparable levels of expression between CHRD814V and CHRD814V-Y719 (Figure 3.10.B).

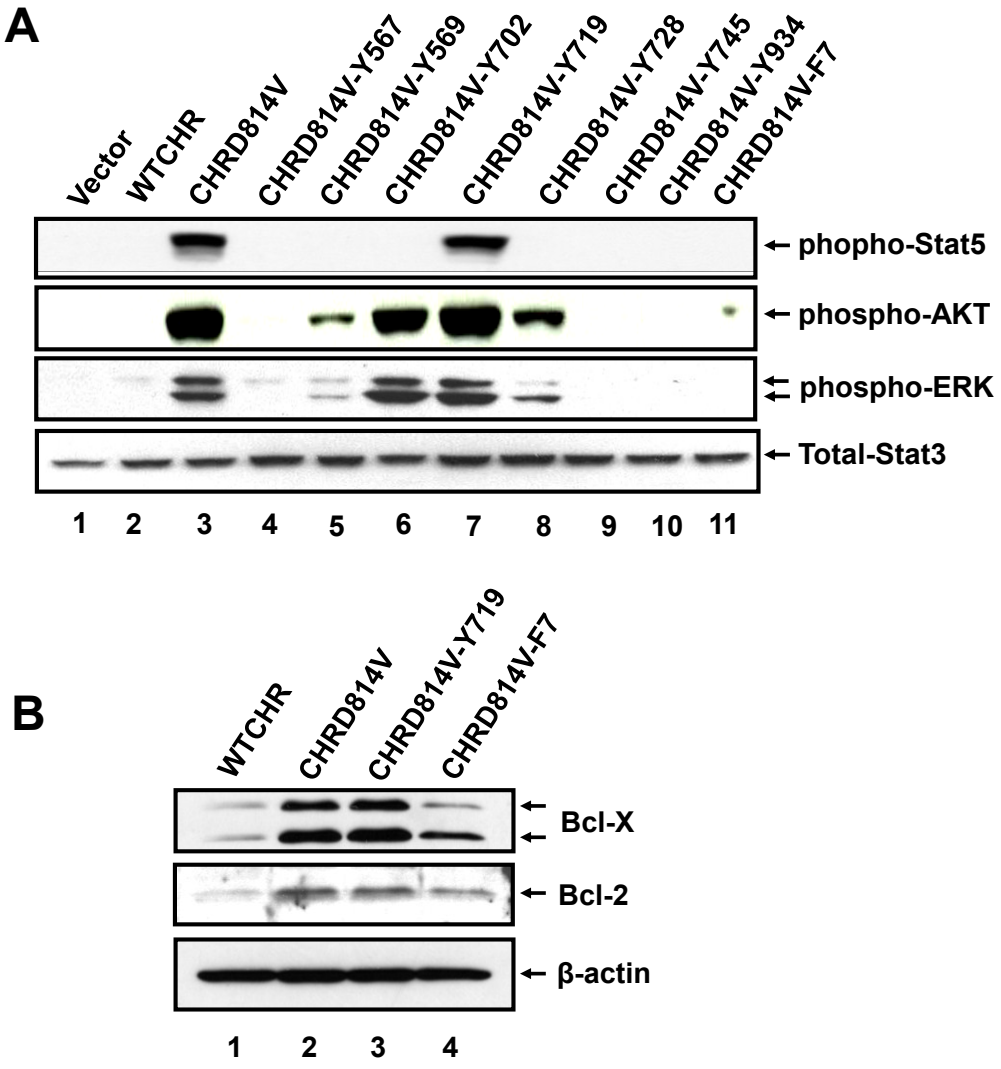

Figure 3.10. Involvement of AKT, ERK and Stat5 signaling in KITD814V-induced ligand-independent growth.

(A) Cells bearing the indicated CHRs were starved in serum-free and growth factor-free medium for 8 hours. Starved cells were lysed and equal amount of protein lysates were subjected to western blot analysis. Immunoblot using an anti-phospho-Stat5, phospho-AKT, phospho-ERK, and total Stat3 antibodies as indicated. Similar results were observed in two in three independent experiments. (B) Immunoblot for anti-apoptotic proteins using anti-Bcl-x, Bcl-2 and β-actin.

As PI3K plays a crucial role in proliferation and signaling of CHRD814V we assessed the binding of p85α to the chimeric receptors through immunoprecipitation. As shown in Figure 3.11, although CHRD814V-F7 does not show binding of p85α, restoration of tyrosine 719 in the CHRD814V-Y719 is sufficient to restore p85α to CHRD814V levels. These observations were further confirmed by binding of KIT to p85α in CHRD814V and CHRD814V-Y719 receptors (Figure 3.11.B). Collectively, these results demonstrate that tyrosine 719 is necessary and sufficient to restore cooperation between KITD814V and the PI3K signaling cascade.

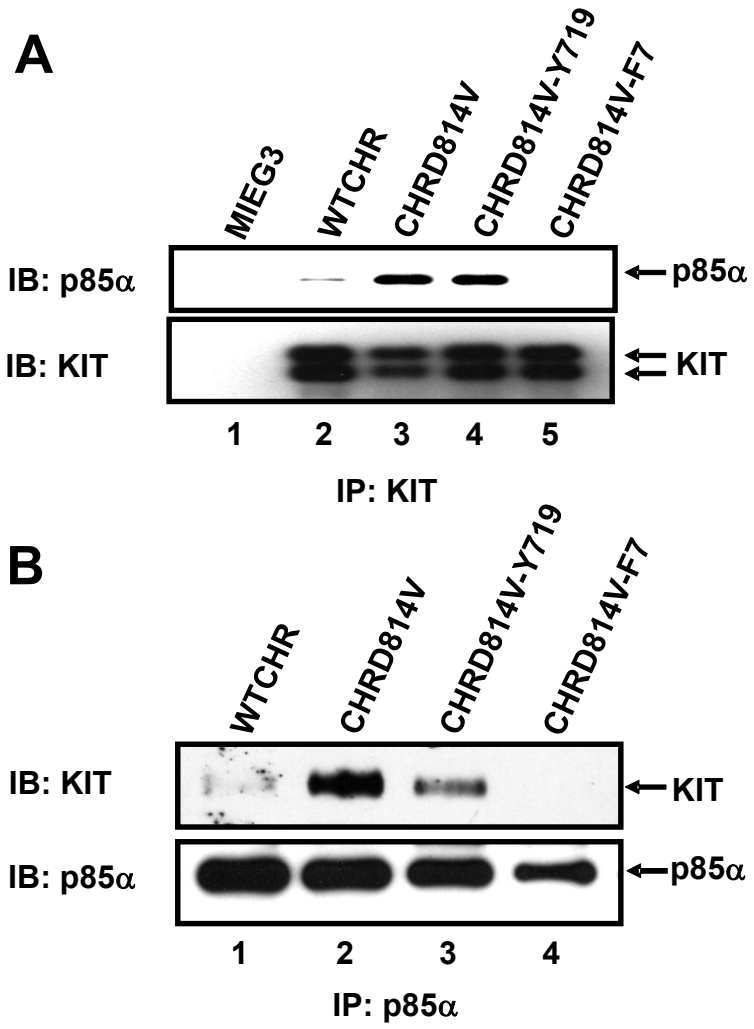

Figure 3.11. Intracellular tyrosines on KITD814V are critical for p85α binding.

Cells bearing the indicated CHRs were starved in serum-free and growth factor-free medium for 8 hours. Starved cells were lysed and equal amount of protein lysates were immunoprecipitated (IP) with (A) anti-KIT or (B) anti-p85α and immunoblotted (IB) with anti-KIT or anti-p85.

3.D CONCLUSIONS

These studies demonstrate that the direct binding of the ligand SCF to KITD814V receptor is not necessary to induce MPN and intracellular tyrosines are critical to this process. Mice bearing either the parental KITD814V receptor or the chimeric CHRD814V receptor, lacking the ability to bind ligand SCF, show similar potency at inducing MPN with similar median survival. Furthermore, no significant difference in disease manifestation was observed between the two transplanted groups. Importantly, intracellular tyrosines show a critical role in CHRD814V-induced MPN and survival. Loss of seven critical tyrosine residues in CHRD814V, which are known to activate phospholipase C-γ (728), (70, 140) PI3K (p85α; 719),(141) Src family kinases (567 and 569), (142-144) Grb2 (702) (144), Grb7 (934) (144) and Ras GTPase activating protein (745) profoundly impaired disease onset and prolonged the survival of transplanted mice. Only 70% mice that were transplanted with cells bearing CHRD814V-F7 succumb to death with very long latency, while 30% mice were free of disease and survived for the remaining duration of the study. Using transduced primary HSC/Ps, we have previously demonstrated that KITD814V not only induces ligand-independent growth or responds to SCF, but also synergizes with interleukin-3 and M-CSF receptors to further enhance the growth of these cells *in vitro*. This suggests that KITD814V-induced ligand-independent growth and its ability to cooperate with other cytokines might also contribute to activating KIT-induced MPN(34). Furthermore, KIT has also been shown to cooperate with granulocyte M-CSF in a kinase-independent manner (145) and to synergize with granulocyte colony-stimulating factor to induce proliferation (146).Therefore, it is conceivable that CHRD814V-F7 is not sufficient to induce robust proliferation without

growth factor *in vitro*, but may cooperate with additional cytokine receptors, such as interleukin-3, M-CSF and/or granulocyte colony-stimulating factor receptors to induce MPN in the 70% transplanted animals in a significantly delayed and milder manner.

In an effort to assess the role of individual tyrosine-mediated signals in KITD814V-induced MPN we employed a unique approach. Although single point mutations in important tyrosine residues have been shown to affect KIT function, (147, 148) we felt that the approach of interfering with the binding of one or two SH2-binding proteins to CHRD814V, when all other SH2 proteins still retain the ability to bind may not allow for complete assessment of the importance of signals emanating from a single pathway, because it is possible that the remaining SH2 consisting proteins may compensate for the loss of a single binding site. A single-add back strategy was used to assess the role of an individual tyrosine-induced pathway in KITD814V-induced MPN. Using a version of the receptor that lacked all seven tyrosines (CHRD814V-F7) as the template, we restored each of the SH2-binding sites from phenylalanine to tyrosine and tested their ability to induce growth *in vitro* and transformation *in vivo*, along with their potential to activate downstream signaling molecules known to be involved in KITD814V-induced MPN, including AKT, ERK and Stat5. A unique role for tyrosine 719 in regulating KITD814V-induced MPN was shown that in restoring this site alone in the CHRD814V-F7 receptor is sufficient to completely rescue ligand-independent growth *in vitro*, MPN *in vivo* and activation of AKT, ERK and STAT5, similar to the CHRD814V receptor. Importantly, while the remaining add-back mutant receptors showed minimal rescue in ligand-independent growth *in vitro*; *in vivo* mice bearing these receptors demonstrated delayed MPN onset and prolonged latency compared with

CHRD814V or CHRD814V-Y719 receptor-bearing mice. These results suggest that although ligand-independent growth is sufficient for KITD814V-induced MPN *in vivo*, presence of SCF and other cytokines might further regulate the MPN phenotype.

The variable onset of MPN and latency in mice transplanted with cells bearing various add-back mutant receptors was associated with differential rescue in the activation of AKT, ERK and STAT5. In general, add-back CHRD814V mutants that lacked the ability to rescue the activation of AKT, ERK and STAT5, such as CHRD814V-F7 receptor-bearing mice, demonstrated prolonged latency, while receptors demonstrating rescue in the activation of AKT and ERK, such as the CHRD814V-Y702 receptor-bearing mice, resulted in MPN, albeit at a slower rate. In contrast, receptors that robustly activated all three signaling molecules (ERK, AKT and Stat5) such as the CHRD814V-Y719 and the CHRD814V receptor, showed the shortest disease latency. Thus, KITD814V-induced MPN *in vivo* is largely dependent on the level of activation of AKT, ERK and STAT5, which to a large extent is regulated by signals emanating from tyrosine 719, suggesting that signals emanating from tyrosine 719 have a dominant role in regulating MPN, while the remaining tyrosine initiated signals contribute to MPN, albeit to a lesser extent. Thus, the impairment in the activation of essential signaling pathways is likely to contribute to a significant delay in the onset of MPN in mice bearing remaining add-back mutants of KITD814V.

Although studies have suggested that persistent activation of PI3K and Stat5 is frequently observed in hematologic malignancies as well as in solid tumors, exactly how PI3K contributes to KITD814V-induced MPN and/or Stat5 activation is unclear (149, 150). We show that tyrosine 719 in KITD814V (binding site for p85α) contributes to the

activation Stat5. In cells expressing CHRD814V-F7, no binding of p85α is observed and consistently no constitutive activation of Stat5 is observed. Furthermore, deficiency of p85α in HSC/Ps expressing KITD814V results in complete inhibition of Stat5 activation (151). These results suggest that PI3K-mediated signaling is essential for constitutive activation of Stat5 in KITD814V-bearing cells. Taken together, our studies determine the contribution of SCF to KITD814V-induced MPN *in vivo*, and also identify critical tyrosine residues and signaling pathways involved in KITD814V-induced MPN.

This research was modified from what was originally published in the journal of *Leukemia*.

Ma P*, Mali RS*, Martin H*, Ramdas B, Sims E, and R Kapur. Role of intracellular tyrosines in activating KIT-induced myeloproliferative disease. *Leukemia*. 2012; **26**: 1499-1506.

*These authors contributed equally to the work.

CHAPTER FOUR

ROLE OF PAK AND RAC GTPASES IN ONCOGENIC KIT INDUCED NEOPLASMS

4.A ABSTRACT

An acquired somatic mutation at codon 816 in the KIT receptor tyrosine kinase is associated with poor prognosis in patients with systemic mastocytosis and acute myeloid leukemia. Treatment of leukemic cells bearing this mutation with an allosteric inhibitor of p21–activated kinase (Pak) or its genetic inactivation results in growth repression due to enhanced apoptosis. Inhibition of the upstream effector Rac abrogates the oncogene-induced growth and activity of Pak. Although both Rac1 and Rac2 are constitutively activated via the guanine nucleotide exchange factor (GEF) Vav1, loss of Rac1 or Rac2 alone moderately corrected the growth of KIT-bearing leukemic cells, whereas the combined loss resulted in 75% growth repression. *In vivo*, the inhibition of Vav or Rac or Pak delayed the onset of myeloproliferative neoplasms (MPNs) and corrected the associated pathology in mice. To assess the role of Rac GEFs in oncogene-induced transformation, we used an inhibitor of Rac, EHop-016, which specifically targets Vav1 and found that EHop-016 was a potent inhibitor of human and murine leukemic cell growth. These studies identify Pak and Rac GTPases, including Vav1, as potential therapeutic targets in MPN and AML involving an oncogenic form of KIT.

4.B INTRODUCTION

Previously, we and others have shown that the regulatory subunit of class IA PI3K, p85α, is required for KITD814V (murine homolog) induced transformation (97, 151). Although p85α is a difficult protein to target therapeutically, the downstream effectors of the PI3K signaling pathway, in particular guanine exchange factors (GEF) such as Vav1, Tiam1 and Trio as well as their downstream targets including Rho family GTPases Rac1 and Rac2 and p21-activated kinase (Pak) might contribute to gain-of-function mutant KIT-mediated transformation.

Expression of the GEF Vav1 is predominantly restricted to the hematopoietic compartment (152). Vav1 consists of multiple domains including calponin homology domain, a Dbl homology domain, a pleckstrin homology domain, and a cysteine-rich region, as well as a Src homology 2 (SH2) domain flanked by two SH3 domains (153). Interestingly, deletion of the N-terminal region of Vav1 alone renders this protein oncogenic (152). Although, Vav has been shown to play an important role in regulating T and B cell signaling as well as neutrophil functions (113, 154); its role in leukemogenesis, in particular in oncogenic KIT induced MPN is unknown. Furthermore, in the context of an oncogene such as KITD814V, it is unclear, to what extent Vav1 regulates the activation of Rac (Rac1 and Rac2) GTPases and to what extent Rac1 and Rac2 contribute to transformation either alone or via their downstream substrate such as Pak.

Rac GTPases cycle between inactive GDP-bound and active GTP-bound states. Rac2 is predominantly expressed in hematopoietic cells whereas Rac1 is ubiquitously expressed (155). Although the role of Rac1 and Rac2 in normal hematopoiesis has been

well documented (155); how these GTPases contribute to oncogenic KIT (KITD814V) induced transformation is poorly understood. A small molecule antagonist of Rac, NSC23766 has been described (156). NSC23766 inhibits the activation of Rac by interfering with the binding of GEFs Tiam1 and Trio (156). More recently, Ortiz et al described a novel Rac inhibitor, EHop-016, which is derived on the basis of NSC23766's structure and inhibits the activation of Rac with a significantly lower IC_{50} compared to NSC23766 (157). How these two drugs impact the relative growth of KITD814V bearing cells and whether they equally inhibit the activation of Rac1 vs. Rac2 in these cells has never been explored.

Paks are serine/threonine kinases (158, 159). As a major downstream effector of Rac, Paks play an essential role in regulating both growth and actin based functions (160-162). Of the three isoforms that belong to group I family, Pak1 is the most well characterized member and is ubiquitously expressed (158). Pak1 expression is up-regulated in several solid tumors including in ovarian, breast and bladder cancers (163-165). While Pak has been shown to function as a potential downstream target of Rac, its relative contribution to MPNs or AML has not been explored. Here, we show that KITD814V (mouse) and KITD816V (human) bearing leukemic cells exhibit constitutive activation of Pak, Rac GTPases, and GEF Vav1. Utilizing a series of experiments using knockout mouse models, mouse models of MPN, dominant negative approaches, an allosteric inhibitor of Pak and a novel small molecule inhibitor of Rac, we provide a mechanism of KITD814V induced transformation and provide potential novel therapeutic targets for treating oncogenic KIT bearing neoplasms.

4.C RESULTS

The guanine exchange factor Vav1 regulates ligand independent growth and Rac1 and Rac2 activation in oncogene KIT (KITD814V) bearing cells

The role of hematopoietic specific GEF and its downstream substrates including Rac1 and/or Rac2 and Pak in KITD814V induced transformation is not known. To assess whether KITD814V bearing cells activate the GEF Vav1, we transduced 32D myeloid cells lacking the expression of endogenous KIT receptor with a bicistronic retrovirus encoding either a wild type (WT) version of KIT or an oncogenic version (KITD814V) (166). Transduced cells were sorted to homogeneity on the basis of EGFP expression and used in experiments described here. As seen in Figure 4.1.A, transduced cells bearing KITD814V following 8 hours of serum and growth factor deprivation demonstrated constitutive phosphorylation of Vav1 relative to WT KIT expressing cells. To assess the functional significance of constitutive Vav1 phosphorylation in KITD814V bearing primary HSC/Ps, we transduced low density bone marrow cells derived from WT or *Vav1-/-* mice with EGFP expressing WT KIT or KITD814V retrovirus. Primary bone marrow (BM) cells transduced at similar efficiency were sorted to homogeneity on the basis of EGFP expression and subjected to ligand independent (in the absence of stem cell factor) growth. As expected and previously shown (166), KITD814V expressing HSC/Ps showed a significant increase in ligand independent growth compared to WT KIT bearing cells, however, lack of Vav1 in these cells resulted in ~75% repression in ligand independent growth (Figure 4.1.B). Loss of Vav2 or Vav3 in primary HSC/Ps did not profoundly impact ligand independent growth of KITD814V bearing cells (Figure 4.2).

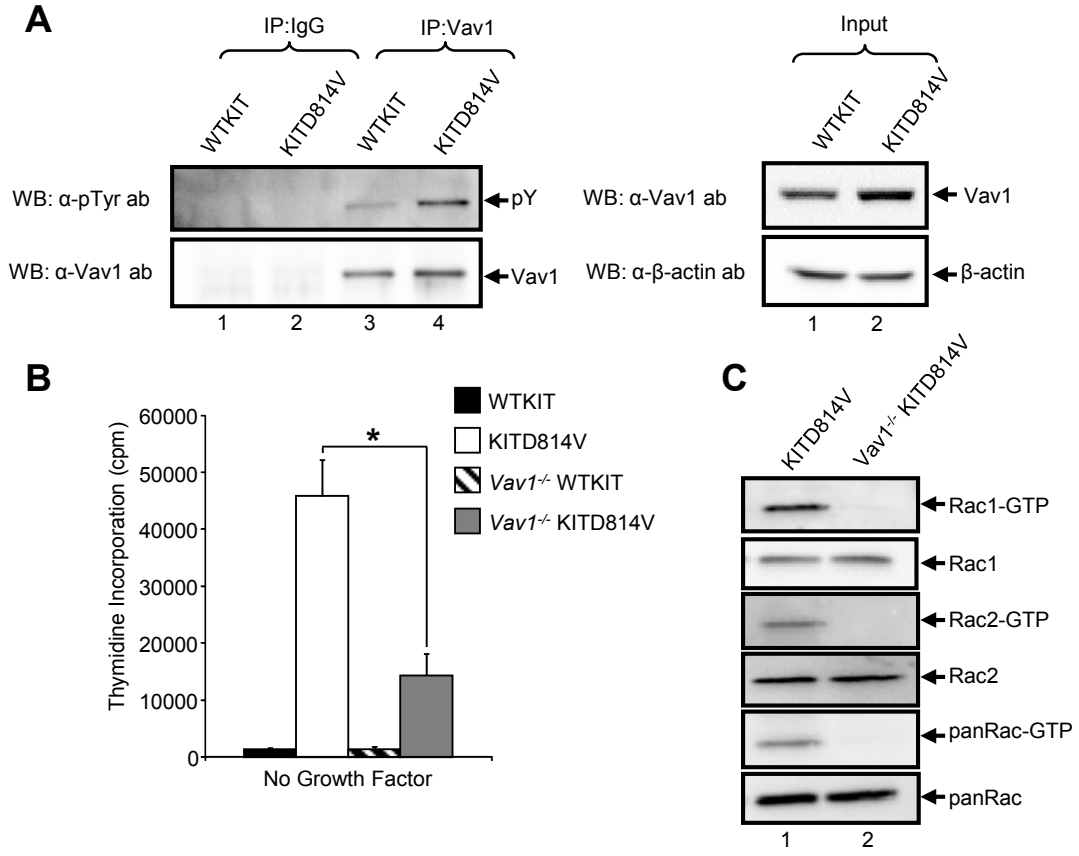

Figure 4.1. Constitutive activation of GEF Vav1 and Rac-GTPase in KITD814V expressing cells.

(A) 32D cells expressing either WT KIT or KITD814V were serum and growth factor starved for 8 hours and equal amount of lysates were subjected to IP using an anti-Vav1 antibody. The position of tyrosine phosphorylated Vav1 is indicated to the right of the blot. The right panel indicates the Vav1 protein whole cell lysate loading control. (B) Proliferation as assessed by thymidine incorporation in KITD814V expressing WT and Vav1 deficient primary BM cells in the absence of growth factors. Bars represent the mean thymidine incorporation (in counts per minute) in primary BM cells expressing the indicated receptors. Similar results were observed in three independent experiments. *p-value<0.05, KITD814V vs. *Vav1*$^{-/-}$ KITD814V. (C) Cell lysates derived in (B) were analyzed for Rac-GTP levels by incubating with agarose beads conjugated to Pak binding domain and subjecting the IPs to western blot analysis using an anti-Rac1, anti-Rac2 or anti-pan Rac antibody. Position of Rac-GTP is indicated to the right of the blot. The bottom panel shows total Rac protein in each lane.

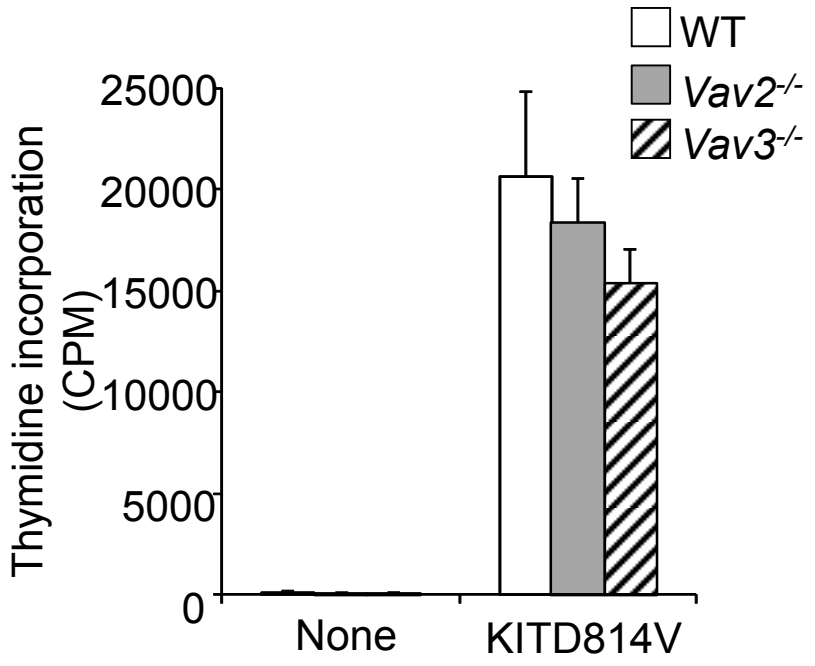

Figure 4.2. Ligand independent growth of KITD814V bearing cells in the absence of Vav2 or Vav3.

Proliferation as assessed by thymidine incorporation in KITD814V expressing WT, Vav2, and Vav3 deficient primary HSC/Ps in the absence of growth factors. Bars represent the mean [^{3}H] thymidine incorporation (in counts per minute) in primary BM cells expressing the indicated receptors. Data are pooled from experiments utilizing BM cells from two independent mice of each genotype.

To assess how Vav1 might contribute to KITD814V induced ligand independent growth, we determined the activation status of downstream substrates of Vav1, Rac family GTPases (155, 167). We examined the activation of both Rac1 and Rac2 in Vav1 deficient KITD814V bearing HSC/Ps. Figure 4.1.C shows constitutive activation of both Rac1 and Rac2 in KITD814V bearing WT HSC/Ps. In contrast, activation of both Rac1 and Rac2 was reduced in KITD814V bearing *Vav1-/-* HSC/Ps. These results suggest that Vav1 regulates Rac1 and Rac2 activation to similar extent in KITD814V bearing cells

and that deficiency of Vav1 in KITD814V bearing cells significantly reduces ligand independent growth normally associated with these cells.

