Title
Interrogating the Roles of Specific Downstream Effector Pathways in the Pathogenesis of Hematopoietic Malignancies Initiated by Oncogenic K-RasG12D

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Interrogating the Roles of Specific Downstream Effector Pathways in the Pathogenesis of Hematopoietic Malignancies Initiated by Oncogenic K-RasG12D

by

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DISSERTATION

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This dissertation is dedicated to my family and to all my friends at Gracepoint Fellowship Church who have walked with me every step of this long journey

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Abstract

Interrogating the Roles of Specific Downstream Effector Pathways in the Pathogenesis of Hematopoietic Malignancies Initiated by Oncogenic K-Ras^{G12D}

Angell Shieh

Mutations that deregulate Ras signaling are prevalent in hematopoietic malignancies. Previous work has shown that expressing oncogenic *Kras*^{G12D} in the hematopoietic compartment of mice leads to growth factor-independent and a hypersensitive pattern of myeloid progenitor colony growth in response to granulocyte-macrophage colony-stimulating factor (GM-CSF), and a fatal myeloproliferative disorder (MPD). This mirrors human myelomonocytic leukemias that are associated with Ras pathway mutations. Oncogenic Ras proteins accumulate in the active GTP-bound form and constitutively activate numerous downstream effector pathways. However, it is unclear which effectors are necessary to initiate and maintain malignant hematologic disease as well as the contributions of specific effector pathways to disease phenotypes.

I performed functional studies utilizing second site K-Ras^{G12D} mutant proteins to explore the role of the three main effector pathways – Raf/MEK/ERK, Phosphotidyl-Inositol-3Kinase(PI3K), RalGDS – in *Kras*^{G12D}-driven oncogenesis. Using primary murine hematopoietic cells transduced with second site mutant constructs, I found that second site mutants maintaining hyperactive signaling down only one effector pathway were unable to confer hypersensitivity to GM-CSF in methylcellulose assays. However, second site mutants that engage two of the three major effector pathways, K-Ras^{G12D,E37G}

and K-Ras^{G12D,Y64G} (which activate PI3K/RalGDS and Raf/RalGDS respectively), retain some growth factor hypersensitivity but do not exhibit cytokine-independent growth.

Transplantation of bone marrow cells transduced with *K-Ras*^{G12D,E37G} or *K-Ras*^{G12D,Y64G} into irradiated recipient mice induced invasive, monoclonal, CD4-CD8 double-positive T-cell acute lymphoblastic leukemias (T-ALLs) that originated in the thymus. A majority of these leukemias show elevated levels of activated Notch1, due to mutations in the PEST domain of *Notch1*. Tumors expressing K-Ras^{G12D,E37G} demonstrated elevated levels of this mutant K-Ras protein but no basal activation of the Raf/MEK/ERK pathway. Interestingly, T-ALLs initiated by K-Ras^{G12D,Y64G} acquired elevated levels of phosphorylated Akt, which was associated with diminished or absent PTEN expression. The strong selective pressure to increase signaling through PI3K by alternative mechanisms, increasing K-Ras^{G12D,E37G} protein levels or decreasing PTEN expression, supports the hypothesis that this pathway plays a central role in T-lineage leukemogenesis. An important therapeutic implication of these data is that inhibiting a single major effector pathway may be insufficient to fully reverse the growth advantage conferred by oncogenic Ras.

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Chapter 1: Introduction

Introduction to Ras

Regulation of cell growth, proliferation, and survival occurs through complex interactions between the extracellular environment and a network of downstream effectors. The Ras family of monomeric G-proteins act as molecular switches that link extracellular inputs through membrane receptors to intracellular signals (Friday and Adjei, 2005). Ras proteins cycle between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformations (Ras•GTP and Ras•GDP). Upon activation of many receptors, phosphorylated tyrosines create docking sites for a complex of adaptor molecules, such as Shc and Grb2, which interact with membrane-localized Ras (Campbell *et al.*, 1998).

One of these proteins, SOS, is a guanine nucleotide exchange factor (GEF) that binds to and facilitates guanine nucleotide dissociation; which is followed by passive nucleotide binding. Because the concentration of free GTP vastly exceeds that of GDP in cells, SOS increases the percentage of Ras•GTP. Ras signaling is terminated when Ras•GTP is hydrolyzed to Ras•GDP through intrinsic GTPase activity. This slow reaction is greatly augmented by GTPase activating proteins (GAPs), which bind to Ras•GTP and accelerate hydrolysis by stabilizing the transition state between Ras•GTP and Ras•GDP. In mammalian cells, the primary GAPs are neurofibromin and p120 GAP. Thus, the activation status of Ras is dependent upon the competing actions of GEFs and GAPs (**Fig. 1**) (Boguski and McCormick, 1993; Bollag and McCormick, 1991; Satoh *et al.*, 1991; Vetter and Wittinghofer, 2001).

