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Differential contributions of c-Kit activating mutations to promotion of AML1-ETO associated neoplasia

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DIFFERENTIAL CONTRIBUTIONS OF C-KIT ACTIVATING MUTATIONS TO PROMOTION OF AML1-ETO ASSOCIATED NEOPLASIA

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ABSTRACT

The t(8;21) translocation, which generates an AML1-ETO fusion protein (also known as RUNX1-ETO), is one of the most frequent cytogenetic abnormalities in acute myeloid leukemia (AML). Murine studies have demonstrated that AML1-ETO promotes the accumulation of myeloid progenitor cells with self-renewal capability and impaired differentiation capacity. However, AML1-ETO^{+} mice do not progress to AML in the absence of additional mutations, suggesting that expression of the translocation is insufficient for leukemogenesis. This hypothesis is supported by studies demonstrating the persistence of *AML1-ETO*-expressing hematopoietic progenitors obtained from patients in long-term clinical remission.

Mutations affecting receptor tyrosine kinases, particularly c-KIT, are commonly detected in t(8;21)$^{+}$ AML. In AML1-ETO^{+} patient samples, differing classes of activating c-KIT mutations have been observed with varying prevalence. The most common (12-48%) involves mutations that occur in the activation loop of the phosphotransferase domain, like D814V, while another involves deletions within exon 8, a region mediating receptor dimerization (2-13% of cases). To formally investigate whether distinct activating c-Kit mutations differ in their capacity to drive AML1-ETO-associated AML, we used a retroviral transduction strategy to co-express AML1-ETO with c-Kit^{D814V} or a representative exon 8 mutant (c-Kit$^{T417I\Delta418\text{-}419}$) in murine bone marrow progenitor cells used to reconstitute lethally irradiated mice. Analysis of reconstituted mice showed that

AML1-ETO;c-Kit^{D814V} co-expression resulted in three non-overlapping phenotypes. In 45% of animals, an AML of relatively short latency and frequent granulocytic sarcoma was noted. Other mice exhibited a rapidly fatal myeloproliferative neoplasm (35%) or a lethal, short-latency pre-B-cell leukemia (20%). In contrast, AML1-ETO;c-Kit$^{T417I\Delta418-419}$ co-expression promoted exclusively AML in a fraction (51%) of reconstituted mice with a median latency nearly double that observed in AML1-ETO;c-Kit^{D814V} mice that developed AML. Analysis of clonality indicated that acquisition of oncogenic events in addition to AML1-ETO and an activated c-Kit receptor were necessary for transformation in all cases. The neoplastic phenotype differences between AML1-ETO;c-Kit^{D814V} and AML1-ETO;c-Kit$^{T417I\Delta418-419}$ mice indicate that distinct activating c-Kit mutants differ in leukemogenic potential, which could account for the disparity in prevalence of each c-KIT mutation concurrent with AML1-ETO in human AML.

Keywords: acute myeloid leukemia, AML1-ETO, c-Kit, hematopoiesis

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LIST OF ABBREVIATIONS

AGM	aorta-gonad-mesonephros
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
ATP	adenosine triphosphate
Bex	blue-excitable GFP
BM	bone marrow
CBC	complete blood count
CBF	core-binding factor
CFU	colony-forming unit
CR	complete remission
C-terminal	carboxy terminal
D	aspartic acid
e	embryonic day
ENU	*N*-ethyl-*N*-nitrosourea
ETO	eight-twenty-one
FAB	French-American-British
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
GFP	green fluorescent protein

GM-CSF	granulocyte-macrophage colony-stimulating factor
H	histidine
HBSS	Hank's balanced salt solution
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
I	isoleucine
Ig	immunoglobulin
IgD5	fifth immunoglobulin-like domain
IL-3	interleukin-3
IL-6	interleukin-6
inv(16)	inversion of chromosome 16
JAK-STAT	Janus kinase-signal transducer and activator of transcription
K	lysine
MAPK	mitogen-activated protein kinase
M-CSFR	macrophage colony-stimulating factor receptor
MFI	mean fluorescence intensity
MPN	myeloproliferative neoplasm
MTG	myeloid transforming gene
N	asparagine
NHR	Nervy homology region
N-terminal	amino terminal
PB	peripheral blood

PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PI3-K	phophoinositide 3-kinase
PLCγ	phospholipase Cγ
RBC	red blood cell
RE	RUNX1-ETO
RHD	Runt homology domain
RTK	receptor tyrosine kinase
SCF	stem cell factor
SFK	Src family kinase
T	threonine
TA	transcriptional activation
tet	tetracycline
TKI	tyrosine kinase inhibitor
V	valine
Vex	violet-excitable GFP
WBC	white blood cell
WT1	Wilm's tumor 1
Y	tyrosine
YFP	yellow fluorescent protein

INTRODUCTION

Hematopoiesis is the process of blood cell development initiated from a rare population of multipotent hematopoietic stem cells (HSCs), immunophenotypically distinguishable by expression of surface molecules, including CD34, c-KIT, and Sca-1, and the absence of mature hematopoietic cell lineage-specific markers.[1-3] Because most mature blood cells are short-lived, HSCs are responsible for ensuring hemostasis throughout the mammalian lifespan by replenishing the pools of multilineage progenitors and more committed precursor cells, which become further restricted in their hematopoietic lineage potential.[2] These progenitors will ultimately differentiate into the numerous functionally distinct mature blood cells including, all myeloid and lymphoid lineages involved in the innate and adaptive immune responses (collectively, white blood cells or WBCs), red blood cells (RBCs), and megakaryocytes (refer to **Figure 1**).[4-8] In the bone marrow (BM) of adult mammals, HSCs reside as undifferentiated cells present at a very low frequency yet are required to generate immense numbers of mature blood cells as needed during the lifetime of an animal. Because of this biological responsibility, HSCs possess these fundamental properties: the ability to self-renew, to proliferate, and to differentiate.[9-11]

During mouse development, primitive hematopoiesis begins around embryonic day 7.5 (e7.5) in the yolk sac blood islands,[12,13] then at about e9 the first definitive HSC are initiated from cells in the aorta-gonad-mesonephros (AGM) region.[14] These cells colonize the fetal liver at e11, where there is extensive expansion of HSC until e14.5.[15]

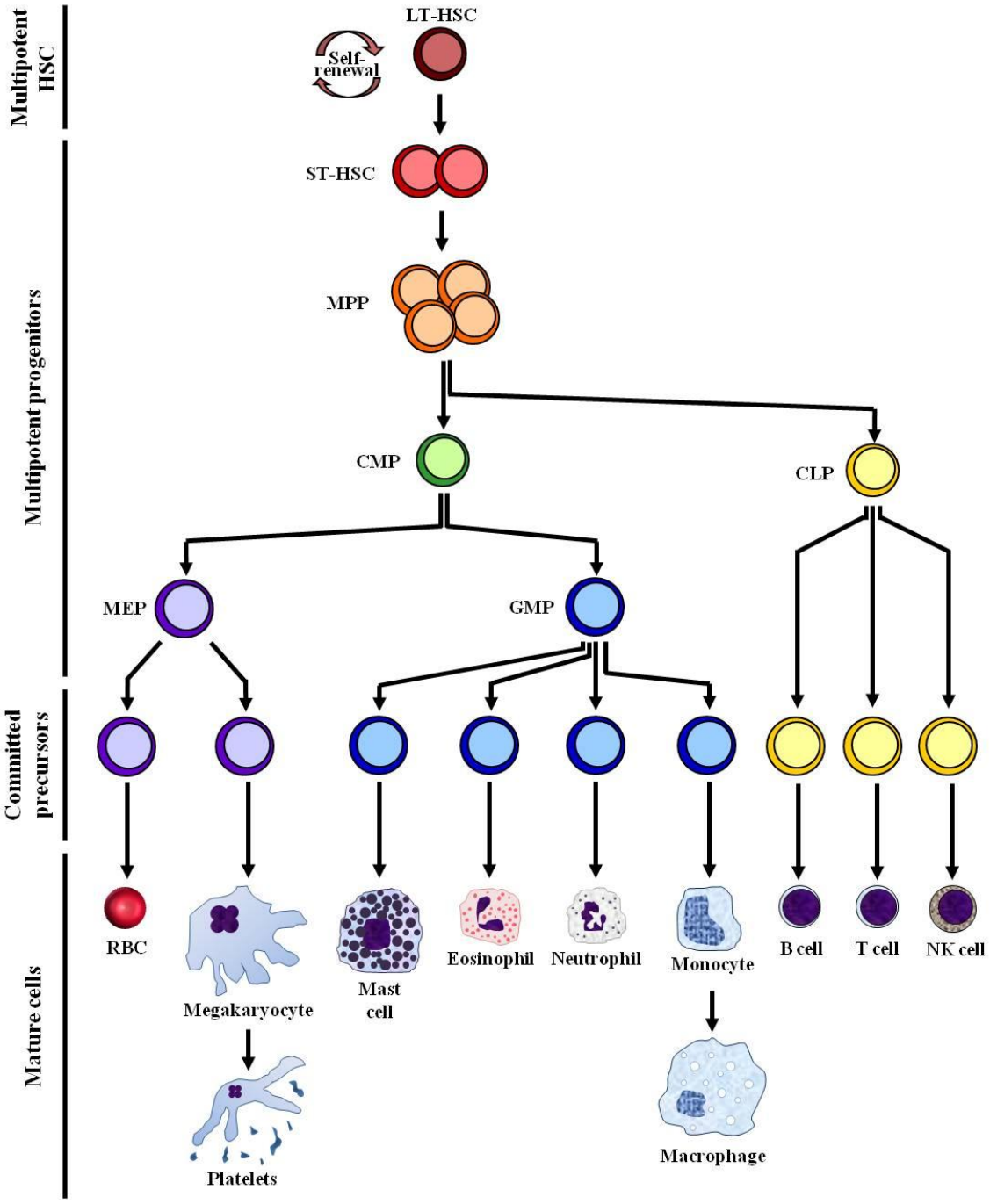

Figure 1. General diagram of hematopoiesis hierarchy, initiated from multipotent HSCs. Abbreviations: LT-HSC, long-term reconstituting hematopoietic stem cell; ST-HSC, short-term reconstituting hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythrocyte progenitor; GMP, granulocyte/macrophage progenitor; RBC, red blood cell.

From about e17, hematopoietic processes begin to take place in the BM, the organ that will continue to be the main source of blood cell production for the rest of post-natal and adult life.[16] In adult life, terminal differentiation and proliferation of the various blood cell types takes place in secondary lymphoid organs, such as spleen, thymus, and lymph nodes, but generation of early myeloid and lymphoid progenitors occurs exclusively in the BM.[16] Overall, hematopoietic system development in humans follows a temporally and anatomically similar pattern as described for mouse.[13,17,18]

The complex and tightly regulated signaling mechanisms critical to hematopoietic system development and establishment of the HSC pool, as well as those which maintain proper balance between HSC self-renewal, proliferation, survival, and differentiation throughout adult life are coordinated by a myriad of proteins including growth factors, cytokines, chemokines, and transcription factors. During development or later in adult life, oncogenic events leading to disrupted or altered function of these critical factors could occur, which has the potential to exert significant changes on the normal physiology of HSCs or other early hematopoietic progenitor cells (HPCs) like blocking maturation ability or activating pathways that lead to excessive cell growth, two of the hallmarks exhibited by cancer cells.[19]

If the collective effects of genetic aberrations on hematopoietic cells are sufficiently transforming, the result is an overt leukemia phenotype characterized by the uncontrolled accumulation of blood cells. Leukemia is categorized into four types: myelogenous or lymphatic, each of which can be acute or chronic.[20] The current mode of thinking holds that development of a leukemia-initiating cell requires the cooperation of at least two different genetic mutation events. One mutation confers impaired hemato-

poietic differentiation while the other results in a proliferative and/or survival advantage.[21] The studies described in this dissertation have focused on a subtype of acute myeloid leukemia (specifically within the granulocytic pathway) associated with the presence of a specific oncogenic fusion protein and are interested in determining whether co-expression of particular secondary mutational events provides the cooperating effect necessary for disease pathogenesis.

Acute myeloid leukemias (AMLs) are a phenotypically and genetically heterogeneous group of rapidly-progressing hematopoietic neoplasia characterized by the clonal expansion of developmentally-arrested myeloid-lineage cells which accumulate in the BM and peripheral tissues. As disease progresses, accumulation of the malignant cells interferes with the production of normal RBCs, WBCs, and platelets and anemia develops in virtually all persons with leukemia. Historically, AML has been classified according to the morphology-based French-American-British (FAB) classification system, which subtypes AML into 9 groups, termed M0 through M7, based on the cell type from which the leukemia developed and its degree of maturity. Frequently, cytogenetic abnormalities can be associated with specific FAB subtypes (see **Table 1**).[22]

The chance of developing AML increases with age and is most common in people over age 60. In the United States, an estimated 12,330 new cases of AML were diag nosed during 2010.[20] The number of patients with AML who enter remission, stay in remission for years or are cured has increased significantly over the past three decades. However, treatment outcomes vary, even among patients with the same diagnosis, and disease relapse remains a significant issue. In fact, from 1999 to 2006, the five-year overall survival rates for AML were 24.2% for adult cases and 60.9% for children under

Table 1. AML subtypes according to the morphology-based FAB classification system and cytogenetic abnormalities associated with specific subtypes.

FAB Subtype	Morphologic description	Cytogenetics
M0	Undifferentiated large granular blasts	
M1	Myeloblastic, without maturation	trisomy 8, -5, -7
M2	Myeloblastic, with granulocytic maturation	t(8;21)(q22;q22), t(6;9)
M3	Promyelocytic	t(15;17)
M4	Myelomonocytic	inv(16)(p13q22), del(16q)
M4Eo	Myelomonocytic with bone marrow eosinophilia	inv(16)/t(16;16)
M5	Monoblastic (M5a) or Monocytic (M5b)	t(9;11), t(8;16), del (11q), t(11;19)
M6	Erythroleukemia	trisomy 8, -5, -7
M7	Megakaryoblastic	t(1;22)

15 years of age.[20]

Initial diagnosis of leukemia includes performing a complete blood count (CBC) which may show elevated or decreased levels of WBCs and platelet and RBC counts below that expected for a healthy individual. BM aspiration and biopsy are subsequently performed to confirm diagnosis, identify the leukemia cell type (by morphologic examination and identification of lineage-specific cell-surface proteins), and look for cytogenetic abnormalities. Patients with AML vary greatly in clinical presentation, blast morphology and degree of maturation, response to therapy and long-term prognosis. Frequently, these differences can be attributed to particular underlying molecular lesions. The most common genetic aberration associated with leukemia is the generation of an oncogenic fusion protein due to a chromosomal translocation event. Up to 55% of adult patients with *de novo* AML display recurrent gene rearrangements.[23-26] Specific chromosomal translocations often correlate with the FAB AML subtype (refer to **Table 1**) as well as with good, intermediate or poor prognosis and differing response to therapy.[24,25]

Translocations involving genes encoding the two subunits of the heterodimeric core-binding factor (CBF) transcription factor complex, *AML1* and *CBFβ*, are the most frequently observed cytogenetic abnormalities in AML and are referred to collectively as CBF-AMLs as they share many clinical features.[23,24,27,28] The rearrangement involving the *AML1* gene, which encodes the DNA-binding subunit of the CBF, occurs between chromosome 8q22 and chromosome 21q22 [t(8;21)(q22;q22), henceforth referred to as t(8;21)].[29-32] It is observed in up to 12% of adult[23,24,33-37] and 12-30% of pediatric[36,38-40] *de novo* AML patients overall. *CBFβ* is commonly a target of the FAB-M4Eo AML subtype-associated inversion of chromosome 16 [inv(16), or t(16;16)] that generates a fusion between CBFβ and the smooth muscle myosin heavy chain.[41]

In particular, the t(8;21) is highly associated with FAB-M2 AML, detected in approximately 40% of patients with this subtype and, in fact, greater than 90% of $t(8;21)^{+}$ leukemias have FAB-M2 AML morphology.[31,42-45] FAB-M2 AML is defined as an acute myeloblastic leukemia with accumulation of at least 20% myeloblast forms in the BM (normal marrow blast composition is <5%) and varying frequencies of granulocyte maturation.[22] Morphologically, leukemic blasts in t(8;21) FAB-M2 AML are described as having prominent Auer rods, strong myeloperoxidase positivity, homogeneous salmon-colored granules, and cytoplasmic vacuolization.[44,46] Additional pathological features of t(8;21)AML include enhanced marrow granulopoiesis with inhibition of erythropoiesis, abnormal myelocytes with increased cytoplasmic granulation and a minority of cases also show BM eosinophilia or mastocytosis.[46-49] Furthermore, t(8;21) AML cases frequently present with an associated granulocytic sarcoma (also referred to as chloroma, or extramedullary leukemia).[50-55] The t(8;21) translocation has also been documented in approx-

imately 6% of AML M1 and, more rarely, in AML M0, M4, M5 and other myeloproliferative neoplasia.[56,57]

Traditionally, most patients with CBF-AML have shown a high rate of complete remission (CR) and lower incidence of relapse, especially those receiving high-dose cytarabine in post-remission treatment, and are thought to have a more favorable prognosis compared to patients with normal karyotype or other chromosomal abnormalities.[24,25,34,36,37,58,59] However, more recent investigations report subsets of CBF-AML patients (40 to 50% of all CBF-AML cases) with a high risk of relapse due to the presence of specific additional genetic mutation events,[26,37,60-66] underscoring the need to further elucidate secondary genetic aberrations that cooperate during CBF leukemogenesis and their impact on CBF-AML presentation and prognosis.

AML1 (also known as RUNX1) is a member of the RUNX family of transcription factors, characterized by an amino-terminal (N-terminal) Runt homology domain (RHD) and carboxy-terminal (C-terminal) transcriptional activation (TA) domains.[67,68] In normal physiology, AML1 heterodimerizes with CBFβ to form the CBF complex which functions as an enhancer-organizing complex by recruiting other transcription factors and co-regulators to activate target gene transcription.[69-72] The RHD of AML1 is required for heterodimerization with CBFβ and binding to the core-enhancer DNA sequence (TGT/cGGT).[70,71,73] CBFβ is important for enhancing the transcriptional activity of AML1 by stabilizing its binding to DNA and protecting it from proteolytic degradation through ubiquitination.[67,74-76]

During development, AML1 is highly expressed by hematopoietic stem cells, endothelial cells of the AGM region from which hematopoietic cells emerge, chondrogenic

centers, olfactory and gustatory mucosa, and neural ganglion cells.[77,78] After organogenesis, high expression of AML1 is detected primarily in hematopoietic cells.[78] Contrastingly, CBFβ is ubiquitously expressed.[75,76] The CBF complex transactivates the expression of numerous genes critical for establishing and maintenance of normal hematopoiesis, including interleukin-3 (IL-3),[79,80] the granulocytic differentiation factor C/EBPα,[81,82] granulocyte-macrophage colony-stimulating factor (GM-CSF),[83,84] the receptor for macrophage colony-stimulating factor (M-CSFR or CSF-1R),[85] myeloperoxidase,[86,87] neutrophil elastase,[86] granzyme B,[88] and subunits of the T-cell antigen receptor.[67,89-91]

Confirmation that both AML1 and CBFβ play critical roles in hematopoiesis was revealed by attempts at generating knock-out mice. Null mutations in either gene resulted in embryonic lethality mid-gestation (e12 to 12.5) due to intracranial hemorrhage and a complete lack of fetal liver-derived hematopoiesis, although primitive yolk sac-derived erythropoiesis appeared normal. Heterozygous AML1 mice ($AML1^{+/-}$), on the other hand, are phenotypically healthy.[92-96] These observations suggest that the CBF complex acts as a master switch regulating the development of a definitive hematopoietic system.