While these results suggest a role for Vav1 in Rac activation and perhaps in KITD814V induced transformation; its direct involvement can only be established by inhibiting the activation of Rac in these cells. To test this, we infected KITD814V bearing 32D myeloid cells with a dominant negative version of Rac (RacN17). A bicistronic retrovirus encoding WT KIT or KITD814V along with a human CD4 antigen as a selectable marker was employed for these studies (Figure 4.3.A). RacN17 was cloned into an EGFP (for selection of transduced cells) encoding retrovirus. 32D myeloid cells were co-infected with virus encoding WT KIT or KITD814V and/or RacN17. Cells were sorted on the basis of hCD4 and/or EGFP expression (Figure 4.3.A). Single or double positive sorted cells were subjected to a proliferation assay. As seen in Figure 4.3B, expression of a dominant negative version of Rac (RacN17) in KITD814V bearing cells significantly repressed ligand independent growth relative to controls. The reduction in the growth of KITD814V and RacN17 co-expressing cells was in part due to reduced survival relative to controls (Figure 4.3.C). Importantly, IL-3 responses in all these cell types including in KITD814V and RacN17 co-expressing cells were comparable (Figure 4.3.B). The reduction in growth and survival of KITD814V and RacN17 co-expressing cells was due to direct repression of Rac1 and Rac2 (Figure 4.3.D). Taken together, these results suggest that downstream from Vav1, Rac1 and Rac2 are likely to play an essential role in regulating KITD814V induced transformation.

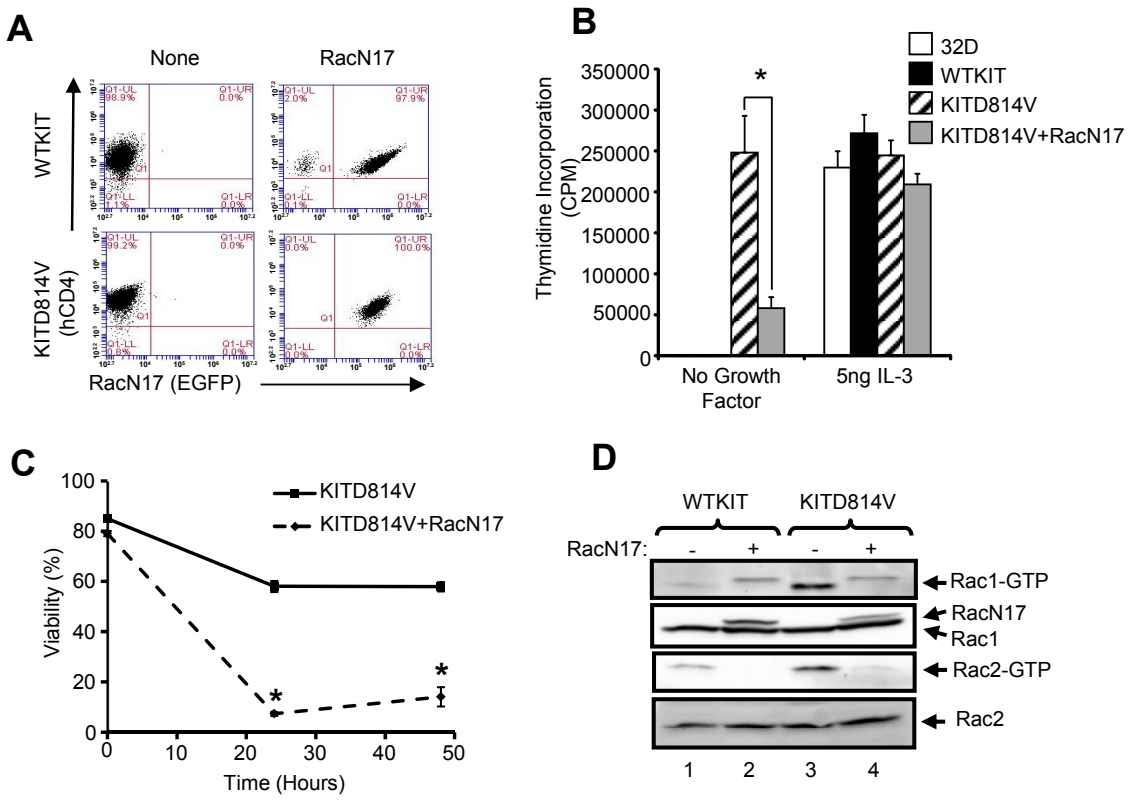

Figure 4.3. Inhibition of Rac activity in KITD814V bearing cells suppresses cell growth and survival.

(A) Flow cytometric analysis of 32D myeloid cells expressing WT KIT-hCD4 or KITD814V-hCD4 (Y-axis) and dominant negative RacN17-EGFP (X-axis). (B) Cells expressing KITD814V with or without RacN17 were cultured for 48 hours in the presence or absence of IL-3 in replicates of four and subjected to a [^{3}H] thymidine incorporation assay. Bars represent the mean ± SD thymidine incorporation (in counts per minute). Similar results were observed in three independent experiments. *p-value<0.05, KITD814V vs. KITD814V+RacN17. (C) Cells in (B) were grown in the absence of growth factors for 24 or 48 hours prior to being subjected to Annexin V and 7-AAD staining. Survival (viability) was determined as the percentage of both Annexin V and 7-AAD negative cells. Bars denote the mean ± SD percentage of total surviving cells from one of two independent experiments performed in quadruplicate. *p-value<0.05, KITD814V vs. KITD814V+RacN17. (D) Cells in (B) were subjected to Pak binding domain (PBD) pull down assay to assess Rac1-GTP, Rac2-GTP, total Rac1 and total Rac2 expression in the indicated genotypes. Data are from a representative experiment performed on 3 independent occasions.

To test this in more detail, we performed transplant studies by injecting myeloid cells co-expressing KITD814V and RacN17 along with control cells into C3H/HeJ syngeneic mice (166). Transplanted mice were observed for the development of MPN and survival relative to controls. Mice transplanted with cells co-expressing KITD814V and RacN17 demonstrated a significantly prolonged lifespan relative to KITD814V only expressing mice (Figure 4.4.A). A cohort of KITD814V and RacN17 co-expressing mice were analyzed in detail at the same time as when KITD814V only bearing mice first began to succumb, to assess the impact of Rac repression on MPN development. As seen in Figure 4.4.B, KITD814V bearing mice showed PB counts in the range of 150 K/μ**l**. In contrast, mice transplanted with cells co-expressing KITD814V and RacN17 demonstrated a profound reduction in PB WBCs and neutrophils counts. Additional analysis was conducted on KITD814V and RacN17 co-expressing mice when KITD814V only mice began to succumb. In other words, KITD814V and RacN17 bearing mice were sacrificed at the same time as moribund KITD814V mice to assess disease progression and the impact of Rac repression on MPN. Around the time of death, up to 90% leukemic cells were detected in the PB, BM and spleen of mice transplanted with cells bearing KITD814V alone (Figure 4.5.A). In contrast, mice transplanted with cells co-expressing KITD814V and RacN17 demonstrated only ~20% leukemic cells as determined by EGFP positivity in all three tissues examined. Figure 4.5.A left panel shows quantitative assessment of the presence of EGFP expressing cells in PB, BM and spleen; and right panel demonstrates representative flow cytometry blots demonstrating the percentage of EGFP expression in various tissues in the indicated genotypes. Figure 4.5.B demonstrates a quantitative reduction in the spleen weight of mice transplanted with cells co-

expressing KITD814V and RacN17 relative to mice transplanted with cells bearing the KITD814V mutation alone. Right panel depicts representative spleens derived from KITD814V bearing mice as well as mice co-expressing both KITD814V and RacN17. Importantly, while gross lung lesions were observed in mice transplanted with cells bearing KITD814V alone; these lesions were nearly absent in the lungs of transplanted mice co-expressing KITD814V and RacN17 (Figure 4.5.C). Histopathological analysis revealed that while massive myeloid cell infiltration was observed in mice transplanted with KITD814V bearing cells in spleen and lungs; mice bearing cells co-expressing KITD814V and RacN17 showed significantly less infiltration of these cells and the splenic architecture in these mice was indistinguishable from control mice (Figure 4.4.C-D).

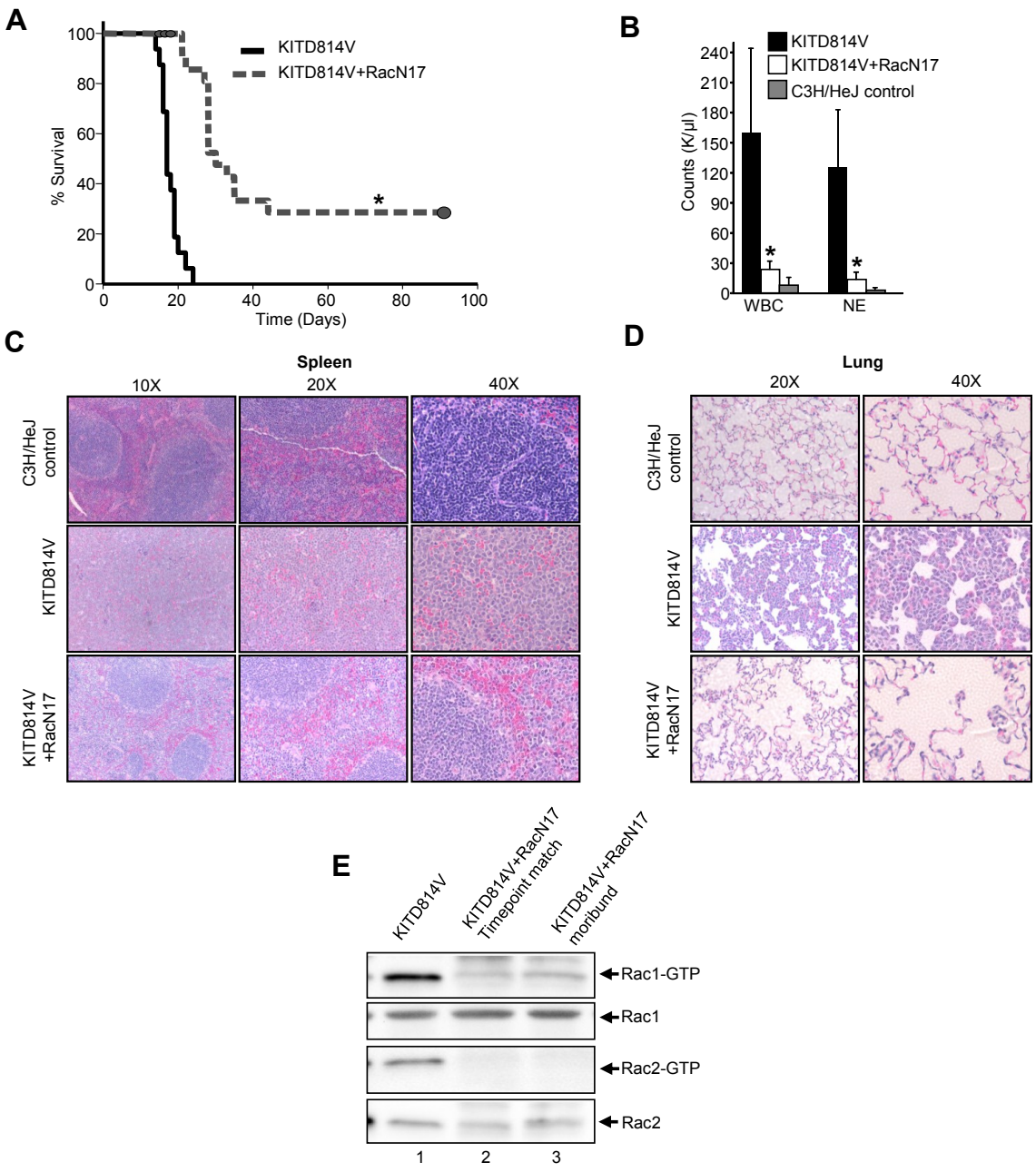

Figure 4.4. *In vivo* inhibition of Rac prolongs the survival and rescues myeloid cell infiltration associated with KITD814V bearing mice.

C3H/HeJ mice were transplanted with 2 million cells bearing KITD814V with or without RacN17. (A) Kaplan-Meier survival analysis of syngeneic C3H/HeJ mice transplanted with cells bearing KITD814V (n=16) or KITD814V+RacN17 (n=26). *p-value<0.001, KITD814V vs. KITD814V+RacN17. (B) White blood cell (WBC) and neutrophil (NE) counts in mice bearing cells transplanted with KITD814V (n=9) or KITD814V+RacN17 (n=12) and C3H/HeJ control mice (n=5). *p-value<0.05, KITD814V vs. KITD814V+RacN17. Histopathologic analysis of spleen (C) and lung (D) from mice

transplanted with cells bearing KITD814V alone or in combination with RacN17. Shown are representative tissue sections (10 to 40 X magnifications) after fixing them in 10% buffered formalin, sectioning and staining with hematoxylin and eosin. Normal erythroid and myeloid components in lungs, liver and spleen were replaced by leukemic cells in KITD814V bearing mice, which were significantly rescued in tissues from mice bearing RacN17 along with KITD814V. (E) Cell lysates derived from spleens of mice described above were subjected to a Rac activity assay as described in Figure 4.1.C. Shown is the level of Rac1-GTP, Rac2-GTP, total Rac1, and total Rac2 in each lane from the indicated genotypes.

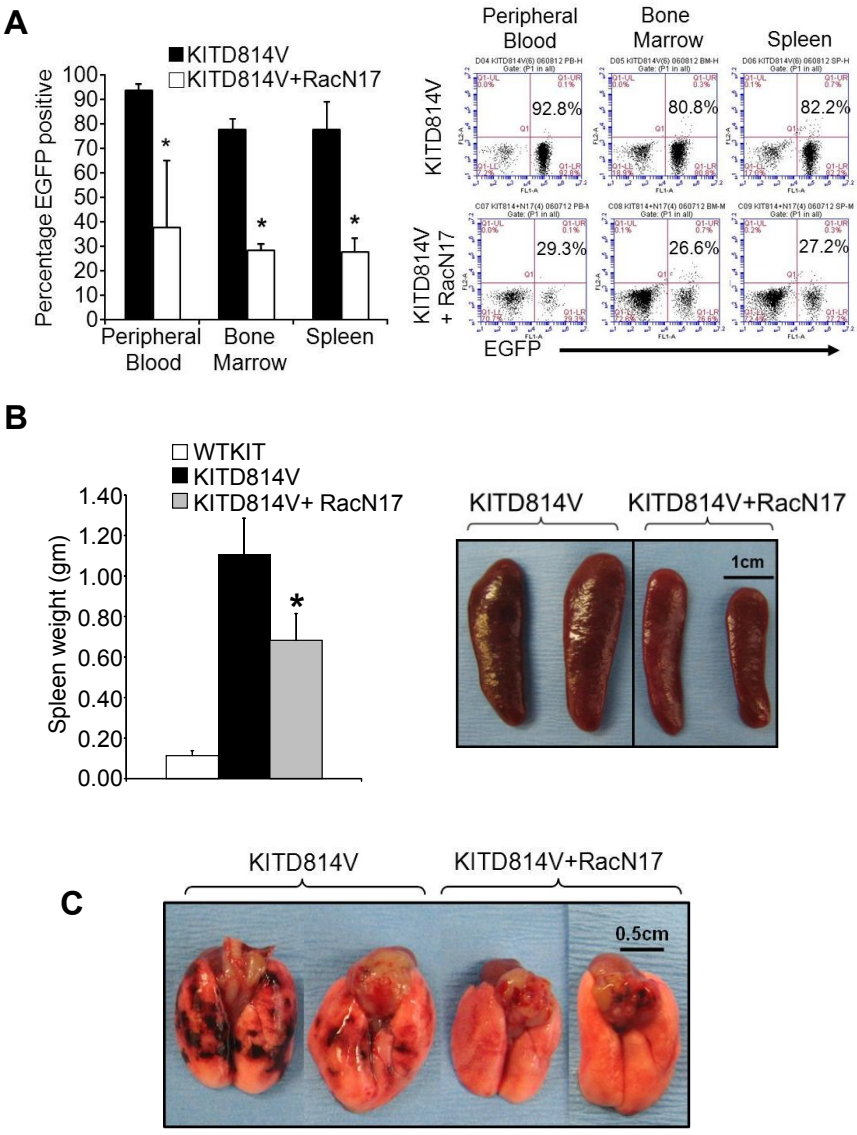

Figure 4.5. Inhibition of Rac rescues the tissue damage associated with mice transplanted with cells bearing KITD814V.

(A) Left panel shows the percentage of EGFP+ leukemic cells in the peripheral blood, bone marrow, and spleen of mice transplanted with cells bearing KITD814V alone or in combination with RacN17 at the time of moribund. n=5, *p-value<0.001, KITD814V vs. KITD814V+RacN17. Right panel shows representative flow cytometric dot blot profiles from mice described in left panel at time of moribund. (B) Weight and size of spleens from various genotypes was assessed at time of moribund. Quantitative differences in the weight of spleens from the indicated genotypes are shown in the left panel and representative pictures of spleens are shown in the right panel. n=5, *p-value<0.001, KITD814V vs. KITD814V+RacN17. (C) Representative pictures of liver from mice transplanted with cells bearing KITD814V alone or in combination with RacN17.

To assess whether the enhanced survival of mice, reduced infiltration of myeloid blasts in various tissues, including lung and liver and lack of destruction of the splenic architecture observed in mice transplanted with cells co-expressing KITD814V and RacN17 was due to reduced Rac activation, we examined the activation status of Rac1 and Rac2 in splenocytes derived from the indicated genotypes. Cellular lysates from spleens of KITD814V bearing mice were harvested at moribund. At the same time, splenic lysates were derived from mice transplanted with cells co-expressing KITD814V and RacN17, designated as time point matched and at moribund, respectively. As seen in Figure 4.4.E, mice transplanted with cells co-expressing RacN17 and KITD814V showed significant repression in the activation of both Rac1 and Rac2 compared to controls. In addition to Rac repression, a significant repression in the activation of Pak, a downstream substrate of Rac was also observed (Figure 4.6). These results suggest that expression of RacN17 in KITD814V bearing cells likely inhibits their growth *in vivo* by inhibiting the activation of Rac1 and Rac2, resulting in prolonged survival and delayed MPN development.

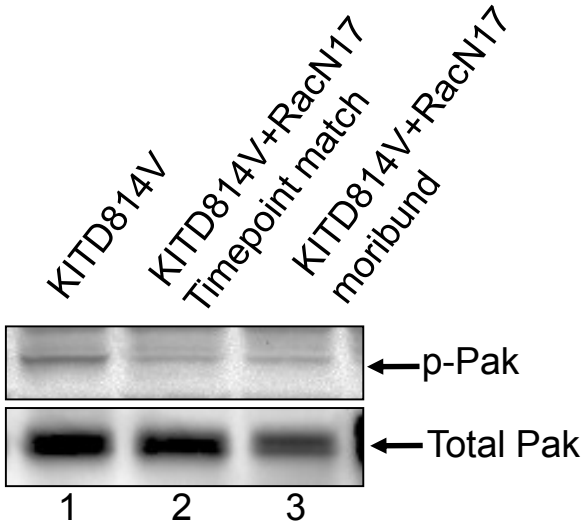

Figure 4.6. *In vivo* reduction of active Pak due to Rac inhibition in KITD814V bearing mice.

Cell lysates derived from spleens of C3H/HeJ mice transplanted with cells bearing KITD814V with or without RacN17 were immunoblotted with antibodies that recognize phospho-Pak and total Pak.

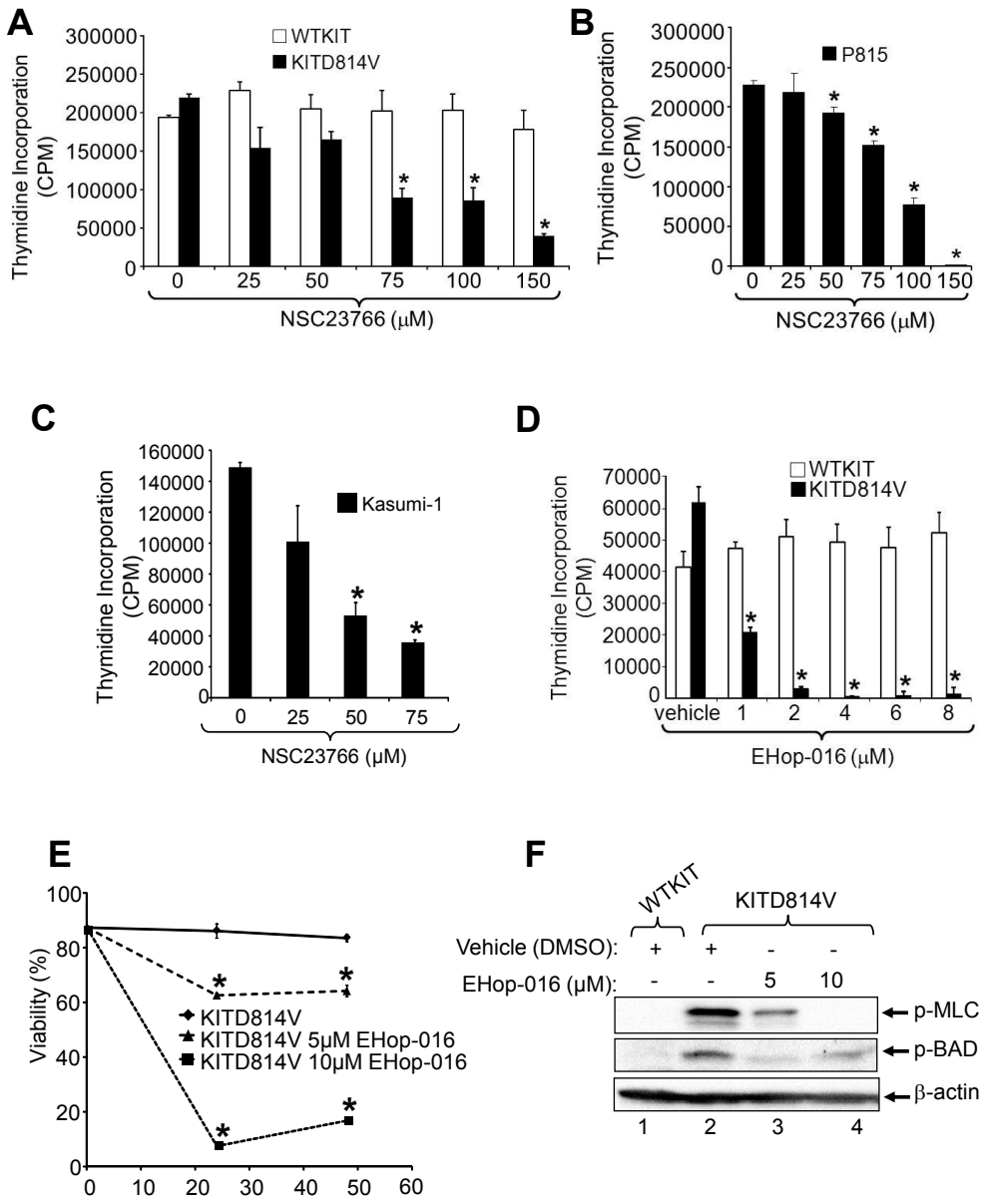

Figure 4.7. Inhibition of Rac GTPases affects KITD814V growth due to increased apoptosis.

(A) 32D cells bearing WT KIT grown in the presence of 5ng/mL IL-3 or KITD814V bearing cells grown in the absence of growth factors and indicated Rac inhibitor (NSC23766) were subjected to a [^{3}H] thymidine incorporation assay. (B) Murine mastocytoma cells, P815, and (C) human AML patient derived Kasumi-1 cells were cultured in the presence of NSC23766 and assessed for proliferation by measuring [^{3}H] thymidine incorporation. Bars represent mean ± SD from 2 to 4 independent experiments performed in replicates of four. *p-value<0.05. (D) 32D cells expressing KITD814V or WT KIT cells grown in the presence of a novel Rac inhibitor, EHop-016, were subjected to a [^{3}H] thymidine incorporation assay as described above. *p-value<0.05 (E)

KITD814V bearing 32D cells were examined for survival in the presence of 5μM or 10μM EHop-016 for 0, 24, or 48 hours prior to subjecting the cells to Annexin V and 7-AAD staining. Survival was assessed by quantitating the percentage of Annexin V and 7-AAD negative (viable) cells. Bars denote the mean ± SD percentage of viable cells from one of two independent experiments performed in quadruplicate. *p-value<0.05, vehicle vs EHop-016 treated. (F) Cells described in (E) were treated with EHop-016 and cell lysates were subjected to immunoblotting with phospho-MLC, phospho-BAD, and β-actin.

Growth of oncogenic KIT (KITD814V in mouse and KITD816V in humans) bearing murine and human mastocytosis patient derived cells is inhibited by a novel Rac inhibitor, EHop-016

Based on the observation that Rac inhibition *in vivo* delays KITD814V driven MPN, we hypothesized that pharmacologic inhibition of Rac might inhibit KITD814V induced proliferation *in vitro*. For these studies, we first utilized a well characterized Rac inhibitor NSC23766 (156). NSC23766 inhibits Rac binding and activation by the Rac specific GEF Trio or Tiam1 (156). Treatment of myeloid cells bearing the WT KIT or KITD814V receptor with NSC23766 demonstrated a dose dependent reduction in hyperproliferation of KITD814V bearing cells but not of WT KIT bearing cells (Figure 4.7.A). Consistently, treatment of a murine mastocytoma cell line P815 that bears the activating KITD814V mutation, as well as AML patient derived Kasumi-1 cells, that expresses the KITD816V mutation, demonstrated a dose dependent growth repression in the presence of NSC23766 (Figure 4.7.B & C). While a dose dependent growth inhibition of oncogene KIT bearing cells in the presence of NSC23766 was observed; the concentration of NSC23766 that resulted in 50% growth inhibition of leukemic cells was nearly 75 to100 μM for all cell types examined. This could potentially be due to a lack of a major role of GEFs Trio and Tiam1 in KITD814V induced transformation.