The transition of Ras between the GDP- and GTP-bound states is accompanied by changes in protein conformation that greatly influence the ability of Ras to bind to

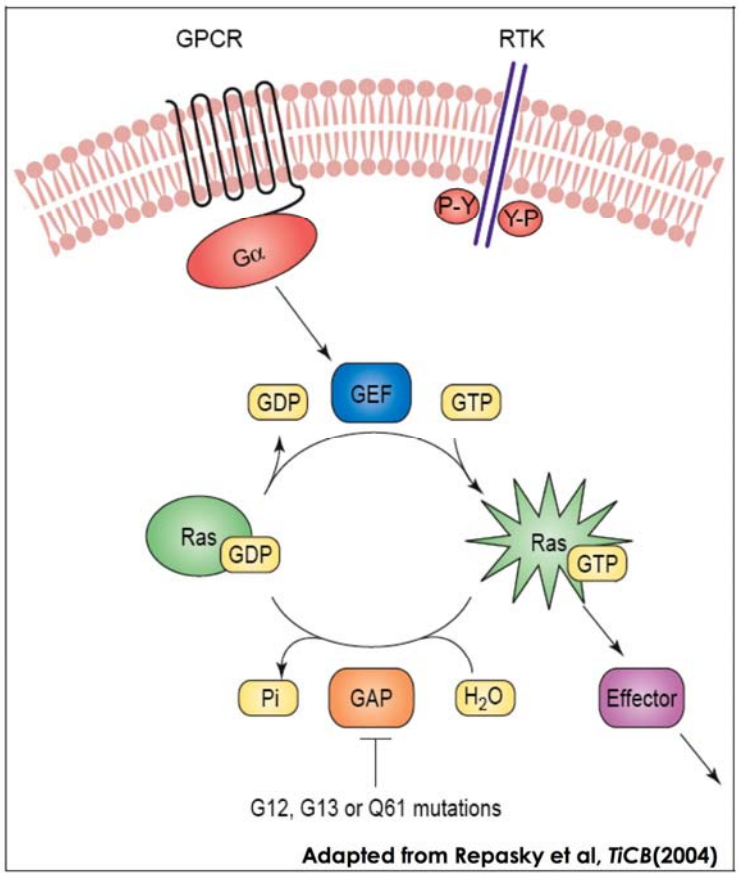

Figure 1 – Ras activation and signaling. *Ras cycles between an inactive GDP-bound and an active GTP-bound conformation. Activation of G-protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs) leads to recruitment of a complex of adaptor molecules to the plasma membrane. One of these molecules is SOS, a Ras guanine-nucleotide exchange factor (GEF) which facilitates dissociation of guanine nucleotides to allow for passive binding of GTP. Activated Ras can then engage downstream effectors. Intrinsic GTPase as well as presence of GTPase activating proteins (GAPs) act to hydrolyze GTP and return it to its inactive conformation. Oncogenic Ras proteins have reduced intrinsic GTPase activity and are resistant to GAPs.*

downstream effector molecules. When Ras is bound to GTP, the highly mobile switch I (residues 32-40) and switch II (residues 60-76) regions adopt a stable conformation that allows for high-affinity binding and activation of effector proteins (**Fig. 2a**) (Vetter and Wittinghofer, 2001). Each effector pathway contributes uniquely to different aspects of Ras activity (Repasky *et al.*, 2004). For example, in fibroblasts, activation of the Raf/MEK/ERK pathway leads to increased cell cycle progression and proliferation, whereas activation of the PI3K/Akt pathway primarily inhibits apoptosis and causes cytoskeletal changes. However, recent studies argue strongly that Ras signaling is modulated through a network of interacting molecules that show extensive cross-talk and feedback rather than simply reflecting the additive effects of self-contained linear pathways that regulate specific phenotypes.

Hyperactive Ras Signaling in Cancer

GTP-bound Ras binds to effector proteins, which, in turn, regulate cell cycle progression, protect against apoptosis, induce cytoskeletal changes, and mediate other functions that influence normal homeostasis. It is therefore not surprising that *RAS* genes are mutated in almost 30% of human cancers with a majority of these mutations encoding amino acid substitutions at codons 12, 13, and 61. Mutations are found most frequently in *KRAS* (~85%), with *NRAS* and *HRAS* accounting for ~15% and <1% of mutations, respectively (Bos, 1989). Mutant Ras proteins accumulate in the GTP-bound state due to defective intrinsic GTPase activity and resistance to GAPs (Donovan *et al.*, 2002; Vetter and Wittinghofer, 2001). Interestingly, the distribution of specific *RAS* mutations shows strong tissue specificity. *KRAS* is the predominant isoform mutated in lung, colon,

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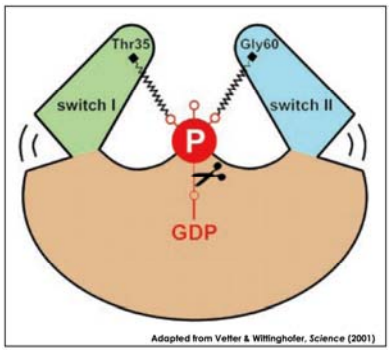

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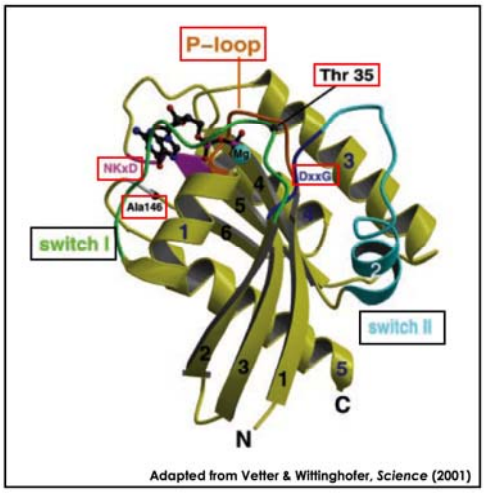