The t(8;21) rearrangement observed in AML results in generation of the chimeric AML1-ETO protein, an in-frame fusion of the N-terminal 177 amino acids of AML1 (encoded on chromosome 21), including the DNA-binding domain, to nearly the entire ETO protein (amino acids 30 to 604, encoded on chromosome 8) (refer to Figure 2).[29,31,32,97] *ETO* (for eight-twenty-one, also known as *AML1T1* or *MTG8*) is member of a small family of genes, called myeloid transforming genes (MTG) that includes *MTGR1* and *MTG16*.[98,99] This gene family is homologous to the *Drosophila* gene *nervy*. Interesting-

ly, MTG16 has also been identified as a translocation target in AML. It is involved in the t(16;21) translocation that results in a fusion with AML1 to generate AML1-MTG16, which is structurally similar to RUXN1-ETO.[99] This observation suggests that conserved domains between the ETO family proteins are important for the transforming activity of these oncogenic protein chimeras. Amino acid sequence comparison of ETO, MTGR1, MTG16, and Nervy revealed four 4 phylogenetically conserved segments, referred to as Nervy homology regions 1-4 (NHR1-4) (see **Figure 2**).[98] The NHR1 domain shares homology with the transcriptional co-activators TAF110, TAF105 and TAF130.[100] The hydrophobic heptad repeat-containing NHR2 domain is critical for ETO homodimerization and heterodimerization with MTGR1 or MTG16.[101] NHR3 contains a small coiled-coil region, specific to ETO family proteins.[102] The NHR4 domain has two predicted zinc finger motifs; however, there is no experimental evidence to suggest ETO can directly bind DNA.[102]

ETO is expressed in $CD34^+$ HSCs/HPCs where it presumably functions as an adaptor protein for transcriptional co-repressor complexes.[103] It has been shown that ETO can directly interact with other transcription factors and, through interactions mediated by its NHR2 and NHR4 domains, serve as a docking site for the nuclear co-repressors N-CoR, mSin3 and SMRT, which in turn leads to recruitment and activation of various histone deacetylases.[104-107] Of note, homozygous disruption of *Eto* in mice did not result in hematopoietic defects, but revealed an essential role in the gastrointestinal system.[108] Aside from this, the functions of ETO, particularly with respect to hematopoiesis, are incompletely understood.

Because the RHD is retained in the AML1-ETO fusion, this protein is able to bind AML1 target DNA sequences, but has gained the capacity to recruit additional cofactors, mostly co-repressors due to replacement of the C-terminal TA domains of AML1 by the ETO moiety (refer to **Figure 2**). Therefore, AML1-ETO has been predicted to act as a transcriptional repressor for AML1 target genes[101,109] and in fact, early work demonstrated that AML1-ETO dominantly interfered with AML1-mediated transactivation.[71,83,109] More recent gene expression profiling and biochemical investigations have uncovered a paradoxical consequence of AML1-ETO expression, whereby the fusion protein was shown to activate as many (or more) genes than it repressed.[110-113] Thus, expression of AML1-ETO significantly alters the normal expression pattern of CBF target genes that are critical for hematopoiesis, providing a likely mechanism of AML1-ETO-mediated initiation of hematopoietic cell transformation.

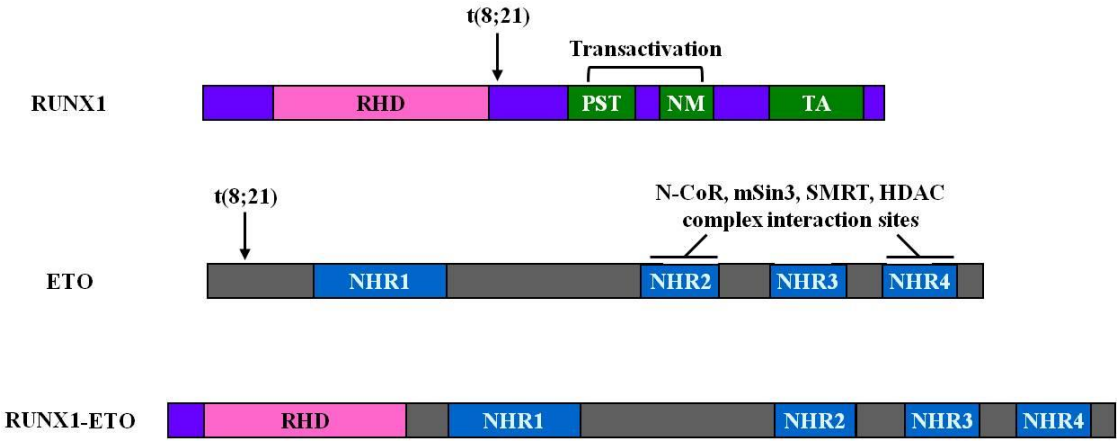

Figure 2. Structure of the AML1, ETO, and t(8;21)-encoded AML1-ETO proteins. AML1 is organized into an N-terminal Runt homology DNA-binding domain (RHD), a proline/serine/threonine-rich region (PST), a nuclear matrix attachment sequence (NM), and a C-terminal transcriptional activation domain (TA). A second transcriptional activation domain has been localized to a region encompassing the PST and NM motifs. ETO contains four regions highly homologous to the *Drosophila* Nervy protein (NHR1-4). The NHR2 and NHR4 domains contain binding sites for N-CoR, mSin3, SMRT, and histone deacetylase (HDAC)-containing transcriptional co-repressor complexes. Chromo-

somal breakpoints in the t(8;21) are indicated by vertical arrows. AML1-ETO consists of the RHD from AML1 and nearly the entire ETO protein.

In effort to study the biology and pathogenesis of t(8;21)-associated AML in the context of a murine model, attempts were made to generate heterozygous AML1-ETO knock-in mice, targeting the fusion sequence to the endogenous murine AML1 locus. In both attempts, the knock-in mice died at e12.5 to 13.5 due to lethal hemorrhage into central nervous system, pericardial sac and soft tissue, mirroring the phenotype of AML1 and CBFβ knock-outs.[114,115] Fetal liver cells from the embryos showed complete lack of definitive hematopoiesis, although dysplastic multilineage hematopoietic progenitors with increased *in vitro* self-renewal capacity were detected. Although primitive yolk sac-derived hematopoiesis appeared normal, these cells demonstrated exclusively macrophage differentiation capability when examined by colony-forming unit (CFU) assays.[114,115] These observations underscored the critical role for AML1 in hematopoiesis and, along with biochemical studies,[71,109] suggest that AML1-ETO exerts dominant-negative effect over wild-type AML1 and the CBF complex-mediated transcription. In addition, expression of the fusion protein interferes with hematopoietic lineage specification and may promote differentiation toward the monocyte/macrophage pathway.[115]

To circumvent the issue of embryonic lethality, several groups have generated conditional AML1-ETO mice by varying methods. Generation of transgenic animals with a tetracycline(tet)-responsive AML1-ETO resulted in high expression of the fusion protein in BM cells and peritoneal macrophages, but no leukemia phenotype.[116] BM cells from these mice had enhanced serial replating capacity *in vitro*, and colonies consisted of higher than normal proportions of myeloblasts and immature myeloid progenitors, as well as mature macrophages and granulocytes. When AML1-ETO was turned off in late pas-

sage cells from tet-AML1-ETO mice, differentiation was restored to normal, but replating capacity was lost.[116] Expressing AML1-ETO from the neutrophil lineage-specific MRP8 promoter (MRP8-AML1-ETO mice) likewise did not result in leukemic transformation during a 24 months observational period, unless additional mutations were induced (see below).[117] Similarly, knock-in mice carrying a cre-inducible loxP-Stop-loxP-AML1-ETO construct did not develop leukemia after cre-mediated activation of the AML1-ETO allele, but myeloid progenitors from these animals showed increased serial replating efficiency *in vitro*, forming mainly granulocyte-macrophage colonies.[118] Again in this model, AML developed only after induction of additional mutation events.

Some investigators have used a retroviral transduction/BM transplantation approach to achieve high-level expression of AML1-ETO in murine HSC/HPCs used to reconstitute lethally irradiated recipient animals. These models resulted in significant expansion of the HSC pool, increased BM myeloblasts and promyelocytes (5 to 14%), increased myeloid CFU ability and some degree of hematopoietic disturbance, including long-latency myeloproliferative or myelodysplastic syndromes *in vivo*.[119-122] Similarly, a nonlethal, long-latency myeloproliferative syndrome was reported for mice in which the *Sca1* locus was used to target AML1-ETO expression to the HSC compartment.[123] However, once again, mice did not present with overt leukemia transformation throughout the duration of the experiments, even despite the high AML1-ETO expression level achieved through the use of retroviral methods.

Importantly, treatment of the MRP8-AML1-ETO and cre-activated AML1-ETO knock-in mice with a highly potent mutagen, *N*-ethyl-*N*-nitrosourea (ENU), resulted in AML and granulocytic sarcomas in 55% and 31% of the mice, respectively, mimicking

many of the features of human t(8;21) AML cases.[117,118] Furthermore, immortalized immature myeloid cell lines derived from ENU-treated knock-in mice were capable of cytokine-independent growth and transplantable into secondary recipient animals.[118] Taken together, evidence from these murine models demonstrate that while expression of AML1-ETO impairs hematopoietic differentiation and imparts increased self-renewal to myeloid progenitors, it is insufficient to induce leukemogenesis in the absence of secondary cooperating mutations.

The idea that AML1-ETO expression is insufficient for leukemogenesis in the context of a murine model is consistent with the hypothesis that t(8;21) is the preceding event in human AML pathogenesis. In fact, retrospective examination of neonatal cord-blood $CD34^+$ hematopoietic progenitor cells showed that the t(8;21) translocation is expressed at relatively high frequencies *in utero*, preceding the development of pediatric AML by as long as 5 to 10 years.[124,125] Furthermore, *AML1-ETO*-expressing BM hematopoietic progenitors have been identified in samples obtained from patients in long-term clinical remission, persisting for years without significant expansion.[126-128] Moreover, AML1-ETO transcripts are detected in peripheral blood (PB) monocytes and B cells and could differentiate into normal erythroid cells *in vitro*, so acquisition of t(8;21) presumably occurs at the level of stem or progenitor cells capable of differentiating into myeloid, erythroid and B cell lineages.[126,129,130]

Based on observations from mouse models and the clinical data from remission patients, it can be inferred that sole expression of AML1-ETO cannot induce or sustain a leukemia phenotype. A probable line of thought is that AML1-ETO expression establishes a developmentally-impaired, pre-leukemic population with enhanced self-renewal

capacity, but lacking a significant growth advantage. Expansion of this progenitor pool due to AML1-ETO-induced biological changes could make this population more susceptible to the acquisition of cooperating mutations that provide the necessary proliferative advantage, resulting in expansion of an overt leukemic clone. In following with this hypothesis, a number of recent investigations have sought to define cooperating mutations that may accelerate progression to AML in association with *AML1-ETO*. For example, acceleration of AML was noted when AML1-ETO was expressed in *ICSBP*[122] or *p21*[131] knockout bone marrow cells. Also, co-expression of AML1-ETO with other oncogenic proteins such as Wilm's tumor 1 (WT1),[132] TEL-PDGFβR,[121] or FLT3-ITD[133] was capable of promoting an AML phenotype. These observations highlighted the synergy between expression of AML1-ETO and a cooperating mutation during leukemogenesis as well as brought to light the potential role of aberrant tyrosine kinase signaling in AML progression.

In terms of human disease, up to 70% of t(8;21) patients carry non-overlapping secondary mutations in specific receptor tyrosine kinases (RTK) such as c-KIT, FLT3, and platelet-derived growth factor (PDGF), or small GTPases such as NRas[35,38,40,61,63,64,134-139] In particular, recent clinical investigations have made clear that the *c-KIT* gene represents the most common secondary mutation target in CBF-AMLs. In fact, although blasts from the majority of AML cases (60 to 80%) express c-KIT,[140-144] mutations in *c-KIT* gene have been documented in up to 48% of patients with t(8;21) versus only 2 to 5% of AML cases overall.[135,137,145,146] Therefore, c-KIT activating mutations are of considerable interest as they likely can cooperate with AML1-ETO to promote AML.

The proto-oncogene *c-KIT* (*c-Kit* in the mouse) encodes a type III RTK, characterized by five immunoglobulin (Ig)-like repeats in the extracellular domain, a single transmembrane domain, a regulatory juxtamembrane domain, and a cytoplasmic kinase domain split by an insert into an ATP-binding region and a phosphotransferase domain.[147,148] c-KIT-mediated signaling, normally under tight control by binding to its ligand, stem cell factor (SCF), is important in regulating the proliferation, differentiation, migration, and survival of hematopoietic stem and progenitor cells, mast cells, melanocytes, germ cells, and intestinal cells of Cajal.[149-154]

In murine models, loss-of-function of c-Kit or SCF causes severe macrocytic anemia, mast cell deficiency,[155] and sterility. In humans, loss-of-function mutations in c-KIT have been linked to autosomal-dominant piebaldism, a disease related to abnormal melanocytes.[156] Activating (or gain-of-function) c-KIT mutations play an oncogenic role in several human malignancies, including the vast majority of gastrointestinal stromal tumors,[157,158] germ cell tumors,[152,159] and subsets of hematologic neoplasia such as mastocytosis,[160,161] sinonasal natural killer/T cell lymphomas,[162,163] and AML.[38,61-63,134,135,137,139,146,164]

In the hematopoietic system, c-KIT is expressed by approximately 70% of $CD34^+$ BM HSCs and HPCs.[149,165,166] Signaling through c-KIT, mediated by interaction with SCF, is key for HSC/HPC survival,[167-169] but is insufficient for maintenance of self-renewal *in vitro*.[170] c-KIT is also expressed on more committed myeloid, erythroid, megakaryocytic, natural killer, and dendritic cell progenitors, as well as pro-B and -T cells, but is generally downregulated upon further differentiation of these cells.[166,171,172] An exception to this is mature mast cells, which continue to express high levels of c-

KIT.[153,154] Synergy between c-KIT/SCF-induced signaling and other cytokine-stimulated pathways is important in HSC mobilization, as well as the proliferation of HSCs and downstream hematopoietic progenitors.[173-178]

Simultaneous binding of dimeric SCF to two receptor monomers induces homo-dimerization,[179,180] mediated by the fourth and fifth Ig-like extracellular domains of c-KIT,[181] and leads to autophosphorylation of tyrosine residues outside the kinase domain. The phosphorylated tyrosines serve as docking sites for signal transduction molecules with Src homology 2 or phosphotyrosine binding domains,[182] consequently activating downstream signaling pathways, including the phosphoinositide 3-kinase (PI3-K), Src family kinase (SFKs), Janus kinase-signal transducer and activator of transcription (JAK-STAT), Ras-mitogen-activated protein kinase (Ras-MAPK), and phospholipase Cγ (PLCγ) pathways.[148] The diverse cellular responses mediated by activation of these signal transduction cascades include cell cycle progression and DNA synthesis, survival and apoptotic resistance, adhesion, chemotaxis, protein trafficking, activation of gene transcription, proliferation, and differentiation.[148,183,184] An important negative regulatory mechanism to control c-KIT signaling is that shortly after its activation, the receptor gets internalized and ubiquitinated by Cbl proteins, marking c-KIT for degradation.[185,186]

Several different activating c-KIT mutations, located in the extracellular domain (exon 8), the juxtamembrane domain (exon 11), and the phosphotransferase domain (exon 17) have been described in association with AML (refer to **Figure 3**).[35,39,40,61-64,133-139,164,187-189] They are strongly associated with FAB-M2 AML, as nearly 70% of c-KIT-mutated AML patients have the M2 phenotype.[35] In the context of $t(8;21)^{+}$ AML patients, the most common class, documented in 11 to 44% of cases, involves mutations

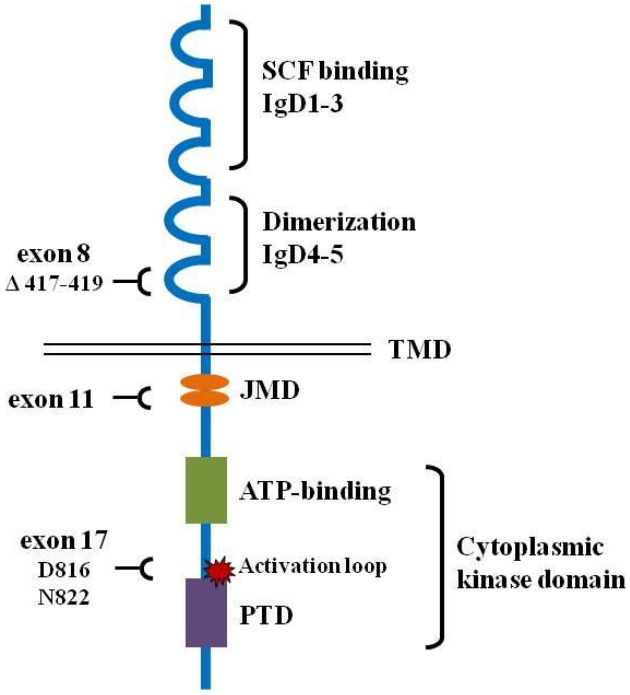

Figure 3. Domain organization of the c-KIT receptor tyrosine kinase and locations of activating mutations associated with AML. Immunoglobulin-like domains (IgD) 1 to 3 in the extracellular portion comprise the ligand-binding region, while IgD4 and 5 mediate receptor dimerization. The juxtamembrane domain (JMD) plays a regulatory role, mediating conformational changes in the presence and absence of stem cell factor (SCF). The cytoplasmic kinase domain is split into a substrate (ATP)-binding region and a phosphotransferase domain (PTD).

that occur in the enzymatic pocket of the activation loop in the phosphotransferase domain, predominantly at D816V (D814V in mice) or N822K (see **Figure 3**). Another class involves heterogeneous deletion/insertion mutations affecting amino acids 417-419 within exon 8, which encodes the fifth Ig-like (IgD5) domain important for c-KIT dimerization (see **Figure 3**).[181] Compared to activation loop c-KIT mutants, exon 8 mutation$^+$ c-KIT forms have been documented less frequently in t(8;21)$^+$ samples, reported in 2-13% of cases.[38,61-63,134,135,137,139,146,164] Genotypically, exon 8 mutations vary from patient to patient, but in most (93%) patients investigated thus far the result is loss of the evolutionarily-conserved D419 residue at the protein level.[38,61,63,134,137,139] One well-

characterized c-KIT mutant (c-KIT$^{T417I\Delta418\text{-}419}$) observed in patients replaces a threonine residue at amino acid 417 with isoleucine and includes a small deletion of amino acids 418-419.[134,190] Hyperactivation of the pathways that mediate signaling downstream of c-KIT as a result of activating mutations in this receptor are a likely mechanism of providing the proliferative and/or survival advantage to developmentally-arrested *AML1-ETO*$^{+}$ myeloid progenitors, thereby contributing to the process of transformation to overt leukemia.