Alternatively, Rac1 and/or Rac2 alone may play only a modest role in KITD814V induced transformation. To test these possibilities and to more precisely identify the consequence(s) of interfering with the function of GEFs and Rac in KITD814V induced transformation, we utilized a novel Rac inhibitor EHop-016, which is a derivative of NSC23766 and inhibits Rac 100-fold more efficiently than NSC23766 in metastatic breast cell line, MDA-MB-231 (157). As seen in Figure 4.8.A & B, KITD816V bearing SM human patient derived HMC1.2 cells, as well as KITD816V and AML1-ETO bearing Kasumi-1 cells derived from an AML patient, demonstrated a significant reduction in the growth of these cells in the presence of EHop-016 at drug concentrations that were significantly less (~50-fold in some cases) than NSC23766, IC_{50}=100 μM. Importantly, primary systemic mastocytosis patient derived cells bearing KITD816V were significantly more susceptible to growth inhibition in the presence of EHop-016 relative to patient samples lacking the expression of KITD816V (Figure 4.8.C). The growth reduction in the presence of EHop-016 was also observed in murine KITD814V expressing cells and was associated with enhanced apoptosis and repression of anti-apoptotic proteins Bad and MLC (Figure. 4.7.D-F). Consistent with these findings, while 20μM of NSC23766 inhibited the activation of Rac1 but not Rac2 in KITD814V bearing cells; 10μM of EHop-016 was sufficient to inhibit the activation of both Rac1 and Rac2 in these same cells, which was associated with reduced binding of Vav for Rac (Figure 4.8.D & 4.8.E). These results suggest that inhibition of both Rac1 and Rac2 is essential for maximal growth repression in KITD814V bearing cells. Further, Rac inhibitor EHop-016 is significantly more efficient in repressing the growth and activation of Rac1 and Rac2 in KITD814V bearing cells compared to NSC23766. Consistent with the *in vit*ro

findings described above, treatment of KITD814V bearing cells with EHop-016 vs. NSC23766 significantly enhances the survival of leukemic mice, which is associated with reduced spleen size and PB counts (Figure 4.9.A-D).

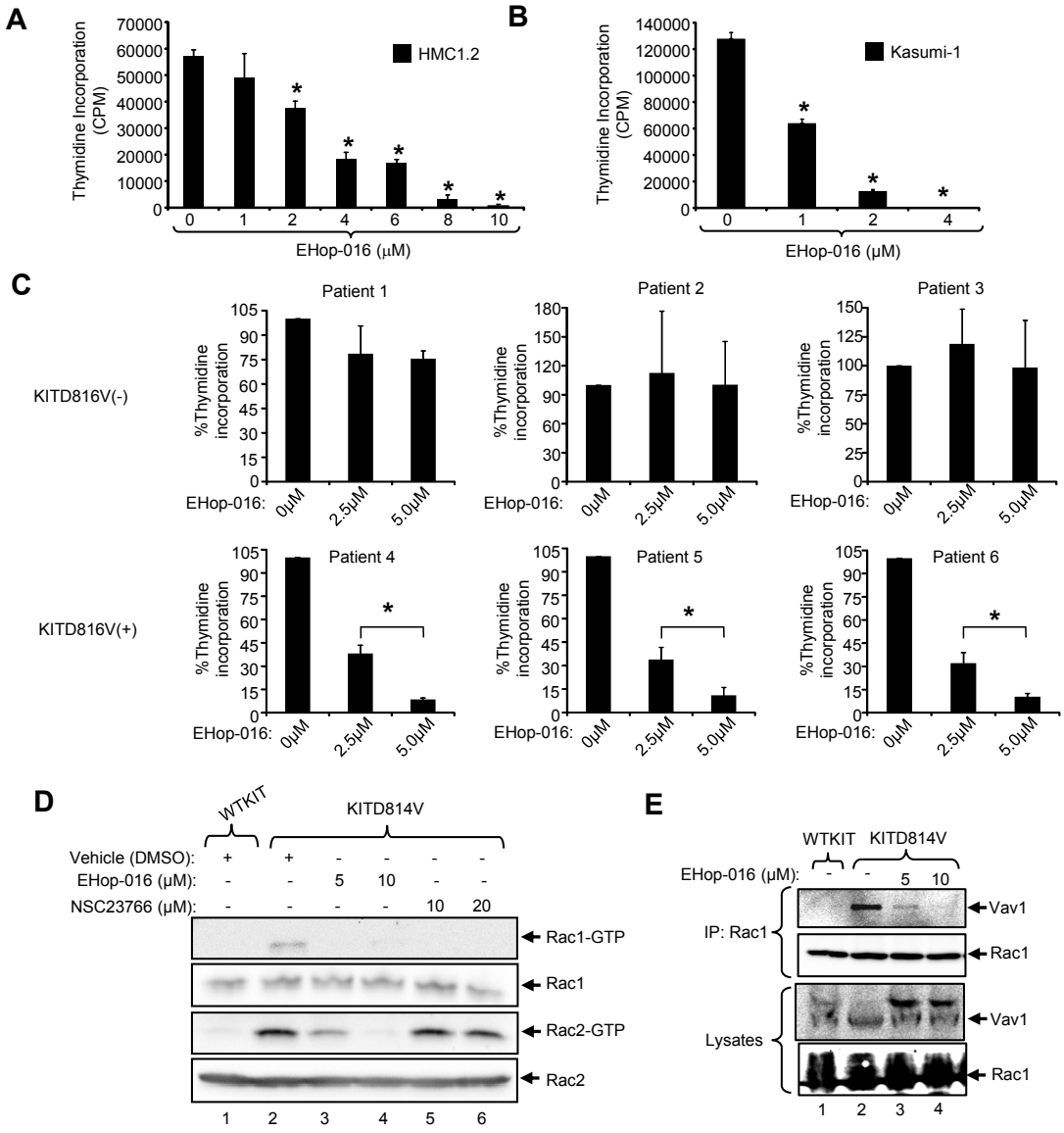

Figure 4.8. A novel Rac inhibitor, EHop-016, is a potent inhibitor of KITD814V induced growth in systemic mastocytosis (SM) and AML patient derived cells.

(A) Human SM patient derived HMC1.2 cells bearing KITD816V and (B) human AML derived Kasumi-1 cells bearing KITD816V were cultured in the presence of EHop-016 and assessed for proliferation by measuring [^{3}H] thymidine incorporation. Bars represent mean ± SD from 4 independent experiments performed in replicates of four. *p-value<0.05 (C) Individual systemic mastocytosis patient derived cells positive or negative for KITD816V expression were cultured in the presence of indicated concentrations of EHop-016. Bars represent mean ± SD performed in replicates of three. *p-value<0.05, 0μM vs indicated concentration. (D) 32D cells bearing WT KIT or KITD814V were starved and treated with indicated concentrations of NSC23766 or EHop-016 and subjected to Rac activation assay. Shown are the levels of active Rac1, Rac2, total Rac1, and Rac2 in each lane. (E) 32D cells expressing either WT KIT or

KITD814V were starved and incubated with vehicle, 5 or 10μM EHop-016; lysates were immunoprecipitated (IP) with anti-Rac1 antibody and immunoblotted with an anti-Vav1 or an anti-Rac1 antibody, respectively. The lower panels indicate the Vav1 and Rac1 protein whole cell lysate loading control.

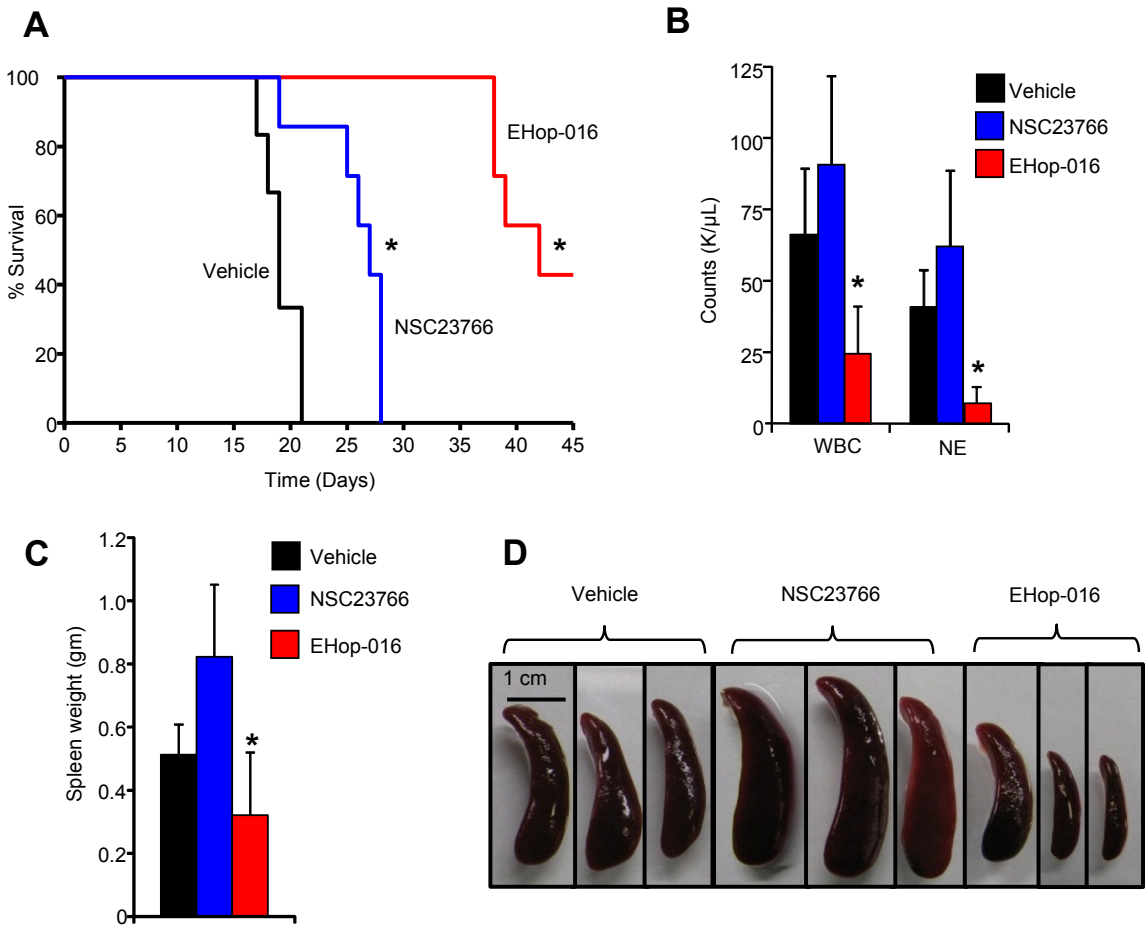

Figure 4.9. Pharmacologic inhibition of Rac GTPases delays disease progression in mice transplanted with cells bearing KITD814V receptor.

C3H/HeJ mice were transplanted with 1 million cells bearing KITD814V following overnight *in vitro* treatment with vehicle (n=6), EHop-016 (2.5 μM, n=7), or NSC23766 (25 μM, n=7) and (A) survival was analyzed by Kaplan-Meier rank log analysis. *p-value<0.05 Vehicle vs EHop-016 or NSC23766. (B) Peripheral blood counts for white blood cells (WBC), and neutrophils (NE) between the different transplanted groups at time of harvest. *p-value<0.05, Vehicle vs EHop-016. (C) Quantitative difference in spleen mass and (D) representative spleens from indicated transplantation groups are shown. *p-value<0.05, Vehicle vs. EHop-016.

Genetic disruption of Rac GTPases in KITD814V bearing HSC/Ps inhibits ligand independent hyperproliferation and MPN in vivo

We next analyzed the specific role of Rac1 and Rac2 and also attempted to confirm our findings using dominant negative Rac (RacN17) and pharmacologic approaches by examining the growth of KITD814V bearing primary BM cells derived from mice lacking the expression of Rac1, Rac2 or both Rac1 and Rac2. Since complete loss of Rac1 results in embryonic lethality, conditional knockout strain of Rac1 was crossed with Mx1-Cre mice allowing for conditional deletion of Rac1 in BM cells. To induce Cre recombinase expression, three doses of 200μg polyI:polyC were injected intraperitoneally at forty-eight hour intervals. As seen in Figure 4.10.A, and has been previously described, conditional deletion of Rac1 results in loss of Rac1 protein (111). Conditional Rac1 mice were crossed with Rac2 transgenic knockout mice and following polyI:polyC treatment a similar loss of Rac1 protein was also observed in mice lacking both Rac1 and Rac2 as assessed by western blot analysis using a Rac1 specific antibody (Figure 4.10.A; lanes 2 & 4). Low density BM cells from all three genotypes were pre-stimulated with cytokines and infected with a retrovirus expressing WT KIT or KITD814V. Figure 4.10.B shows the transduction efficiency of the two viruses in BM cells derived from different genotypes. Transduced cells were sorted to homogeneity and utilized in a proliferation assay as well as to assess survival in the absence of Rac proteins. As seen in Figure 4.10.C, while loss of Rac2 resulted in ~50% inhibition in ligand independent growth of KITD814V bearing cells; loss of Rac1 showed only a modest reduction (~10 to 15%) in ligand independent growth. In contrast, BM cells

deficient in both Rac1 and Rac2, showed a more profound reduction in the growth of KITD814V bearing cells (~75%).

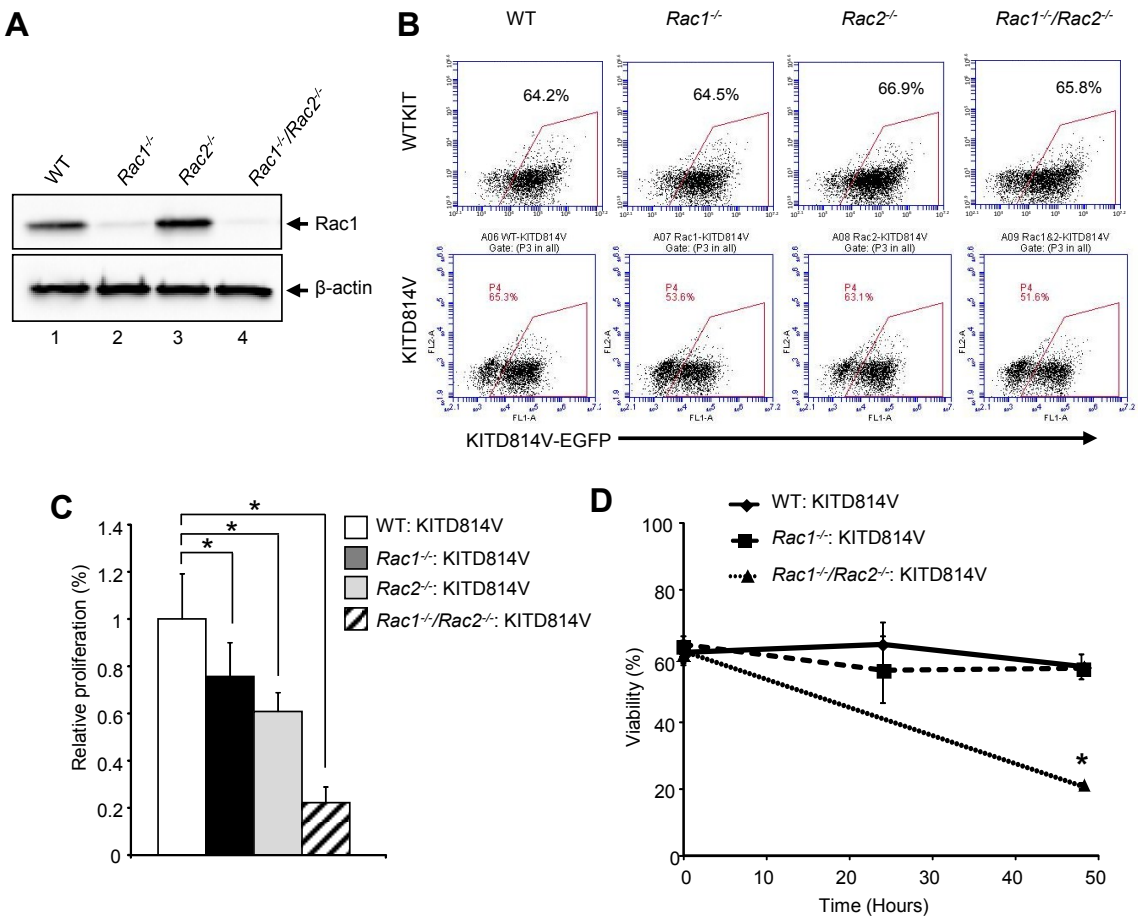

Figure 4.10. Deficiency of Rac1 and Rac2 in KITD814V bearing primary HSC/Ps represses ligand independent growth.

(A) Peripheral blood cells were collected from WT, *Rac1 flox/flox, Rac2-/-* and *Rac2-/-:Cre:Rac1 flox/flox* mice following four consecutive intraperitoneal injections of polyI:polyC given at 48 hour intervals. Red blood cells were lysed, and equal amount of protein were subjected to western blotting using an anti-Rac1 antibody. Levels of Rac1 in each lane are shown. (B) Representative flow cytometric dot plots of low density bone marrow cells derived from WT, *Rac1-/-, Rac2-/-* and *Rac1-/-:Rac2-/-* mice transduced with WT KIT or KITD814V expressing retrovirus. EGFP expression on X-axis is reflective of the transduction efficiency in the indicated genotypes. (C) WT, *Rac1-/-, Rac2-/-* and *Rac1-/-:Rac2-/-* primary HSC/Ps expressing WT KIT or KITD814V were subjected to a thymidine incorporation assay. *p-value<0.05, KITD814V in WT vs. *Rac2-/-, Rac1-/-* or *Rac1-/-:Rac2-/-* cells. Combined data from three independent experiments in replicates of four is shown. (D) WT, *Rac1-/-* and *Rac1-/-:Rac2-/-* primary HSC/Ps expressing KITD814V were grown in the absence of growth factors for 0, 24 or 48 hours prior to being analyzed by Annexin V and 7-AAD staining. Apoptosis was determined as percentage of both Annexin V and 7-AAD positive staining. Bars denote the mean ± SD percentage of Annexin V and 7-AAD negative (viable) cells. *p-value<0.05, WT, *Rac1-/-* vs. *Rac1-/-:Rac2-/-*.

The reduction in the growth of Rac1/Rac2 double knockout cells bearing KITD814V was due in part to reduced survival of cells relative to controls (Figure 4.10.D). These findings suggest that combined loss of Rac1 and Rac2 results in most growth repression in KITD814V bearing cells. Restoring the expression of activated Rac in the setting of Rac1/Rac2 deficiency rescued ligand independent growth *in vitro* (Figure 4.11.A). Furthermore, no compensatory increase in the expression of Rac3 was noted in these cells (Figure 4.11.B). To further assess the role of Vav1 and Rac1/Rac2 in KITD814V induced MPN, we infected WT or *Vav1-/-* or *Rac1/Rac2-/-* 5-FU treated BM cells with a virus expressing WT KIT or KITD814V, sorted the cells on the basis of EGFP expression and transplanted into lethally irradiated hosts. Figure 4.12 shows the transduction efficiency in 5-FU treated cells of all three genotypes. As seen in Figure 4.13.A, a significant increase in the survival of mice bearing *Vav1-/-* or *Rac1/Rac2-/-*KITD814V expressing cells was observed relative to controls. Further, a rescue in the peripheral blood counts and splenomegaly in these mice relative to controls was also observed (Figure 4.13.B-D). Loss of Rac2 alone in the setting of KITD814V expression also prolonged the survival of leukemic mice but to a much lesser degree compared to mice transplanted with KITD814V bearing cells lacking the expression of Vav1 or Rac1/Rac2 (Figure 4.14).

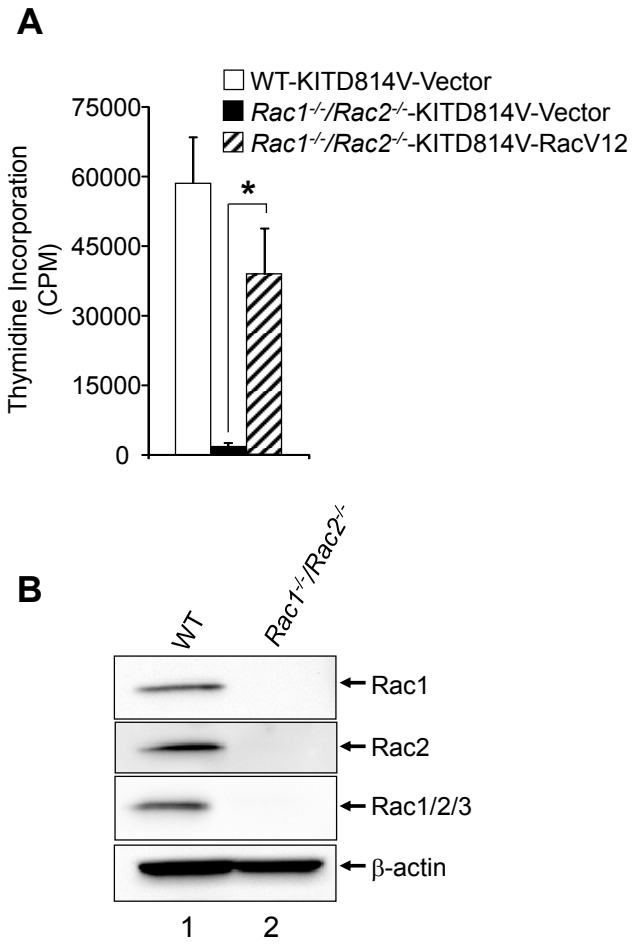

Figure 4.11. Expression of active RacV12 restores KITD814V induced hyperproliferation in Rac1/Rac2 deficient HSC/Ps.

(A) Primary HSC/Ps were collected from WT and *MxCreRac1$^{flox/flox}$Rac2$^{-/-}$* mice following three consecutive intraperitoneal injections of polyI:polyC given at 48 hour intervals. These cells were transduced with KITD814V in the presence of empty vector or RacV12, sorted on EGFP and selected in puromycin and assessed for proliferation by [^{3}H] thymidine incorporation in the absence of growth factors. Bars represent the mean [^{3}H] thymidine incorporation (in counts per minute) in primary HSC/Ps expressing the indicated receptors. *p-value<0.05, empty vector vs RacV12. (B) KITD814V bearing WT or *Rac1-/-:Rac2-/-* HSC/Ps were lysed, and equal amount of lysates were subjected to western blotting using total Rac1, Rac2, pan-Rac, and ß actin antibody.

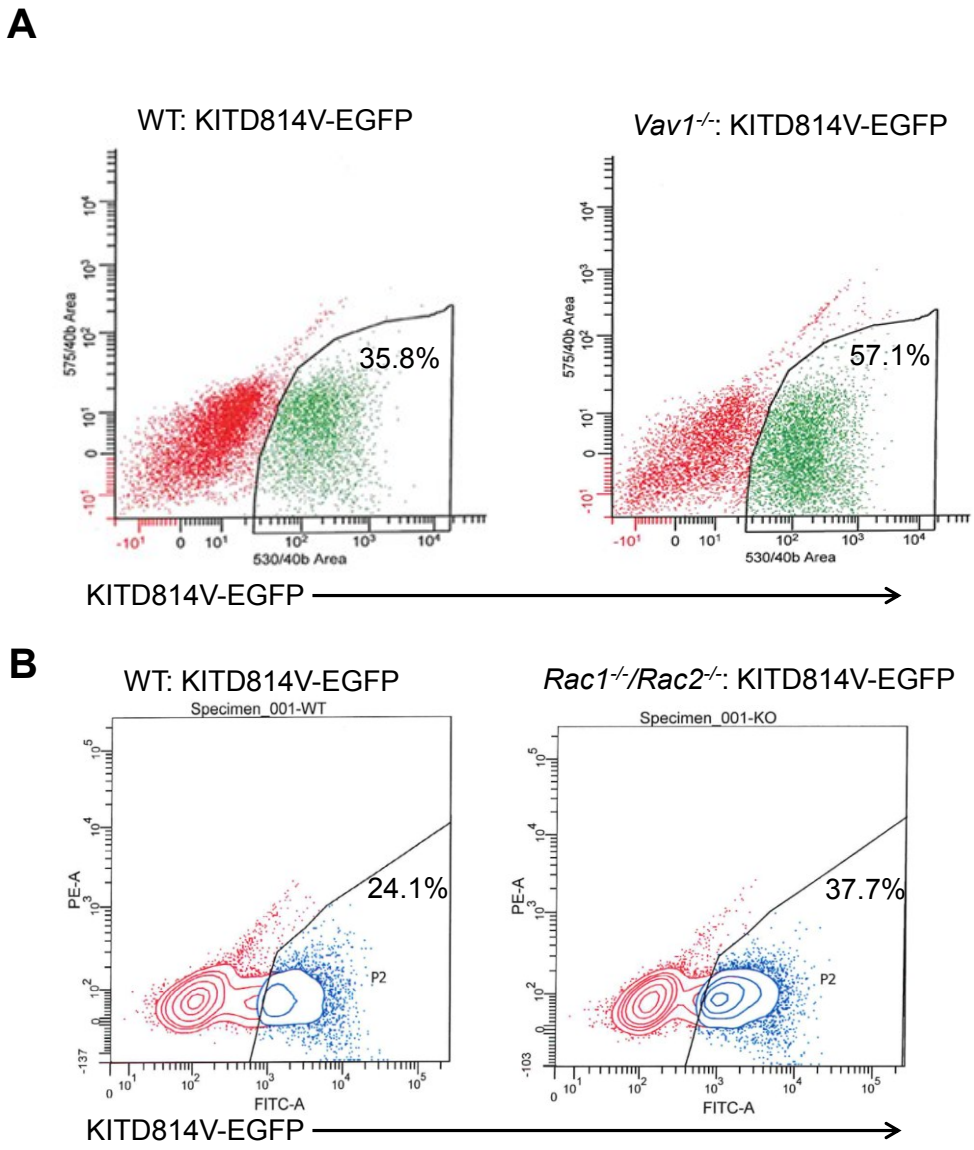

Figure 4.12. Transduction efficiency of primary 5-FU treated bone marrow (BM) cells.