Figure 2 – Structure Models of Ras. (A) *"Loaded spring" model of switch I and switch II stabilization when Ras is bound to GTP. The oxygens of the γ-phosphate of GTP form hydrogen bonds with the amine groups of Threonine-35 and Glycine-60, which stabilizes Ras in a conformation that permits productive effector binding. Release of the γ-phosphate by GTP hydrolysis allows the switch regions to spring back open and relax in the GDP-specific conformation.* ***(B)*** *Ribbon plot of Ras G-domain bound to GTP and Mg2+. Conserved regions are boxed in red and are responsible for specific binding of the guanine nucleotide as well as facilitating the GTPase reaction. Switch regions are boxed in black.*

Tissue	%of all cancers	H-Ras	K-Ras	N-Ras
Lung	12%	1%	17%	1%
Breast	11%	1%	5%	1%
Large Intestine	9%	0%	32%	3%
Stomach	9%	4%	6%	2%
Prostate	6%	6%	8%	1%
Liver	6%	0%	7%	4%
Hematopoietic	6%	0%	5%	12%
Cervix	5%	9%	8%	1%
Oesophagus	4%	1%	4%	0%
Bladder	3%	12%	4%	3%
Oral cavity	3%	16%	4%	0%
Pancreas	2%	0%	60%	2%
Kidney	2%	0%	1%	0%
Ovary	2%	0%	15%	4%
Uterus	2%	1%	14%	0%
Brain and CNS	2%	0%	1%	2%
Skin	2%	5%	2%	19%
Larynx	2%	0%	5%	0%
Thyroid	1%	4%	3%	7%
Other	14%			
• *Biliary tract*		*0%*	*32%*	*1%*
• *Eye*		*0%*	*4%*	*1%*
• *GI tract*		*0%*	*19%*	*0%*
• *Small intestine*		*0%*	*20%*	*25%*
• *Thymus*		*0%*	*15%*	*0%*

Table 1 – Ras mutations in Human Cancer. *Data were derived from Catalogue of Somatic Mutations in Cancer (COSMIC) of the Wellcome Trust Sanger Institute, Cambridge UK and adapted from (Karnoub and Weinberg, 2008). Tissues were arranged by worldwide cancer frequency as of 2002 as described in (Parkin et al., 2005).*

and pancreatic cancers whereas *NRAS* accounts for most of the mutations found in hematopoietic malignances, liver cancer, and melanoma. *HRAS* mutations predominate in bladder cancer (Table 1) (Downward, 2003; Karnoub and Weinberg, 2008; Rodriguez-Viciana *et al.*, 2005). It is not yet clear why there are these differences. It is possible that they may be due to varied expression levels of the isoforms within different cell types as evidenced by differences in viability of Ras isoform knockouts (Esteban *et al.*, 2001; Johnson *et al.*, 1997; Umanoff *et al.*, 1995), differences in post-translational modification and membrane targeting (Hingorani and Tuveson, 2003; Mor and Philips, 2006), and/or differential abilities to activate downstream effectors and induce biological responses (Voice *et al.*, 1999; Walsh and Bar-Sagi, 2001; Yan *et al.*, 1998).

The importance of aberrant Ras signaling in cancer is further supported by mutations that affect proteins that interact with Ras biochemically. Loss-of-function mutations and deletions of the *NF1* gene, which encodes the Ras-GAP neurofibromin (NF1), result in increased levels of Ras-GTP due to impaired GTP hydrolysis. Persons with germline mutations in the *NF1* gene are predisposed to a variety of tumors including benign neurofibromas, plexiform neurofibromas, optic nerve gliomas, and myeloid leukemias/myelodysplastic syndromes (Dasgupta and Gutmann, 2003; Weiss *et al.*, 1999). Genetic analysis of these benign and malignant tumors frequently reveals inactivation of the normal *NF1* allele, thereby proving that *NF1* functions as a classical tumor suppressor gene. In myeloid leukemia, loss of *NF1* is rarely seen concomitantly with a *RAS* mutation, which is consistent with the idea that *NF1* inactivation and oncogenic *RAS* mutations play similar roles in myeloid transformation (Side *et al.*, 1998; Side *et al.*, 1997).

Hyperactive Ras signaling can also result from upstream mutations. For example, somatic mutations in *FLT3*, a gene encoding a receptor tyrosine kinase present on immature hematopoietic cells, are found in over 30% of acute myeloid leukemia (AML) (Gilliland and Griffin, 2002) and have been shown to activate the Ras/Raf/MEK/ERK and STAT signaling pathways. The observation that *RAS* and *FLT3* mutations are rarely found in the same AML and that dominant negative *RAS* inhibits *FLT3* mediated transformation infers that Ras activation is essential for *FLT3*-induced transformation (Hayakawa *et al.*, 2000; Mizuki *et al.*, 2000).