The D816V c-KIT mutant was first identified in the human mast cell leukemia line, HMC-1[191] and identification of a D816 mutation in an AML patient by Beghini et al.[189] provided the first direct evidence for involvement of this mutant in AML. Subsequently, it was demonstrated that D816V c-KIT, and the murine equivalent D814V c-Kit, is constitutively phosphorylated on tyrosine and activated in the absence of SCF.[191-193] Introduction of c-Kit^{D814V} into various IL-3-dependent lymphoid or myeloid cell lines resulted in SCF- and IL-3-independent growth *in vitro* and these cells become tumorigenic after subcutaneous injection into nude mice.[190,192,194,195] Retroviral expression of c-Kit^{D814V} in primary murine BM cells resulted in mixed erythroid/myeloid, granulocyte/macrophage, and mast cell colonies in CFU assays.[196] Furthermore, transplantation of c-Kit^{D814V}-transduced BM progenitors caused leukemia in 6/10 mice within 1.5 to 5 months post-transplant. Leukemia cells had an undifferentiated blast morphology and were largely positive for B220, but negative for Mac-1 and Gr-1, suggesting a disease of B lymphocyte origin.[196] Similarly, 4/15 transgenic mice expressing c-Kit^{D814V} from the H-2L^{d} promoter developed B-acute lymphocytic leukemia (ALL), T-ALL, or lymphoma.[196] In another study, expression of either a chimeric receptor consisting of the extra-

cellular and transmembrane domains of murine c-Kit fused to the intracellular signaling domain of human c-KIT^{D816V} or murine c-Kit^{D814V} resulted in rapidly lethal myeloproliferative neoplasm (MPN) (median latency of 1.5 to 2.5 months) characterized by PB leukocytosis, splenomegaly, and accumulation of mature granulocytic cells (Mac-1^{+}) in BM and peripheral tissues in 100% of reconstituted mice.[197] Finally, a very recent study using a retroviral transduction/transplantation approach showed that retroviral expression of the activation loop mutant c-Kit^{N822K} resulted in lethal MPN with a median survival of approximately 6 months.[198] In comparison, approximately 70% of mice transplanted with cells that co-expressed c-Kit^{N822K} with AML1-ETO developed an AML with a median latency similar to that observed in mice transplanted with c-Kit^{N822K}-expressing cells. Mice with AML exhibited high frequencies of c-Kit^{+}Mac-1^{-}Gr-1^{-}B220^{-}CD19^{-} myeloblasts in bone marrow and the periphery, anemia, and high WBC counts,[198] mimicking the pathology observed in human t(8;21)$^{+}$ AML cases. This study demonstrated for the first time that an activating c-Kit mutant is capable of cooperating with AML1-ETO to promote leukemogenesis.

Exon 8 c-KIT mutations in AML patients were identified Gari et al.[134] and subsequent work *in vitro* suggests that these mutations cause hyperactivation of the receptor in response to SCF.[190,194,195] Expression of exon 8 mutants with a substitution at residue 417 (T to I, Y, or V) and deletion of residues 418 and 419 in various hematopoietic cell lines led to factor-independent growth, enhanced proliferation, and apoptotic resistance *in vitro*, albeit at a lower rate than similar transforming effects conferred by expression of c-KIT^{D816V}.[190,194,195] Prior to the studies which will be described in this dissertation, a murine model carrying an activating c-Kit exon 8 mutant had not been generated.

Although the data are still preliminary and contrasting, clinical reports suggest that t(8;21) patients with c-KIT mutations may have higher risk of relapse than those without c-KIT mutations (80% vs 13.5%) and lower 6-year relapse-free survival (18% vs 60%).[63,64] In particular, several groups have observed that t(8;21) patients with exon 17 mutations (such as D816V) present with higher WBC count at diagnosis,[62] and have more adverse outcome with regard to event-free survival, overall survival, relapse risk and salvage after relapse.[35,139,164]

Importantly, specific c-KIT mutants show varying sensitivity to therapeutic agents such as small-molecule tyrosine kinase inhibitors (TKI). For example, exon 8 mutants and the N822K activation loop mutant are sensitive to the TKI Imatinib *in vitro*, whereas the D816V mutant is not.[38,137,190,195,198] In contrast, leukemias bearing D816V mutations may respond to the TKI Dasatinib (BMS-354925)[35,199] or Nilotinib (AMN 107).[200,201] Because of their contrasting prognostic and therapeutic implications, it is important to formally test whether distinct c-KIT mutants cooperate with AML1-ETO in leukemogenesis as this knowledge could eventually be translated to the clinic allowing better patient risk assessment and improved treatment plans.

The research presented in this dissertation sought to examine the relative ability of two activating c-KIT mutations, one in exon 8 (T417IΔ418-419) and one in the activation loop (D814V), to promote AML pathogenesis coincident with AML1-ETO expression using a murine model. Most clinical studies of t(8;21) cases have reported a higher prevalence of c-KIT^{D816V} than c-KIT exon8 mutations in these patients and some suggested that a D816 mutation, but not exon 8 mutations, in t(8;21)$^{+}$ AML is linked to unfavorable prognosis. Additionally, *in vitro* studies demonstrated that c-Kit D814 mutants confer

more potent proliferation and survival signals than c-Kit exon 8 mutants. These observations suggest that distinct classes of activating c-KIT mutations possess variable transforming potential. For these reasons, we hypothesized that the particular c-Kit mutants we investigated would differ in their capacity to accelerate AML1-ETO-associated AML. By combining our observational data with that of other recent studies, we were able to gain insight into the ability of distinct classes of activated c-Kit receptor molecules to function in driving the evolution of AML1-ETO^{+} AML.

DISTINCT CLASSES OF C-KIT ACTIVATING MUTATIONS DIFFER IN THEIR ABILITY TO PROMOTE RUNX1-ETO-ASSOCIATED ACUTE MYELOID LEUKEMIA

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Abstract

The t(8;21) *RUNX1-ETO* translocation is one of the most frequent cytogenetic abnormalities in acute myeloid leukemia (AML). In *RUNX1-ETO*$^{+}$ patient samples, differing classes of activating c-KIT receptor tyrosine kinase mutations have been observed. The most common (12-48%) involves mutations like D816V that occur in the tyrosine kinase domain, while another involves deletions within exon 8 in a region mediating receptor dimerization (2-13% of cases). To test whether distinct subtypes of activating c-KIT mutations differ in their leukemogenic potential in association with RUNX1-ETO, we used a retroviral transduction/transplantation model to co-express RUNX1-ETO with either c-Kit^{D814V} or c-Kit$^{T417I\Delta418\text{-}419}$ in murine hematopoietic stem/progenitor cells used to reconstitute lethally irradiated mice. Analysis of reconstituted mice showed that RUNX1-ETO;c-Kit^{D814V} co-expression resulted in three non-overlapping phenotypes. In 45% of animals, a transplantable AML of relatively short latency and frequent granulocytic sarcoma was noted. Other mice exhibited a rapidly fatal myeloproliferative phenotype (35%) or a lethal, short-latency pre-B-cell leukemia (20%). In contrast, RUNX1-ETO;c-Kit$^{T417I\Delta418\text{-}419}$ co-expression promoted exclusively AML in a fraction (51%) of reconstituted mice. These observations indicate that c-Kit^{D814V} promotes a more varied and aggressive leukemic phenotype than c-Kit$^{T417I\Delta418\text{-}419}$, which may be due to differing potencies of the activating c-Kit alleles.

Introduction

Chromosomal translocations involving genes encoding the two subunits of the heterodimeric core-binding factor (CBF) transcription factor complex, *RUNX1* and *CBFβ*, are the

most frequent cytogenetic abnormalities in acute myeloid leukemia (AML).[1,2] The t(8;21)(q22;q22) rearrangement results in an in-frame fusion of the N-terminal 177 amino acids of RUNX1, including the DNA-binding Runt domain, to nearly the entire ETO protein generating RUNX1-ETO.[3,4] ETO (also known as *RUNX1T1*, or *MTG8*) can interact with the nuclear corepressors N-CoR, mSin3, SMRT, and various histone deacetylases[5-8] so it may function in RUNX1-ETO by altering the normal expression pattern of CBF target genes. Observations showing that *RUNX1-ETO* heterozygous knock-in mice phenocopy *RUNX1* homozygous knockout mice support the view that RUNX1-ETO functions as a dominant inhibitor of normal CBF activity.[9-12] The t(8;21) is thought to be an early event in AML pathogenesis as the translocation can be detected at relatively high frequencies *in utero*.[13] Murine studies have also shown that RUNX1-ETO promotes gradual but significant accumulation of myeloid progenitor cells that have enhanced serial replating potential *in vitro* and some capacity for myeloid differentiation.[9,14-16] In these studies, mice rarely progress to AML. This is consistent with observations showing that cells from patients in long-term clinical remission continue to express *RUNX1-ETO* transcripts.[17]

A number of studies have defined cooperating mutations that significantly accelerate progression to AML in association with *RUNX1-ETO*,[18] including treatment of RUNX1-ETO^{+} mice with ENU[15] and co-expression of RUNX1-ETO with TEL-PDGFβR,[19] FLT3-ITD[20] and Wilm's Tumor 1.[21] Acceleration of AML was also noted when RUNX1-ETO was expressed in *ICSBP*[22] or *p21*[23] knockout bone marrow cells. In up to 70% of human t(8;21)$^{+}$ patient samples, non-overlapping mutations in receptor tyrosine kinases (RTK), particularly c-KIT, but also FLT3 to a lesser degree, or mutations in NRAS have been

noted.[24-34] *c-KIT* encodes a type III RTK, characterized by five immunoglobulin-like repeats in the extracellular portion of the molecule, a transmembrane domain, and a cytoplasmic region that includes a juxtamembrane domain encoded by exon 11 and a kinase domain split by an insert into an ATP-binding region and a phosphotransferase domain encoded by exon 17.[35,36] Although c-KIT activating mutations have only been observed in 2-5% of total AML cases, they are disproportionately present in 12-48% of $t(8;21)^+$ patient samples.[26,31] The most common class of c-KIT mutation in AML associated with CBF translocations occurs in the activation loop of the kinase domain predominantly at D816V (D814V in mice) or N822K in 11-44% of cases. Exon 8 mutations within the extracellular portion of the receptor that encodes the c-KIT dimerization domain occur in 2-13% of $t(8;21)^+$ samples.[18,25-27,29-31,33,37] The D816V and N822K mutations in the activation loop may not be functionally analogous given that each is differentially sensitive to the tyrosine kinase inhibitor imatinib.[31,38] Exon 8 mutations leading to hyperactivation of the receptor are heterogeneous and involve small deletion/insertion mutations affecting amino acids 417-419. One well-characterized c-KIT mutant ($c\text{-}Kit^{T417I\Delta418\text{-}419}$) observed in patients replaces a threonine residue at amino acid 417 with isoleucine and includes a small deletion of amino acids 418-419.[25,39] The ability of exon 8 mutations to promote progression to AML in association with RUNX1-ETO *in vivo* has not been explored.

In murine studies, retroviral expression of $c\text{-}Kit^{D814V}$ in a number of hematopoietic progenitor cell lines resulted in factor-independent growth *in vitro* and leukemia within 6-19 weeks in 6/10 transplanted animals. Leukemic cells were largely positive for B220 and lacked expression of Gr-1 and Mac-1, suggesting that the leukemias were largely immature B-ALL.[40] Transgenic mice expressing $c\text{-}Kit^{D814V}$ from an MHC Class I $H\text{-}2L^d$

promoter similarly developed B-ALL, T-ALL, or lymphomas in 4/15 transgenic animals.[40] In another study, retroviral expression of either a chimeric c-Kit molecule fusing the extracellular and transmembrane portions of murine c-Kit in-frame with the intracellular signaling domain of human c-KIT^{D816V}, or full-length murine c-Kit^{D814V}, resulted in rapidly lethal myeloproliferative disease (MPD) (median latency of 40-70 days) characterized by leukocytosis, splenomegaly and infiltration of Mac-1^{+} cells in peripheral tissues in 100% of reconstituted mice.[41] Finally, a very recent study using a retroviral transduction/transplantation approach showed that retroviral expression of the c-Kit^{N822K} activation loop mutant resulted in lethal MPD with a median survival of 171 days.[38] Mice reconstituted with cells that only expressed a c-Kit juxtamembrane mutant developed a rapidly fatal B220^{+}CD19^{+} B-ALL in two-thirds of transplanted mice or a fatal MPD in the remaining one-third of mice. Co-expression of RUNX1-ETO with c-Kit^{N822K} resulted in lethal AML in approximately 70% of transplanted mice with a median latency of 177 days, which was similar to the latency observed in mice transplanted with c-Kit^{N822K}-expressing cells.[38]

In this study, we used a retroviral transduction/transplantation approach to compare the relative ability of an activating c-Kit mutation in exon 8 (c-Kit$^{T417I\Delta418\text{-}419}$) versus a c-Kit mutation in the activation loop (c-Kit^{D814V}) to cooperate with RUNX1-ETO in promotion of AML. Analysis of reconstituted mice showed that RUNX1-ETO;c-Kit^{D814V} co-expression resulted in three non-overlapping phenotypes, including AML (45%), a myeloproliferative neoplasm (35%), and pre-B-ALL (20%). RUNX1-ETO changed both the latency and the neoplastic phenotype of mice compared with animals that only expressed c-Kit^{D814V}, which predominantly developed lethal MPD or T-ALL. In contrast, RUNX1-

ETO;c-Kit$^{T417I\Delta418\text{-}419}$ mice developed exclusively AML in a fraction (51%) of reconstituted animals with a median latency that was nearly double that observed in RUNX1-ETO;c-Kit^{D814V} mice that developed AML. Analysis of clonality showed that additional genetic changes were likely necessary for leukemic progression in all reconstituted mice, suggesting that these two "hits" were not sufficient for AML. Differences in neoplastic phenotype between RUNX1-ETO;c-Kit^{D814V} and RUNX1-ETO;c-Kit$^{T417I\Delta418\text{-}419}$ mice indicate that different classes of c-Kit activating mutations differ in their ability to promote AML, which may account for the observed differences in prevalence of distinct c-Kit activating mutations associated with t(8;21) leukemia.

Methods

Cloning

Wild-type murine c-Kit cDNA was PCR-amplified from pEF-BOS-Wt-c-Kit (kindly provided by Dr. Itaru Matsumura, Osaka, Japan) and cloned into MSCV-IRES-Vex (MIV)[42,43] generating MSCV-c-Kit^{WT}-IRES-Vex. Retroviral vectors expressing c-Kit^{D814V} or c-Kit$^{T417I\Delta418\text{-}419}$ were made by site-directed mutagenesis of MSCV-c-Kit^{WT}-IRES-Vex using QuikChange II XL (Agilent Technologies, Santa Clara, CA). The MSCV-IRES-Bex (MIB) and MSCV-RUNX1-ETO-IRES-Bex (MIB-RE) vectors have been described.[16]

Retroviral transduction and transplantation assays

Bone marrow (BM) was harvested from C57BL/6-Ly5.1 mice (7-8 weeks old) treated 3.5-4 days previously with 150 mg/kg 5-fluorouracil (5-FU; Sigma-Aldrich, St. Louis,

MO) was pre-stimulated for 24 hours at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 50 ng/mL stem cell factor (SCF), 5 ng/mL IL-6, and 1 ng/mL IL-3 (PeproTech, Rocky Hill, NJ). BM cells were transduced in the presence of 5 µg/mL polybrene (Sigma-Aldrich, St. Louis, MO) for 48 hours at 37°C by co-culture with BOSC23 retroviral producer cells co-transfected one day prior with MIB;MIV, MIB-RE;MIV-c-Kit^{D814V}, or MIB-RE;MIV-c-Kit$^{T417I\Delta418\text{-}419}$. Retrovirally transduced BM cells were removed from the co-cultures, washed and resuspended in phosphate buffered saline (PBS). C57BL/6-Ly5.1 mice (~8-12 weeks old) were lethally irradiated with a split dose of 9.0 Gy spaced by 4 hours and then transplanted with transduced cells. Secondary recipient mice (C57BL/6-Ly5.1; ~8-12 weeks old) were lethally irradiated as described above and transplanted with $5x10^4$ to $1x10^6$ whole bone marrow cells from primary recipients. Recipient mice were monitored for leukemia development by observation of physical symptoms and by peripheral blood analysis using FACS to detect Bex or Vex single-positive or Bex;Vex double-positive cells. Primary recipient animals remaining disease-free were sacrificed at 50-60 weeks post-transplant and disease-free secondary recipients were sacrificed at 25 weeks post-transplant. Animal research was performed in accordance with the Institutional Animal Care and Use Committee at UAB under the supervision of ALAC-accredited veterinary staff and housing facilities.

Flow cytometry analysis of membrane-bound and intracellular c-Kit

NIH3T3 cells were transduced with MIV, MIV-c-Kit^{WT}, MIV-c-Kit^{D814V}, or MIV-c-Kit$^{T417\Delta418\text{-}419}$ in the presence of 5µg/mL polybrene for 24hr. Transduced cells were stained with anti-c-Kit^{biotin} (2B8), followed by streptavidin^{FITC} for detection of membrane

c-Kit. Intracellular FACS was performed using BrdU Flow Kit and anti-c-Kit^{APC} (2B8) according to manufacturer's protocol (BD Pharmingen). Flow cytometry was performed using a 3-laser LSRII (BD Biosciences) and data analyzed using FlowJo software (TreeStar, Ashland, OR).

BM, PB, spleen and sarcoma flow cytometry and cell sorting

Beginning at 3-4 weeks post-transplant, peripheral blood (PB) was collected from the lateral tail vein of recipient mice. Red blood cells (RBCs) were removed by sedimentation in 2% dextran/PBS for 30 minutes at 37°C followed by lysis with ACK (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2 – 7.4). The remaining leukocytes were washed in PBS and resuspended in HBSS/2% FBS. BM cells were isolated from tibias and femurs by flushing in cold PBS and resuspended in HBSS/2% FBS. Splenocytes and granulocytic sarcoma cells were obtained by mechanical disruption and resuspended in HBSS/2% FBS. Cells were stained with: anti-Mac-1^{APC} (M1/70), anti-Gr-1$^{PE\text{-}Cy7}$ (RB6-8C5), anti-CD115^{PE} (AFS98), anti-B220$^{eFluor®450}$ (RA3-6B2), anti-CD19$^{PE\text{-}Cy7}$ (1D3), anti-CD43^{PE} (S7), anti-IgM^{APC} (RMM-1; BioLegend, San Diego, CA), anti-CD5^{PE} (53-7.3) anti-CD4$^{PE\ or\ APC}$ (GK1.5 or RM4-5), anti-CD8$^{PE\ or\ PE\text{-}Cy7}$ (53-6.7), anti-CD25^{PE} (PC61), anti-CD44$^{PE\text{-}Cy5\ or\ APC}$ (IM7). FACS analysis was performed as described above. Sorting was performed using a 3-laser MoFlo (Dako Cytomation, Fort Collins, CO) to purity greater than 95%. Dead cells were excluded by propidium iodide. Antibodies were purchased from eBiosciences or BD Pharmingen unless noted.