5-fluorouracil treated BM cells derived from WT, *Vav1*$^{-/-}$, and *Rac1*$^{flox/flox}$*Rac2*$^{-/-}$ mice were infected with retrovirus encoding the KITD814V receptor. EGFP expression on the x-axis is indicative of the transduction efficiency for each genotype. Transduced cells were sorted to homogeneity prior to being transplanted into recipients for MPN development and survival studies described in Figure 4.13.

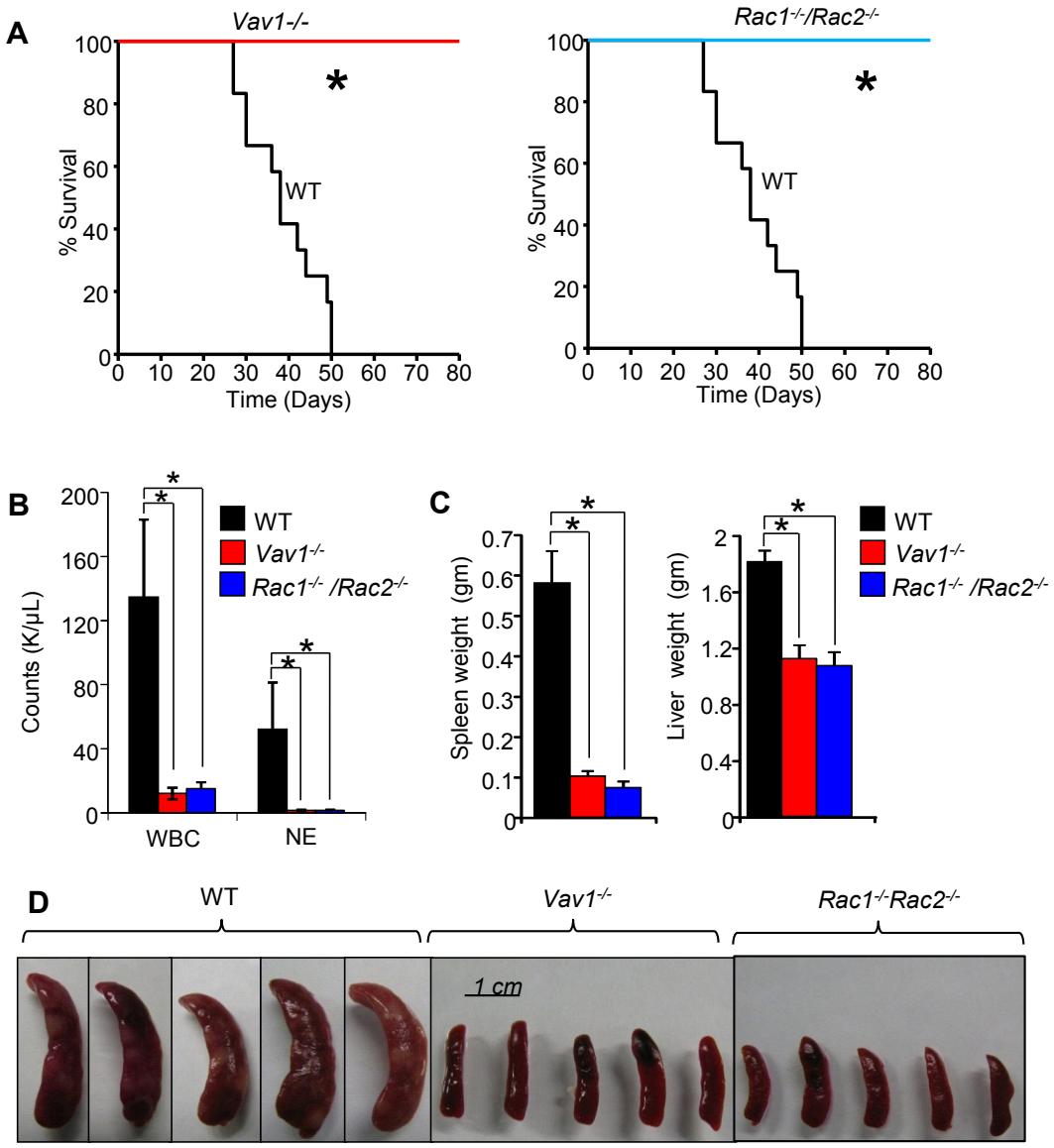

Figure 4.13. Genetic disruption of GEF Vav1 and Rac GTPases enhances the survival of KITD814V bearing mice.

(A) Cumulative survival analysis using Kaplan-Meier log-rank test of recipient mice bearing KITD814V expressing wild type (WT; n=10), *Vav1*$^{-/-}$ (n=5), or *Rac1*$^{-/-}$*;Rac2*$^{-/-}$ (n=5) HSC/Ps. *p-value<0.05 WT vs *Vav1*$^{-/-}$ or *Rac1*$^{-/-}$*/Rac2*$^{-/-}$. (B) Peripheral blood counts were significantly elevated in recipients of KITD814V bearing WT HSC/Ps (n=10) relative to KITD814V bearing *Vav1*$^{-/-}$ (n=5), or *Rac1*$^{-/-}$*;Rac2*$^{-/-}$ (n=5) HSC/Ps. *p-value<0.05 for WT vs *Vav1*$^{-/-}$ or *Rac1*$^{-/-}$*;Rac2-/-:* KITD814V. Data are mean ± SEM. (C) Quantitative difference in the size of the spleen and liver between different transplanted groups. *p-value<0.05. WT vs *Vav1*$^{-/-}$ or *Rac1*$^{-/-}$*/Rac2*$^{-/-}$. (D) Representative spleen pictures derived from mice transplanted with HSC/Ps bearing KITD814V in the setting of the indicated genotypes.

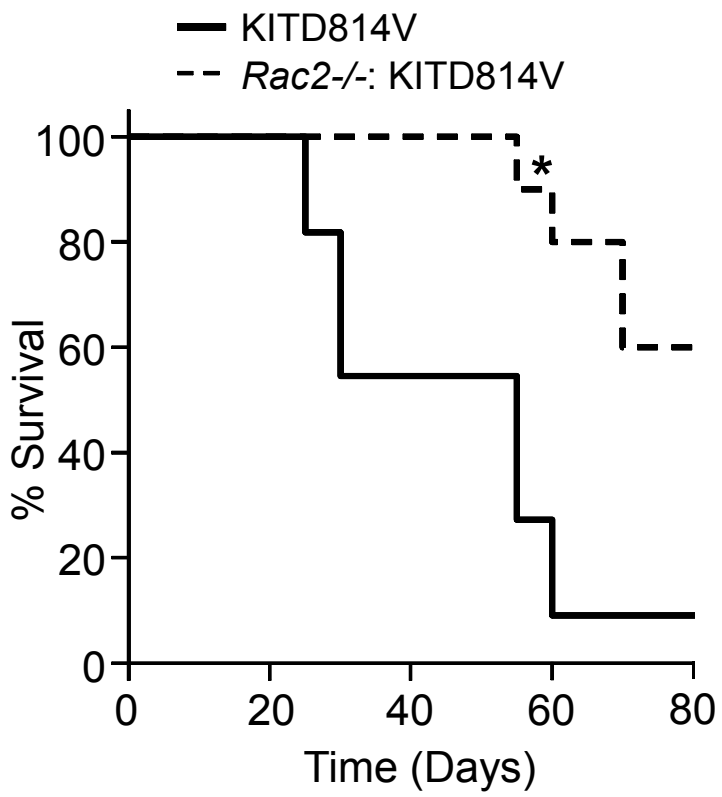

Figure 4.14. Genetic disruption of Rac2 enhances survival of KITD814V bearing mice.

Cumulative survival using Kaplan-Meier log-rank test of recipient cohorts bearing KITD814V expressing wild type (WT; n=11) (97) or *Rac2*$^{-/-}$ (n=10) BM. *p-value<0.05. Data showing the survival of KITD814V bearing mice has been previously shown (97).

Role of p21-activated kinase in KITD814V induced transformation

As noted earlier, expression of dominant negative Rac (RacN17) in KITD814V bearing cells derived from the spleens of transplanted mice, not only resulted in reduced Rac1/Rac2 activation but also inhibited the activation of Pak. Paks involvement in MPNs or in leukemogenesis has never been reported, although recent studies have implicated Pak in regulating the growth and actin based functions in solid tumors (168-170). To assess the role of Pak in KITD814V induced transformation and to determine if Pak functions downstream from Vav1 and Rac1/Rac2 in KITD814V bearing cells, we

examined Pak activation in KITD814V bearing *Vav1-/-, Rac1/Rac2-/-,* RacN17 or EHop-016 treated primary HSC/Ps. As seen in Figure 4.15.A-D, a reduction in the activation of Pak was observed in every scenario. To assess Paks role directly in regulating ligand independent growth of KITD814V bearing cells, we utilized myeloid cells bearing the expression of KITD814V and infected them with a retrovirus expressing a dominant negative version of Pak (PakK299R). 100% EGFP (reflecting the expression of PakK299R) and hCD4 (reflecting the expression of KITD814V) double positive cells were sorted to homogeneity and subjected to a biochemical and proliferation assay as described in Figure 4.2 (Figure 4.16.A). As seen in Figure 4.15.E, while expression of KIT and its constitutive phosphorylation was observed in both KITD814V and KITD814V and PakK299R co-expressing cells, activation of Pak was completely inhibited in cells co-expressing KITD814V and PakK299R relative to controls. Importantly, Pak repression in KITD814V bearing cells completely abrogated ligand independent proliferation normally observed in KITD814V bearing cells but not of cells stimulated via the IL-3 signaling pathway (Figure 4.15.F). Expression of an activated version of Pak (PakT423) in Rac1/Rac2 deficient KITD814V bearing primary HSC/Ps rescued ligand independent growth (Figure 4.16.B).

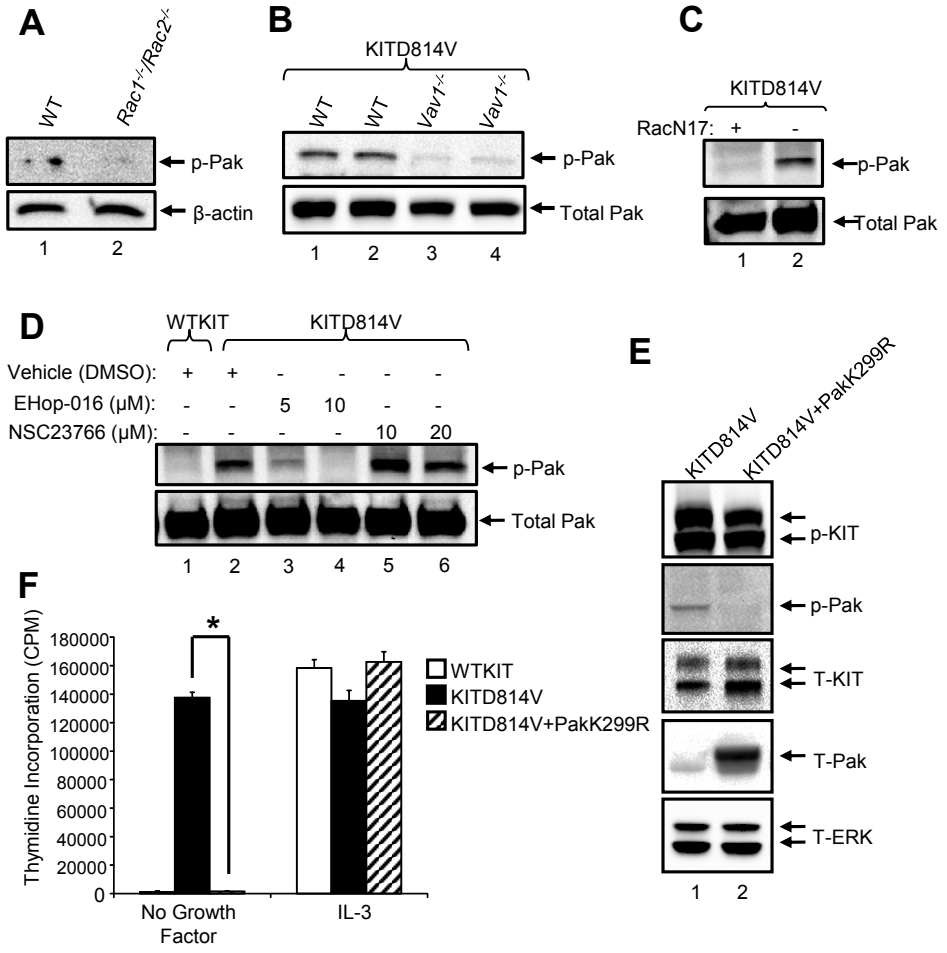

Figure 4.15. KITD814V induces the activation of Pak in a Vav1 and Rac dependent manner.

KITD814V expressing WT or Rac1/Rac2 (A) or Vav1 (B) deficient primary HSC/Ps from two independent animals were starved and lysed and immunoblotted for active Pak. (C) KITD814V bearing 32D cells expressing RacN17 were starved, lysed and equal amounts of lysates were subjected to immunoblotting using a phospho-Pak antibody and total Pak. (D) Cells bearing WT KIT or KITD814V were starved and treated with indicated concentrations of NSC23766 or EHop-016 and subjected to Rac activation assay. Shown are the levels of phosphorylated and total Pak. (E) 32D cells were transduced with KITD814V with or without dominant negative PakK299R and starved, lysates were subjected to western blot analysis using a phospho-KIT, phospho-Pak, total KIT, total Pak and total ERK antibodies. (F) Cells in (E) were starved for 6 hours before being plated in quadruplicate in the presence or absence of growth factors (IL-3, 5ng/ml) for 48 hours. Cells were pulsed with [^{3}H] thymidine for 6 hours and harvested. Bars denote the mean thymidine incorporation (cpm ± SD) from one of four independent experiments performed in quadruplicate. *p-value<0.05, KITD814V vs. KITD814V plus PakK299R.

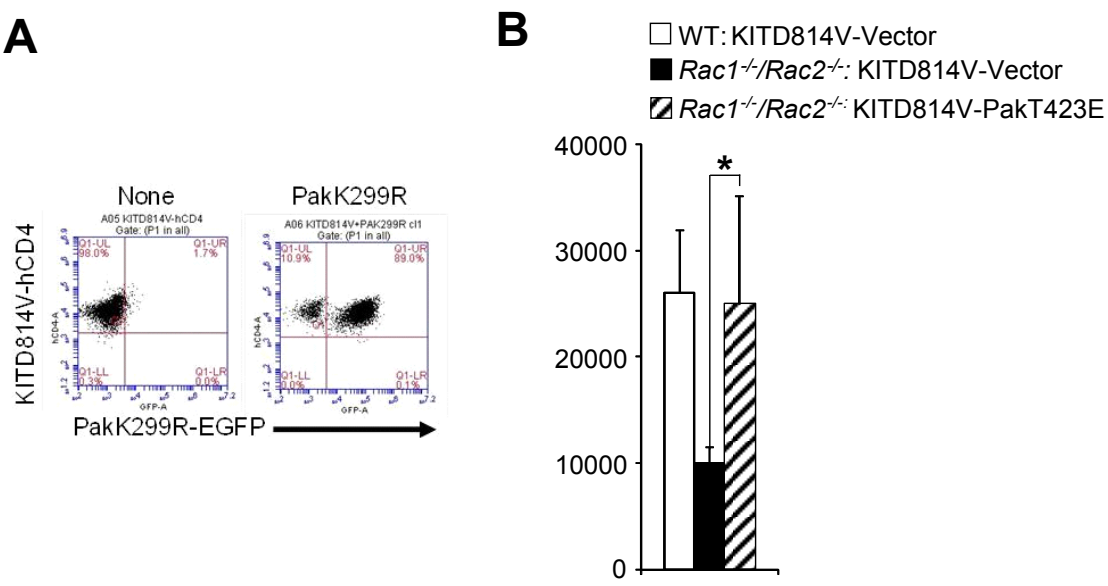

Figure 4.16. Activated Pak in the absence of Rac GTPases rescues hyperproliferation in primary LDMNCs bearing KITD814V.

(A) 32D cells were transduced with KITD814V with or without dominant negative PakK299R. Flow cytometric dot plot profiles from a representative experiment are shown. (B) Primary HSC/Ps from WT and *MxCreRac1*$^{flox/flox}$*Rac2*$^{-/-}$ mice were harvested following three consecutive intraperitoneal injections of polyI:polyC given at 48 hour intervals. These cells were transduced with KITD814V in the presence of empty vector or PakT423 and assessed for growth by thymidine incorporation in the absence of growth factors. Bars represent the mean thymidine incorporation (in counts per minute) in primary HSC/Ps expressing the indicated receptors. *p-value<0.05, empty vector vs PakT423.

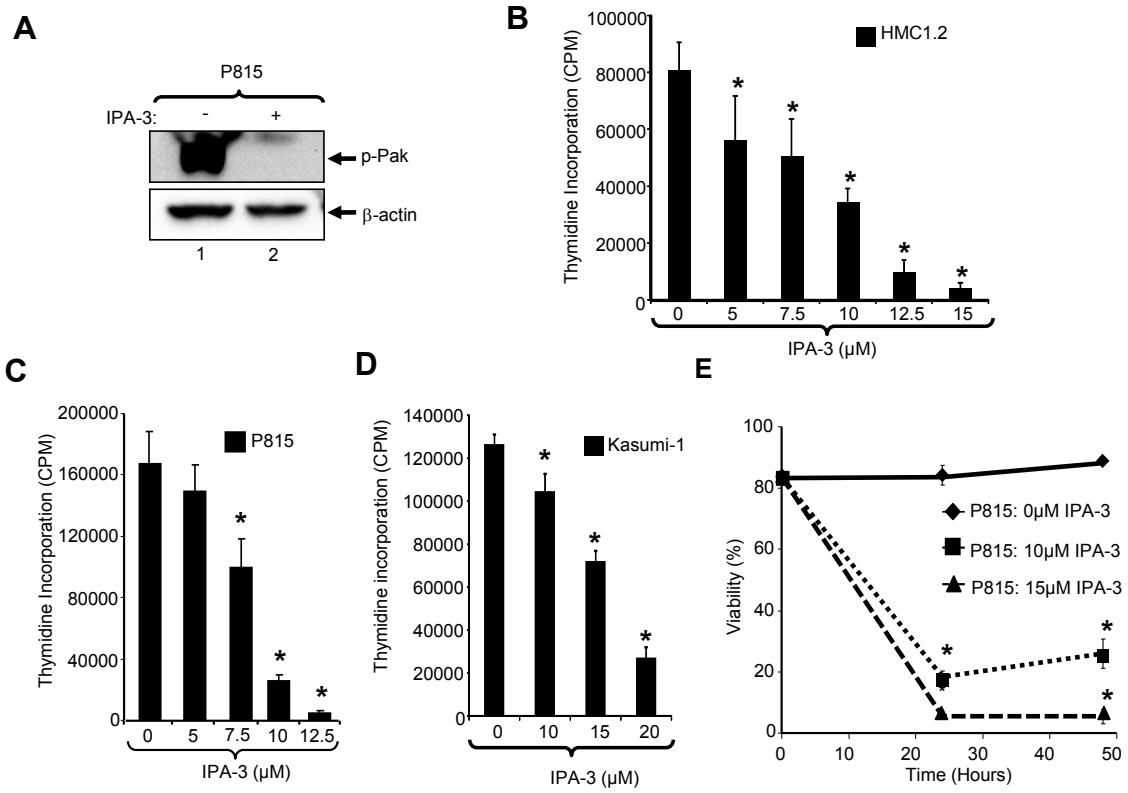

Figure 4.17. Inhibition of constitutively active Pak by IPA-3.

(A) KITD814V bearing murine mastocytoma cells P815 were incubated in the presence or absence of 10μM IPA-3 and subjected to immunoblotting with phospho-Pak antibody. Levels of phospho-Pak and total protein in each lane are shown. HMC1.2 (B), P815 (C) and Kasumi-1(D) cells bearing an activating KIT mutation exhibited dose dependent inhibition when grown in the presence of IPA-3 and assessed for proliferation by measuring [^{3}H] thymidine incorporation. Bars represent mean ± SD from 2 to 3 independent experiments performed in replicates of three. *p-value<0.05. (E) P815 cells were subjected to an apoptosis assay after treating them with 10 or 15μM IPA-3 under no growth factor conditions for 24, or 48 hours prior to analyzing them by Annexin V and 7-AAD staining. Apoptosis was assessed as percentage of Annexin V and 7-AAD positive cells. Bars denote the mean ± SD percentage of total viable cells from one of two independent experiments performed in quadruplicate. *p-value<0.05, 0μM vs.10 or 15μM.

We next assessed the consequence of pharmacologic inhibition of Pak on the growth of KITD814V bearing cells using a cell permeable allosteric inhibitor of Pak, IPA-3 (171). As seen in Figure 4.17.A, treatment of KITD814V bearing P815 cells with IPA-3 resulted in inhibition of Pak activation and treatment of HMC1.2 cells, P815 cells and Kasumi-1 cells with the same inhibitor led to a dose dependent inhibition in the growth of KITD816V and KITD814V-bearing cells, respectively with IC_{50} of 10-15 μM (Figure 4.17.B-D). Repression in the growth of oncogene KIT bearing cells in the presence of IPA-3 was largely due to reduced survival (Figure 4.17.E).

To directly assess the impact of Pak inhibition on KITD814V induced MPN development, we transplanted C3H/HeJ mice with cells bearing KITD814V and PakK299R as described (166). Transplanted mice were observed for the development of MPN and survival. Although all KITD814V bearing mice die by day 15 post-transplantation; all KITD814V and PakK299R co-expressing mice survived this period and showed a significant delay in disease onset (Figure 4.18.A). To assess the changes in tissue pathology in mice bearing cells co-expressing KITD814V and PakK299R with those bearing only KITD814V, we sacrificed a cohort of mice on days when KITD814V only bearing mice succumb to death. As seen in Figure 4.18.B and 4.18.C, compared to KITD814V bearing control mice, mice co-expressing KITD814V and PakK299R demonstrated a significantly lower spleen weights and peripheral blood counts. In addition, these mice demonstrated a significant correction in splenic architecture (Figure 4.18.E), myeloid cell infiltration in the lung (Figure 4.18.D) and liver (Figure 4.18.F) as well as lacked lesions on lungs normally associated with KITD814V bearing mice (Figure 4.18.G). All of these findings were associated with reduced percentage (as

assessed by EGFP expressing cells) of leukemic cells in tissues of mice transplanted with cells bearing KITD814V and PakK299R (Figure 4.18.H).

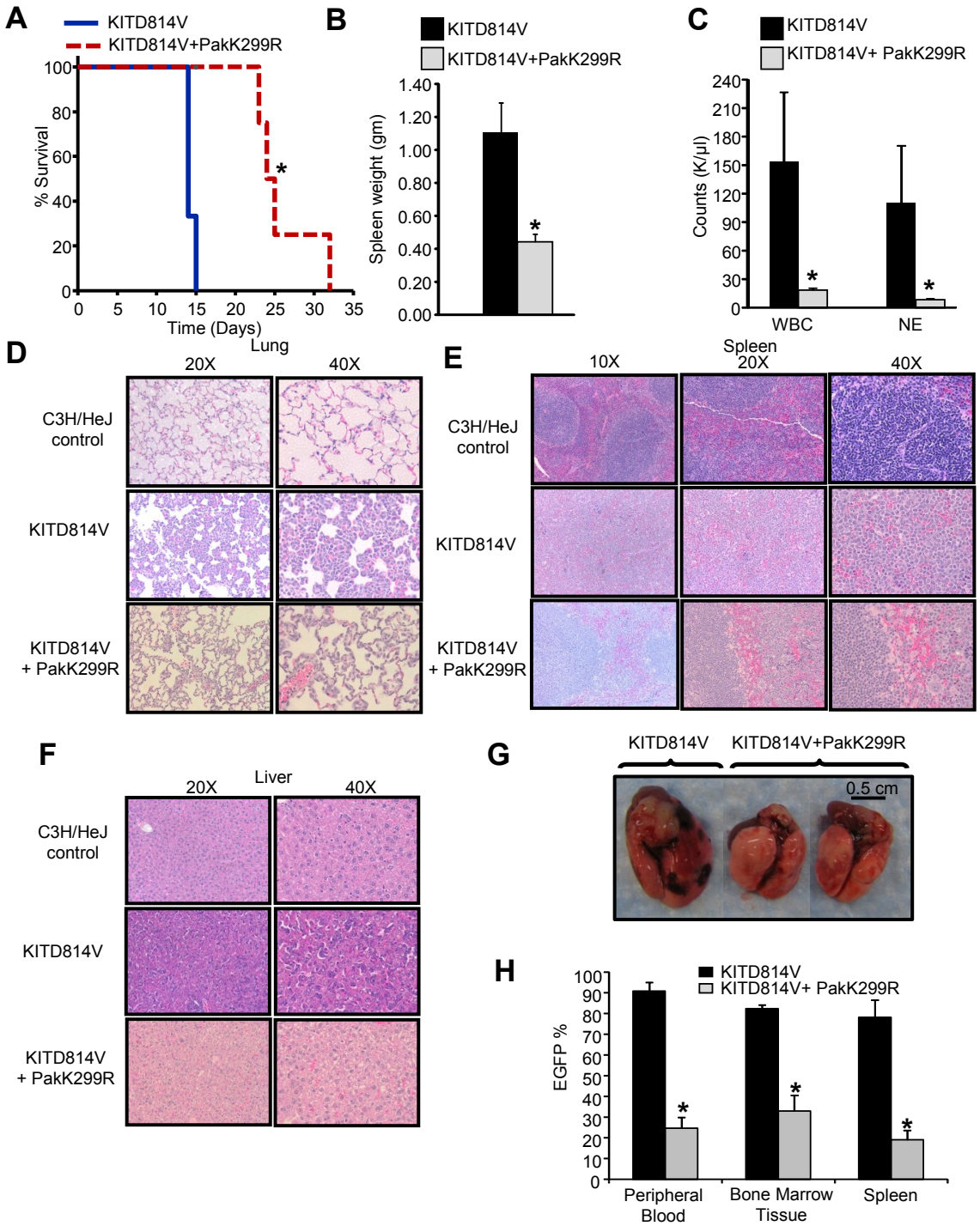

Figure 4.18. Inhibition of Pak *in vivo* in KITD814V bearing mice enhances the survival and significantly delays development of MPN.