Lastly, activating mutations in downstream effectors of Ras-GTP also contribute to tumorigenesis. *BRAF* is mutated in a majority of melanomas and the most common amino acid substitution (V600E) results in a constitutively active kinase that stimulates the Raf/MEK/ERK pathway to levels similar to those seen in oncogenic Ras. Furthermore, melanomas with $BRAF^{V600E}$ mutations do not show *NRAS* mutations, suggesting that this mutation is functionally equivalent to oncogenic Ras in melanocyte transformation (Davies *et al.*, 2002; Wan *et al.*, 2004).

Structural Basis of Ras Activation and Effector Binding

Crystallographic studies of Ras in its GDP and GTP bound states have been instrumental for understanding how Ras acts as a molecular switch as well as how it activates different effector pathways. Ras belongs to a family of guanine nucleotide-binding proteins that includes the heterotrimeric G proteins with α, β, and γ subunits and proteins involved in protein synthesis such as EF-Tu. These proteins all contain a

common G domain that carries out the nucleotide binding and hydrolysis and confer a switch-like mechanism (Vetter and Wittinghofer, 2001).

This G domain consists of six β sheets and five α helices interconnected by ten loops, among which are four conserved sequence elements important for guanine-nucleotide binding (**Fig. 2b**, red boxes). A conserved P-loop with the motif GXXXXGK(S/T) (residues 10-17 in Ras) forms bonds with the α- and β-phosphates of both GTP and GDP. Threonine-35 contains an amide that forms a hydrogen bond to γ-phosophate oxygens as well as side chain hydroxyls that stabilize a catalytic Mg^{2+} ion in coordination with oxygens of β- and γ-phosphates of GTP. The conserved DXXG motif (residues 57-60 in Ras) contains an invariant aspartate that binds to the catalytic Mg2+ as well as gives specificity for guanine. The amide of the invariant Glycine-60 forms a hydrogen bond with the γ-phosphate of GTP much like Threonine-35. Lastly, the (N/T)KXD motif (residues 116-119 of Ras) and Alanine-146 give specificity for the guanine-nucleotide and prevent binding of an adenine amino group (Bourne *et al.*, 1991; Karnoub and Weinberg, 2008; Pai *et al.*, 1990; Vetter and Wittinghofer, 2001).

When the GDP-bound and GTP-bound states of Ras were compared, large structural differences were mainly confined to two segments of Ras, which were therefore called "switch regions" (**Fig. 2b**, black boxes) (Milburn *et al.*, 1990). In Ras, switch I consists of residues 32-40 and switch II is comprised of residues 59-67. In the GDP-bound state, these segments take on many different conformations and are shown to have increased flexibility on NMR (Farrar *et al.*, 1997; Ito *et al.*, 1997). However, upon binding of GTP, the conformation becomes more rigid. Threonine-35 in switch I and Glycine-60 in switch II form hydrogen bonds to the γ-phosphate of GTP and thereby

"lock" the switch regions into a stable conformation. Upon release of the γ-phosphate by GTP hydrolysis, both switch regions are released and relax back into their GDP-bound conformations (**Fig. 2a**) (Vetter and Wittinghofer, 2001; Wittinghofer and Nassar, 1996).

Conformational stabilization of the switch I and switch II regions upon binding of GTP are essential for efficient binding of Ras to downstream effectors. For example, the Ras binding domain (RBD) of Raf-1 has 1,300-fold lower affinity for Ras-GDP when compared with Ras-GTP (Herrmann *et al.*, 1995). PI3K and other effectors also show much higher affinity for Ras-GTP versus Ras-GDP (Wittinghofer and Herrmann, 1995). In addition, the switch I and switch II regions contain the main residues that comprise the interface layer of many Ras-effector complexes (Corbett and Alber, 2001). This interface layer is formed when a ubitquitin fold motif (ββαββα) in the effectors binds to Ras in an intermolecular β sheet interaction that is stabilized by amino acid sidechains (Huang *et al.*, 1998; Nassar *et al.*, 1995; Pacold *et al.*, 2000; Vetter *et al.*, 1999).

It has been shown that effectors only bind to the switch regions when the switch regions are in a specific conformation. If that is perturbed, they are no longer able to bind efficiently (Spoerner *et al.*, 2001). When effectors bind to the switch regions, they increase stability of the switch-effector conformation and reduce nucleotide dissociation rate without affecting GTP hydrolysis (Herrmann, 2003; Herrmann *et al.*, 1996). In addition, binding of downstream effectors stabilizes the binding of a highly mobile Tyrosine-32 in Ras to the γ-phosphate of GTP. Loss of the γ-phosphate releases the Tyrosine-32 and destabilizes binding of the effector (Geyer *et al.*, 1996; Nassar *et al.*, 1995). Based on these data, the following model of Ras-effector binding has been proposed. When Ras is bound to GDP, the switch regions take on many conformations of

which only one is specific for effector binding. Consequently, downstream effectors are unable to bind efficiently to Ras-GDP. However, when Ras is bound to GTP, the switch regions are locked into a conformation that the effectors can recognize, bind to, and become activated (Wittinghofer and Nassar, 1996). The activation of these downstream effectors is extinguished upon GTP hydrolysis.