Histology and cytology

Tissues (spleen, liver, lung, femur, sarcoma, lymph node, thymus) from moribund mice were fixed in 10% neutral buffered formalin and femur samples decalcified in 8% HCl/dH_2O for 12hr after fixation. Fixed tissues were dehydrated in ethanol, cleared in xylene, embedded in paraffin, and specimen sections stained with hematoxylin and eosin (H&E). For morphologic analysis, $3x10^4$ Bex^+Vex^+ myeloid scatter-gated bone marrow cells were FACS-purified into PBS/12% FBS then cytospun onto glass slides and stained with Wright-Giemsa. Images were acquired using a Zeiss Axio Imager A1 microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss AxioCam MRc camera and Zeiss AxioVision 4.5 software. Zeiss EC-Plan-NEOFLUAR 10x/0,3 and Zeiss Plan-APOCHROMAT 63x/1,4 oil objectives were used for image capture.

Colony-forming assays

Bex^+Vex^+ myeloid scatter-gated bone marrow cells were FACS-purified into DMEM/10% FBS and $1x10^4$ cells plated in duplicate in methylcellulose media containing SCF, IL-3, IL-6, and erythropoietin (M3434; StemCell Technologies; Vancouver, BC). Total numbers of myeloid colony-forming units (CFU) per plate were counted after 7-12 days of culture in 5% CO_2 at 37°C.

Southern blot

Genomic DNA was prepared from whole splenocytes of moribund RUNX1-ETO;c-Kit^{D814V} or RUNX1-ETO;c-$Kit^{T417I\Delta418\text{-}419}$ animals. 10 µg of each genomic DNA sample was digested with *NcoI*, separated on a 0.75% agarose gel, then transferred to Amersham

Hybond XL nylon membranes (GE Healthcare, Buckinghamshire, UK). Blots were hybridized with α-^{32}P[dCTP]-labeled probes complementary to *c-Kit* or *ETO* sequences. Probes were radioactively labeled with Amersham Rediprime II Random Prime Labeling System (GE Healthcare). Retroviral vectors contained a single *NcoI* site localized downstream of the probe-binding region, allowing detection of unique proviral integrants. Genomic DNA isolated from splenocytes of wild-type C57BL/6-Ly5.1 mice served as controls for detection of endogenous sequences.

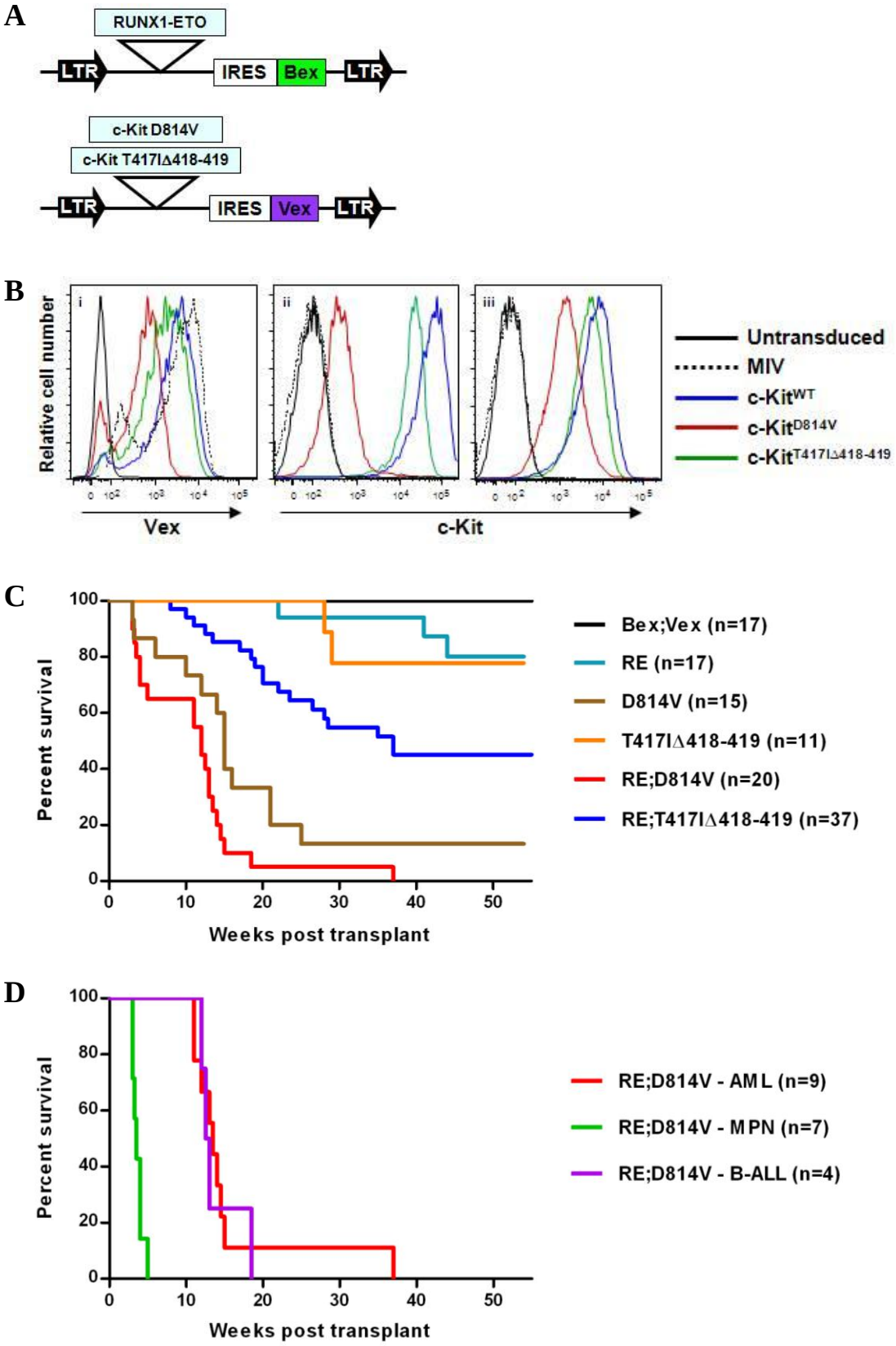
Results

Differing potencies of c-Kit^{D814V} or c-Kit$^{T417I\Delta418\text{-}419}$ to promote AML when co-expressed with RUNX1-ETO

Since distinct subtypes of activating mutations in c-Kit occur at different frequencies in *RUNX1-ETO*$^{+}$ patient samples, we wished to test whether this might impact their ability to cooperate with RUNX1-ETO in promotion of AML. To address this, we co-transduced an enriched population of hematopoietic stem/progenitor cells with two MSCV-based retroviral vectors,[42] with one construct co-expressing RUNX1-ETO (RE) and a blue-excited GFP variant (Bex) and the other retrovirus co-expressing either c-Kit^{D814V} or c-Kit$^{T417I\Delta418\text{-}419}$ in conjunction with a violet-excitable GFP reporter, Vex (**Figure 1A**).[42,43] c-Kit activating mutations were verified by DNA sequencing and protein expression was confirmed by FACS analysis and Western blotting prior to generation of high-titer retrovirus used in the transductions (**Figure 1B and data not shown**). These results showed that Vex protein levels correlated with levels of co-expressed c-Kit mutants and that the c-Kit^{D814V} mutant was expressed at lower levels on the cell-surface than wild-type c-Kit

or the c-Kit exon 8 mutant, even though it is expressed at proportionally higher levels in the cytoplasm. These results confirm previous studies showing that in contrast to wild-type c-Kit, the c-Kit^{D814V} mutation is expressed at lower levels on the plasma membrane but is abundantly expressed in the Golgi, where it maintains its signaling and transforming capabilities.[38,41] Transduced cells were transplanted into lethally irradiated, C57BL/6-Ly-5.1 congenic recipient mice, which were then monitored for hematopoietic defects and leukemia development in both single- and double-transduced cells beginning at 3 weeks post-transplantation.

We observed that all mice co-expressing RE and c-Kit^{D814V} (RE;c-Kit^{D814V} mice) developed lethal hematopoietic neoplasms of diverse phenotype between 2-4 months post-transplant that included AML in 45% of mice, myeloproliferative neoplasm (MPN) in 35% of mice and a pre-B cell neoplasm in 20% of animals (**Figure 1C and Table 1**). Moribund RE;c-Kit^{D814V} mice presenting with AML or pre-B cell ALL progressed with similar kinetics at 3-4 months post-transplant, while mice with MPN exhibited a much more aggressive phenotype, dying within 3-5 weeks of transplant (**Figure 1D**). Animals reconstituted with cells only expressing RE (n=17) rarely progressed to lethal disease within 1 year post-transplant, as noted previously (**Figure 1C**).[14-16,45] However, ~90% of mice reconstituted with cells only expressing the c-Kit^{D814V} mutant allele died from either MPN in half of the moribund mice, or ALL that was predominantly T-ALL (**Figure 1C and Table 1**).The co-expression of RE with c-Kit^{D814V} accelerated the onset of death by approximately 1 month compared with c-Kit^{D814V} mice (**Figure 1C**) and significantly altered the type of hematologic malignancy that developed since AML was the predominant phenotype in RE;c-Kit^{D814V} mice (**Table 1**). AML was characterized by high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RUNX1-ETO
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Relative cell number
i
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MIV
c-KitWT
c-KitD814V
c-KitT417IΔ418-419
C
Percent survival
Weeks post transplant
Bex;Vex (n=17)
RE (n=17)
D814V (n=15)
T417IΔ418-419 (n=11)
RE;D814V (n=20)
RE;T417IΔ418-419 (n=37)
D
Percent survival
Weeks post transplant
RE;D814V - AML (n=9)
RE;D814V - MPN (n=7)
RE;D814V - B-ALL (n=4)

Figure 1. Generation of animals transplanted with RE;c-Kit^{D814V}- or RE;c-Kit$^{T417I\Delta418-419}$-expressing cells. (A) Representation of MSCV-based retroviral constructs. LTR, long-terminal repeats; IRES, internal ribosome entry site; Bex, blue-excitable GFP; Vex, violet-excitable GFP. (B) FACS analysis of NIH 3T3 cells transduced with MIV, c-Kit^{WT}, c-Kit^{D814V}, or c-Kit$^{T417I\Delta418-419}$ retroviruses, demonstrating Vex expression (i), surface c-Kit (ii), and intracellular c-Kit (iii) levels. (C) Kaplan-Meier survival analysis of mice transplanted with bone marrow cells expressing control Bex;Vex (n=17), RUNX1-ETO (RE) (n=17), D814V (n=15), T417IΔ418-419 (n=11), RE;c-Kit^{D814V} (n=20), or RE;c-Kit$^{T417I\Delta418-419}$ (n=37) retroviruses. (D) Kaplan-Meier survival analysis depicting differing latencies of RE;c-Kit^{D814V}-associated neoplasia, which included AML (n=9), MPN (n=7), and pre-B-ALL phenotypes (n=4).

Table 1. Hematopoietic neoplasia in reconstituted mice

Retroviral construct(s)	No. of mice	AML (Sarcoma)	MPN	B-ALL	T-ALL
Bex;Vex	17	0	0	0	0
RE	17	3 (nd)	0	0	0
D814V	15	0	7	1	5
T417IΔ418-419	11	0	0	2	0
RE;T417IΔ418-419	37	19 (3)	0	0	0
RE;D814V	20	9 (7)	7	4	0

AML: Acute myeloid leukemia; MPN: Myeloproliferative neoplasm; B-ALL: pre-B-cell acute lymphocytic leukemia; T-ALL: T-cell acute lymphocytic lymphoma; nd: not determined

frequencies of myeloid blasts that generally represented 60-80% of Bex^{+}Vex^{+} nucleated white blood cells (WBC) in bone marrow of moribund animals (**Figure 4A**). In contrast to RE;c-Kit^{D814V} mice, approximately 50% of animals transplanted with RE;c-Kit$^{T417I\Delta418-419}$-expressing cells never developed a lethal hematologic neoplasm within the one year post-transplant that mice were observed (**Figure 1C**). In addition, AML with a median onset of ~4-5 months was the only phenotype noted among the 50% of RE;c-Kit$^{T417I\Delta418-419}$ mice that became moribund, although there were discernable differences in the frequencies of differentiated myeloid cells between moribund RE;c-Kit$^{T417I\Delta418-419}$ mice,

with animals displaying minimally differentiated and partially differentiated phenotypes (**Figure 6B**). RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice could display high levels of Bex^{+}Vex^{+} expression for long periods of time (months) before any noticeable changes in chimerism indicative of additional genetic changes leading to clonal expansion were observed (note differences between 2 subsets of Bex^{+}Vex^{+} cells in Mouse A,**Figure 2**).

The reduced latency and penetrance of the AML phenotype in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice compared with RE;c-Kit^{D814V} animals that progressed to AML was not due to reduced levels of RE or c-Kit$^{T417I\Delta418\text{-}419}$ expression compared with levels observed in RE;c-Kit^{D814V} mice. Prior to the onset of changes leading to more aggressive malignancy (pre-leukemic phase), the mean fluorescence intensity (MFI) of Bex^{+}Vex^{+} cells in peripheral blood of 8-10 weeks post-transplant RE;c-Kit^{D814V} animals that would ultimately progress to AML was 3,151 for Bex (RE expression, n=8) and 1,333 for Vex (c-Kit^{D814V} expression, n=8), while the MFI for pre-leukemic 8-10 weeks post-transplant RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice was 3,060 for Bex (RE, n=18) and 2,858 for Vex (c-Kit$^{T417I\Delta418\text{-}419}$; n=18). The difference in RE levels between genotypes was not statistically significant (p=0.89, unpaired *t*-test), while higher c-Kit levels in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice were significant (p=0.02, unpaired *t*-test). In addition, the reduced penetrance and delayed onset of AML in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice was not due to reduced levels of peripheral blood chimerism in pre-leukemic RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice compared with age-matched RE;c-Kit^{D814V} animals (Bex^{+}Vex^{+} chimerism levels at 8-10 weeks averaged 5.5% in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that progressed to AML, n=18, versus 1.6% in RE;c-Kit^{D814V} animals, n=8, p=0.04, Mann Whitney test). These results suggest that intrinsic differences between

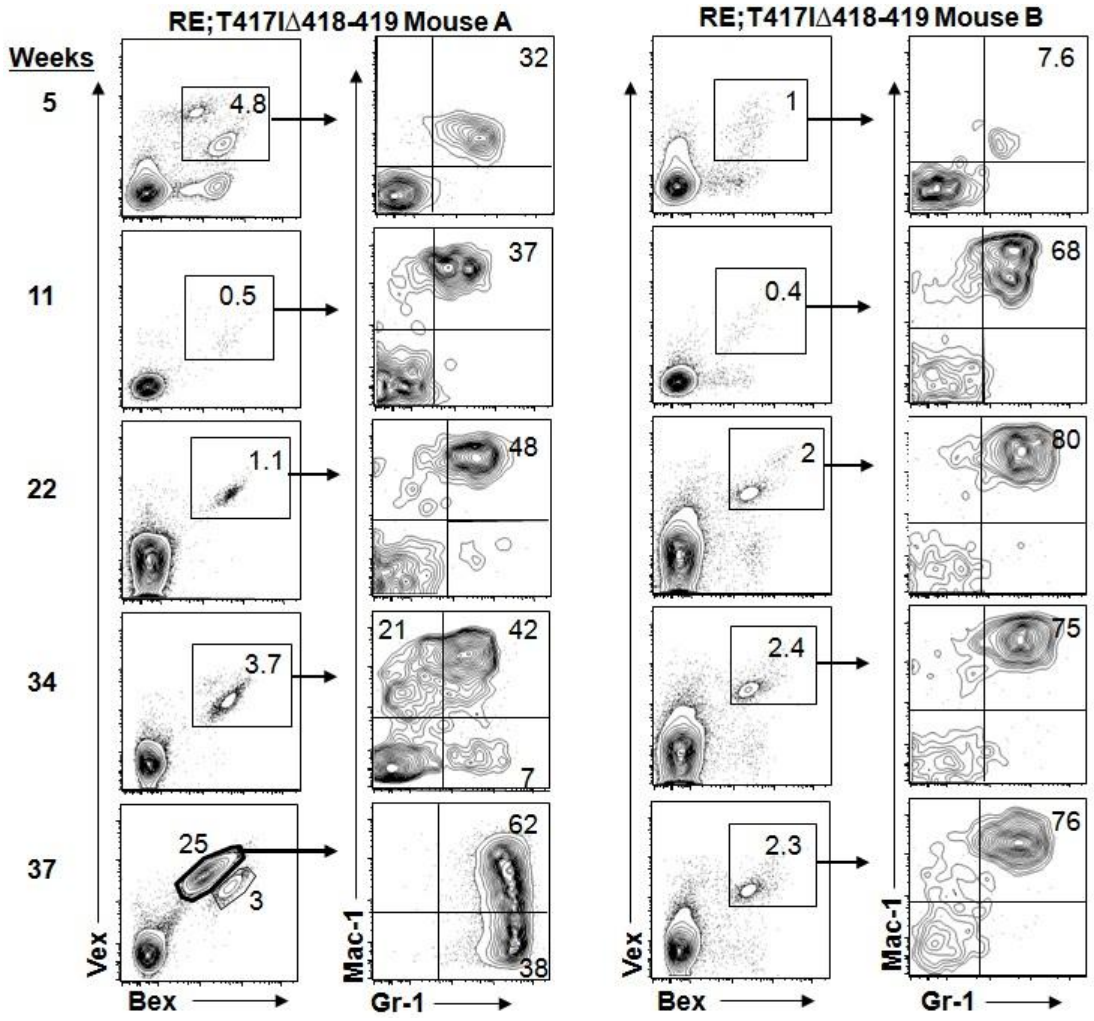

Figure 2. Co-expression of c-Kit$^{T417I\Delta418\text{-}419}$ and RUNX1-ETO promotes AML with significantly delayed kinetics. Peripheral blood analysis of RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice at various time points post-transplant. Left panels for representative Mouse A and Mouse B show persistence of chimerism in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice over time without selective expansion, indicating that this mutational combination is not sufficient to promote clonal proliferation (n=18). Mouse A (left column) is representative of an animal that likely acquired additional genetic changes in a RE;c-Kit$^{T417I\Delta418\text{-}419}$ progenitor cell which promoted selective expansion of this clone at 37 weeks post-transplant.

distinct subtypes of c-Kit activating mutations were responsible for the observed differences in both penetrance and onset of lethal AML. In addition, since both mutant c-Kit retroviruses would be expected to integrate randomly into the genome, it is highly unlikely that differences in hematologic malignancy observed between RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice were due to cooperating mutations caused by random retroviral integration. Furthermore, the initial (at 8-10 weeks) expression levels of RE or c-Kit$^{T417I\Delta418\text{-}419}$ between RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that progressed to AML and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that never progressed during the one year of observation were not different, indicating that other factors promoted progression to AML (MFI at 8-10 weeks for RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that progressed to AML was 3,060 and 2,858 for RE and c-Kit$^{T417I\Delta418\text{-}419}$, respectively, n=18; and 2,608 and 2,003 for RE and c-Kit$^{T417I\Delta418\text{-}419}$ in age-matched mice that never progressed to AML, n=15; p=0.43 for RE and p=0.27 for c-Kit$^{T417I\Delta418\text{-}419}$, unpaired *t*-test). Although pre-leukemic levels of RE and c-Kit$^{T417I\Delta418\text{-}419}$ expression remained stable over time, we noted a 8.8-fold amplification of c-Kit$^{T417I\Delta418\text{-}419}$ expression levels in 44% of moribund c-Kit$^{T417I\Delta418\text{-}419}$ mice with AML that was not accompanied by a similar increase in RE expression (**Figure 3, right plot of middle panel**). Although it is difficult to make a strong point from this observation since FACS parameters like laser alignment and compensation can change over time, we set an arbitrary cut-off of changes that were greater than 2-fold when comparing fluorescence changes between pre-leukemic and leukemic mice to minimize these variables. That said, the data may suggest that selective amplification of c-Kit$^{T417I\Delta418\text{-}419}$ expression might play a role in acceleration of malignant progression in a subset of RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice.