(A) Kaplan-Meier survival curves of mouse cohorts transplanted with either 32D cells bearing KITD814V alone or in combination with PakK299R. Survival of recipients with PakK299R was significantly prolonged compared to KITD814V alone transplanted mice

(n=3 to 4; *p-value<0.01). Repression of Pak *in vivo* in KITD814V bearing cells results in significant lower spleen weights (B) and peripheral blood counts (C) relative to controls. n=3, *p-value<0.05. (D), (E) & (F) lungs, spleens and livers from the indicated genotypes were subjected to histopathological analysis. Shown are representative tissue sections at 10 to 40X magnification. (G) Representative pictures of intact lungs from mice bearing KITD814V alone or in combination with PakK299R. (H) Percentage of EGFP+ leukemic cells in mice bearing KITD814V or KITD814V in combination with PakK299R. Quantitative assessment of EGFP positive cells (n=3, *p-value<0.05).

4.D CONCLUSIONS

Rac and its downstream effector Pak are frequently overexpressed and/or hyperactive in solid cancers including in breast cancer (104). In these cells, Rac and Pak have been implicated in regulating invasion and metastasis (172, 173). While significant progress has been made in our understanding of the role of these molecules in solid cancers; how these molecules regulate oncogene induced MPN is poorly understood. We provide *in vitro* and *in vivo* genetic, biochemical, as well as pharmacologic evidence to suggest that the Vav/Rac/Pak pathway plays an essential role in regulating transformation via an oncogenic form of KIT (KITD814V) associated with MPN and AML and for which no efficacious therapies are currently available. While gain of function KIT mutations localized to the juxtamembrane region associated with GISTs are highly sensitive to inhibition by imatinib, KIT mutations within the second catalytic domain found in patients with AML and SM, including KITD814V, interfere with the binding of imatinib, thus rendering this mutation insensitive to imatinib inhibition (83, 136, 174). Our results suggest that inhibition of constitutively active Vav, Rac or Pak downstream from KITD814V is a highly efficient alternative approach for treating hematologic malignancies involving this mutation.

To date, several GEFs for Rac have been identified (175, 176); however, GEFs such as those that belong to the Vav family as well as Tiam-1 have been primarily implicated in tumorgenesis (177-179). Although, the precise mechanism(s) by which these GEFs regulate cancer progression is still unclear; studies suggest that Vav GEFs are significantly more diverse in their ability to activate Rho family GTPases and can regulate the activation of Rac, Rho and Cdc42, compared to Tiam-1 and Trio, which

primarily activate Rac (156, 180-182). While it is unclear how this occurs, presence of Src homology and cysteine rich domains in Vav are likely to contribute to this diversity. Our findings utilizing HSC/Ps derived from Vav1 deficient mice engineered to express KITD814V suggest an essential role for Vav1 in regulating ligand independent growth of oncogene bearing cells as well as activation of both Rac1 and Rac2.

Our findings demonstrating correction of myeloid cell infiltration in the lungs of mice co-expressing RacN17 or PakK299R along with KITD814V, suggests that KITD814V induced metastasis of myeloid cells in these tissues is dependent on Rac and Pak. Furthermore, our results also demonstrate that prolonged survival of KITD814V bearing mice in the setting of RacN17 expression correlates with significant repression of Rac1, Rac2 and Pak in these cells and loss of Rac1, Rac2 or both Rac1 and Rac2 significantly represses ligand independent growth in these cells. These findings were also confirmed by utilizing mice bearing KITD814V in the setting of Vav1 and Rac1/Rac2 deficiency and transplanted into lethally irradiated WT recipients. Interestingly, our data implicating Rac1 and Rac2 in KITD814V induced transformation are similar to those reported by Thomas et al using the p210BCR-ABL translocation (183). In both instances, Rac1 and Rac2 deficiency collectively shows a more prolonged survival of leukemic mice.

In addition to the genetic approaches to test the role of Rac in MPN, we also employed pharmacologic approaches to test the possibility of targeting Rac GTPases for the treatment of KITD814V induced MPN and AML. To this end, we first utilized an established Rac inhibitor, NSC23766. Previous studies have shown that NSC23766 functions by binding in the pocket of Rac1 that interact with GEFs such as Trio and

Tiam1 but not Vav (156, 184). Our results using this drug suggest that Rac1 does not significantly contribute to KITD814V induced transformation as we see only 50% reduction in the growth of both murine and human oncogene KIT bearing cells at very high doses of NSC23766. While reasonable, an alternate explanation may involve a lack of robust involvement of Trio and Tiam1 in KITD814V induced transformation. Therefore, in an effort to assess if Rac GTPase could be targeted more efficiently in KIT oncogene bearing cells, we utilized a newly described Rac inhibitor, EHop-016 (157). This drug is a derivative of NSC23766 and inhibits Rac 100-fold more efficiently relative to the parental compound (157). EHop-016 like NSC23766 binds to the surface groove of Rac, which is critical for interacting with GEFs. Studies have shown that EHop-016 is more potent than the parental compound in terms of its interaction with the effector domains of Rac (157). This presumably allows EHop-016 to block the activation of additional Rac-GEFs in addition to those blocked by NSC23766. Our head to head studies comparing NSC23766 and EHop-016 show that EHop-016 is a more potent Rac inhibitor than NSC23766.

Although we have no direct evidence to suggest which additional GEFs might be inhibited by EHop-016 in oncogene KIT bearing cells, previous studies in MDA-MB-435 metastatic breast cancer line has shown that EHop-016 inhibits the interaction of Vav2 with Rac (157). It is therefore likely that in KITD814V bearing HSC/Ps, EHop-016 inhibits the interaction of Vav1 with Rac1 and Rac2. We show that treatment of oncogene bearing cells with EHop-016 results in inhibition of both Rac1 and Rac2. Consistent with these findings, deficiency of Vav1 in oncogene bearing cells impairs the activation of both Rac1 and Rac2 and substantially corrects the ligand independent

growth observed in WT oncogene bearing HSC/Ps, very similar to the growth repression observed in the presence of EHop-016.

Downstream from Rac, we show that Pak plays an essential role in KITD814V induced transformation. We utilized both an allosteric inhibitor of Pak, IPA-3, as well as a genetic approach to implicate Pak in KITD814V induced transformation. Although IPA-3 selectively inhibits the activation of Pak1 (171), at higher doses it has been shown to also inhibit the activation of other isoforms including Pak2 and Pak3 (171). Our studies utilizing a dominant negative form of Pak (K299R) confirmed IPA-3 findings demonstrating that Pak indeed contributes to KITD814V induced transformation; whether other isoforms of Pak also contribute to leukemogenesis remains to be determined. As animal models deficient in the expression of Pak isoforms become available, the role of specific isoforms of Pak in transformation can be more readily and efficiently addressed in future studies. Taken together, our studies identify a novel pathway involving Vav/Rac and Pak in regulating non-classical MPNs such as SM in which activating mutation of KIT plays an essential role.

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Martin H*, Mali RS*, Ma P*, Chatterjee A*, Ramdas B*, Sims E, Munugalavadla V, Ghosh J, Mattingly RR, Visconte V, Tiu RV, Vlaar CP, Dharmawardhane S, and R Kapur. Pak and Rac GTPases promote oncogenic KIT-induced neoplasms. *The Journal of Clinical Investigation*. 2013; 123 (10): 4449-4463. © American Society for Clinical Investigations

*These authors contributed equally to the work.

CHAPTER FIVE

SYNERGISTIC COOPERATION OF ONCOGENIC KIT IN CORE BINDING FACTOR (CBF) LEUKEMIAS

5.A ABSTRACT

12% of acute myeloid leukemia (AML) patients and 40% of patients with subtype M2 AML have the cytogenetic abnormality t(8; 21) (21). This chromosomal rearrangement generates the fusion protein AML1-ETO, leading to the disruption of the core binding factor complex that regulates the transcription of several genes in the hematopoietic stem and progenitor cells (32, 34, 185). AML patients harboring this translocation alone usually have a favorable prognosis; however, a substantial portion of patients bearing an additional oncogenic receptor tyrosine kinase, KIT, mutation have significantly worse prognosis (186, 187). A secondary mutagenic event of an activating mutation of aspartic acid to valine in the KIT receptor activation loop (KITD814V) which results in altered substrate recognition and utilization, constitutive tyrosine autophosphorylation, and promiscuous signaling. While clinical studies suggest poor prognosis due to the presence of both these mutations in AML patients; little however is known concerning the possible mechanisms of cooperation between AML1-ETO and KITD814V. In this study, we show that growth of AML1-ETO bearing myeloid cells remains ligand dependent, while myeloid cells that express both AML1-ETO and KITD814V demonstrate ligand independent proliferation. Furthermore, functional assays show that expression of AML1-ETO and KITD814V in the same cells results in increased cycling and decreased apoptosis, which may contribute to the observed ligand

independent proliferation. Interestingly, while the expression of KITD814V alone in myeloid cells results in significant activation of STAT3, STAT5, JAK2, Akt, and ERK MAPK pathway; the activation of these molecules is significantly dampened in the presence of AML1-ETO expression. To confirm that AML1-ETO alone is not sufficient to induce leukemia; but needs the presence of KITD814V, a syngeneic murine transduction/transplantation model for myeloproliferative neoplasm (MPN) was established. *In vivo* results demonstrated that mice transplanted with AML1-ETO and KITD814V bearing cells succumbed to a fatal MPN-like phenotype, while AML1-ETO expressing mice remained disease free. Although, no significant differences in the phenotypic manifestation was observed in cohorts of mice bearing KITD814V mutation alone or combination of both AML1-ETO and KITD814V (similar splenomegaly and hepatomegaly), there were significant differences in survival of the two groups of mice (median survival 23 days vs. 39 days, respectively). These studies suggest that the fusion protein AML1-ETO is not sufficient to induce ligand independent growth *in vitro*, nor MPN *in vivo*, but requires cooperation with a secondary mutagenic event, KITD814V, to promote leukemogenesis.

5.B INTRODUCTION

Acute myeloid leukemia is thought to occur as a result of cooperation between alterations in genes that encode for transcription factors and genes that regulate the growth and survival of hematopoietic cells (188). Genetic alterations in TFs constitute chromosomal translocations which inhibit the differentiation potential of hematopoietic progenitors. In contrast, alterations in genes involved in growth and survival of

hematopoietic progenitors include single point mutations in growth factor receptors or their downstream kinases (189-191). In general, leukemic clones only possesses a single chromosomal translocation in TFs. Likewise, leukemic cells in which more than one mutation in the receptor tyrosine kinases occur are also rare. However, there are several examples of leukemic clones in which both a chromosomal translocation in a TF can be found along with a point mutation in the receptor tyrosine kinase, such as KIT(192).

The cooperative model of AML has been substantiated by a significant number of experimental models. For example, the fusion protein AML-1-ETO is one of the most frequently seen chromosomal translocation in patients with core binding factor acute myeloid leukemia (CBF-AML) (32, 193). However, in several murine models of leukemogenesis, the presence of AML-1-ETO in itself does not appear to be sufficient for disease development (194, 195). Under conditions when mice have been targeted to express AML1-ETO in hematopoietic cells; mice only develop a myeloproliferative neoplasm but never result in the development of AML (196). However, treating AML1-ETO expressing mice with ethylnitrosourea (ENU) renders these mice susceptible to AML (197, 198). This data strongly support the hypothesis that mutations in TFs alone are not sufficient to develop leukemia. To this end, emerging clinical data suggests that the molecular events leading up to AML might involve activating mutations in the receptor tyrosine kinases including KIT (132, 199-201). Clinical reports strongly suggest cooperation between activating KIT mutations and core binding factor TFs in a subset of AMLs. Approximately 12.8 to 46.1% of adults with core binding factor leukemias express activating KIT mutations (29-31). Furthermore, 12% of pediatric non-acute pro-myelocytic leukemia (APL) AML carry an activating mutations of KIT (31). Therefore,

determining the extent to which activating KIT mutations and CBF-TFs cooperate in AML and identifying the mechanism(s) by which they cooperate is critical to the development of targeted therapies for patients with this subset of AML.

Of all AML patients, 15% express alterations in the core binding factor TFs. In general, the median age of these patients is significantly lower and their over prognosis is more favorable than AML patients with other chromosomal alterations (202-204). In contrast, a higher incidence of elevated WBC counts, greater relapse incidence (RI) and worse overall survival (OS) has been observed in AML patients co-expressing CBF TFs (AML1-ETO or CBFβ-MYH11) and KIT mutations (29-34, 205-208). Importantly, KIT mutations are not observed in other subsets of AML (32).

Although these studies strongly support the notion of cooperation between KIT mutations and CBF-TFs in a subset of AML, and suggest that KIT mutations have a negative overall impact on the outcome of CBF-AML in both adult and pediatric patients, they do not directly prove the notion that KIT mutations cooperate with CBF TFs in CBF-AML. While correlations made in clinical studies suggest poor prognosis due to the presence of both these mutations in AML patients; little however is known concerning the possible mechanisms of cooperation between AML1-ETO and KITD814V. Expanding our understanding of the molecular pathogenesis of CBF-AML and associated mutations would open potential venues for possible therapeutic intervention. In this study we investigate the molecular mechanisms of growth and leukemic potential of activating KITD814V mutation and AML1-ETO. Our results suggests that the fusion protein AML1-ETO alone is insufficient to induce ligand independent growth *in vitro*, nor MPN

in vivo, but requires cooperation with a secondary mutagenic event, KITD814V, to promote leukemogenesis.

5.C RESULTS

Transforming potential of KITD814V and AML1-ETO in vitro

Although there is a clinical association concerning AML patients that harbor both a CBF gene rearrangement and a KITD816V mutation, cooperation between the two genetic aberrations has not been established. To assess whether KITD814V bearing cells that co-express a CBF-translocation, either AML1-ETO or CBFß-MYH11 show an cooperation in growth, constructs expressing KITD814V and the CBF-translocations were first generated. To establish our cell lines, cDNAs encoding KITD814V was isolated from a bicistronic ecotropic retroviral, pMIEG3, vector expressing EGFP using restriction enzymes *NotI* and *EcoR1*. The KITD814V cDNA was then subcloned into a pMIEG3 vector expressing a human (h) CD4 tag, pMIEG3-hCD4. The core binding factor gene rearrangements were expressed in plasmids bearing an EGFP marker, pMIEG3-*CBFß-MYH11*-EGFP, and pMIGR1-*AML1-ETO*-EGFP (Figure 5.1.A).

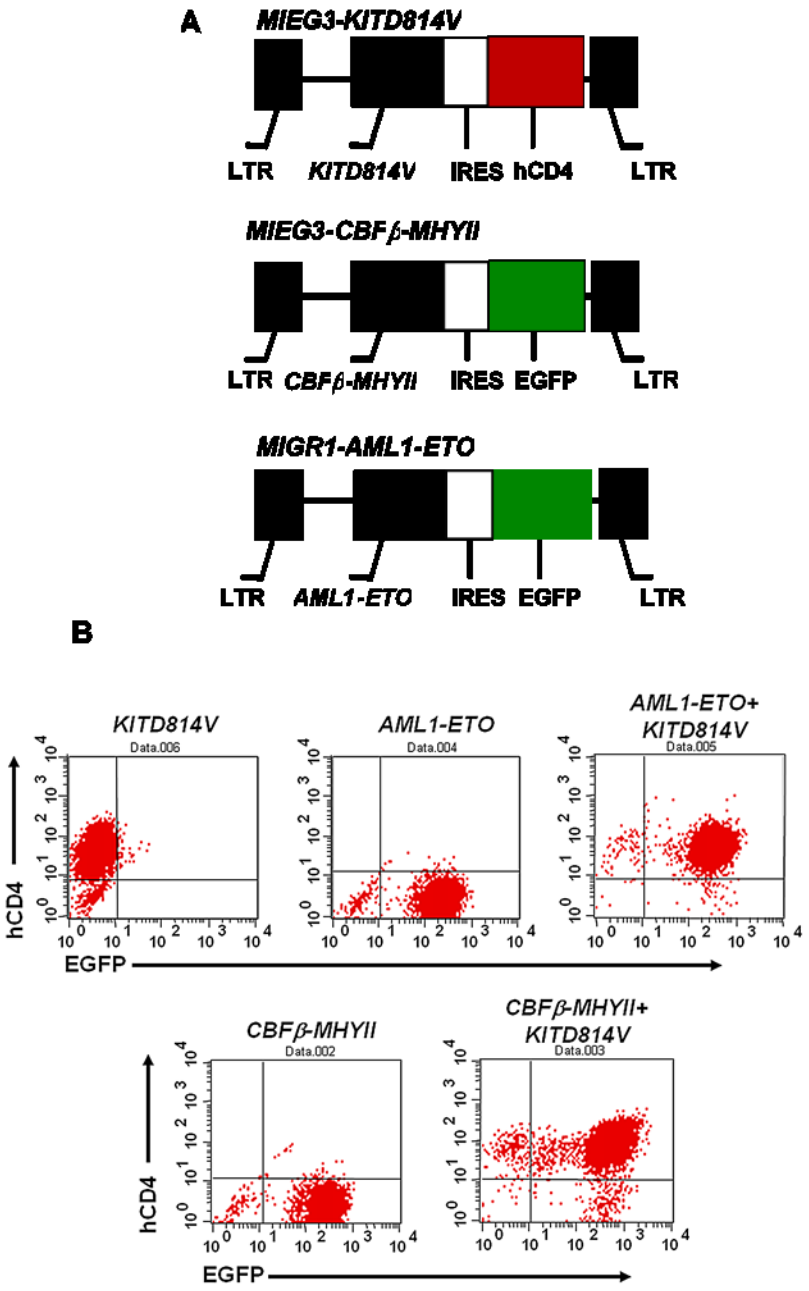

Figure 5.1. A schematic diagram of retroviral constructs and analysis of KIT and AML1-ETO expression in myeloid cells.

(A) MIEG3-based retroviral constructs used to express KITD814V, AML1-ETO and CBFβ-MYH11 with EGFP and human (h) CD4 markers. (B) Representative dot blots showing the expression of 32D cells bearing either pMIEG3-KITD814V-hCD4, or pMIGR1-AML1-ETO-GFP, CBFβ-MYH11 alone or in combination with KITD814V. Expression of hCD4 was determined by hCD4-phycoerythrin (PE) antibody and flow cytometric analysis.

Retroviral supernatants were prepared and 32D cells were stably infected with a single plasmid or a combination of activating KITD814V and a CBF translocation expressing plasmids. Cells were sorted by FACS to homogeneity based on the expression of EGFP, hCD4, or both (Figure 5.1.B).

To determine the extent of ligand-independent proliferation of 32D cells harboring CBF translocations and KITD814V, cells were deprived of growth factors and cytokines for 8 hours prior to being cultured for 48 hours in a 96-well plate. When grown in the absence of growth factors AML1-ETO and CBFβ-MYH11 expressing cells demonstrated little growth, while cells bearing the KITD814V mutation grew in a ligand-independent manner as previously described in this thesis. Cells harboring a core binding factor translocation, either AML1-ETO or CBFβ-MYH11, in the presence of KITD814V showed a significant increase in ligand-independent proliferation compared to cells bearing KITD814V alone (Figure 5.2). This significant increase in the proliferation of cells bearing both a CBF translocation and an acquired activating KIT mutation provides experimental support that cooperation exists between KITD814V and CBF fusion proteins.

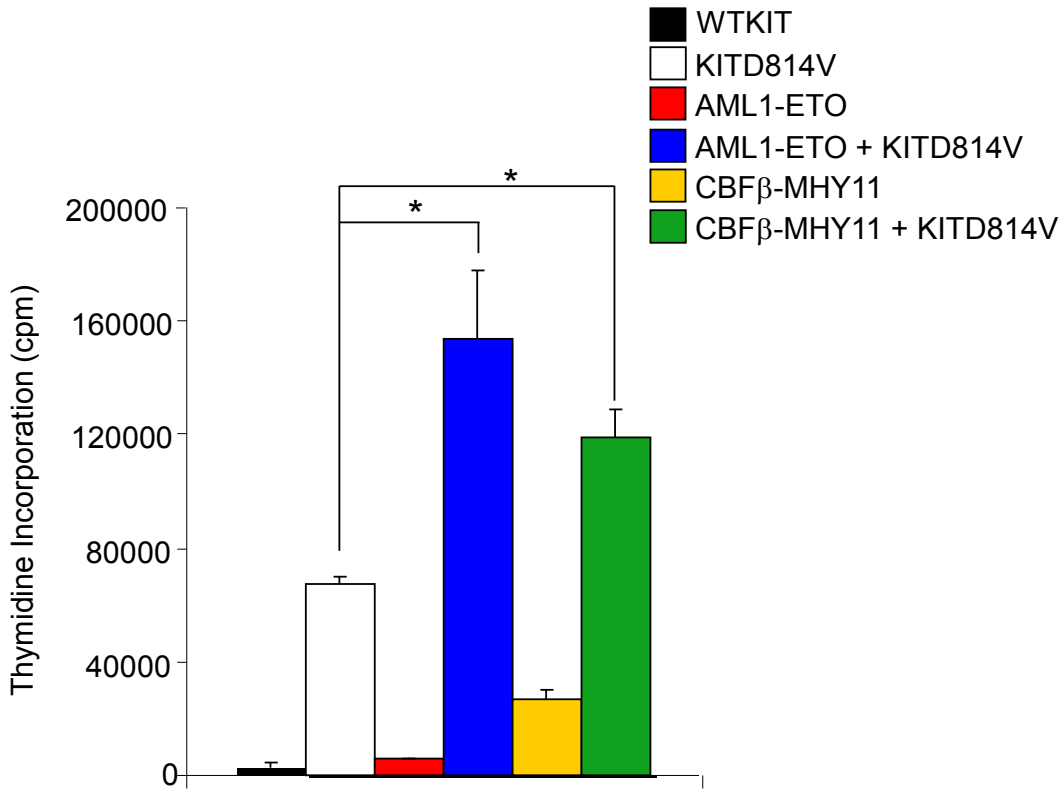

Figure 5.2. Cells bearing KITD814V and CBF TFs cooperate to enhance growth.

Proliferation was assessed by thymidine incorporation in 32D cells expressing KITD814V, AML1-ETO, AML1-ETO and KITD814V, CBFβ-MYH11, or CBFβ-MYH11 and KITD814V in the absence of growth factors. Bars denote the mean thymidine incorporation (cpm ± SD) from one of three independent experiments performed in quadruplicate. *p-value<0.05, Cells co-expressing AML1-ETO or CBFβ-MYH11 and KITD814V vs KITD814V expressing cells alone.

Cells expressing AML1-ETO and KITD814V exhibit an increase in cell cycle and survival

We demonstrated that AML1-ETO and KITD814V co-expressing cells show increased proliferation. It has previously been reported that expression of the core binding factor transgene, AML1-ETO, in murine myeloid cells leads to an accumulation of cells in G1 phase (209, 210). To examine the effect of AML1-ETO expression on cell cycle regulation in KITD814V-expressing cells, we utilized a propidium iodide assay. 32D cells bearing AML1-ETO and/or KITD814V, or WT KIT were starved of growth factors and serum for 6 hours. These cells were cultured in a 24-well plate ($2x10^5$ cells/well) in replicates of four for up to forty-eight hours at 37°C. Cells were collected and DNA content was determined by staining the cells with propidium iodide and analyzed by flow cytometry. Consistent with prior studies, cells bearing *AML1-ETO* transcripts showed an increase in the accumulation of cells in G1 phase of cell cycle as compared to 32D cells bearing the wild type form of the KIT receptor (Figure 5.3.A). The percentage of AML1-ETO or WT KIT expressing cells in S-phase steadily decreased over 24 hours before undergoing quiescence (Figure 5.3.B). Notably, AML1-ETO with KITD814V bearing cells maintained a significantly increased percentage of cells in S-phase as compared to KITD814V alone (Figures 5.3.B). To determine whether other functional phenotypic changes occurred, we performed an apoptosis assay in which cells were collected and stained with Annexin V-PE and 7-AAD and subjected to flow cytometry analysis (Figure 5.4.A). While cells bearing WT KIT or AML1-ETO had undergone complete apoptosis, there was a modest increase in survival of cells co-expressing AML1-ETO and KITD814V compared to KITD814V alone (Figure 5.4.B).

These findings suggest that AML1-ETO is not sufficient to induce ligand-independent growth and proliferation of 32D cells in the absence of growth factors. However, KITD814V bearing cells exhibit a significant increase in ligand-independent growth in the presence of AML1-ETO, which is due to an increase of cells in S-phase and increased survival.