Oncogenic Ras mutations deregulate cell growth by increasing Ras-GTP levels and subsequently increasing the signaling through effector pathways. The two most common oncogenic mutations found in human cancers alter Glycine-12 and Glutamine-61. In Glycine-12 mutants, replacement of the glycine with any residue other than proline results in oncogenic activation (Seeburg *et al.*, 1984). Co-crystallization of Ras with GAP showed that the arginine finger of GAP, which is essential for accelerating GTP hydrolysis, forms very close van der Waals contact with Glycine-12 (**Fig. 3a**). Therefore, although binding to GAP is unaffected in these mutants, any other amino acid in residue 12 blocks the arginine finger from entering into the GTP pocket and increasing the rate of GTP hydrolysis (Scheffzek *et al.*, 1997). Recent studies have revised this model by showing that inhibition of GAP activity is less a result of steric block as much as displacement of critical catalytic residues from the arginine finger of GAP (Ahmadian *et al.*, 1999; Rodriguez-Viciana *et al.*, 2005).

In Glutamine-61 mutations, the kcat of the RasGAP GTPase reaction is reduced by $10^{-3} – 10^{-7}$ depending upon mutation (Shurki and Warshel, 2004). In a structural analysis of Ras, Glutamine-61 was found to form a hydrogen bond with a water molecule and activate it for a nucleophilic substitution reaction with the γ-phosphate of GTP (Pai *et al.*, 1990). Data from co-crystal structures of Ras and RasGAP, showed that RasGAP

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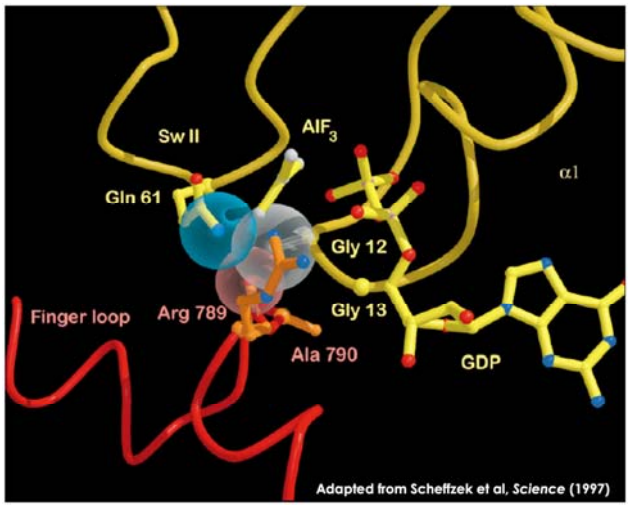

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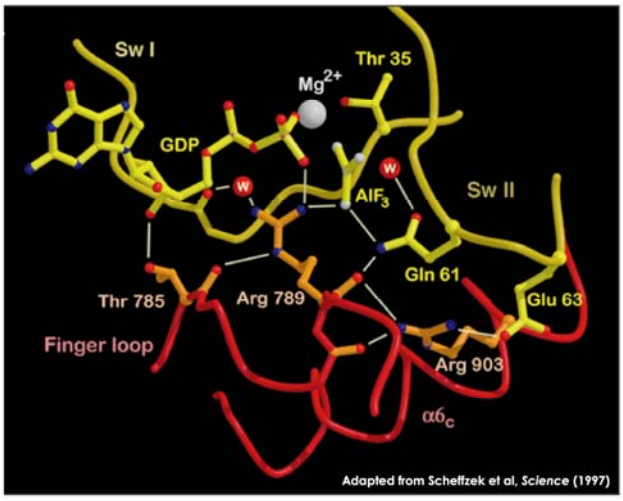

Figure 3 – Active Site Structure of Ras. (A) *Co-crystallization of RasGAP with the P-loop of Ras. In the P-loop of Ras, substituting any other amino acid for Glycine-12 (in this case alanine, gray sphere) leads to steric clashes with both Arginine-789 of RasGAP (red sphere) and Glutamine-61 of Ras (blue sphere). This underlies resistance to GAP-mediated GTPase acceleration seen in oncogenic Ras proteins.* ***(B)*** *Structural view of the active site for GAP-mediated hydrolysis of GTP. Presence of the RasGAP arginine finger (Arginine-789) acts to stabilize the transition state by neutralizing partial negative charges during the phosphoryl transfer reaction and by helping stabilize the catalytic glutamine.*

accelerates γ-phosphate hydrolysis by stabilizing the transition state as well as stabilizing the Glutamine-61 residue in its catalytic conformation (**Fig. 3b**) (Scheffzek *et al.*, 1997). Therefore, mutation of the glutamine results in loss of the catalytic residue that is essential for GTP-hydrolysis.

Downstream Effector Pathways

When Ras is bound to GTP, the switch I and switch II regions are stabilized into conformations that have high affinity for downstream effector proteins. Mutational analysis within and surrounding the switch I region has identified amino acid substitutions that destroy the ability of Ras-GTP to signal, but do not alter GTP-binding, Ras stability, or plasma membrane localization. These mutations define the residues that are required for the binding and activation of all effectors and have been generally defined as the core effector domain (residues 32-40) (Marshall, 1996; McCormick and Wittinghofer, 1996; Nassar *et al.*, 1995). Mutations in the switch II region have identified residues that are important for binding of neurofibromin and PI3K (Moodie *et al.*, 1995).