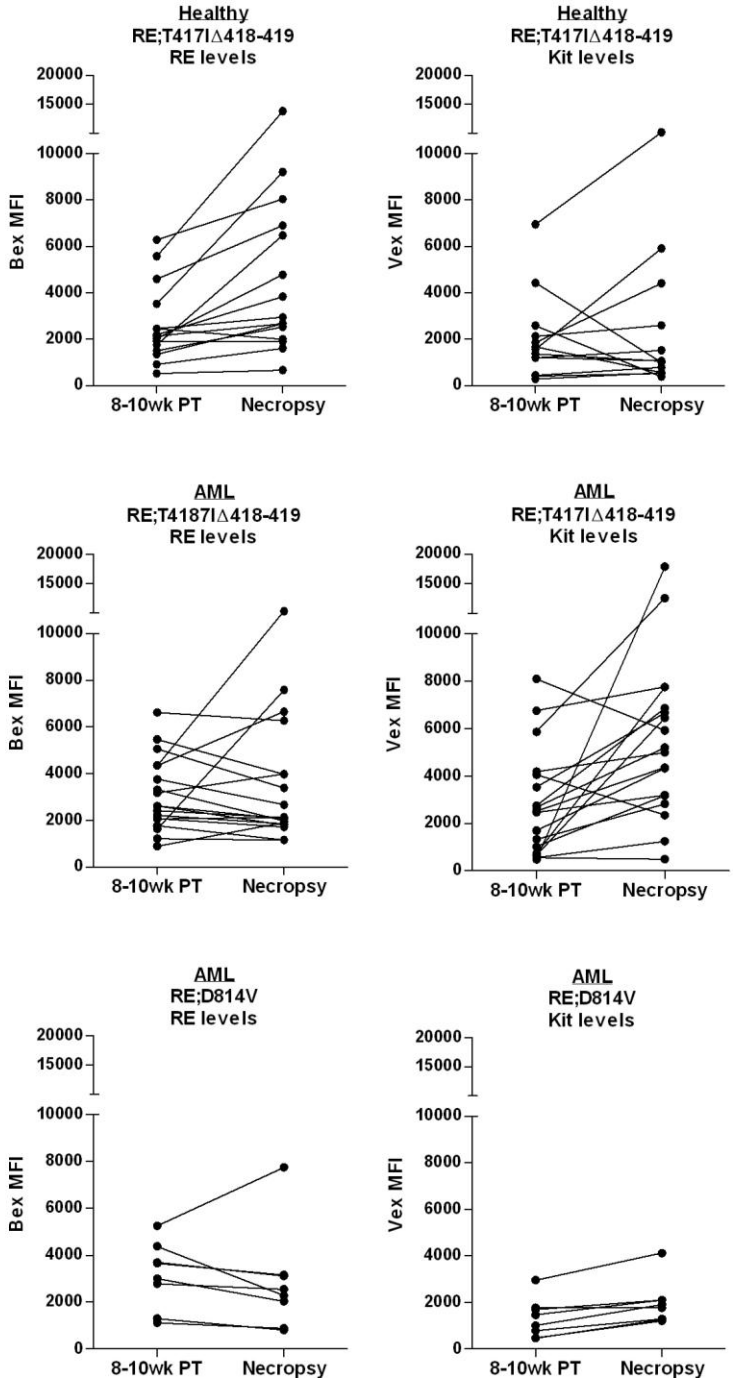

Figure 3. Selective amplification of c-Kit$^{T417I\Delta418\text{-}419}$ in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice may contribute to leukemic progression in some cases. Mean fluorescence intensities (MFI) of Bex or Vex were used as relative measures of RE, c-Kit^{D814V} or c-Kit$^{T417I\Delta418\text{-}419}$ expression levels in peripheral blood leukocytes from RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice. Each line represents Bex and Vex fluorescence levels in a given animal during a pre-leukemic phase (8-10 weeks post-transplant) and when the same animal became moribund.

Characteristics of AML observed in RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice

In addition to high blast cell frequencies in bone marrow of moribund mice (**Figures 4A and 4B**), all animals with AML showed extensive blast infiltration in lung, liver, and spleen (**Figure 4B**) and splenomegaly (**Figure 4C**). In addition, lymph node involvement was notable in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice (**data not shown**). We observed a high incidence of granulocytic sarcomas in RE;c-Kit^{D814V} animals (7/9 mice), which was less common in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice (3/19 animals). Granulocytic sarcomas all had high frequencies of Mac-1^{+}Gr-1^{+} and/or Mac-1$^{-/lo}$Gr-1^{+} blasts (**Figure 5**). Platelet counts were significantly reduced in moribund RE animals co-expressing either subtype of c-Kit activating mutation but in AML cases, only RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice exhibited significantly reduced red blood cell counts and hematocrit (**Table 2**).

FACS analysis of bone marrow and spleen in moribund RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice showed the presence of differing frequencies of Mac-1^{+}Gr-1^{+} and/or Mac-1$^{-/lo}$Gr-1^{+} blast cells as was noted in the granulocytic sarcomas (**Figure 5, Figure 6A**). Interestingly, bone marrow cells expressing RE;c-Kit^{D814V} lacked CD115 (M-CSF receptor) expression (**Figure 6A**), which was a distinguishing marker that characterized the c-Kit^{D814V} mutation from exon 8 deletions. CD115^{+} bone marrow cells in control and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice were always Mac-1^{+}Gr-1^{+} (**data not shown**). It is not clear whether RE;c-Kit^{D814V} co-expression blocked myeloid differentiation prior to induction of CD115, which seems unlikely since we observed differentiated myeloid cells within bone marrow cytospin preparations from RE;c-Kit^{D814V} mice (**Figures 4A and 6B**), or whether the c-Kit^{D814V} mutation blocks expression of the M-CSF receptor at some level. Consistent with reduced CD115 expression, RE;c-Kit^{D814V} mice

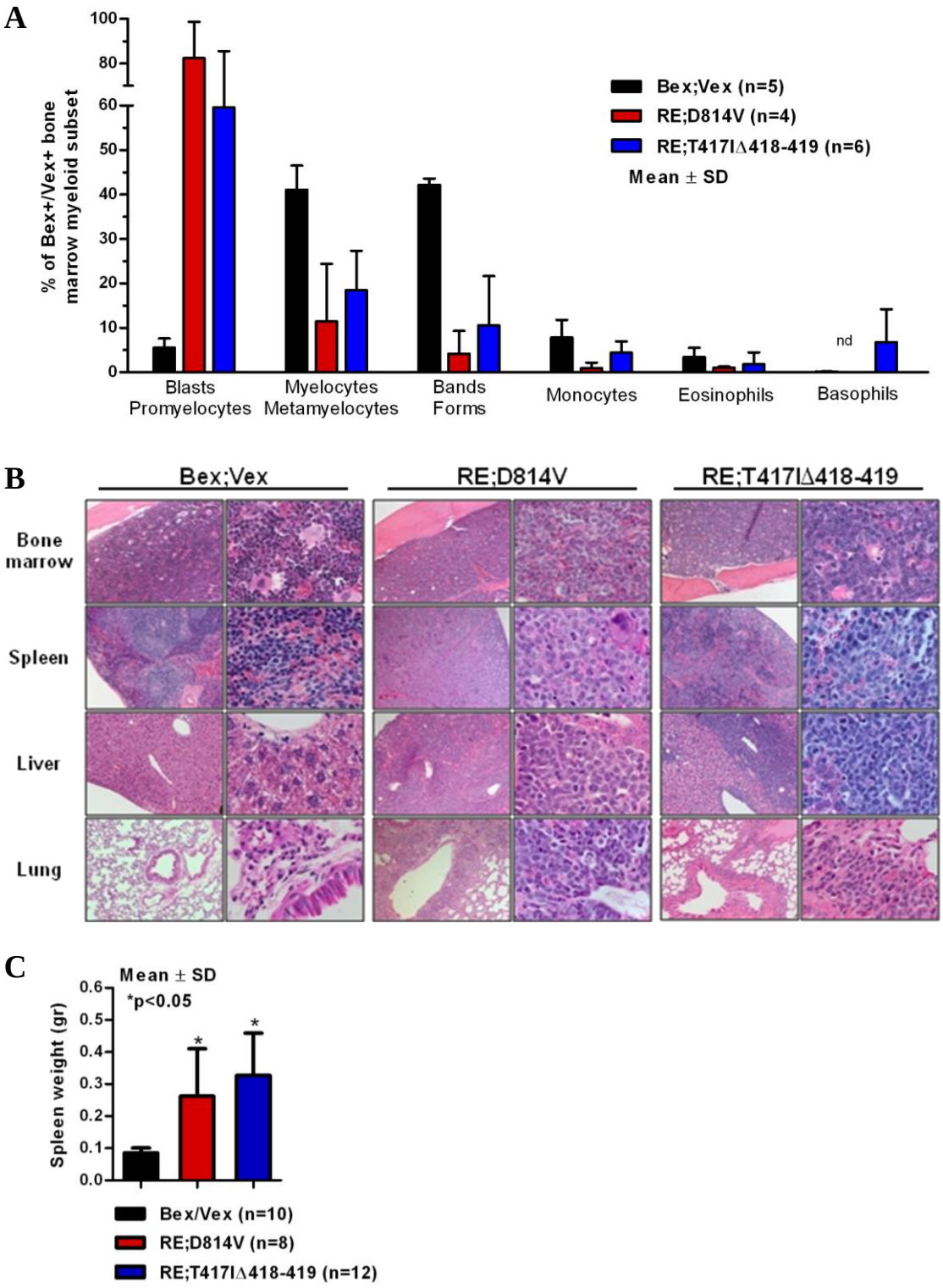

Figure 4. AML blasts in bone marrow and peripheral tissues. (A) Differential counts were performed on Wright-Giemsa stained cytospins of FACS-purified $Bex^{+}Vex^{+}$ myeloid scatter-gated cells isolated from bone marrow of control Bex;Vex (n=5), moribund RE;c-Kit^{D814V} (n=4) and moribund RE;c-$Kit^{T417I\Delta418\text{-}419}$ (n=6) mice. Data are mean per-

centages plus or minus SD of the indicated myeloid cell subsets determined by typing 350 – 500 cells per sample. (B) Representative hematoxylin and eosin (H&E)-stained tissue sections of bone marrow, spleen, liver, and lung from Bex;Vex control, leukemic RE;c-Kit^{D814V} and leukemic RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice. Data are representative of a minimum of 5 moribund RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice. Magnifications in the left and right columns of Bex;Vex, RE;c-Kit^{D814V}, and RE;c-Kit$^{T417I\Delta418\text{-}419}$ images were x100 and x630, respectively. (C) Splenomegaly was observed in all moribund RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice. Spleen weights (grams) are presented as means plus or minus SD for Bex;Vex controls (n=10), RE;c-Kit^{D814V} (n=8), and RE;c-Kit$^{T417I\Delta418\text{-}419}$ (n=12). *p<0.01, when compared to Bex;Vex controls, unpaired t test.

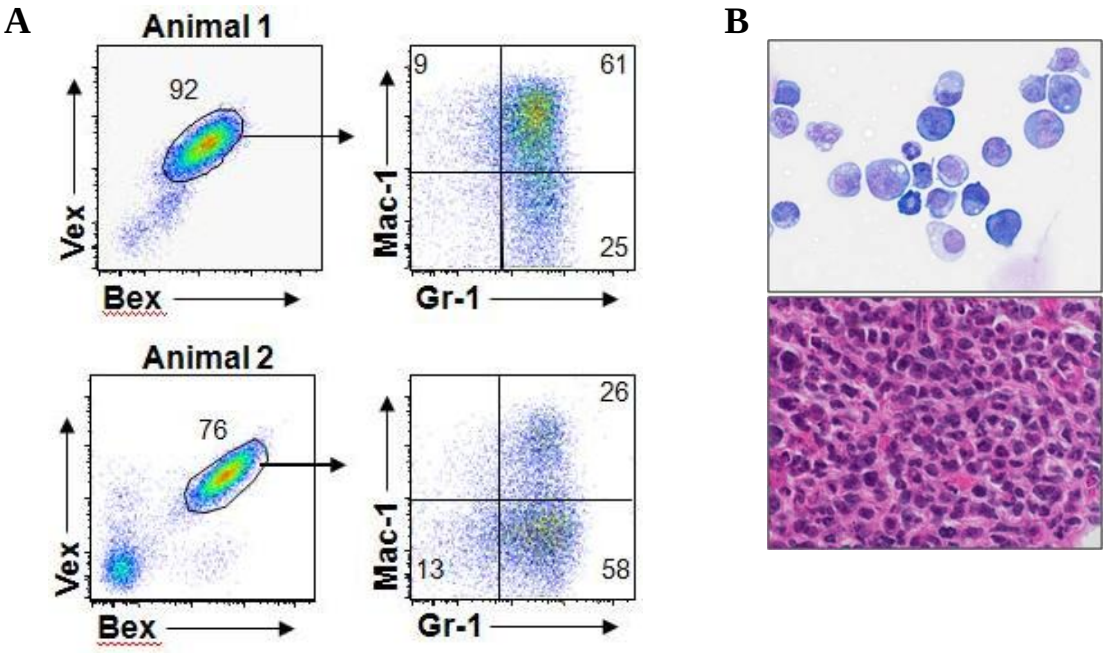

Figure 5. Granulocytic sarcomas are commonly detected in RE; c-Kit^{D814V} mice with AML. (A) Flow cytometric profiles of granulocytic sarcoma cells from two representative moribund RE;c-Kit^{D814V} mice. (B) Representative Wright-Giemsa stained cytospin of FACS-purified Bex^{+}Vex^{+} myeloid scatter-gated sarcoma cells and H&E stained sarcoma tissue section obtained from moribund animals (magnifications x630).

Table 2. Peripheral blood cell counts in reconstituted mice.

Retroviral construct(s) - Phenotype	No. mice analyzed	WBC (K/µl)	RBC (M/µl)	Hematocrit (%)	PLT (K/µl)
Bex;Vex	13	6.39 ± 4.00	8.11 ± 2.22	34.6 ± 10.0	580 ± 295
RE – Non-leukemic	5	6.43 ± 4.00	8.15 ± 2.12	37.2 ± 3.2	644 ± 333
D814V – MPN	3	**>200**	**4.30 ± 1.28**	23.4 ± 10.2	686 ± 328
D814V – T-ALL	3	3.41 ± 1.72	9.90 ± 1.68	45.8 ± 11.8	360 ± 228
D814V – B-ALL	1	12.34	5.07	20.9	191
T417IΔ418-419 – B-ALL	1	80.29	6.51	29.7	270
RE;T417IΔ418-419 – AML	11	15.59 ± 19.83	**2.77 ± 2.12**	**15.6 ± 11.1**	**157 ± 151**
RE;D814V – AML	8	11.39 ± 11.55	7.11 ± 2.37	30.9 ± 9.8	**142 ± 97**
RE;D814V – MPN	6	**22.14 ± 15.89**	**3.78 ± 1.47**	**16.9 ± 9.4**	**132 ± 21**
RE;D814V – B-ALL	4	19.94 ± 29.64	7.20 ± 2.81	29.6 ± 8.0	**140 ± 84**

Peripheral blood was collected by cardiac puncture from non-leukemic or moribund mice. A 50µl sample was subjected to complete blood cell counting using a Hemavet 950 instrument. Data are means plus or minus SD. For values in bold, $p<0.01$ when compared to Bex;Vex controls (Unpaired t test).