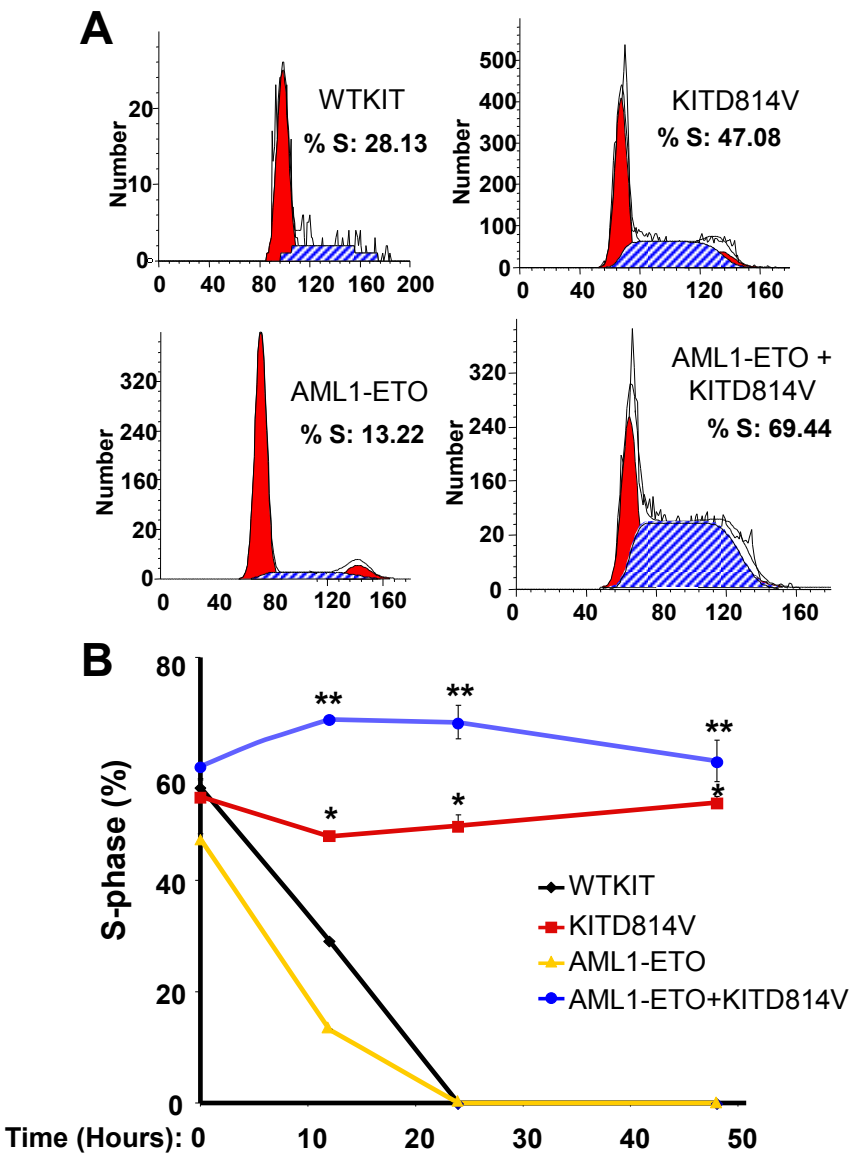

Figure 5.3. Cells bearing AML1-ETO and KITD814V show enhanced percentage of cells in S-phase of cell cycle relative to AML1-ETO and KITD814V expressing cells.

WT KIT, KITD814V, AML1-ETO, and AML1-ETO and KITD814V expressing cells were grown under no growth factor conditions for 0, 12, 24 or 48 hours prior to being stained with propidium iodide and analyzed by flow cytometry. Cycling was determined by assessing cells in S-phase if cell cycle. A) Representative cell cycle data showing the total percentage of cells in S-phase. B) Line graph shows S-phase cycling at various time points in the absence of serum and cytokines. Bars denote the mean percentage of cells in S-phase (%± SD) from one of three independent experiments performed in quadruplicate. *p-value<0.05, KITD814V and/or AML1-ETO vs. WT KIT. **p-value<0.05, KITD814V and AML1-ETO co-expressing cells vs. KITD814V.

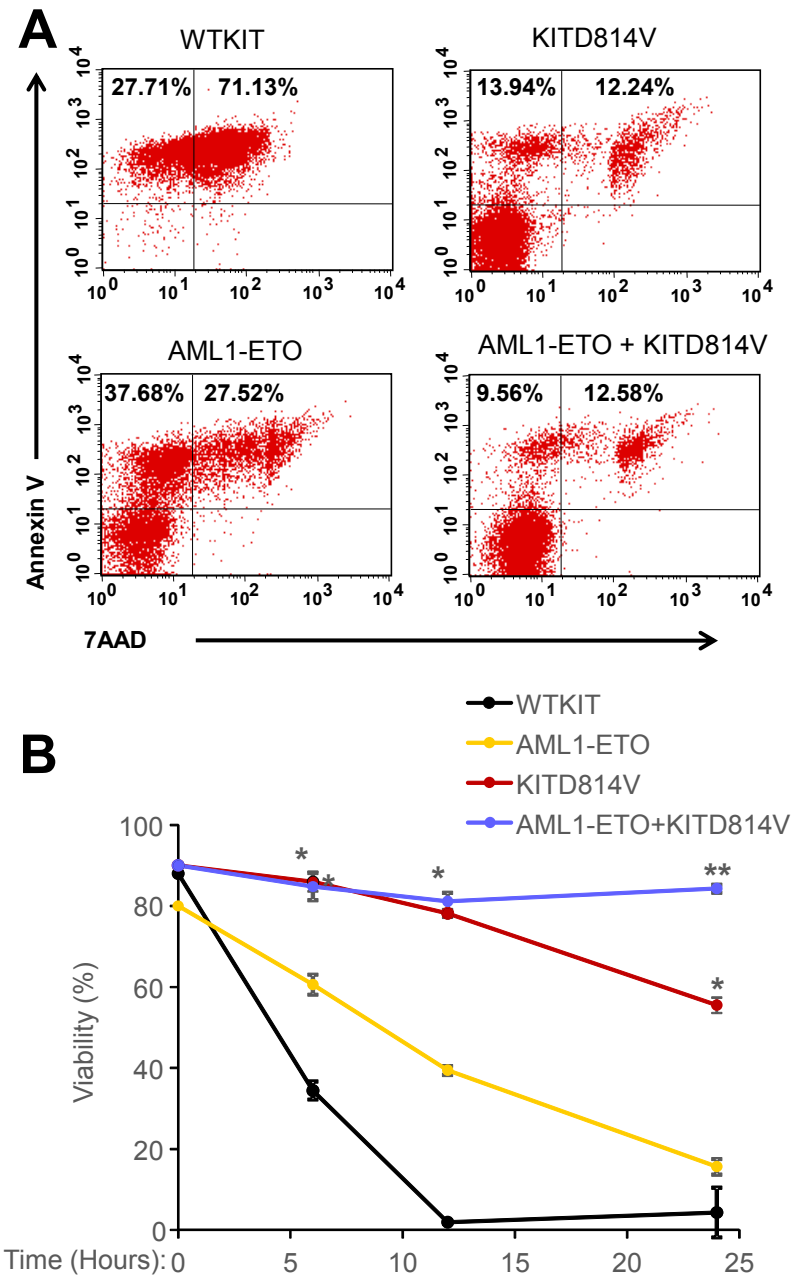

Figure 5.4. Survival of AML1-ETO and KITD814V expressing cells.

WT KIT, KITD814V and/or AML1-ETO cells expressing cells were grown in no growth factor conditions for 0, 6, 12, 24 or 48 hours prior to being analyzed by flow cytometry after staining with Annexin V-phycoerythrin and 7-AAD. A) Representative dot blot showing the percent apoptosis. (B) Bars denote the mean percentage of Annexin V and 7-AAD negative (viable) cells (mean % ± SD) from one of three independent experiments performed in quadruplicate.

To determine the extent of contribution that downstream pathways impart on AML1-ETO and KITD814V-mediated hyperproliferation, pharmacological inhibitors were used to assess sensitivity of these cells to inhibition of downstream pathways. As there was a significant decrease in KITD814V-induced constitutive activation of Jak2 in the presence of AML1-ETO, we examined if pharmacological inhibition of Jak2 would have a differential affect among the cell lines. Treatment of various cells with a JAK2 inhibitor showed a dose-dependent reduction in ligand-independent hyperproliferation of KITD814V bearing cells, but only a modest reduction in proliferation other cell lines. Inhibition was more pronounced in KITD814V bearing cells than those expressing AML1-ETO and KITD814V (Figure 5.5.A). Furthermore inhibition of PI3K by, Ly294002, showed a dose-dependent response in to KITD814V bearing cells, and to cells co-expressing KITD814V and AML1-ETO at higher doses (Figure 5.5.B). While, in the presence of MEK inhibitor, PD98059, ligand-independent hyperproliferation of KITD814V-expressing cells was significantly reduced in AML1-ETO co-expressing cells compared to KITD814V only expressing cells (Figure.5.5.C). These studies suggest that AML1-ETO cells co-expressing KITD814V may utilize signal transduction pathways that are different from those activated by KITD814V only expressing cells.

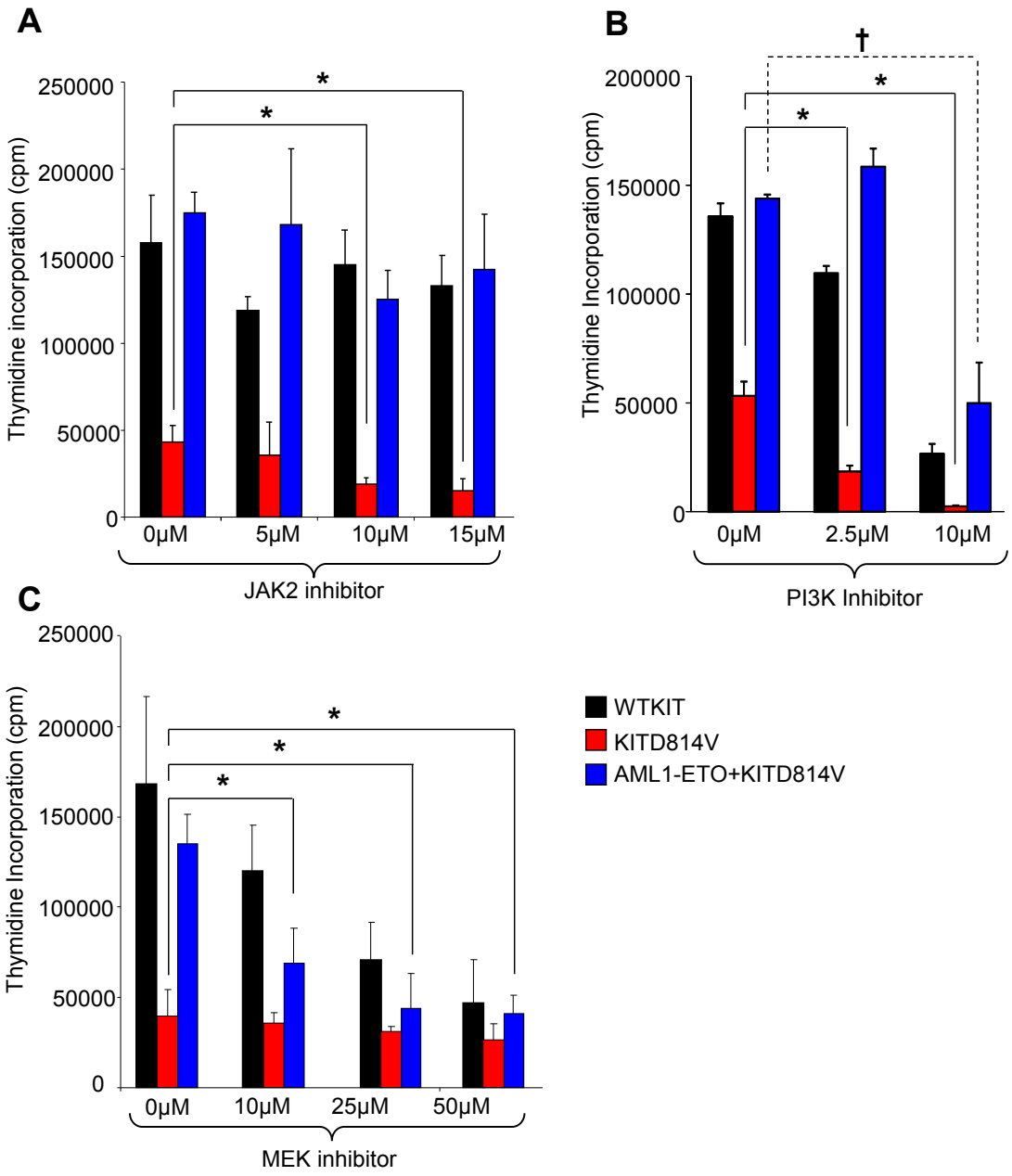

Figure 5.5. Differential effects of various inhibitors on the growth of KITD814V or KITD814V and AML1-ETO bearing cells.

32D cells bearing WT KIT grown in the presence of IL-3 or KITD814V or KITD814V with AML1-ETO bearing cells grown in the absence of growth factors and (A) Jak2 inhibitor, (B) PI3K inhibitor, Ly294002, or (C) MEK inhibitor, PD98059, were subjected to a [^{3}H] thymidine incorporation assay. Bars represent mean cpm ± SD from 2 independent experiments performed in replicates of four. *,p-value<0.05, 0μM vs. various drug concentrations.

Transplantation of mice with cells co-expressing AML1-ETO and KITD814V develop myeloproliferative neoplasms (MPN)

Previous studies have shown that AML1-ETO does not promote leukemia in murine models without a secondary mutagenic event, yet the role of the secondary mutation by oncogenic KIT is poorly understood. To determine the extent to which AML1-ETO and KITD814V cooperate in the generation of a myeloproliferative neoplasm–like disease, we used a C3H/HeJ syngeneic murine model. Two weeks post-transplantation of the C3H/HeJ mice with cells bearing KITD814V and AML1-ETO, mice were monitored for signs of leukemia development, including physical appearance and percentage of EGFP+ and/or hCD4+ cells in the peripheral blood. Thirty days after post-transplantation mice began to display symptoms of MPN including increase white blood cell counts and rapid decline in health. At signs of moribund, these mice were sacrificed. Splenomegaly and hepatomegaly was observed in every animal transplanted with cells bearing AML1-ETO and KITD814V (Figure 5.6.A and data not shown). We analyzed the percentage of transplanted cells via FACS analysis of EGFP+ and hCD4+ in peripheral blood, bone marrow, and spleen suspensions, and observed donor cells bearing AML1-ETO and KITD814V in all tissues (Figure 5.7). All recipients transplanted with AML1-ETO and KITD814V co-expressing cells succumbed to fatal MPN within 65 days of transplantation (Figure 5.6.B). In contrast, mice transplanted with WT KIT or AML1-ETO alone did not show obvious signs of disease progression during the course of the study. We further confirmed engraftment of transplanted cells by western blot analysis in which lysates were prepared from harvested bone marrow and spleen cells in leukemic mice (data not shown).

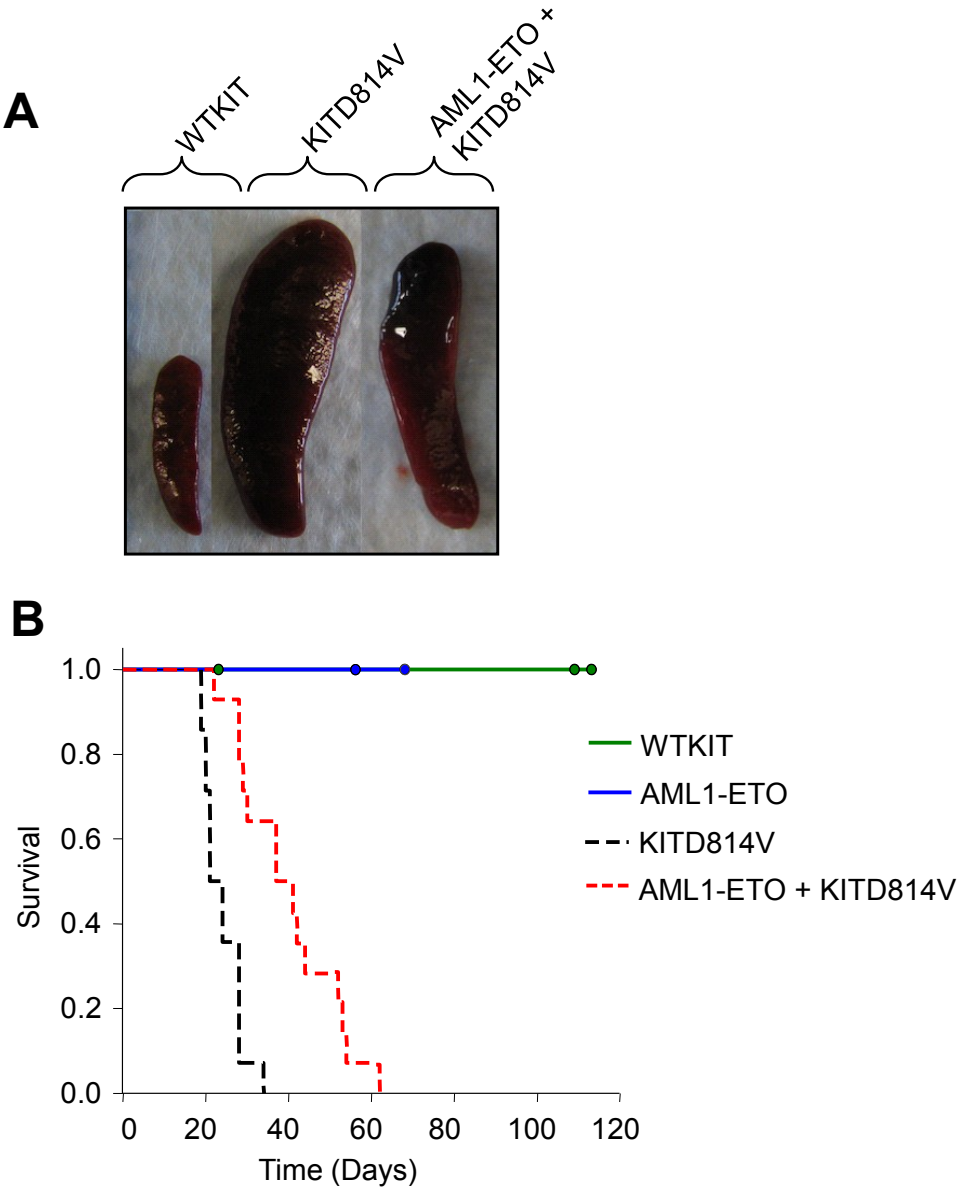

Figure 5.6. AML1-ETO and KITD814V co-expressing cells confer a MPN phenotype in syngeneic murine transplantation model.

(A) Representative pictures of spleen from mice bearing WT KIT, KITD814V, or KITD814V and AML1-ETO. (B) Cumulative survival analysis using Kaplan-Meier log-rank test of recipient mice bearing WT KIT (n=5), AML1-ETO (n=3), KITD814V (n=15) or AML1-ETO plus KITD814V (n=14) expressing 32D cells.

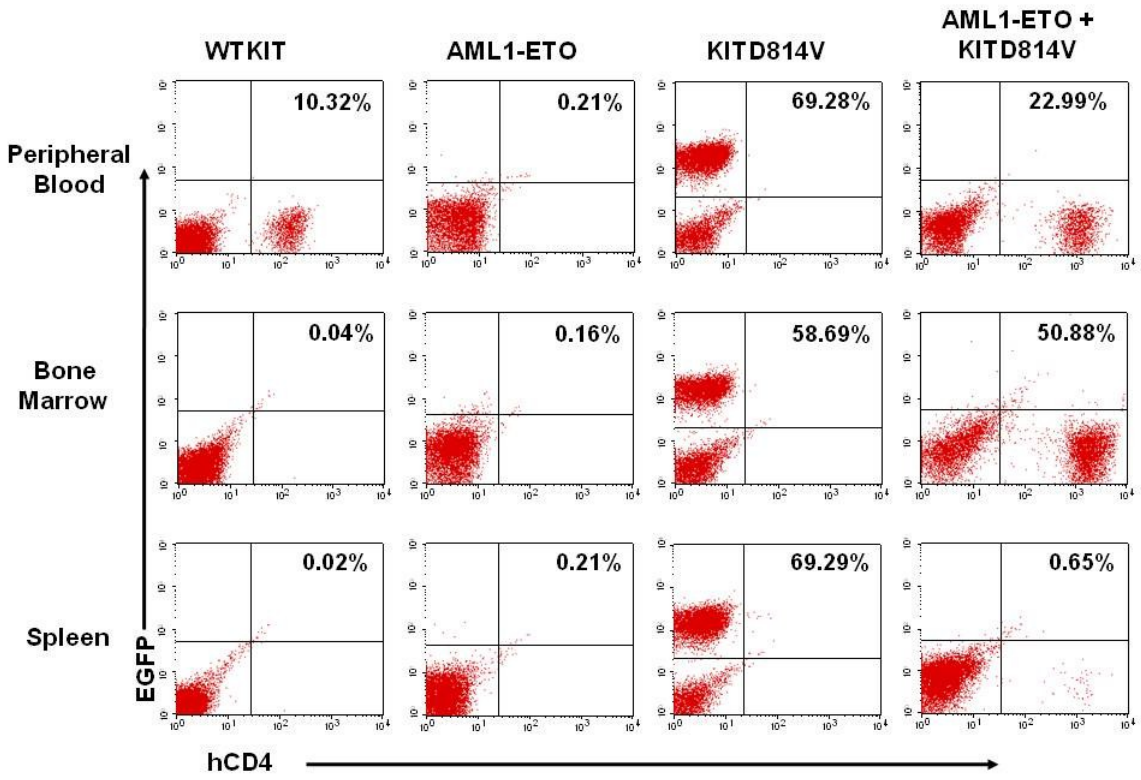

Figure 5.7. Engraftment of cells in KITD814V and/or AML1-ETO transplanted recipients.

Representative engraftment flow cytometry dot plots of peripheral blood, bone marrow, and spleen cells demonstrating the presence of $EGFP^+$ and $hCD4^+$ of WT KIT, AML1-ETO, KITD814V or AML1-ETO plus KITD814V transplanted mice upon sacrifice.

Histological analysis of bone marrow from mice transplanted with WT KIT-bearing cells showed normal marrow composition with a mixed population of immature myeloid and erythroid progenitors (Figure 5.8**.** Panel 1). A distinct area of red pulp and white pulp that is indicative of normal spleen tissue was observed (Figure 5.8. Panel 2). Normal liver and healthy normal lung was observed in control mice or AML1-ETO mice (Figure 5.8. Panel 3 & Panel 4). Tissues from mice transplanted with cells bearing a combination of AML1-ETO and KITD814V were hyperplastic with disruption of normal

tissue architecture in the bone marrow, spleen, liver and lung. Multiple mitotic events were observed in the bone marrow with the majority of marrow infiltrated with tumor cells and only a few normal myeloid and erythroid progenitors (Figure 5.8. Panel 1). The architecture of the spleen was disrupted by tumor cells with little red or white pulp. Again, many mitotic events were observed (Figure 5.8. Panel 2). Also, tumor cells were abundant in periportal area and in liver sinusoids (Figure 5.8. Panel 3). Pulmonary vessels were filled with tumor emboli, resulting in widened alveolar septa including partial thickening of alveolar walls, accompanied by several mitotic events (Figure 5.8. Panel 4).

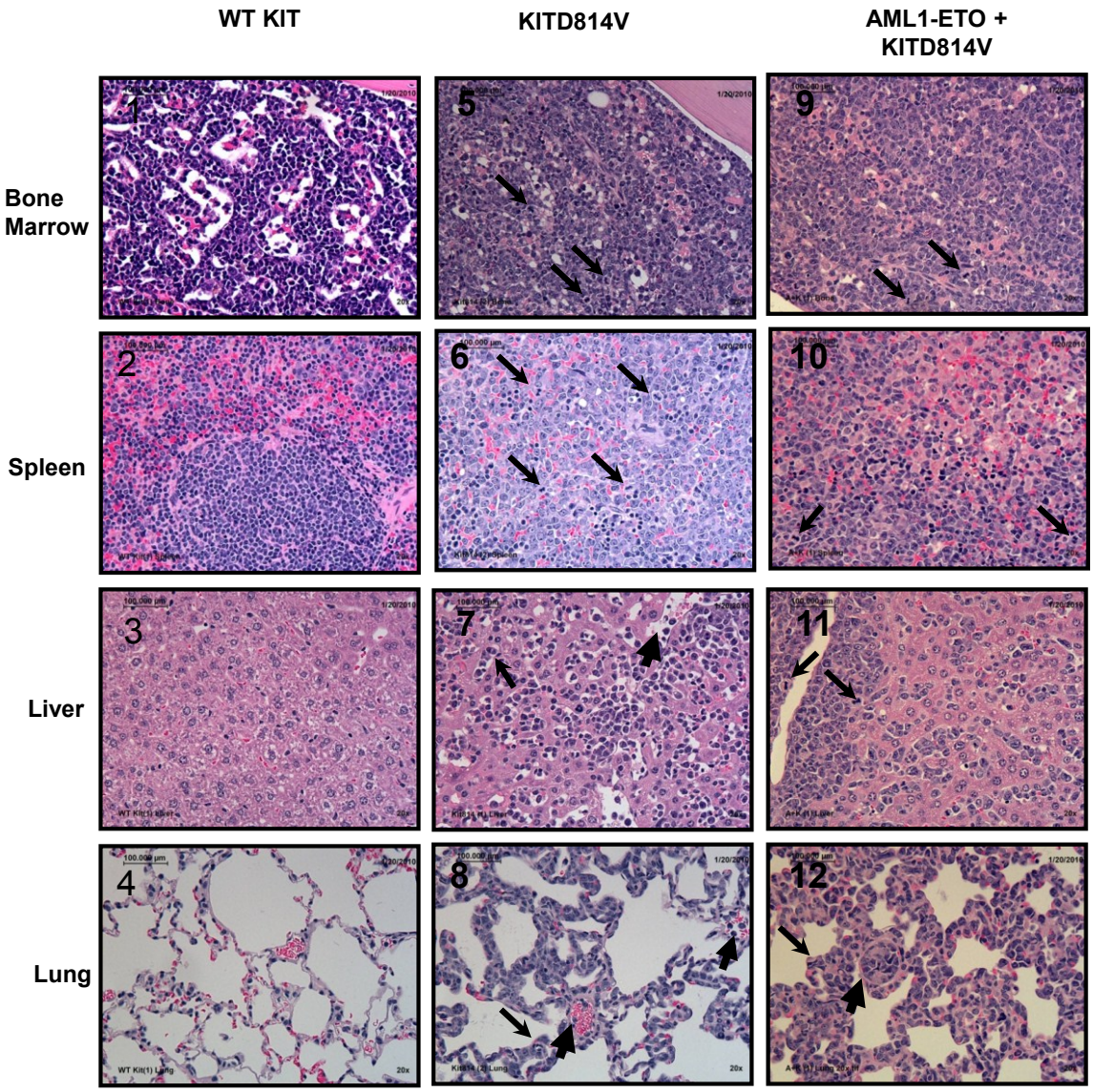

Figure 5.8. Histopathological analysis of mice transplanted with 32D cells expressing the indicated retroviral constructs.