A Ras effector is defined as a protein that has strong preferential selectivity for Ras-GTP versus Ras-GDP and whose binding is affected by mutations in the core effector domain. Many potential Ras effector proteins have been isolated through identification of Ras-binding domains. However, this feature alone is insufficient to characterize a protein as a Ras effector. Several other criteria are also used to determine whether a Ras-binding protein functions as a Ras effector. First, binding of endogenous Ras to the full-length protein must be validated. Measurement of affinity *in vitro* utilizing Ras-binding domains may not reflect what happens *in vivo* near the plasma membrane

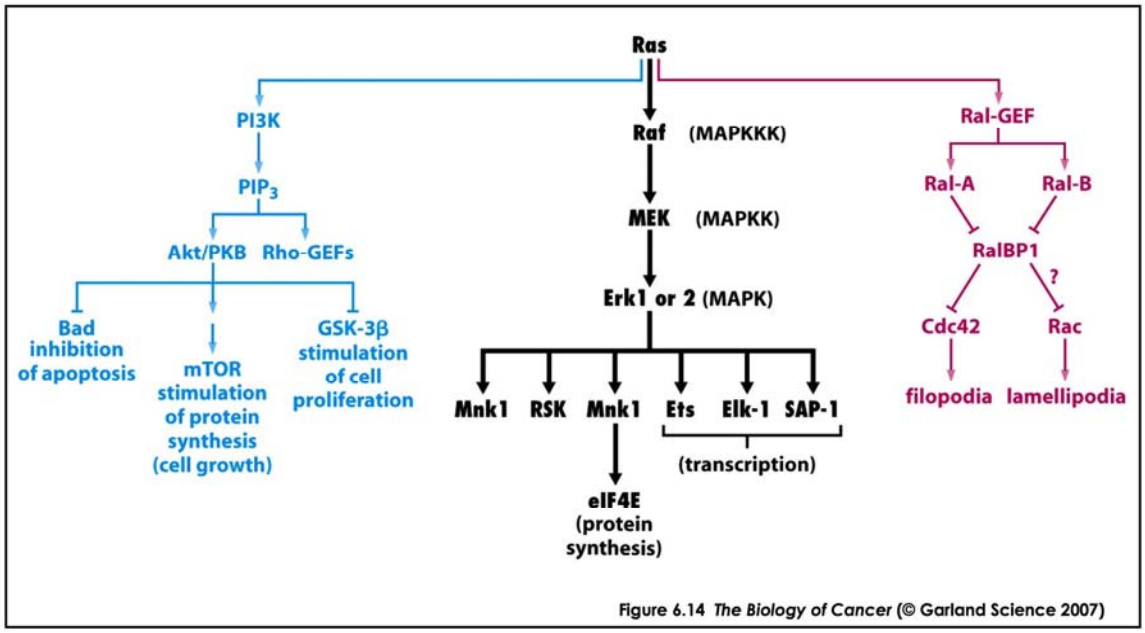

Figure 4 – Downstream Ras effector pathways*. The three major Ras effector pathways are Raf, PI3K and Ral-GEF (of which RalGDS is a member). After the effectors bind to Ras and are activated, they set off a series of downstream signaling cascades that together contribute to the sum total of activated Ras phenotype. Though they act in concert to transduce the Ras signal, each effector pathway has its own unique contribution to Ras activity. PTEN and other components of the PI3K pathway and BRAF in the MAPK cascade are commonly altered by mutations in human cancers.*

and in a particular ionic environment. Immunoprecipitation and immunofluorescence in the context of activated Ras are useful for demonstrating *in vivo* binding (Hallberg *et al.*, 1994). Second, interaction with Ras-GTP should modulate the function of the effector either by localizing it to the membrane, causing allosteric modification, or stimulating formation of a complex. Lastly, the effector protein should regulate a Ras-regulated phenotype. Approaches that have been helpful for defining the roles of Ras effectors include pharmacologic inhibitors, mutations within the Ras binding domain or the effector RBD, knockouts of effectors, or siRNA (Marshall, 1996; Repasky *et al.*, 2004). Through use of these criteria, there have been at least 10 functional classes of Ras effectors identified. However, three of the classes, the Raf, PI3K and RalGDS effector pathways are the major effectors involved in Ras signaling and Ras-mediated transformation and are understood best (**Fig. 4**) (Shields *et al.*, 2000). These pathways are discussed individually in the following sections.

Raf and the Raf/MEK/ERK Pathway

The family of Raf serine/threonine kinases was the first downstream effector pathway of Ras to be identified and is also the most well-characterized and best understood in terms of its interaction with Ras and biological function. Activated Ras recruits Raf to the membrane and partially activates it. Full activation of Raf requires phosphorylation at specific sites in the N-region, C-terminus, and activation segments. Activation of Raf leads to phophorylation/activation of MEK and then subsequently ERK which can then translocate to the nucleus and influence expression of genes that regulate proliferation, survival, and cytoskeletal changes (**Fig. 5a**) (Mercer and Pritchard, 2003).