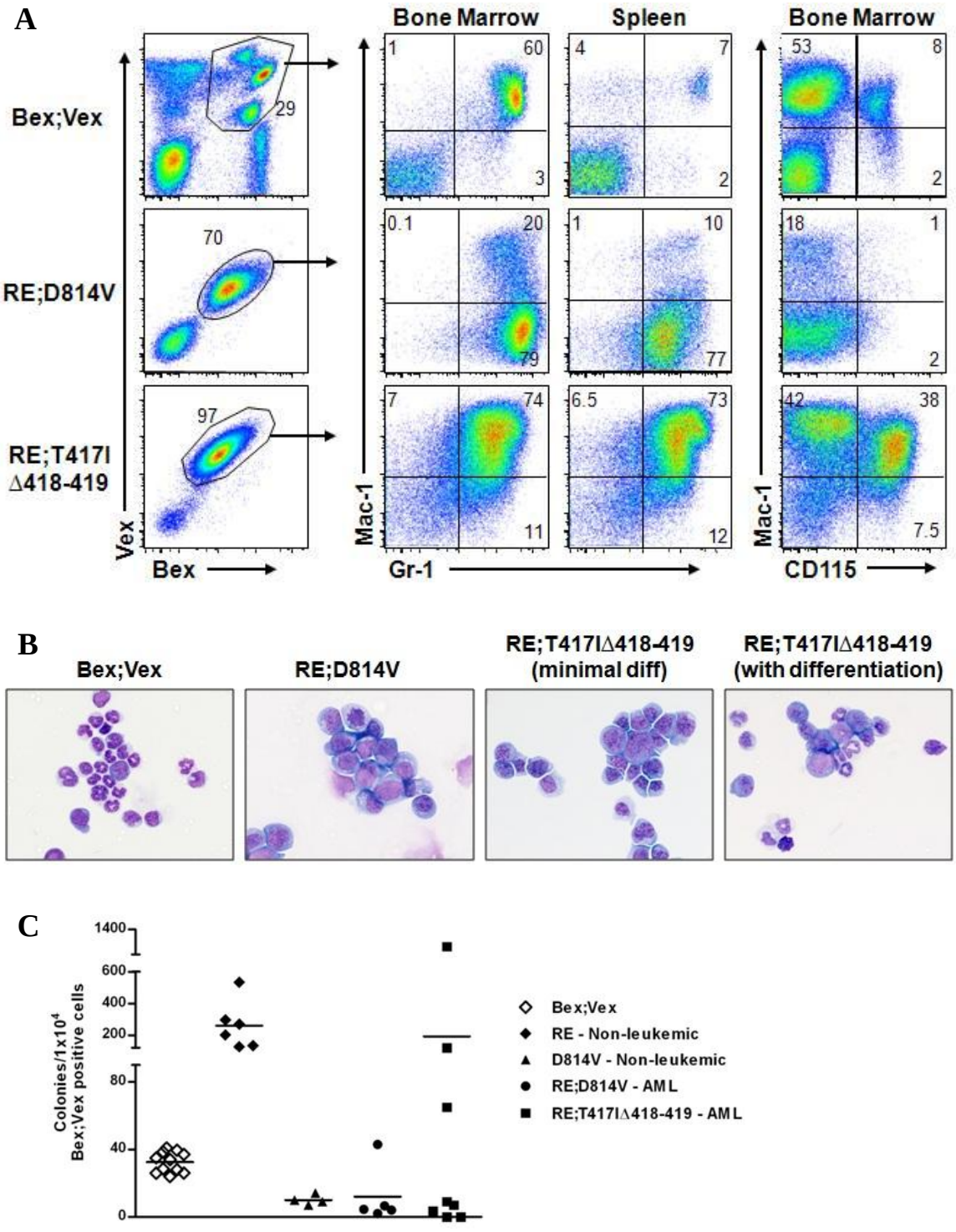

Figure 6. Myeloid-lineage phenotype of leukemic cells in RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418-419}$ mice. (A) Representative FACS analysis of AML cells harvested from bone marrow and spleen of Bex;Vex control and moribund RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418-419}$ mice. Cells were gated for Bex^{+}Vex^{+} expression then analyzed for Mac-1, Gr-1, and CD115 (M-CSFR) expression. (B) Wright-Giemsa stained cytospins of

FACS-purified Bex^+Vex^+ myeloid-gated bone marrow cells from control or moribund animals demonstrating a high frequency of blast forms in leukemic RE;c-Kit^{D814V} and RE;c-$Kit^{T417I\Delta418\text{-}419}$ mice (magnification x630). (C) Myeloid colony forming unit (CFU) assays performed by plating $1x10^4$ FACS-purified Bex^+Vex^+ myeloid scatter-gated bone marrow cells from Bex;Vex control, non-leukemic RE (39–45 weeks post-transplant), non-leukemic D814V (3–17 weeks post-transplant), moribund RE;c-Kit^{D814V}, and moribund RE;c-$Kit^{T417I\Delta418\text{-}419}$ mice in M3434 methylcellulose. Each data point represents the total number of myeloid CFU from an independent animal counted 7-12 days after plating.

with AML had significantly lower frequencies of monocytes than RE;c-$Kit^{T417I\Delta418\text{-}419}$ or control mice among Bex^+Vex^+ cells (**Figure 4A**). Analysis of 32 RE^+ human AML microarray samples showed no significant reduction in CD115 mRNA expression in 9 samples that also possessed D816V mutations (**P. Valk, personal communication**), which suggests that CD115 expression may be post-transcriptionally regulated in RE;c-Kit^{D814V} blasts or our observations are unique to murine cells.

Quantification of myeloid colony-forming units (CFU) in methylcellulose supplemented with SCF, IL-6, IL-3 and Epo showed that RE alone increased CFU numbers 8-10-fold compared with plating equivalent numbers of Bex^+Vex^+ control bone marrow cells (**Figure 6C**). Co-expression of RE;c-Kit^{D814V} (AML phenotype) or c-Kit^{D814V} expression alone completely abrogated myeloid CFU. Curiously, RE;c-$Kit^{T417I\Delta418\text{-}419}$ expression inhibited myeloid CFU in a fraction (5/8) of RE;c-$Kit^{T417I\Delta418\text{-}419}$ mice with AML (**Figure 6C**). In the 3 RE;c-$Kit^{T417I\Delta418\text{-}419}$ samples where colony numbers were similar to what was observed with RE alone, the average MFI for c-$Kit^{T417I\Delta418\text{-}419}$ expression was 2,738, whereas the average c-Kit MFI for the 5 samples where CFU were blocked was 4,342. Although the trend toward lower c-$Kit^{T417I\Delta418\text{-}419}$ expression correlated with reduced ability to block CFU formation, this was not statistically significant ($p=0.24$, unpaired t-test). This may suggest that a threshold level of activated c-Kit sig-

naling is necessary to alter gene expression or impact differentiation in such a way that leads to a block in myeloid CFU formation potential.

AML is clonal and transplantable from moribund RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice

The relatively long latency for development of AML in both RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice suggests that other genetic changes occur to promote AML progression. To test clonality, Southern blots were generated using genomic DNA isolated from the spleens of 5 moribund RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice (lanes 1-5, **Figure 7**). Blots were hybridized with radiolabeled probes complementary to *ETO* or *c-Kit* sequences to detect unique retroviral integrants distinct from germline bands observed in control C57BL/6 genomic DNA. As shown in **Figure 7**, all RE proviral integrants (*ETO* probe) showed a clonal pattern where one unique integrant (see asterisks) was responsible for all donor hematopoietic cells in the spleen. Analysis of c-Kit mutant retroviral integrants from the same mice showed 1-2 integrants per sample, again indicating clonal expansion of a selected Bex^{+}Vex^{+} leukemic clone for all moribund RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice.

To test whether AML was transplantable, we transferred varying doses of whole bone marrow cells into lethally irradiated secondary recipient mice. We observed a dose-dependent, rapid (between 3-10 weeks) progression to lethal AML with similar kinetics using equivalent doses of either RE;c-Kit^{D814V}- or RE;c-Kit$^{T417I\Delta418\text{-}419}$-expressing cells (**Table 3**). FACS profiles of AML cells in hematopoietic tissues of secondary recipient mice were identical to what was observed in the primary recipients (**Figure 8**). The fre-

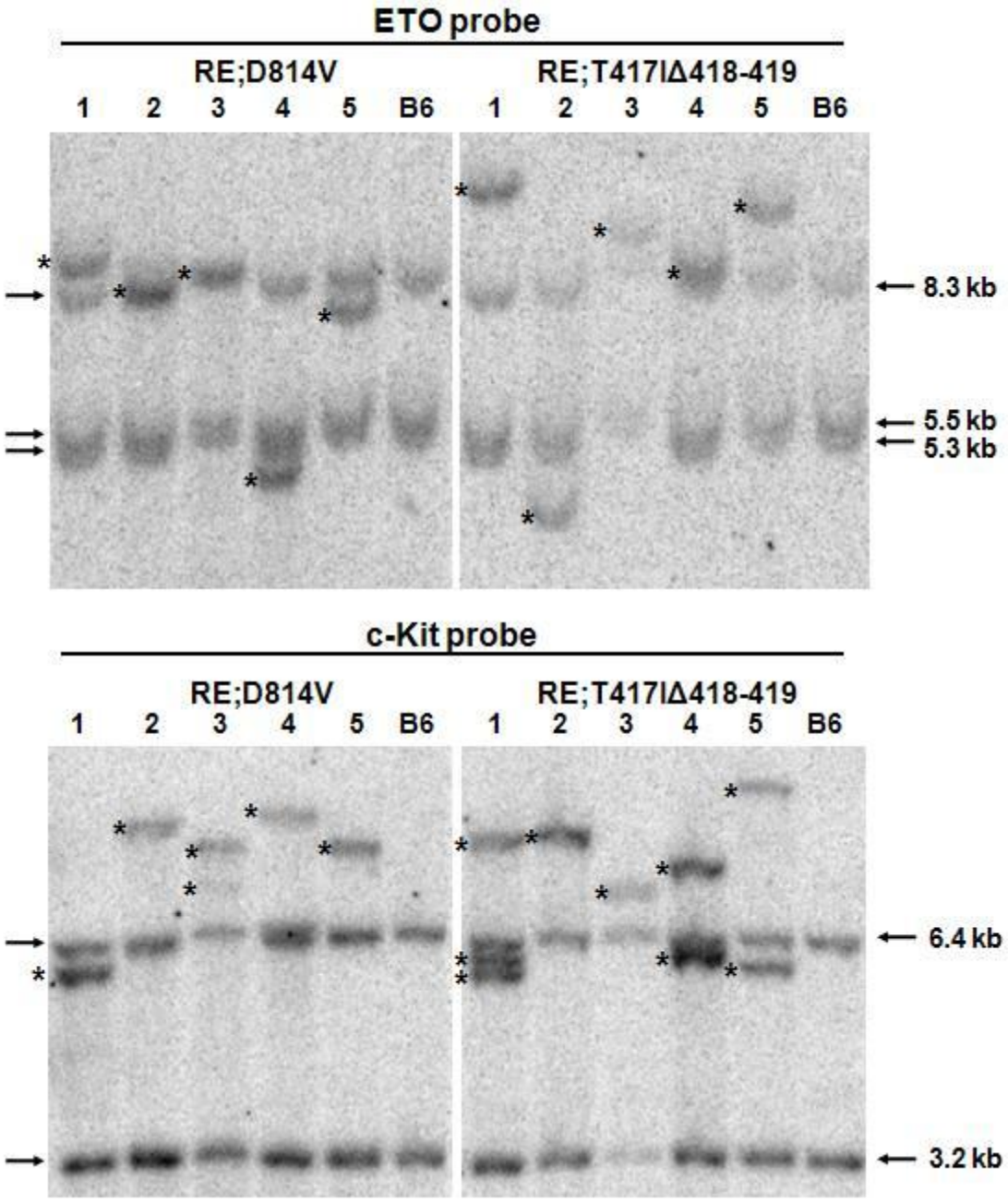

Figure 7. RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ AML phenotypes are clonal. Southern blot analysis using genomic DNA isolated from whole splenocytes obtained from 5 independent, moribund primary RE;c-Kit^{D814V} or RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice. Lane numbers represent the same DNA sample used for both blots. Blots were hybridized with radiolabeled probes complementary to *ETO* or *c-Kit* sequences to detect unique retroviral integrants. Arrows indicate the endogenous murine *Eto* and *c-Kit* bands. Wild-type C57BL/6 (B6) splenocytes served as control samples for each blot. Asterisks indicate unique retroviral integrants in each sample.

Table 3. Transplantability of AML phenotypes into secondary recipient animals.

Cell dosage	RE;D814V Moribund/Total	RE;T417IΔ418-419 Moribund/Total
$1x10^6$	4/4	6/6
$1.5x10^5$	8/13	8/9
$5x10^4$	9/14	1/5
$1x10^4$	0/3	0/6

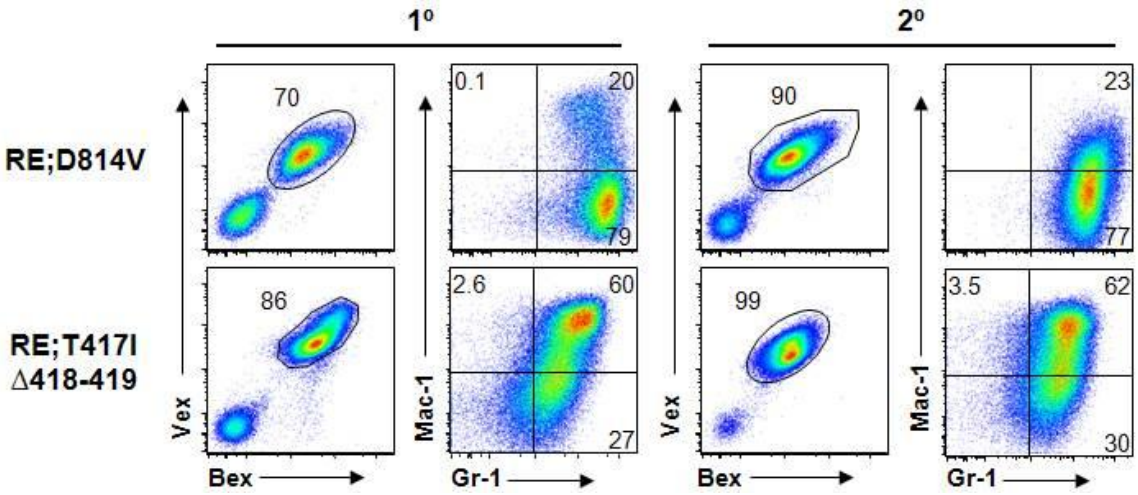

Figure 8. RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ blasts are malignant in secondary recipients. Whole bone marrow from moribund primary animals (RE;c-Kit^{D814V}, n=4; RE;c-Kit$^{T417I\Delta418\text{-}419}$, n=5) was transplanted at various doses ($5x10^4$ – $1x10^6$ cells) into lethally (9Gy) irradiated secondary hosts. Representative bone marrow flow cytometry profiles of primary and secondary recipient mice. Cells were gated for Bex$^+$Vex$^+$ expression then analyzed for Mac-1 and Gr-1 expression.

quency of leukemia-initiating cells (LIC) for both RE;c-Kit mutant genotypes was similar, with 100,000-500,000 whole bone marrow cells being equivalent to a limiting dilution dose of LIC (**Table 3**).

Characterization of MPN and pre-B ALL in RE;c-Kit^{D814V} mice

RE;c-Kit^{D814V} mice with lethal MPN (35% of mice) had ~4-fold higher WBC counts in moribund mice than control animals (22.14 ± 15.89 K/μl versus 6.39 ± 4.0 K/μl, respectively, **Table 2**) and showed ~2-fold reductions in hematocrit and red blood cell counts versus controls. Total platelet counts in moribund RE;c-Kit^{D814V} mice with an MPN phenotype were reduced ~4-fold versus platelet counts in control mice (**Table 2**). The increased WBC count in moribund mice with MPN was characterized by high percentages of more differentiated Mac-1^{+}Gr-1^{+} myeloid cells in bone marrow and spleen (**Figure 9A** and data not shown) and splenomegaly (**Figure 10A**). The splenic architecture was significantly effaced with extensive myeloid infiltrates in the red pulp, as well as infiltrates of more mature myeloid cells in the parenchyma of the lungs and liver (**Figure 10B**). The 20% of RE;c-Kit^{D814V} mice that presented with pre-B cell leukemias had Bex^{+}Vex^{+} cells that showed a distinctly lymphocyte forward (FSC) and side-scatter (SSC) profile in bone marrow that was readily distinguishable from myeloid scatter profiles observed in all cases of AML or MPN (**Figure 9A**). The reduced granularity of the lymphoid blasts observed in the FSC/SSC profiles was also noted in cytospin preparations of bone marrow cells from control and moribund RE;c-Kit^{D814V} mice with pre-B ALL (**Figure 9B**). Leukemic pre-B cells expressed the B-cell markers B220 and CD19 but did not express IgM on the cell surface (**Figure 9A** and data not shown).

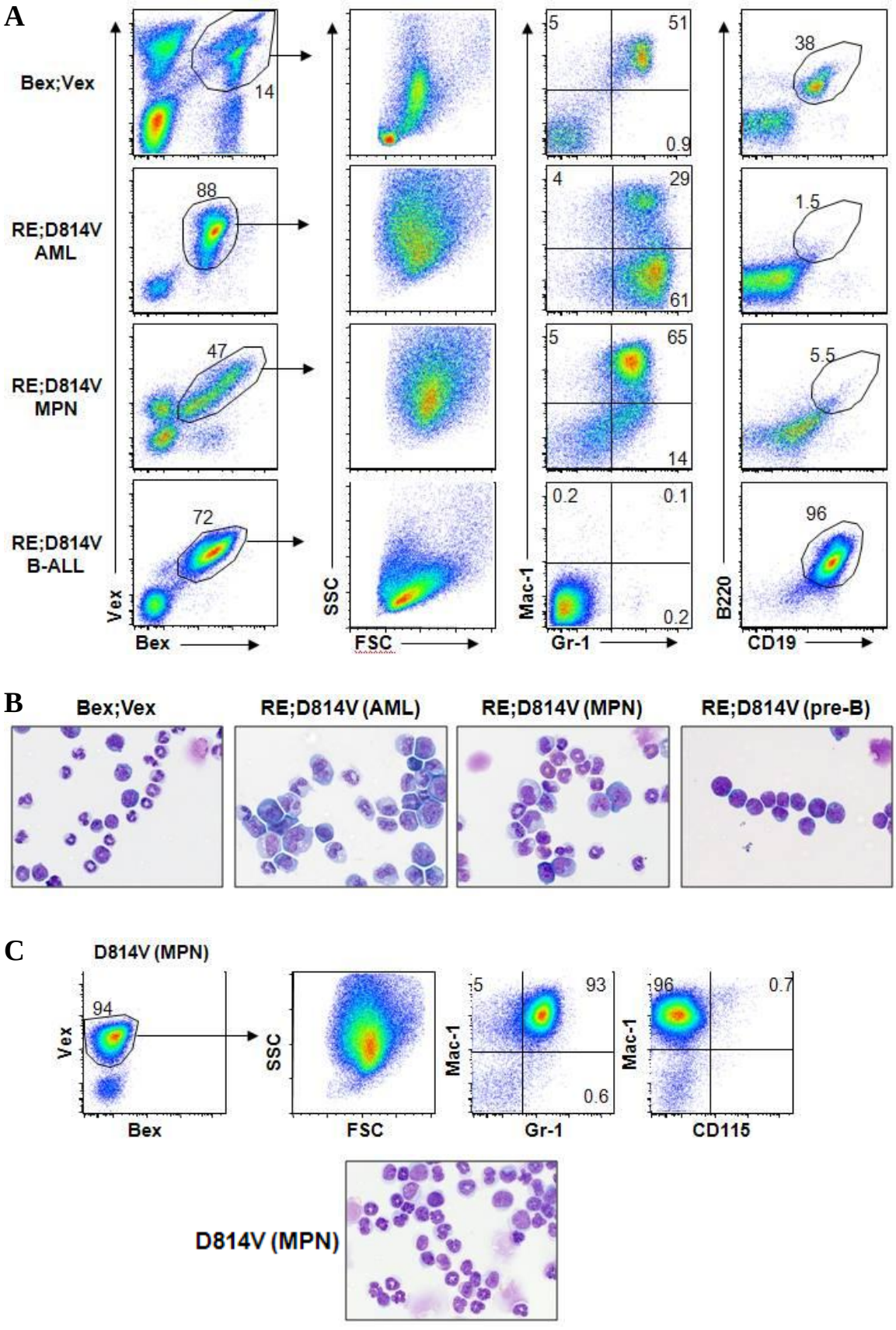
A
Bex;Vex
RE;D814V AML
RE;D814V MPN
RE;D814V B-ALL
14
88
47
72
5
51
0.9
4
29
61
5
65
14
0.2
0.1
0.2
38
1.5
5.5
96
Vex
Bex
SSC
FSC
Mac-1
Gr-1
B220
CD19
B
Bex;Vex
RE;D814V (AML)
RE;D814V (MPN)
RE;D814V (pre-B)
C
D814V (MPN)
94
Vex
Bex
SSC
FSC
5
93
0.6
Mac-1
Gr-1
96
0.7
Mac-1
CD115
D814V (MPN)

Figure 9. Immunophenotypic and morphologic comparison of neoplastic cells from RE;c-Kit^{D814V} mice with AML, MPN, or pre-B-ALL. (A) Representative FACS analysis of neoplastic cells harvested from bone marrow of Bex;Vex control and moribund RE;c-Kit^{D814V} mice with the indicated disease phenotype. Cells were gated for Bex^{+}Vex^{+} expression then analyzed for forward/side scatter profiles and Mac-1, Gr-1, B220 and CD19 expression. (B) Wright-Giemsa stained cytospins of FACS-purified Bex^{+}Vex^{+} myeloid scatter-gated or Bex^{+}Vex^{+} lymphoid blast scatter-gated bone marrow cells from control or moribund animals (magnification x630). Expansion of metamyelocytes, band forms, and eosinophilic progenitors was noted in RE;c-Kit^{D814V} mice with MPN. Morphology of pre-B-ALL cells in RE;c-Kit^{D814V} mice was predominantly lymphoblastic. (C) FACS analysis of myeloid-lineage surface markers and Wright-Giemsa stained cytospin preparation of FACS-purified Bex^{+}Vex^{+} myeloid-gated bone marrow cells from a representative moribund animal with c-Kit^{D814V+} MPN showing a predominance of maturing granulocytes.