Bone marrow, spleen, liver, and lungs from the recipient mice upon sacrifice were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Representative tissue from transplanted mice. Top panels are from representative WT KIT transplanted mice. Middle panels are from KITD814V transplanted mice. Bottom panels are from AML1-ETO plus KITD814V transplanted mice. Panel 1: Normal bone marrow with mixed population of immature myeloid and erythroid progenitors. Panel 2: Normal spleen with distinct red pulp and white pulp. Panel 3: Normal liver. Panel 4: Normal lung. Panel 5: Bone marrow hypercellular with tumor cells with scattered normal bone marrow cells and many mitotic figures (small arrows indicate individual cells undergoing mitosis). Panel 6: Normal splenic architecture disrupted and replaced with large number of tumor cell and many mitotic events. Panel 7: Large areas of liver

necrosis with surrounding tumor cells with many undergoing mitotic events (arrowheads indicate tumor cells circulating in blood vasculature). Panel 8: small venule with circulating tumor cells, partial thickening of alveolar walls with tumor cells and some mitotic events. Panel 9: Few normal erythroid and myeloid components replaced by tumor cells with some mitotic figures. Panel 10: Disruption of red and white pulp by large number of tumor cells, many undergoing mitotic events. Panel 11: Periportal area plus liver sinusoids filled with tumor cells. Panel 12: Pulmonary vessel filled with tumor emboli, resulting in widened alveolar septa. Partial thickening of alveolar walls with tumor cells, several mitotic events. Bar: 100 μM. Similar results were seen in several tissue sections from other transplanted mice.

5.E CONCLUSIONS

Clinical reports have suggested a strong association between CBF-AML and the presence of activating KIT mutations in up 50% of AML cases. In patients in which KITD816V is present along with the fusion proteins AML1-ETO or CBFβ-MYH11; these patients present with poor overall prognosis and overall survival. Although KITD816V mutations confer a poor prognosis to AML1-ETO or CBFβ-MYH11 bearing patients, at this time there is no direct evidence as to how these two mutations may possibly cooperate.

Our results show that cells bearing a CBF translocation, AML1-ETO or CBFβ-MYH11, and KITD814V, demonstrate a significant increase in ligand independent proliferation compared to KITD814V bearing cells alone. Growth of cells bearing WT KIT, AML1-ETO, or CBFβ-MYH11 remains ligand dependent. Furthermore, cells co-expressing AML1-ETO and KITD814V demonstrate an increase in cell cycle and a decrease in apoptosis, suggesting that the enhanced proliferation of these cells may be due to increased cell cycle and increased survival. Inhibitor studies demonstrated that signaling between KITD814V bearing cells alone and those bearing the two mutations, AML1-ETO and KITD814V, may be unique, as Jak and PI3K inhibition preferentially leads to a significant decrease in the growth of KITD814V bearing cells as compared to KITD814V and AML1-ETO co-expressing cells.

In vivo, we transplanted cohorts of animals with cells bearing KITD814V, which developed MPN by median day 23, while cohorts co-expressing AML1-ETO and KITD814V showed a delayed latency with median MPN development at day 39.

Although *in vitro* studies showed an increase in the proliferation in cells co-expressing the two mutations, the *in vivo* delay in survival of mice bearing the two mutations may be due to several factors. 32D cells may not be capable of differentiating abnormally when expressing KITD814V and AML1-ETO together. Also, the presence of AML1-ETO may result in poor engraftment or homing of these cells.

Overall, our results support the hypothesis that AML1-ETO and KITD814V interact in a cooperative manner, resulting in increased growth and survival *in vitro* and development of MPN *in vivo.* Also our study shows differential signaling pathways utilized by cells expressing KITD814V and AML1-ETO. In summary, AML involving CBF TFs, and KIT may utilize a distinct strategy from patients with SM bearing activating KIT mutations alone.

CHAPTER SIX

GENERATION OF HUMAN XENOGRAFT MODELS TO STUDY THE DEVELOPMENT AND PROGRESSION OF MASTOCYTOSIS AND ACUTE MYELOID LEUKEMIA

6.A INTRODUCTION

Acute myeloid leukemia (AML) is a devastating disease predominantly affecting elderly individuals and accounts for about 25% of all leukemia in adults in the western world (211, 212). Patients with AML often present with an overabundance of myeloid cells, anemia, and thrombocytopenia. Furthermore, patients under 65 years of age demonstrate a 34.4% 5-year survival rate, while the outlook for patients over 65 years of age is a dismal survival rate of 4.3% (211). It is extremely important to recognize the difficulty in treating older patients with AML. As patients age, they tend to develop multiple co-morbidities and, thus, become less tolerant of harsh chemotherapy and radiation (211-213). Because of these complications, older patients are often ineligible for cytotoxic therapy and are only treated with supportive, rather than curative care. Therefore, it is imperative that therapies with reduced toxicity and increased efficacy are developed to treat AML patients. Internal tandem duplications in FMS-like tyrosine kinase (FLT3-ITD) are insertions of several amino acids into or around the juxtamembrane domain and are found in approximately 20-25% of all AML patients, usually as an isolated anomaly (214-218). The presence of FLT3-ITDs contributes to an unfavorable prognosis. Despite promising *in vitro* studies using FLT3 inhibitors, such as PKC412, SU5614, and sorafenib, the clinical response of AML patients with FLT3-ITD

are moderate and temporary (88, 219). Thus, targeting FLT3-ITD in AML is a reasonable approach.

Systemic mastocytosis (SM) is a hematological neoplasm characterized by the abnormal deposition and accumulation of mast cells in various organs including skin, bone marrow and visceral organs (38, 39). A somatic point mutation has been identifies after genetic sequencing in the receptor tyrosine kinase, KIT, at codon 816 and plays a critical role in the pathogenesis and diagnosis of SM as it's detectable in >90% of SM patients (220). Along with KIT mutations patients with more aggressive SM frequently have mutations in genes such as TET2, SRSF2, ASXL1, RUNX1, and CBL that may contribute to the SM (51). KITD816V has also been observed in a subset of CBF-AMLs, resulting in overall poor prognosis. As of yet there are no clinically efficacious therapies for malignancies harboring an activating KITD816V mutation.

Over the years, numerous transgenic murine models have been designed to investigate target molecules that are involved in AML and SM as well as the response of these targets to therapeutic inhibition. These models have provided valuable insight into the mechanisms and targets of therapeutic intervention. However, the only way to know whether a patient's leukemia will respond to a similar therapeutic regime, one must examine the response of that human leukemia. A model examining the response of these factors in human neoplasms, rather than a murine neoplasm, would provide clinically relevant model of disease and therapeutic treatment. The most biologically relevant model is the *in vivo* use of human xenografts of hematological malignancies in humanized murine models.

Over the past two decades, the humanized murine models have been utilized and refined allowing for the transplantation of human hematopoietic cells into recipient animals without graft rejection (221). Generation of a NOD/SCID model has allowed for leukemic cell lines and primary patient samples to be engrafted and resemble the distribution and course of the human disease (222). Further refinement of the strain through defects of the interleukin-2 receptor gamma chain [NOD/SCID IL2Rnull (NSG)] further impaired the murine immune system by depleting innate immunity and natural killer (NK) cell activity leading to better engraftment and differentiation of transplanted human hematopoietic stem cells (115, 223). Additional improvements have been made on this model in which transgenic expression of human stem cell factor hSCF, hGM-CSF, and hIL-3 in the NSG background led to improvements in engraftment of AML samples and pre-leukemia modeling (116).

While investigators are beginning to use these models by developing them at their respective institutions; there is significant amount of variability in the engraftment of studies have been described so far (Figure 6.1). Furthermore, no xenograft models of human MPNs have been described. These models thus have to be generated independently in one's own institutions before detailed additional studies can be pursued. The purpose of these studies was to utilize NOD/SCID, NSG and NSG with human cytokine mice to establish murine xenograft models of human hematologic malignancies including AML and SM.

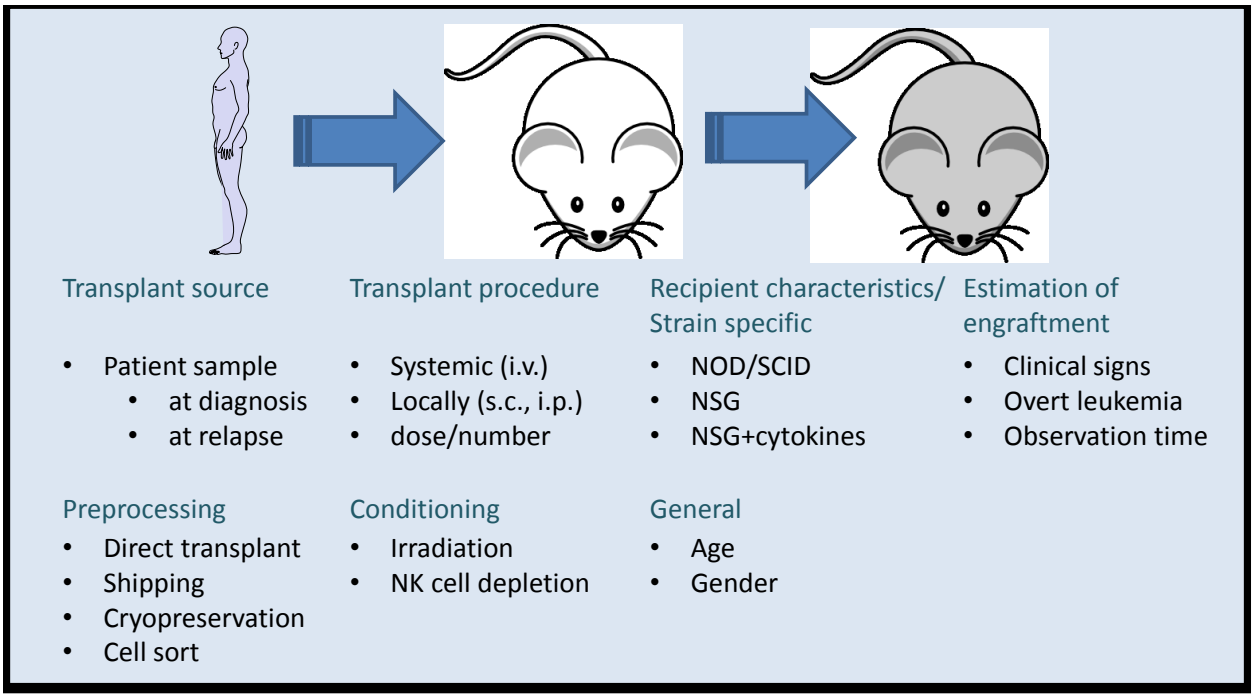

Figure 6.1. Factors affecting leukemia xenograft engraftment, growth and outcome. Adapted from Meyer and Debatin (224).

6.B RESULTS

Pharmacological inhibition of focal adhesion kinase (Fak) and p21-activated kinase (Pak) represses growth of FLT3-ITD positive patient derived cells in NSG mice

Our lab has recently shown that focal adhesion kinase (Fak) and p21-activated kinase (Pak) to be constitutively active downstream effectors of FLT3-ITDs. Inhibition of these effectors represses the growth of murine leukemic cells *in vivo* and primary AML cells *in vitro* (225). However, the impact of inhibition of these molecules *in vivo* on human leukemia cell has not been investigated. We investigated whether inhibition of downstream effectors of FLT3-ITD, Fak and Pak, can suppress engraftment of patient-derived MV4;11 cells bearing FLT3-ITD *in vivo.*

Engraftment and growth capabilities of a patient-derived MV4;11 cells was investigated using an immunodeficient xenograft model. Six-week old non-obese diabetic severe combined immunodeficient gamma (NSG) (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) recipient mice were sub-lethally irradiated (300 rad) and intravenously (i.v.) injected with $1x10^7$ MV4;11 cells. Drug treatments were initiated 14 days post-transplantation. Recipient mice were divided into multiple groups including vehicle (10% DMSO in PBS, n=3), Fak inhibitor (F-14, 20mg/kg/day in vehicle, n=3), and Pak inhibitor (PF-3758309, 25mg/kg/day in vehicle, n=3) groups. The mice received intraperitoneal (i.p.) injections of the above drugs for five consecutive days. Treated mice were harvested at day 30 post-transplantation. Engraftment of MV4;11 cells in the bone marrow was assessed by analyzing the expression of human (h) CD4 expression by flow cytometry (Figure 6.2.A). Previous studies have shown the expression of hCD4 antigen on MV4;11 cells (226). *In vivo* transplantation of FLT3-ITD bearing patient-derived MV4;11 cells resulted in

leukemogenesis as characterized by splenomegaly and hepatomegaly (Figure 6.2.C). Importantly, treatment with F-14 or PF-3758309 showed a reduced presence of hCD4 positive cells in the bone marrow compared vehicle-treated controls (5.51% and 4.68% to 27.4% respectively) (Figure 6.2.B). While vehicle-treated mice exhibited splenomegaly, this was significantly decreased in F-14 and PF-3758309-treated mice (Figure 6.2.C). These findings indicate that AML patient-derived cells can be successfully transplanted into NSG mice and that they respond to FAK and Pak inhibitors *in vivo* by repressing the presence of leukemic cells.

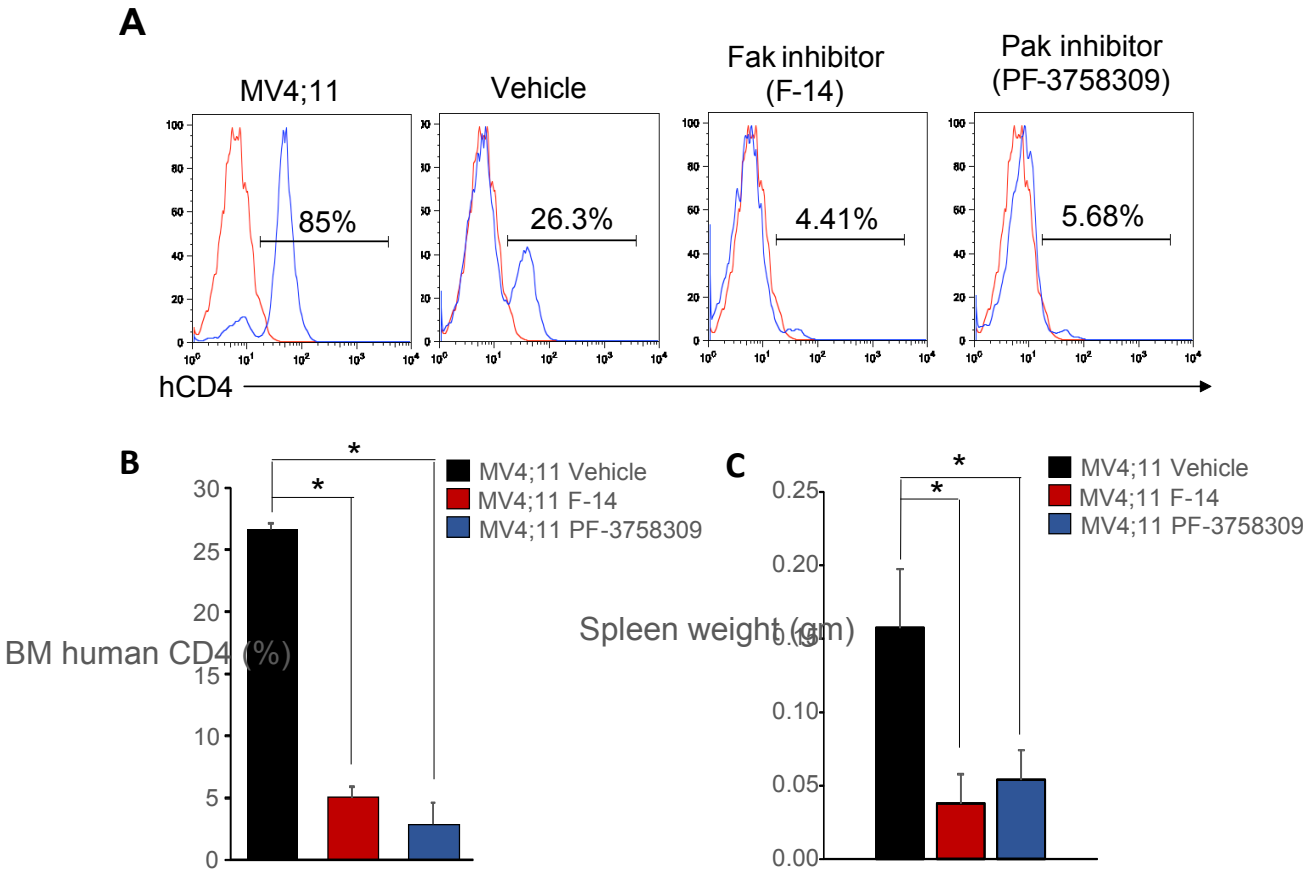

Figure 6.2. Impact of FAK and PAK inhibition on MV4;11 cell engraftment.

(A) Representative flow cytometry staining for human CD4 positive MV4;11 cells vehicle, FAK inhibitor (F-14) and PAK inhibitor (PF-3758309) treated recipients, respectively. (B) Mean human CD4 positive cells in bone marrow. (C) Quantitative differences in spleen mass of vehicle, F-14 or PF-3758309 treated cohorts. *p-value<0.05 vehicle vs F-14 or PF-3758309 (n=3 mice per cohort).

Subcutaneous growth of human systemic mastocytosis patient-derived KITD816V bearing cells

We next generated a murine model of human SM. Although oncogenic KIT is present in a vast majority of systemic mastocytosis patients, development and severity of the disease may be affected by additional mutations. In this regard TET2, a tumor

suppressor that has an enzymatic function in DNA demethylation may contribute significantly to SM (52, 53).

To investigate the cooperative effects of loss of TET2 in the presence of oncogenic KIT, we transplanted systemic mastocytosis patient derived cells, HMC1.2, bearing KITD816V in the presence or absence ofTET2 using a plasmid bearing a hTET2 shRNA. Two cohorts in which $1x10^7$ HMC1.2 cells alone or HMC1.2 cells expressing hTET2 shRNA were transplanted subcutaneously into the right flank of non-obese diabetic severe combined immunodeficient (NOD/SCID) recipient mice. Tumor growth was monitored by external caliperation in which tumor volume [*Tumor volume=1/2 (length x width^2)*] was monitored following six weeks of transplantation. Mice were harvested at week 10 post-xenotransplantation and tumor growth was assessed. Tumor growth and mass were significantly enhanced in mice bearing HMC1.2 cells with shRNA TET2 suppression relative to HMC1.2 cells (Figure 6.3.A and 6.3.B). Representative pictures of each cohort are shown in Figure 6.3.C. These results suggest that loss of Tet2 in conjunction with the presence of KITD814V mutation enhances the tumorigenic potential of these cells.

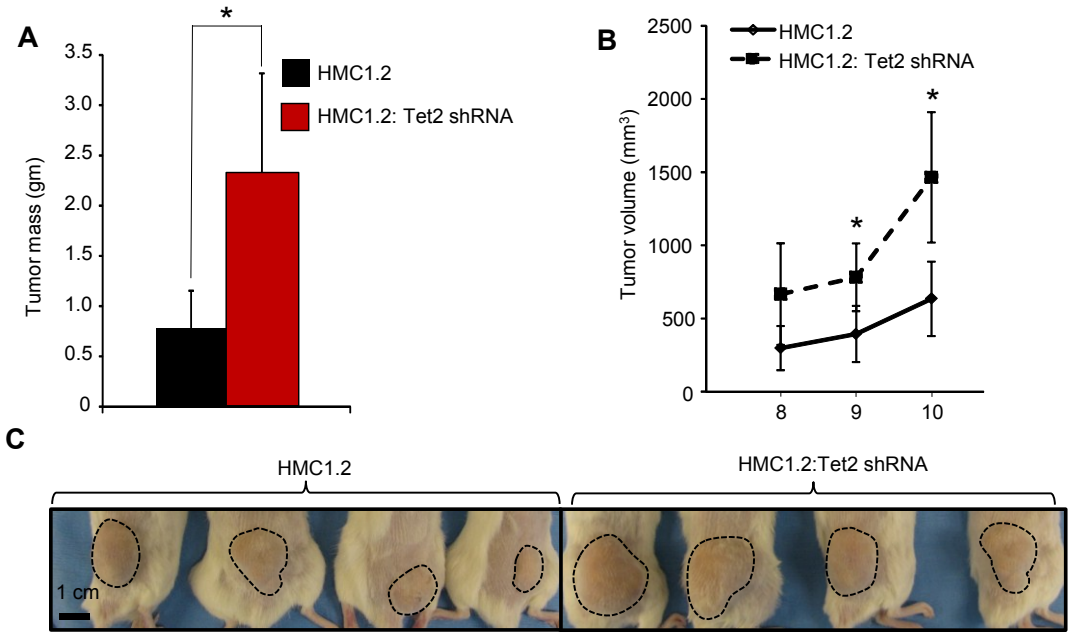

Figure 6.3. HMC1.2 cells with or without hTET2 shRNA expression subcutaneously were injected in the right flank of NOD/SCID mice.

(A) Tumor mass following sacrifice of recipient animals at 10 weeks post-transplantation. *p-value<0.05, HMC1.2 vs. HMC1.2 with hTET2 shRNA. n=5 mice (B) External caliperation measurements of tumor growth determined by volume. *p-value<0.05, HMC1.2 vs. HMC1.2 with hTET2 shRNA. n=5 mice (C) Representative images of tumor volume in HMC1.2 cells bearing NOD/SCID and HMC1.2 cells expressing hTET2 shRNA.

NSG and NSG plus cytokine mice show engraftment of primary AML patient-derived cells

We obtained five frozen AML patient samples with >75% leukemic blasts from Cleveland Clinic (Cleveland, OH). NSG and NSG with cytokine recipient mice were sub-lethally irradiated (300 rad) 24 hours prior to transplantation. Patient samples were thawed and viability was assessed by trypan blue staining and viable cells were counted. A small sample of cells from each vial was subjected to flow cytometry to assess the overall content of hCD33 and hCD45 positive cells. Recipient mice were transplanted intravenously with 1.8-5x10^6 $hCD33^+$/$hCD45^+$ mononuclear cells. Two patient samples, Patient 12111502 and 13051501, were transplanted into NSG recipient animals that expressed hSCF, hGM-CSF, and hIL-3, and three patient samples (Patient 13071701, 13040401, and 12111501) were transplanted into NSG recipient mice (Table 6.1). To determine the engraftment efficiency of these cells, quantitative analysis of peripheral blood, bone marrow, and spleen was performed at time of harvest. Engraftment of human AML patient-derived cells was determined as the percentage of cells expressing $hCD45^+hCD33^+$ (Figure 6.4.B). Mean engraftment percentages in the peripheral blood, bone marrow and spleen from five mice injected with cells derived from Patient 13051501 showed significant levels of engraftment in NSG mice bearing human cytokines (Figure 6.4.C).

Table 6.1. Primary AML patient-derived samples for xenograft transplantation in NSG or NSG with cytokines sublethally irradiated recipient hosts.

AML patient samples					
Case ID	Specimen	MNC sent	Description	Institution	Murine recipient
12111502	75340.0.10	20 million/vial	78% blasts	Cleveland Clinic	NSG+cytokines
13051501	86380.1.10	20 million/vial	78% blasts	Cleveland Clinic	NSG+cytokines
13071701	88640.2.9	20 million/vial	78% blasts	Cleveland Clinic	NSG
13040401	82960.1.10	30 million/vial	78% blasts	Cleveland Clinic	NSG
12111501	75320.1.6	16.5 million/vial	78% blasts	Cleveland Clinic	NSG

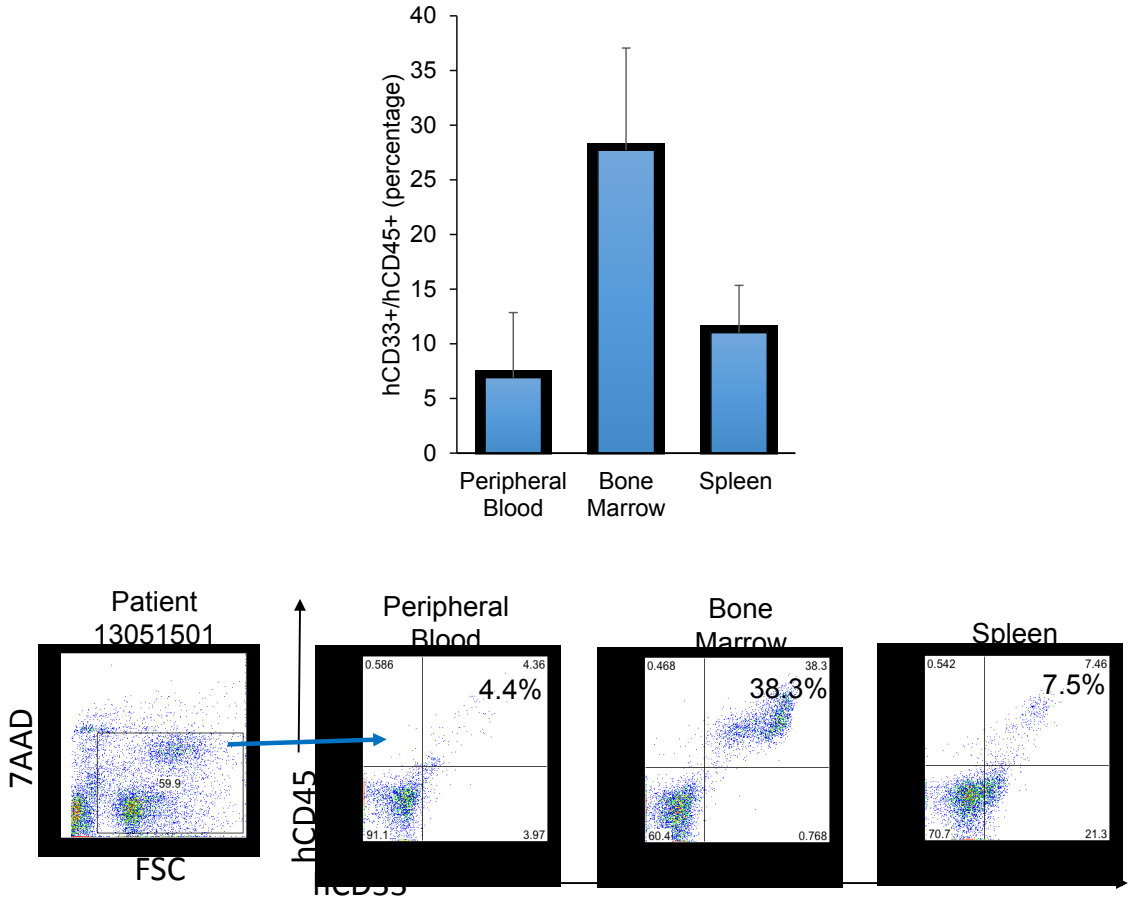

Figure 6.4. Engraftment of AML-derived patient 13051501 cells.