While A-Raf and Raf-1 mutations are rare in cancer, somatic *BRAF* mutations are common in melanoma, thyroid, colorectal, and ovarian cancers (Wellbrock *et al.*, 2004). B-Raf has strongly elevated basal kinase activity compared with the other Raf isoforms because of its different structure and that is thought to prime B-Raf so that single point mutations are able to constitutively stimulate its kinase activity. Expression of B-Raf^{V600E} activates MEK/ERK signaling to similar levels as oncogenic Ras and can transform rodent fibroblasts and melanocytes (Repasky *et al.*, 2004). The Raf-MEK-ERK cascade, which is also known as the classical mitogen activated protein kinase (MAPK) pathway, has also been characterized by several groups as being sufficient and necessary for Ras-induced transformation of murine cell lines thereby emphasizing its importance in Ras-mediated oncogenesis (Karnoub and Weinberg, 2008).

Phosphatidylinositol 3-Kinase

PI3Ks are heterodimers consisting of a p85 regulatory subunit and p110 catalytic subunit. Upon receptor tyrosine kinase or Ras activation, PI3K becomes activated through localization to the plasma membrane and relief of an inhibitory p85-p110 interaction. There, it catalyzes the conversion of PIP_2 to the second messenger PIP_3, which functions as a ligand for PH-domain containing proteins of which the most prominent is Akt (**Fig. 5b**). When Akt is recruited to the plasma membrane by PIP_3, it becomes activated through phosphorylation on T308 and S473 by PDK1 and the mTOR-Rictor complex respectively. Phophorylated Akt is the most important known mediator of PI3K's effects on survival through inhibition of the apoptotic pathway, increased proliferation through cyclin D1, and increased cell growth through mTOR (Vivanco and

Sawyers, 2002). Perhaps not surprisingly, activating mutations in the p110 catalytic subunit of PI3K have been found in colorectal and breast cancer (Bader *et al.*, 2005). In addition, mutations and deletions of *PTEN*, which encodes a negative regulator of PI3K, occur in 30-40% of all human cancers, reflecting the importance of this pathway in tumor formation (Downward, 2003).

Ral and RalGDS

Like Ras, RalA and RalB are small GTPases that cycle between their inactive GDP-bound state and active GTP-bound state. Cycling between these two states is facilitated by RalGEFs which promote the dissociation of GDP and allow for passive binding of GTP, and RalGAPs which accelerate hydrolysis of GTP to GDP. One of the RalGEFs is Ral-GDP Dissociation Stimulator (RalGDS). When Ras is activated, RalGDS is recruited to the membrane through its Ras association (RA) domain and interacts with membrane-bound Ral-GDP to induce its activation. Ras-induced activation of PI3K leads to recruitment of PDK1 to the membrane which then functions to relieve N-terminal auto-inhibition of RalGDS (**Fig. 5c**) (Feig, 2003). RalA plays a major role in secretory vesicle trafficking through the exocyst and can also regulate gene expression and protein translation. It has also recently been shown to be a target of the tumor suppressor PP2A. RalB also plays a role in exocyst formation, facilitates host immune defense responses, and may contribute to cell survival (Bodemann and White, 2008). Large genome-wide studies of human cancer have only found a few *Ral-GEF* mutations and it is not yet known whether these are functionally relevant or represent "passenger" mutations that do not contribute to tumorigenesis (Bodemann and White, 2008). However, the role of Ral