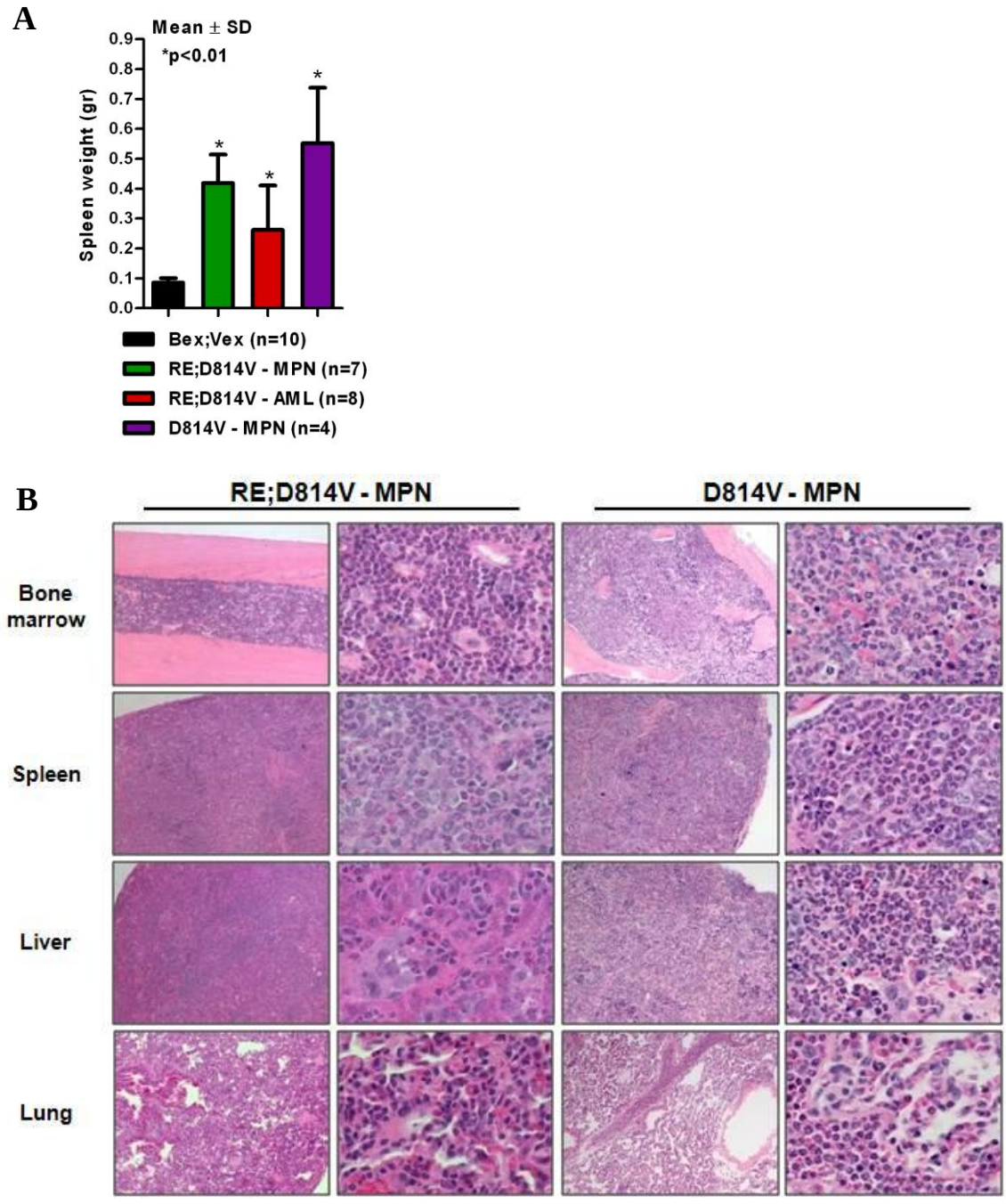

Figure 10. Neoplastic cells associated with RE;c-Kit^{D814V} and c-Kit^{D814V} myeloproliferative neoplasia are disseminated to peripheral tissues. (A) Significant splenomegaly was observed in moribund RE;c-Kit^{D814V} and c-Kit^{D814V} mice with MPN. Spleen weight (grams) are means plus or minus SD. *p<0.01, when compared to Bex;Vex controls, unpaired *t*-test. (B) H&E-stained tissue sections demonstrates extensive infiltration of peripheral tissues by MPN cells. Data are representative of a minimum of 4 moribund RE;c-Kit^{D814V} and 3 moribund c-Kit^{D814V} mice with MPN. Original magnifications in the left and right columns of RE;c-Kit^{D814V} and c-Kit^{D814V} were x100 and x630, respectively.

c-Kit^{D814V} animals that developed highly aggressive MPN had even higher WBC counts (>200K/μl) than RE;c-Kit^{D814V} mice with MPN (**Table 2**) but normal platelet counts, suggesting that RE was primarily responsible for the reduced platelet counts in RE;c-Kit^{D814V} mice. MPN in c-Kit^{D814V} animals was Mac-1^{+}Gr-1^{+}CD115^{-} and displayed a myeloid FSC/SSC profile (**Figure 9C**).

Discussion

In this study, we compared the ability of two classes of c-Kit activating mutations to cooperate with RUNX1-ETO in promotion of AML. Mice reconstituted with cells co-expressing either RE;c-Kit^{D814V} or RE;c-Kit$^{T417I\Delta418\text{-}419}$ both developed AML, although with markedly different kinetics and penetrance (**Figure 1C**). Consistent with clinical observations showing higher coincident expression of c-KIT^{D816V} with RUNX1-ETO (12-48% of cases) than exon 8 mutations like c-KIT$^{T417I\Delta418\text{-}419}$ (2-13% of cases), we observed more robust progression to AML in RE;c-Kit^{D814V} mice than RE;c-Kit$^{T417I\Delta418\text{-}419}$ animals (**Figures 1C and 1D**).[24-28,30,31,33,34,39] One explanation for the delayed onset and incomplete penetrance of AML observed in RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice could be reduced potency and/or altered signaling associated with mutations in the extracellular domain of c-Kit. Previous studies have shown that c-Kit exon 8 mutations promote spontaneous receptor dimerization, hyperresponsiveness to stem cell factor, growth factor-independent proliferation of IL-3-dependent cell lines like Ba/F3 and FDC-P1, and phosphorylation of downstream signaling molecules like mitogen-activated kinase and PI3-kinase.[39,46] In contrast to activating c-Kit kinase domain (exon 17) mutations represented by c-Kit^{D814V}, three representative c-Kit exon 8 mutant alleles were not autophosphorylated in one

study,[39] although significant autophosphorylation of a similar exon 8 receptor mutant was observed by another group.[47] The latter group also noted that each class of c-Kit mutation activated both overlapping and distinct signaling pathways downstream of c-Kit.[47] This may be responsible for the observed overlap, and yet distinct difference, between gene expression signatures in human AML blasts with the core-binding factor mutations (t(8;21)(q22;q22) or inv(16)(p13.1q22)) and c-Kit activating mutations in either exon 8 or exon 17.[48]

In addition to inherent differences in the signaling potential of distinct classes of c-Kit activating mutations, it is also possible that expression differences in c-Kit between RE;c-Kit$^{T417I\Delta418\text{-}419}$ and RE;c-Kit^{D814V} mice might contribute to the observed variability in leukemic penetrance. As noted in **Figure 1B**, the level of intracellular c-Kit^{D814V} protein was only ~3-fold lower than the level of c-Kit$^{T417I\Delta418\text{-}419}$ protein. However, cell-surface expression of c-Kit^{D814V} was ~50-fold lower than c-Kit$^{T417I\Delta418\text{-}419}$ protein. The impact of this difference is not clear, since c-Kit^{D814V} (in contrast to exon 8 mutations) was shown to accumulate in the Golgi where it remained constitutively active and able to stimulate potent myeloproliferative disease in mice.[41] Since we did not observe a significant correlation between the levels of mutant c-Kit expression in AML blasts (*in vivo* MFI determinations) and the ability of each c-Kit mutant to stimulate more rapid progression to AML (the highest expression was seen for the less potent c-Kit$^{T417I\Delta418\text{-}419}$ allele), it seems unlikely that expression differences between the c-Kit mutants would account for the phenotypic differences in the animals. However, it was interesting to note that of the RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that progressed to AML, ~44% (8/18) showed significant amplification (greater than 2-fold) of c-Kit$^{T417I\Delta418\text{-}419}$ levels (8.8-fold MFI increase) that

was not accompanied by a similar increase in RE MFI levels in the same cells (**Figure 3**, middle panel). Greater than 2-fold amplification of c-Kit$^{T417I\Delta418\text{-}419}$ was only observed in 2/13 RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that never progressed to AML (**Figure 3**, right plot of top panel). The specificity for c-Kit$^{T417I\Delta418\text{-}419}$ amplification and the correlation with progression to AML may suggest that amplification of c-Kit expression may be one mechanism for selectively increasing the oncogenicity of a more weakly active c-Kit mutant allele.

The relatively long latency (**Figure 1C, Figure 2**) and the clonal nature of the leukemic blasts in RE;c-Kit^{D814V} and RE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that progressed to AML (**Figure 7**) suggests that additional events are occurring that are essential for leukemic progression in the context of both classes of c-Kit activating mutations. This is likely true for other receptor tyrosine kinase mutations, like FLT3-ITD, that are observed more rarely with RE since RE;FLT3-ITD mutant mice progress with a relatively long mean latency of 233 days and retroviral integration patterns based on Southern blotting were monoclonal/oligoclonal in this retroviral transduction/transplantation model.[20] Mice that developed AML within 1-4.5 months when RE was co-expressed with Wilm's Tumor protein (WT1) also exhibited monoclonal/oligoclonal hematopoiesis in leukemic blasts but polyclonal hematopoiesis among cells that only expressed RE.[21] A possible exception to the need for additional genetic events for AML progression may be RE co-expression with a TEL/PDGR□R fusion, where a transplantable, rapidly lethal AML was observed within 2 months of reconstitution in 19/19 mice and blasts were oligoclonal based on Southern analysis of retroviral integration patterns,[19] although additional hits caused by insertional mutagenesis might also be involved in any of these retroviral models. Although 60-70% of human t(8;21)$^{+}$ samples have non-overlapping RTK or activating *RAS* mutations, addi-

tional recurrent genetic abnormalities have also been noted in a high percentage of cases including loss of a sex chromosome in ~50% of cases and deletions in 9q,[32] which indicates that additional genes regulating AML progression remain to be identified.

An interesting difference between the mouse modeling of RE;c-Kit^{D814V} and human patients with RE and c-KIT^{D816V} lesions is the broad spectrum of both lymphoid and myeloid malignancies observed in transplanted mice compared with the exclusively myeloid leukemias seen in humans. One likely explanation for this difference relates to the temporal order in which mutations occur during leukemic progression in humans, which is not typically modeled in murine studies using retroviral or gene-targeted approaches. Sequential occurrence of oncogenic insults may limit the target cell that secondary AML-promoting events occur in if the primary event restricts developmental potential, as is the case with RUNX1-ETO, which efficiently blocks early T-cell development resulting in the absence of *RUNX1-ETO* transcripts in T cells in *RUNX1-ETO* knock-in mice or in human t(8;21)$^{+}$ patient samples.[14,49] RUNX1-ETO expression in mice also results in a significant expansion (on the order of 50-100-fold) of myeloid progenitor cells in bone marrow over time,[16] which would result in a more likely target population for subsequent randomly occurring AML-promoting changes like activating RTK mutations given the relative abundance of myeloid progenitor cells compared with lymphoid progenitors that do not expand. This expansion in myeloid progenitor cells is not modeled when two mutations are simultaneously introduced into HSC/progenitors cells used to reconstitute mice, which could account for the fraction of RE;c-Kit^{D814V} mice presenting with pre-B ALL in our study. Consistent with this interpretation, RE;FLT3-ITD mice developed B-ALL in 3/11 transplanted mice.[20] There is also good evidence that the *RUNX1-ETO* trans-

location is a primary event in leukemogenesis based on observations that *RE* can be detected *in utero* at relatively high frequencies[13] and observations showing that patients diagnosed with both RE and c-KIT activating mutations only retain RE^{+} cells during clinical remission.[31] This may lead to myeloid progenitor cell expansion in humans during a $t(8;21)^{+}$ pre-leukemic phase that could predispose an individual to further progression-associated changes within the myeloid lineage.

The results of this study functionally demonstrate that two major classes of c-Kit activating mutations can cooperate with RE to promote AML in mice. In a very recent study, two different c-Kit mutants were co-expressed with RE in a retroviral transduction/transplantation model.[38] One c-Kit mutant in this study was representative of a juxtamembrane c-Kit mutation encoded by exon 11, while the other (N822K) is a commonly observed activation loop mutant that is distinct from c-Kit^{D814V} in that it retains sensitivity to imatinib.[38] Similar to what we observed in c-Kit^{D814V} mice, animals expressing only c-Kit^{N822K} developed lethal myeloproliferative disease. When co-expressed with RE, RE;c-Kit^{N822K} mice developed a transplantable AML with a median latency of 177 days in approximately 70% of reconstituted mice, which contrasts with the 100% penetrance that we observed at a median latency of 94 days for RE;c-Kit^{D814V} mice that developed AML (**Figure 1D**). Curiously, the RE;c-Kit^{N822K} mice in their study more closely resembled the malignant phenotype of RE;c-$Kit^{T417I\Delta418\text{-}419}$ (exon 8 mutant) animals with respect to leukemic penetrance and uniquely AML presentation. Together, these analyses demonstrate that the three most common activating c-KIT mutations observed in RE^{+} patient samples function as driver mutations that promote progression to AML. Further work and whole-genome sequencing of $t(8;21)^{+}$ patient samples will be necessary to de-

fine the entire spectrum of changes that allow progression to AML in conjunction with RE.

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Authorship

Contribution: H.J.N. and C.A.K designed the research and wrote the manuscript; H.J.N., H.K., and V.R. performed the research.

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SUMMARIZING CONCLUSIONS

Using retroviral transduction/transplantation approach, we co-expressed AML1-ETO with an activating c-Kit exon 8 mutation (c-Kit$^{T417I\Delta418\text{-}419}$) or an activation loop c-Kit mutant (c-Kit^{D814V}) in murine HSCs/HPCs used to reconstitute lethally irradiated recipient mice. We observed a lethal AML phenotype resulting from mice that co-expressed AML1-ETO;c-Kit^{D814V} and those that co-expressed AML1-ETO; c-Kit$^{T417I\Delta418\text{-}419}$, whereas mice that carried only AML1-ETO rarely developed a lethal hematologic malignancy. The resultant AML in our mice recapitulated many of the pathological features demonstrated in human t(8;21)$^{+}$ cases, including: accumulation of high frequencies of BM blasts that varied in size and nuclear to cytoplasmic ratio, displayed increased cytoplasmic granulation, and showed abnormal cytoplasmic vacuolization, abnormal myelocytes and eosinophilic progenitors with increased granulation, accumulation of basophils or mast cell progenitors, frequent presentation of granulocytic sarcoma, and disruption of erythropoiesis.

The AML phenotypes we observed were similar to that reported in a recent investigation by Wang et al. which used a retroviral transduction/transplantation strategy to co-express AML1-ETO with c-Kit^{N822K}, another kinase domain mutant detected in human t(8;21) AML.[198] In their study, approximately 70% of AE;c-Kit^{N822K} mice developed a lethal AML with a median latency of around 6 months,[198] which contrasts with the 100% penetrance and approximately 3.5 month latency to death we observed for AE;c-Kit^{D814V} -associated AML. In terms of kinetics and frequency of presentation, the

AE;c-Kit^{N822K} -associated AML observed by that group was in fact more similar in to what we reported for AE;c-Kit$^{T417I\Delta418\text{-}419}$ mice. By combining our data with that from the study by Wang et al., we may conclude that two common activation loop c-Kit mutants can contribute to the progression of AML1-ETO-associated AML, but with differing latency and penetrance, which could reflect distinct transforming potencies inherent to each c-Kit mutant. Importantly, the N822K c-Kit mutant appears functionally dissimilar from the D814V mutant, as it retains sensitivity to the TKI imatinib.[137,198]

Clinical studies have demonstrated a higher incidence of activation loop c-KIT mutants than exon 8 c-KIT mutations in t(8;21)$^{+}$ AML cases (11 to 44% versus 2 to 13%, respectively).[35,61,63,64,134-137,139,190] In accordance with those results, the kinetics and frequency of AML presentation we observed in mice varied with respect to the particular c-Kit mutant being co-expressed with AML1-ETO. Specifically, we noted a rapidly fatal and completely penetrant AML phenotype in animals that carried AML1-ETO concurrent with c-Kit^{D814V} compared to a relatively slower progressing disease that developed in only a fraction (~50%) of mice that co-expressed AML1-ETO and c-Kit$^{T417I\Delta418\text{-}419}$.

A possible explanation for the longer latency and reduced penetrance of AML observed in AE;c-Kit$^{T417I\Delta418\text{-}419}$ mice could be inherent differences in signaling activation through c-Kit exon 8 mutants versus kinase domain mutants such as D814V. Crystal structure examinations posit that mutations affecting residues 417 to 421 of c-KIT exert their oncogenic effect because they enhance the binding affinity and homotypic interactions between neighboring IgD5 domains.[181] Thus, it is believed that mutations in IgD5 (i.e. exon 8) might lower the threshold of ligand concentration that is necessary for

receptor dimerization and subsequent activation.[181,190] While a study by Kohl et al.[190] did not detect autophosphorylation of three representative exon 8 mutants (including a T417IΔ418-419 variant as was used in our studies) in the absence of SCF, constitutive phosphorylation and activation of similar exon 8 mutation variants (T417VΔ418-419 and T417YΔ418-419) was observed by other groups.[194,195] Contrastingly, mutations at residue D816 of the c-KIT kinase domain increase the affinity of the receptor for ATP,[202,203] resulting in enhanced tyrosine kinase enzymatic activity and significant autophosphorylation of the receptor via a dimerization-independent mechanism in the absence of SCF.[191-195] This may explain the different biological effects of exon 8 mutations that cause hyperactivation in response to SCF in contrast to exon 17 mutations (i.e. at D816) that lead to ligand-independent, cis-activation of the kinase domain. For instance, it has been well documented that expression of c-KIT^{D816V} (and c-Kit^{D814V}) or various representative c-Kit exon 8 mutants in IL-3-dependent hematopoietic cell lines such as Ba/F3 and FDC-P1 confers cytokine-independent proliferation and increased survival, but the rate of these transforming effects are lower for exon 8 mutants than c-KIT^{D816V}.[190,192,194-197] Of note, in a recent gene expression profiling study by Lück et al., leukemic blasts from CBF-AML patients showed distinct and non-overlapping gene expression signatures depending on the presence of an exon 17 or exon 8 c-KIT mutation.[66] It is quite possible that this is due to differential activation of signaling pathways downstream of constitutively activate versus hyperactive c-KIT receptor mutants.