(A) Mean ± SD of human CD33 and human CD45-positive engrafted cells in peripheral blood, bone marrow, and spleen. (B) Representative $hCD45^{+}/CD33^{+}$ dot blots of peripheral blood, bone marrow and spleen from patient 13051501-transplanted NSG with human cytokine mice.

6.C CONCLUSIONS

Our data suggests a significant reduction in the leukemic burden of mice treated with Fak or Pak inhibition. Likewise, we demonstrate that loss of TET2 contributes to an aggressive tumor growth in human mast cell leukemic line harboring a KITD814V mutation. Although the mechanism by which TET2 mutation contributes to this aggressive growth is poorly understood, this observation is likely to be instrumental in designing novel therapeutic strategies for SM patients that bear both of these mutations.

In the present study, we have used external measurements with calipers to establish tumor size, which can be a subjective. In the future, we intend to follow tumor progression through bioluminescent imaging to more accurately analyze the tumor volume and response to therapy. These studies have since been initiated and involve HMC1.2 cells bearing a firefly luciferase reporter transplanted into NOD/SCID recipient mice. In collaboration with Paul Territo, M.D. at the Office of Research Imaging at IUSM, tumor growth will be imaged following luciferin dosing. In this manner, a tumor regression model can be established in which tumors can be grown to baseline volumes prior to therapeutic treatments, and growth or repression of the tumors can be accurately assessed.

In addition to the above described models, we also demonstrate engraftment of primary human patient AML samples in NSG mice expressing human cytokines. While these studies are somewhat preliminary our results indicate great potential in understanding and treating AML utilizing these models. Some benefits include clinical manifestation of the disease in peripheral blood, bone marrow and spleen with minimal

variance from animal to animal with the same patient samples, which provides an ideal model for therapeutic regimes in the future.

CHAPTER SEVEN

DISCUSSION

Despite efforts to develop efficacious treatments and therapies for CBF-AML and SM patients bearing KITD816V mutations, current treatment options are limited and at best modestly effective. While AML patients harboring a CBF translocation have relatively successful treatment and positive prognosis, those patients harboring an additional mutation of an oncogenic KIT have a worse overall outcome (29, 30, 34). The significance of our studies is derived from the realization that despite the success of therapies such as imatinib, sunitinib, and dasatinib, for treating activating KIT induced GISTs; activation loop mutations of KIT are largely resistant to such available therapies. Further, the aberrant molecular mechanisms that regulate transformation *in vivo* via this mutation are poorly understood. Therefore, the overall goal of this project was to define the molecular mechanism(s) underlying the transformation capacity of KITD814V in HSC/Ps, with the long-term goal of defining novel therapeutic targets in CBF-AML and SM.

As previously described, KIT encodes a receptor tyrosine kinase characterized by the presence of five Ig-like repeats in the extracellular domain and an insert that splits the cytoplasmic kinase domain into the triphosphate (ATP)-binding and phosphotransferase domain (67). The binding of SCF promotes dimerization of wild type KIT receptor and activates intrinsic protein kinase activity, resulting in transphosphorylation at critical tyrosine residues (67). In contrast, activation loop mutations of KIT (e.g. KITD816V in humans, KITD814V in mice) do not result in dimerization, but are found to result in

receptor self-association in the cytoplasmic region in absence of ligand stimulation. Although it is unclear how KITD814V undergoes self-association, rather than receptor dimerization; it has been hypothesized that KITD814V may stabilize a conformation equivalent to that induced by ligand binding to the wild type receptor. Therefore, KITD814V may not require its extracellular domain for constitutive activation and growth promoting signals. To examine this, we developed a chimeric KITD814V receptor in which the extracellular domain of the murine KIT receptor was replaced by the extracellular domain of the human macrophage colony-stimulating receptor. We were able to demonstrate that an acquired KITD814V mutation was sufficient to induce ligand independent growth and cellular hyperproliferation. By replacing the extracellular domain of KIT, the chimeric receptor is impervious to endogenous murine cytokine binding and stimulation. Our findings show that a KITD814V receptor devoid of its extracellular domain is constitutively activated and capable of ligand-independent growth and sufficient to induce MPN *in vivo*. Therefore it is possible that self-association of KITD814V may result from the D814V mutation itself and that the D814V mutation may activate KIT signaling by creating a novel receptor that is not reliant on the extracellular domain.

Although a significant advance has been made in our understanding of the KITD814V receptor at the structural level; little is known about the aberrant signals that contribute to transformation by this mutation. To identify signaling pathways that are involved in KITD814V-induced MPN, we used a chimeric model to examine the role of seven specific intracellular tyrosine residues in KITD814V-induced MPN. Our findings show that intracellular tyrosine residues are important for KITD814V-induced MPN,

albeit to different degrees. Using 32D cells expressing CHRKITD814V or CHRD814V-F7 cells we show that by mutating the tyrosine residues within the JM and/or kinase insert domain of KITD814V completely abrogates ligand-independent growth and transformation *in vivo*. Additionally our studies suggest that the signals initiated from tyrosine 719 appear to sufficiently contribute to KITD814V ligand independent growth *in vitro*, transformation *in vivo,* and activation of AKT, ERK, STAT5 and anti-apoptosis proteins Bcl-x and Bcl-2 to a similar extent as CHRD814V receptor. To a lesser extent, signals initiated from tyrosines 567, 569, 702, 728, or 934 appear contribute to KITD814V induced transformation. Our findings suggest that tyrosine 719 has a dominant role in regulating KITD814V-induced MPN, and that downstream signaling pathways from tyrosine 719 are crucial for KITD814V pathology.

Additional studies suggest that persistent activation of PI3K is observed in hematological malignancies and are necessary for KITD814V-induced transformation (133, 150). We show that tyrosine 719 is necessary for the p85α regulatory subunit of PI3K to bind to KITD814V. Evidence suggests that PI3K activates GEF Vav that facilitates the conversion of Rac GTPases from an inactive GDP bound state to an active GTP bound state (72, 96, 97). Therefore we hypothesized that the hematopoietic specific GEF, Vav1, contributes to KITD814V induced transformation. Using 32D cells expressing WT KIT or KITD814V, we demonstrated constitutive hyperactivation of Vav1. To examine the relevance of Vav1 in meditating oncogenic KIT-induced ligand independent growth. We examined the effect of genetic disruption of Vav1 on the proliferative capacity of HSC/Ps expressing KITD814V. Comparing wild type or Vav1 deficient primary murine bone marrow LDMNCs transduced with WT KIT or

KITD814V, we showed loss of Vav1 corrected ligand-independent hyperproliferation of KITD814V by 70%. As only modest reduction in growth was observed in Vav2 or Vav3 null LDMNCs, this suggests that KITD814V signaling occurs through the hematopoietic-specific expressed Vav1 rather than the other Vav family members. These findings suggest that ligand independent growth observed in LDMNCS expressing KITD814V is preferentially dependent on Vav1 since deficiency of Vav1 almost completely rescues this response. Furthermore, using an *in vivo* model, we found that loss of Vav1 in bone marrow of KITD814V transplant recipients resulted in a prolonged survival of leukemic mice. These observations validate the hypothesis that Vav1 contributes to KITD814V transformation.

Since Vav1 is a critical GEF for Rho GTPases in particular Rac family of Rho GTPases, we examined whether Rac GTPases are also constitutively activated in cells expressing KITD814V. We demonstrated that activation of all Rac GTPases isoforms in the presence of KITD814V-expressing LDMNCs were dependent of Vav1 expression. To test the role of Rac GTPase activation on KITD814V-induce ligand independent growth and transformation dominant negative form of Rac was used and demonstrated significant rescue of ligand-independent hyperproliferation and MPN in a syngeneic transplantation model. The involvement of individual Rac isoforms was determined using primary murine cells with loss of Rac2, Rac1 (polyI:polyC-treated $Rac1^{flox/flox}$;$Mx1\text{-}Cre^{+}$) or both. Both *in vitro* and *in vivo* studies suggest that both Rac1 and Rac2 are involved in KITD814V-induced transformation. Loss of both resulted in rescue of KITD814V-induced hyperproliferation and transplanted recipients had prolonged survival, while loss

of individual Rac GTPase isoforms modestly, yet significantly abrogated hyperproliferation of KITD814V bearing cells.

Given the involvement of Rac GTPases in transformation, we used a first generation small molecule inhibitor that inhibits the activation or Rac2. The drug NSC23766 suppresses the activation of GEF Trio and Tiam1 induced cell transformation. In human prostate cancer cell line, NSC23766 suppresses endogenous Rac1 activity and inhibits the proliferation, anchorage independent growth and invasion phenotypes that are Rac1 dependent. NSC23766 was subsequently shown to function by inhibiting Rac protein binding and activation by Rac specific GEF Trio and Tiam1 in a dose-dependent manner. Thus NSC23766 was initially thought to be a Rac-specific small molecule inhibitor that is capable of reversing cancer cell growth associated with Rac dysregulation. Although promising in regard to inhibiting the activation of Trio and Tiam1, our results using NSC23766 in murine 32D and p815 cell lines expressing KITD814V and Kasumi-1 cell lines expressing AML1-ETO and KITD816V demonstrated only a modest reduction in ligand independent growth. Furthermore, the inhibition in growth was observed at very high doses of NSC23766. Thus, although we have established that the Rac inhibitor NSC23766 (specific for GEF Trio and Tiam1) modestly inhibits KITD814V induced ligand independent growth *in vitro*, its utility as a drug for treating hematological malignancies involving KITD814V is likely to be limited due in part to low expression of Trio and Tiam1 in hematopoietic cells and in part due to this drug's high IC_{50}. So although a useful drug to establish proof of principal, additional Rac specific drugs that inhibit binding between hematopoietic specific GEF Vav1 and Rac would be beneficial. We then used a newly described Rac inhibitor, EHop-016,

which is a NSC23766 analog (157). EHop-016 functions by binding to Rac GTPases and inhibiting interactions between Rac GTPases and GEFs Vav. Indeed upon treatment with EHop-016, Vav1 binding with Rac1 was interrupted in KITD814V-expressing cells. EHop-016 also inhibited growth of cells bearing KITD814V 100-fold more effectively than the parental compound. This further validates the hypothesis that GEF Vav1 and its downstream effectors, Rac GTPases, contribute to KITD814V induced transformation.

In targeting Vav1 and Rac GTPases we further identified Pak as a downstream effector of Rac GTPases that is involved in KITD814V-induced transformation. Dominant negative and pharmacological approaches were used to assess the role of Pak in KITD814V induced transformation. In 32D cells, we observed that dominant negative suppression of Pak abrogated KITD814V-induced hyperproliferation. In targeting Pak pharmacologically with Pak inhibitor, IPA-3, we were able to demonstrate that inhibition of PAK activity in cells expressing KITD814V or KITD816V resulted in a dose-dependent and statistically significant reduction in hyperproliferation. Under no growth factor conditions cells expressing KITD814V had an increase in apoptosis upon IPA-3 treatment compared to untreated KITD814V bearing cells. Syngeneic KITD814V transplant recipients succumbed to fatal KITD814V-induced malignancies by day 15 post-transplantation, while mice bearing dominant negative PakK299R in the presence of KITD814V had a significantly delayed latency compared to KITD814V alone. This data suggests that Pak inhibition *in vitro* with dominant negative suppression of Pak or pharmacological inhibition by IPA-3 successfully inhibited KITD814V and KITD816V-induced hyperproliferation. Our *in vivo* data supports that suppression Pak delays KITD814V-induced MPN onset.

While these *in vitro* and *in vivo* data are informative, we further tested the potential of pharmacological suppression of Rac GTPases or Pak in activated KIT bearing human neoplasms. We cultured patient-derived SM cells bearing either wild type form of KIT or with an activating KITD814V mutation with EHop-016 or IPA-3. We observed a significant dose-dependent response to EHop-016 and IPA-3 in patient-derived SM cells bearing KITD816V, but no significant response in those SM patient samples bearing wild type KIT. These data suggest that targeting Rac GTPases and Pak may have potential therapeutic benefits in treatment of KITD816V-bearing AML and SM.

Significance

Oncogenic mutations of the receptor tyrosine kinase, KIT, are associated with poor prognosis in human neoplasms. However, the molecular mechanism by which an activating KIT mutation acts to induce leukemogenesis is poorly understood. We discovered several novel findings regarding the molecular mechanisms of KITD814V-induced malignancies. In the process of understanding these molecular mechanisms, we observed that intracellular tyrosines are crucial for ligand independent growth and MPN development. Specifically, signaling through tyrosine 719 was sufficient in itself to induce MPN. Furthermore, activation of PI3K through tyrosine 719 is critical for KITD814V-induced proliferation, activation of its downstream effectors Vav1, Rac GTPases and Pak, and for the development and progression of KITD814V-induced MPN in vivo. Collectively we demonstrated the novel role of Rac GTPases and Pak in activating KIT mutation induced neoplasms and show convincing evidence that targeting

Rac GTPases and Pak may provide therapeutic benefit to treat KITD816V-induced AML and SM.

Drug development that targets KIT and its downstream effectors are suitable therapeutic targets, as KIT contributes to the initiation and progression of many human malignancies. Dysregulation of KIT through the mutation D816V is a common molecular aberration observed in advanced SM and CBF-AML patients with poor prognosis. The propensity of such human malignancies to be resistant to tyrosine kinase inhibitors emphasizes the importance of a deeper understanding of KIT signaling in cells expressing oncogenic mutants of KIT. With this knowledge novel target molecules for pharmacological intervention, such as Rac GTPases and Pak, can be discovered and novel therapeutic modalities can be developed. If the specific pathways that are utilized by the oncogenic mutants of KIT can be targeted, the risk of side effects should be reduced. It is also likely that simultaneously targeting different signaling molecules will circumvent the resistance to tyrosine kinase inhibitors. In many human malignancies multiple molecular aberrations can be observed. In CBF-AML, chromosomal rearrangements resulting in AML1-ETO or CBF-MYH11 result in a block of differentiation by acting in a dominant negative manner blocking transcription of genes important for hematopoietic differentiation. Advanced systemic mastocytosis patients have also been observed to also have mutations in genes that affect epigenetic regulation, such as TET2, ASXL1, and DNMT3A. As these diseases result from complex molecular aberrations having combinational therapies that target the effects of these multiple mutations may be necessary to demonstrate sustained activity. Therefore, furthering our understanding of the molecular mechanisms of oncogenic KIT mutations are of the

upmost importance and will translate into major advances on the treatment of SM and CBF-AML.

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B.S. in Chemistry, A.C.S. with Biochemistry emphasis

TRAINING

2003-2005 Research Technician III, Breast Care Center
Baylor College of Medicine, Houston, TX
Mentor: Suzanne Fuqua, Ph.D.
Project- "Oncogenic mutations of estrogen receptor α and Her-2 neu in Breast Cancer"

FUNDING

NIH/NIA 1F31 AG040974-01 (Martin 01/01/12-12/31/13)
"Novel therapeutic targets for Leukemia in Elderly"

NIH/NIDDK 2T32 DK007519-25 (Broxmeyer 7/01/07 –12/31/11)
"Regulation of Hematopoietic Cell Production"

AWARDS AND HONORS

2013 Poster Award, 2nd place Basic Science Research, IUSCC Cancer Research Day, Indianapolis, IN

2012 ASH Abstract Achievement Award, Graduate student category, 54th Annual Meeting of the American Society of Hematology (ASH), Atlanta, GA

2012 2012 St. Jude National Graduate Student Symposium participant, Memphis, TN

2012	Poster Award, Graduate student category, 10th Annual Midwest Blood Club, Indianapolis, IN
2011	Paradise Award, 1st place at 2011 Sigma Xi Graduate Biomedical Research Competition, Indiana University School of Medicine, IN
2011	Honorable Mention in the Translational / Clinical Research, IUSCC Cancer Research Day, Indianapolis, IN
2007-2011	Regulation of Hematopoietic Cell Production (DK 007519) Training Grant, Indiana University School of Medicine, IN
2003	Senior Undergraduate Sigma Xi award Biology, Hope College, MI
2003	Senior Undergraduate Sigma Xi award Chemistry, Hope College, MI
2003	Hope Outstanding Woman Award, Hope College, MI
2003	USRP Merck Scholar, Hope College, MI
2001-2002	National Science Foundation Summer Research Participant, Hope College, MI
1999-2003	Endowed Scholarship recipient, Hope College, MI
1999-2001	Jaecker C. Chemistry Scholarship recipient, Hope College, MI

TEACHING AND TRAINING

1999-2003 Researcher/Technician, Department of Biology
Hope College, Holland, MI
Mentor: Glenda Gentile, Ph.D. and James Gentile, Ph.D.
Project- "Effects of chronic exposure to aflatoxin B_1 and Fasciola hepatica in Hepatocellular Carcinomas"

2001-2003 Teaching Assistant, Departments of Biology and Nursing
Hope College, Holland, MI
Supervisor: Lori Hertel

2007-2013 Supervisor for summer research students, rotations students, and technicians in Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, IN

RESEARCH PUBLICATIONS

Chatterjee A, Ghosh J, Ramdas B, Mali RS, **Martin H**, Vemula S, Chan R, and Kapur R. Unraveling a novel mechanism of Stat5 regulation by FAK, Rac1 and PAK1 in oncogenic Flt3 and Kit induced acute myeloid leukemia and systemic mastocytosis. [In revision for Cell Reports]

Martin H*, Mali RS*, Ma P*, Chatterjee A*, Ramdas B*, Sims E, Munugalavadla V, Ghosh J, Mattingly RR, Visconte V, Tiu RV, Vlaar CO, Dharmawardhane, and Kapur R.

Pak and Rac GTPases promote oncogenic KIT-induced neoplasms. J Clin Invest. 2013 Oct 1; 123 (10):4449-446. (* authors contributed equally to this study)

Mali RS*, Ma P*, Zeng L*, **Martin H***, Ramdas B, He Y, Sims E, Ghosh J, Nabinger S, Li S, Munugalavadla V, Chatterjee A, Sandusky G, Craig AW, Bunting KD, Feng G, Chan RJ, Zhang Z and Kapur R. Role of SHP2 phosphatase in KIT induced transformation: Identification of SHP2 as a druggable target in diseases involving oncogenic KIT. Blood 2012 Sep 27; 120 (13):2669-78. PMID: 22806893 (* authors contributed equally to this study) PMC Journal-In Progress

Ma P*, Mali RS*, **Martin H***, Ramdas B, Sims E, and Kapur R. Role of Intracellular tyrosines in activating KIT-induced myeloproliferative disease. Leukemia 2012 Jul; 26(7): 1499-506. (* authors contributed equally to this study)

Ma P, Mali RS, Munugalavadla V, Krishnan S, Ramdas B, Sims E, **Martin H**, Ghosh J, Li S, Chan RJ, Krystal G, Craig AW, Takemoto C, Kapur R. The PI3K pathway drives the maturation of mast cells via microphthalmia transcription factor. Blood. 2011 Sep 29;118(13):3459-69. PMC: 318628

Motorna OO, **Martin H**, Gentile GJ, and Gentile JM. Analysis of lacI Mutations in Big Blue® Transgenic Mice Subjected to Parasite-induced Inflammation. Mutat Res. 2001 Dec 12;484(1-2):69-76. 11733073

ABSTRACTS

Oral presentations:

Martin H, Ma P, Chatterjee A, Ramdas B, Sims E, Vlaar CP, Dharmawardhane, and Kapur R. *Role of p21 Activated Kinase and Specific Guanine Exchange Factors of Rac Gtpases in Oncogenic KIT Induced Systemic Mastocytosis and Acute Myeloid Leukemia.* 54th Annual Meeting of the American Society of Hematology. Atlanta, Georgia. December 8-11, 2012.

Martin H, Ma P, Chatterjee A, Ramdas B, Sims E, and Kapur R. *Genetic Disruption and Pharmacologic Inhibition of the Vav-Rac-Pak Signaling Pathway Suppresses Oncogenic KIT Mediated Leukemogenesis.* 2012 St. Jude National Graduate Student Symposium participant, Memphis, Tennessee. March 20-23, 2012.

Martin H, Ma P, Ramdas B, and Kapur R.*,p21 Activated Kinase (PAK) as a Potential Therapeutic Target for Oncogenic KIT in Acute Myelogenous Leukemia.* Sigma Xi Research Competition. Indianapolis, Indiana. August 12, 2011.

Martin H, Ma P, Ramdas B, and Kapur R. *Cooperation of AML1-ETO and oncogenic Kit in Acute Myelogenous Leukemia,* Sigma Xi Research Competition. Indianapolis, Indiana. May 10, 2010.

Poster presentations:

Martin H, Mali R., Ma P., Chatterjee A., Ramdas B., Sims E., Vlaar C. P., Dharmawardhane S., and Kapur R. *P21-Activated Kinase and Specific Guanine Exchange Factor of Rac GTPases in Oncogenic Kit Induced Systemic Mastocytosis and Acute Myeloid Leukemia***.** IUSCC Cancer Research Day. Indianapolis, Indiana. May 22, 2013.

Martin H, Ma P, Chatterjee A, Ramdas B, Sims E, and Kapur R. *Genetic Disruption and Pharmacologic Inhibition of the Vav-Rac-Pak Signaling Pathway Suppresses Oncogenic KIT Mediated Leukemogenesis.* 10th Annual Midwest Blood Club meeting, Indianapolis, Indiana. March 15-16, 2012.

Martin H, Chatterjee A, Ma P, Ramdas B, Sims E, and Kapur R. *p21 Activated Kinase (PAK) as a Potential Therapeutic Target for Oncogenic KIT in Acute Myelogenous Leukemia.* 40th annual Society for Hematology and Stem Cells (ISEH) meeting. Vancouver, Canada, August 2011.

Martin H, Chatterjee A, Ma P, Ramdas B, and Kapur R. *Rac GTPases and Pak as Potential Therapeutic Targets of Oncogenic KIT in Acute Myelogenous Leukemia.* IUSCC Cancer Research Day. Indianapolis, Indiana. May 26, 2011.
Chatterjee A, Vemula S, Ramdas B, **Martin H**, Nabinger S, Chan R, and Kapur R. *Novel FAK-Rac-PAK signaling pathway in Flt3ITD mediated leukemogenesi*s. IUSCC Cancer Research Day. Indianapolis, Indiana. May 26, 2011.

Ma P, Singh R, Zeng Li-Fan, **Martin H**, Ramdas B, He Y, Sims E, Ghosh J, Nabinger S, Li S, Munugalavadla V, Sandusky G, Craig A, Bunting K, Feng G, Chan R, Zhang Z, and Kapur R. *Kit induced Myeloproliferative Disease is dependent on PI3K and SHP2 Phosphatase: Identification of SHP2 as a novel druggable target for treating MPN and AM*. IUSCC Cancer Research Day. Indianapolis, Indiana. May 26, 2011.

Martin H, Chatterjee A, Ma P, Ramdas B, and Kapur R.*Rac GTPases and Pak as Potential Therapeutic Targets of Oncogenic KIT in Acute Myelogenous Leukemia.* Midwest Blood Club Symposium Conference, Cincinnatti, Ohio. April 21-22, 2011.

Chatterjee A, Vemula S, Ramdas B, **Martin H**, Nabinger S, Chan R, and Kapur R. Novel *FAK-Rac-PAK signaling pathway in Flt3ITD mediated leukemogenesis.* Midwest Blood Club Symposium Conference, Cincinnatti, Ohio. April 21-22, 2011.

Martin H, Ma P, Ramdas B, and Kapur R. *Cooperation of AML1-ETO and oncogenic Kit in Acute Myelogenous Leukemia,* Midwest Blood Club Symposium Conference. Indianapolis, Indiana. May 6-7, 2010.

Ma P, **Martin H**, Sims E, Ramdas B, Ghosh J, and Kapur R. *Role of Intracellular Tyrosine Residues of KIT on Oncogenic Transformation.* Midwest Blood Club Symposium Conference. Indianapolis, Indiana. May 6-7, 2010.

Martin H, Ma P, Ramdas B, and Kapur R. *Cooperation of AML1-ETO and oncogenic Kit in Acute Myelogenous Leukemia. Indiana University Cancer Research Day.* Indianapolis, Indiana. May 5, 2010.

Ma P, **Martin H**, Sims E, Ramdas B, Ghosh J, and Kapur R. *Role of intracellular tyrosine residues in oncogenic Kit-induced transformation.* 2009 Annual Meeting of the American Society of Hematology, New Orleans, Louisiana. December 5-8, 2009.

Martin H, and Kapur R. *Cooperation of AML1-ETO and oncogenic KIT in association with Acute Myelogenous Leukemia.* IUSM Medical and Molecular Genetic Department Research Day, Indianapolis, Indiana. September 1, 2009.

Martin H, Gentile GJ, Gentile JM. *Genotoxic effect of aflatoxin in animals with parasite-associated inflammation.* 34th Annual Environmental Mutagen Society Meeting. Miami, Florida. 2003.

Martin H, Gentile GJ, and Gentile JM. *Mutation sequence specificity of Big Blue transgenic mice exposed to aflatoxin B_1 and Fasciola hepatica.* 16th National Conference on Undergraduate Research. Whitewater, Wisconsin. 2002.