A second possible explanation for the observed difference in potency of leukemogenesis promotion is that the transforming effects of an exon 8 mutant require a

higher expression level of this receptor variant than an exon 17 mutation variant such as D816V. This hypothesis was first suggested in a report by Cammenga et al.[195] which found 3.5- to 6-fold higher expression, both at the level of transcription and protein, from retroviral vectors in IL-3-independent FDC-P1 cultures expressing c-Kit exon 8 mutants, as compared to c-Kit^{D814V}. Interestingly, using Vex MFI as a relative measure for c-Kit expression level in our studies, we observed significant amplification (mean 8.76-fold MFI increase) of c-Kit$^{T417I\Delta418\text{-}419}$ levels in approximately 44% of AE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that progressed to AML, which was not accompanied by a similar increase in AML1-ETO levels in the same cells. Furthermore, only 2/13 AE;c-Kit$^{T417I\Delta418\text{-}419}$ mice that never progressed to AML displayed c-Kit$^{T417I\Delta418\text{-}419}$ amplification and the mean MFI increase of these was only 3.08-fold. Also, Southern blot analysis of leukemic cells from moribund animals demonstrated that whereas all AML1-ETO proviral integrants were monoclonal in nature, 3/5 AE;c-Kit$^{T417I\Delta418\text{-}419}$ mice carried two or more unique c-Kit$^{T417I\Delta418\text{-}419}$ retroviral integrations, but only 1/5 AE; c-Kit^{D814V} mice showed more than one c-Kit^{D814V} proviral integrant. Although interpretation of these observations is complicated by the fact that potential differences in receptor turnover rates at the protein level may occur, the Southern blot and MFI data indicate that clonal selection for AML1-ETO^{+} hematopoietic progenitors expressing a high level of c-Kit$^{T417I\Delta418\text{-}419}$ may occur during progression to AML and amplification of c-Kit$^{T417I\Delta418\text{-}419}$ expression is a potential mechanism of leukemia progression in a subset of AE; c-Kit$^{T417I\Delta418\text{-}419}$ mice. Importantly, the pre-leukemic expression levels of c-Kit$^{T417I\Delta418\text{-}419}$ were significantly higher than that of c-Kit^{D814V}, and we did not observe significant amplification of c-Kit^{D814V} during progression to AML, suggesting that intrinsic differences in potency

between these two activating c-Kit mutations are contributing to variation in leukemogenic potential.

Additional evidence that an activating mutation in exon 17 of c-Kit is a more potent oncogene than an exon 8 mutation of c-Kit came from our observations of mice reconstituted with cells that expressed only c-Kit^{D814V} or c-Kit$^{T417I\Delta418\text{-}419}$. Approximately 90% of mice carrying c-Kit^{D814V} developed either a lethal MPN (half of moribund mice) or an ALL that was predominantly T-ALL. The median latency to death was around 2.5 months for c-Kit^{D814V}-associated MPN and 4 months for c-Kit^{D814V}-associated T-ALL. The MPN and ALL phenotypes we observed in c-Kit^{D814V} mice were similar to those reported by other groups[192,196,197] and similar to the MPN that resulted from expression of the N822K activation loop c-Kit mutant, although the latency of c-Kit^{N822K}-induced MPN was relatively longer than that of c-Kit^{D814V}-associated MPN (~5.7 versus ~2.5 months, respectively).[198] Contrastingly, only 2/11 (18%) of mice that expressed c-Kit$^{T417I\Delta418\text{-}419}$ developed a lethal hematopoietic neoplasm in our study. This leukemia appeared to be of pre-B cell origin and was of much longer latency, not presenting until 7 months post-transplant. Additionally, the relative expression level of c-Kit$^{T417I\Delta418\text{-}419}$ in mice that became moribund was approximately 3-fold higher than that of moribund c-Kit^{D814V} mice, again suggesting that transforming effects of exon 8 mutants require high expression levels. Collectively, our observations of the varied latency and penetrance of hematopoietic neoplasia that developed in mice co-expressing AML1-ETO with c-Kit$^{T417I\Delta418\text{-}419}$ or c-Kit^{D814V}, and those that solely carried c-Kit$^{T417I\Delta418\text{-}419}$ or c-Kit^{D814V} may reflect weaker oncogenicity of exon 8 versus exon 17 c-Kit mutants.

Previous mouse models of AML1-ETO expression either alone or in conjunction with other oncogenic factors such as TEL-PDGFRβ, FLT3-ITD, or WT1, along with cytogenetic observations from clinical reports on human $t(8;21)^{+}$ AML have clearly demonstrated that AML1-ETO is a critical, albeit insufficient factor for leukemogenesis. The currently-accepted model suggests that leukemia development requires the cooperative effects of two distinct genetic "hits".[21] In our studies, Southern blot analysis determined that leukemic blasts from both the AE;c-Kit$^{T417I\Delta418\text{-}419}$- and AE;c-Kit^{D814V} -associated AML phenotypes were clonal in nature, suggesting that these two genetic mutation events were insufficient for leukemogenesis and acquisition of further genetic changes is necessary for progression to AML. However, the relatively shorter latency AML that developed from co-expression of AML1-ETO with c-Kit^{D814V} may indicate that fewer additional cooperating genetic mutations are required for promoting overt leukemia transformation in this context, presumably due to the stronger oncogenicity already inherent to c-Kit^{D814V} as compared to c-Kit$^{T417I\Delta418\text{-}419}$. This is further supported by our observations that AE;c-Kit$^{T417I\Delta418\text{-}419}$ mice could display high levels of AML1-ETO and c-Kit$^{T417I\Delta418\text{-}419}$ expression (presence of $Bex^{+}Vex^{+}$ PB cells) for many months before any noticeable increases in chimerism indicative of additional genetic changes leading to clonal expansion were detected and approximately 50% of mice reconstituted with AE;c-Kit$^{T417I\Delta418\text{-}419}$-expressing cells never developed a lethal hematologic neoplasm during the one year post-transplant observational period. In terms of clonality, similar observations were documented in other murine models of t(8;21). For instance, retroviral integration patterns were monoclonal or oligoclonal in the AE;FLT3-ITD and AE;WT1

models, but polyclonal hematopoiesis was observed for cells only expressing AML1-ETO.[132,133]

Secondary mutational events affecting growth factor receptors, proto-oncogenes, and transcription factors are commonly identified in t(8;21) AML patients (up 70% of cases)[57] and it has become increasingly evident that additional recurrent cytogenetic abnormalities occur in a significant number of these cases. For instance, loss of a sex chromosome has been documented in ~35% of female and ~56% of male t(8;21) patients.[60,138,204-208] While loss of sex chromosome is a natural phenomenon associated with aging, it occurs at a significantly younger age and higher frequency in t(8;21) patients compared to the general population and is observed in less than 5% of patients with other types of AML.[204,209] In addition, chromosome 9q deletion, trisomy 4, and trisomy 8 are commonly detected in t(8;21) AML.[60,138,205,210] Taken together, the data from murine models of AML1-ETO-associated AML and cytogenetic studies of human $t(8;21)^{+}$ patients indicate that the "two-hit" model may be an oversimplification of AML pathogenesis and additional genetic events essential to leukemogenesis remain to be identified.

Unexpectedly, in addition to an AML, we observed two other neoplastic phenotypes in mice reconstituted with AE;c-Kit^{D814V}-expressing hematopoietic progenitors. A rapidly fatal myeloproliferative neoplasm developed in 35% of transplanted animals (median latency ~1 month), while another 20% of mice showed a pre-B-ALL that presented with a latency similar to the AML phenotype (3 to 4 months post-transplant). This contrasts with the exclusively myeloid leukemia seen in humans. A probable explanation for the observed phenotypic disparity relates to the temporal

order of mutational acquisition during AML progression in humans. This important aspect of disease pathogenesis is not accurately reproduced in mouse models of leukemia that are generated using retrovirally-mediated oncogene expression strategies.

An emerging theme in acute leukemia pathogenesis is that one of the earliest events during disease progression involves loss of function of a transcription factor critical for hematopoietic lineage programming/commitment.[47] These lineage commitment factor defects lead to expansion of progenitor pools, which may then be poised for acquisition of additional transforming mutation events. AML1 is one such factor that has been demonstrated to play an essential role in hematopoietic development and there exists significant evidence that alteration of its normal function (due to generation of AML1-ETO) by the t(8;21) is the earliest event during leukemogenesis, likely acquired at the stage of a HSC or early HPC. For example, *AML1-ETO* transcripts have been detected *in utero*, preceding the development of pediatric AML by up to 10 years,[124,125] are identified in BM progenitors subsets obtained from patients that have been in clinical remission for many years,[126-128] and in fact, patients diagnosed with both AML1-ETO and c-Kit activating mutations only retain AML1-ETO$^+$ cells during clinical remission.[137]

AML1-ETO expression was shown to significantly expand the BM HSC and HPC populations, substantially increase the frequency of early myeloid progenitors and impart self-renewal capability to myeloid progenitor cells, which normally do not possess this characteristic.[114,115,119,120] Furthermore, in biochemical assays and gene expression studies, AML1-ETO effectively repressed the expression of other transcription factors and microRNAs critical for programming not only neutrophil maturation, but also B cell,

T cell, erythroid, megakaryocyte, mast cell, and eosinophil development.[81,211-215] In sum, this oncogenic fusion protein imparts a myeloid bias to progenitor subsets in the BM and strongly limits their differentiation capacity to early stages of lineage commitment. Hematopoietic stem and progenitor cell expansion and sequential acquisition of mutations are not modeled when we simultaneously introduce AML1-ETO and a c-Kit mutant into HSC/HPCs used to reconstitute mice. It is quite likely that introduction of an activated c-Kit mutant, especially a potent variant such as D814V, at the HSC/HPC stage could sufficiently override some of AML1-ETO's lineage-restricting effects and instead push that population toward a lymphoid fate. A lymphoid-biased effect of c-Kit^{D814V} expression is evidenced by the frequent development of B- and T-cell neoplasia in mice reconstituted with HSC/HPC cells that expressed only c-Kit^{D814V}, including approximately 50% of the animals in our study.[192,196] Furthermore, several studies have reported a critical role for c-Kit-mediated signaling in pro-B and pro-T cell development.[216-218] This could explain the fraction of AE;c-Kit^{D814V} mice that presented with pre-B-ALL in our study. Consistent with this interpretation, AE;FLT3-ITD mice developed B-ALL in 3/11 transplanted mice.[133]

The proposed mechanism of leukemia induction due to AML1-ETO and activated c-KIT signaling remains a topic of consideration. The exact ways in which AML1-ETO expression mediates the induction HPC proliferation/expansion and alteration of self-renewal are not completely understood, but several AML1-ETO-regulated genes are proposed to play a role. For example, AML1-ETO directly suppressed transcription of *p19^{ARF}* (p14^{ARF} in humans), a critical mediator of the p53 checkpoint pathway for oncogene-induced apoptosis and senescence,[219,220] whereas it caused upregulation of

BCL-2, a potent anti-apoptotic factor.[221] A possible influence of AML1-ETO on slowing the G_1-to-S phase transition is suggested, possibly because it changes the expression of *$p21^{waf1/cip1}$* and *cyclin D3*, alterations in which have been implicated in regulation of HSC quiescence/proliferation and differentiation.[222-225] AML1-ETO also induced Jagged1, a Notch ligand known to drive stem cell proliferation.[111] Finally, it caused activation of *Plakoglobin* and *β-catenin*, mediators of Wnt signaling which promotes stem cell self-renewal.[112] Mechanisms of AML1-ETO-mediated inhibition of differentiation include altering the transcriptional activity of genes necessary for proper megakaryocyte-erythroid progenitor, granulocyte-monocyte progenitor, and common lymphoid progenitor commitment, as well as those needed for terminal maturation. This growing list of genes includes *E2A, PU.1. GATA-1, C/EBPα, GM-CSF, myeloperoxidase*, *neutrophil elastase*, and subunits of the T-cell antigen receptor[47] Recent studies have also begun to elucidate an epigenetic role for AML1-ETO, as it can cause silencing of microRNAs that are needed for myeloid differentiation.[214,215] In addition, expression of AML1-ETO deregulated DNA repair base excision repair genes, thereby potentially contributing to genomic instability.[111] Interestingly, AML1-ETO has also been linked to failure to downregulate c-KIT in the expanded progenitor pool.[137] In fact, that study suggested that AML1-ETO directly regulates c-KIT expression. Using inducible expression in the monoblastic U937 cell line, the investigators found significant upregulation of c-KIT at mRNA level after AML1-ETO was induced.[137]

In terms of the mechanisms of transformation mediated by c-KIT activating mutations, these events lead to hyperactivation of the PI3-K/AKT, JAK-STAT, and/or MAPK pathways, which are responsible for regulating numerous processes including cell

cycle progression, survival, apoptosis, activation of gene transcription, proliferation, and differentiation.[148,183,184] Induced alterations in the activation status of these pathways, therefore, has many consequences for the biology of a cell such as excessive proliferation, uncoupled from regulatory growth factors; the ability to bypass oncogene-induced apoptosis pathways; and increased survival signal frequency causing longer lifespan. With respect to the specific gene expression signatures downstream of distinct classes of activated c-KIT receptors, studies remain in the initial stages of investigation. Thus far, several interesting findings of genes differentially regulated by expression of exon 17 or exon 8 c-KIT mutants have been reported, including genes that encode factors of the NF-κB signaling pathway, IL-1R signal transduction molecules, CD40L signaling pathway components, molecules regulating granulocyte adhesion and diapedesis, TNF/stress related signaling factors, tyrosine kinases that initiate T-cell receptor activation, T helper 2 cytokine genes, various cytokines involved in the inflammatory response, as well as several others.[66]

Although much remains to be discovered in terms of fundamental biology and chemistry of the process, we can envision a general model for t(8;21) leukemogenesis as follows: The initiating translocation event occurs in the HSC/HPC compartment, resulting in restriction of developmental potential to the stage of myeloid progenitors and imparting mechanisms of self-renewal, causing primary clonal expansion of these progenitor populations. This pool of myeloid-biased cells is a more likely target for sequential acquisition of additional randomly-occurring oncogenic events simply by statistical chance due to its expanded and self-renewing nature or, alternatively, as a result of defective DNA repair mechanisms. Continued expression of c-KIT by this

population provides a target for an activating RTK mutation providing the second "hit" in AML progression. Altered signaling pathways downstream of an activated RTK such as c-KIT likely provide the important proliferative or survival advantage subsequently leading to further expansion of the pre-neoplastic myeloid progenitors. Upon accumulation of more genetic lesions one of these cells becomes the fully-transformed clone.

Over the past several decades, cytogenetic and molecular studies have greatly contributed to our understanding and classification of AML and an ever-widening range of therapeutic agents has served to increase overall patient survival. However, disease relapse remains a significant issue for subsets of $t(8;21)^{+}$ patients and this is likely due to the presence of certain secondary genetic abnormalities such as c-KIT activating mutations. Small-molecule TKIs are a well-studied group of therapeutic agents with demonstrated efficacy in treating certain types of leukemia, thus activated RTKs are attractive targets for the development of novel TKIs. In addition, numerous molecules activated in the signaling cascades downstream of RTKs could be targets for therapy.

For these reasons, several studies have sought to define exactly which signaling pathways are involved downstream of distinct c-Kit mutants and determine the effects of activating and, subsequently, inhibiting these pathways on hematopoietic cells. These reports demonstrated that PI3-K is constitutively phosphorylated downstream of c-KIT^{D816V} and exon 8 c-Kit mutants.[190,191,194,226-228] Interestingly, constitutive activation of AKT (one of the best known targets downstream of PI3-K) was observed only in cases of c-Kit exon 8 mutant expression.[190,191,194,226-228] Inhibition of PI3-K or AKT in exon 8 mutant-expressing hematopoietic cells abolished the transforming effect of this

mutant.[190,194,195] In contrast, blocking PI3-K in c-KIT^{D816V}-expressing cells significantly inhibited the growth and tumorigenicity conferred by this mutant, but inhibition of AKT had little effect.[190,194,195,226-228]

The majority of previous reports suggest no or weak phosphorylation of MAPK factors downstream of D816 mutants (D816 to V, H, or N) and inhibition of this pathway did not block the proliferative effect exerted by c-KIT^{D816} mutant expression.[194,195,226,227,229-231] Although the data are still contrasting, some investigators have reported MAPK activation associated with c-Kit exon 8 mutant expression, and use of a MAPK inhibitor significantly repressed the growth of cells expressing this type of mutant.[190,195] Finally, expression of either an exon 8 or D816 mutant led to phosphorylation of STAT1, 3, and 5.[194,195,229,231] In summary, these observations have begun to highlight the similarities and differences in signaling pathways through which distinct oncogenic c-Kit mutants exert their transforming effects.

Due to the increasing knowledge that various cytogenetic aberrations can greatly impact the clinical features, progression and prognosis of t(8;21) AML, there remains considerable interest in better defining the molecular genetic events essential to and sufficient for pathogenesis of this AML subtype, from a clinical as well as basic science standpoint. Toward this end, we used murine models to compare the leukemogenic potential associated with concurrent expression of AML1-ETO and either of two distinct activating c-Kit mutants, one involving a small deletion within the extracellular domain encoded by exon 8 and the other involving a point mutation in the phosphotransferase domain. Our results functionally demonstrate that both types of activating c-Kit mutations can cooperate with AML1-ETO to promote AML in mice, although with

varying kinetics and frequency, likely due to inherent differences in the potency of each c-Kit mutant. By combining our observations with that of the recent report by Wang et al.,[198] we can conclude that the three most common activating c-Kit mutations observed in t(8;21)$^{+}$ patients function to accelerate progression to AML.

The models we developed were important not only for examining the basic pathology of AML resulting from expression of these particular genetic lesions, but may also be used as tools in future studies to test novel therapeutic approaches and investigate the specific biochemistry of cooperation between AML1-ETO and activated c-Kit. In addition, future work should be done to properly address the temporal order of acquiring these mutations. Finally, in the age of gene expression profiling and whole-genome sequencing, global cytogenetic investigations of samples from t(8;21) patients will likely continue to assist in determining the nature of additional molecular changes that arise during AML evolution.

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APPENDIX

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: February 23, 2011

TO: CHRISTOPHER A KLUG, Ph.D.
SHEL-510 2182
FAX: (205) 934-1875

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Analysis of a Murine Model of the AML1-ETO Translocation
Sponsor: Internal
Animal Project Number: 110207254

As of February 23, 2011, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	0
Mice	C	0

Animal use must be renewed by February 22, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 110207254 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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