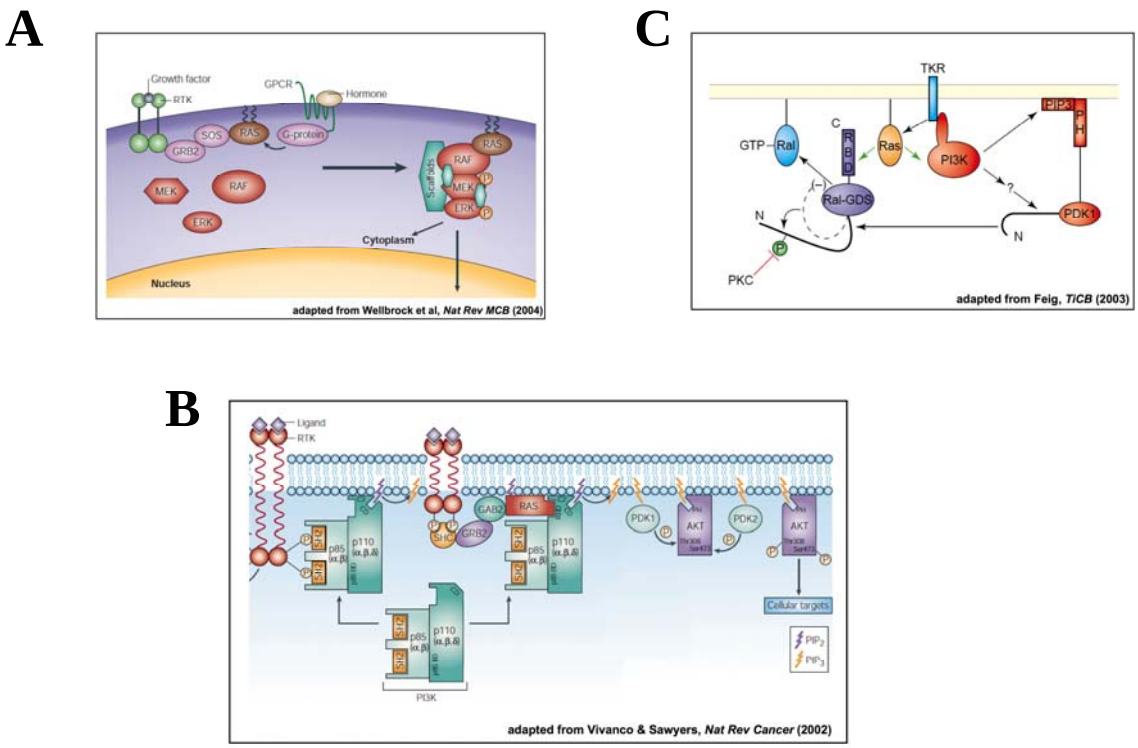

Figure 5 – Ras activation of downstream effectors. ***(A)*** *Raf/MEK/ERK pathway. Stimulation of receptor tyrosine kinases or G-protein coupled receptors leads to activation of Ras. This in turn initiates membrane recruitment and activation of Raf, which in turn activates MEK, and MEK subsequently activates ERK. ERK has a variety of substrates in both the cytosol and nucleus. The efficiency of this activation cascade is facilitated by scaffold proteins, which stabilize and coordinate assembly of the signaling complexes.* ***(B)*** *PI3K pathway. Upon activation of RTKs, the SH2 domains of p85 bind to phosphotyrosine residues and localize PI3K to the surface as well as relieve p85's inhibition of p110. Alternatively, binding of PI3K to Ras leads to membrane localization and activation of the kinase domain. Once at the cell membrane, PI3K converts PIP_2 to PIP_3. Akt becomes localized to the membrane upon binding to PIP_3 via its PH domain and is subsequently activated through phosphorylation by PDK1 and PDK2 (now identified as the mTOR-Rictor complex). Activation of Akt leads to regulation of cellular targets that result in cell growth, survival, and proliferation.* ***(C)*** *RalGDS pathway. Activated Ras binds to and localizes RalGDS, a Ral-GEF, to its target Ral in the plasma membrane. Alternatively, the activation of PI3K promotes binding of PDK1 to the N-terminus of RalGDS and relieves the inhibitory effect of the N-terminus on the catalytic domain of RalGDS. Ral-GTP plays a major role in the exocyst pathway as well as regulates gene expression and protein translation*

in Ras-mediated oncogenesis is supported by studies showing that expression of dominant-negative *RalA* impaired Ras-mediated transformation and co-expression of *RalGDS* with activated *Raf* induced synergistic focus formation (Repasky *et al.*, 2004). In addition, Ral was shown to play a crucial role in Ras-driven transformation of immortalized primary human fibroblasts (Hamad *et al.*, 2002).

Approaches for Assessing the Contributions of Individual Effectors to Ras-Induced Transformation

Based on the high prevalence of *RAS* mutations in many different cancers and evidence that oncogenic Ras plays an essential role in tumor maintenance (Chin *et al.*, 1999), it is not surprising that many groups are attempting to develop anti-cancer therapeutics to reverse the effects of hyperactive Ras. Rational targeting of Ras requires a comprehensive understanding of how Ras functions in normal cells as well as how it is deregulated in cancer. Over time, immense knowledge has been gained in terms of Ras biochemistry, signal transduction, and specific mechanisms by which it regulates cell growth. However, even with all these advances, it has proved extremely difficult to implement potent, effective anti-Ras therapeutic strategies. Since Ras binds to GTP with high affinity, it is unlikely that competitive analogs could be used to selectively displace GTP in oncogenic mutants. Early studies therefore focused upon correcting the defective oncogenic Ras protein by restoring its GTPase activity or hydrolyzing GTP through an alternate method. Screens for small molecules that can facilitate hydrolysis of GTP bound to Ras failed to identify any compounds (Rodriguez-Viciana *et al.*, 2005). This was perhaps not surprising given the structural constraints of the Ras-GTP-GAP interface

near the P-loop and the need to restore GTPase activity rather than to inhibit an overactive enzyme.

Since oncogenic Ras is an exceptionally challenging biochemical target for drug discovery, attention has turned to indirect approaches that do not involve restoring GTPase activity. Post-translational modifications of Ras and subsequent localization to the plasma membrane are essential for Ras-mediated transformation (Kohl *et al.*, 1993; Kohl *et al.*, 1995). As a result, much work has been done in developing farnesyl-transferase inhibitors (FTIs) as a way of inhibiting Ras. However, it was quickly discovered that both K-Ras and N-Ras, the isoforms that are mutated in most cancers, have an alternate method of post-translational processing through geranylgeranyl transferase and are therefore unaffected by FTIs (Whyte *et al.*, 1997). With the initial failure of targeting Ras by targeting the post-translational modification pathway, attention has shifted to blocking downstream effectors.

To determine which effectors to design targeted therapeutics against, it is important to understand the role each plays in Ras signaling and to identify effectors that are integral to the malignant phenotype. In addition, oncogenic Ras-driven malignancies are often characterized by a wide range of co-morbidities that may originate from the effects of hyperactivated Ras. For example, in Ras-driven myeloid malignancy, there is concomittant severe anemia that contributes to morbidity. Interestingly, the loss of functional red blood cells in *Kras* mutant bone marrow is not due to crowding of the niche by hyperproliferative leukocytes, but by an erythroid differentiation block (Braun *et al.*, 2006; Zhang *et al.*, 2007). *In vitro* studies have implicated deregulated MAPK signaling as the downstream effector pathway that mediates the differentiation arrests

(Zhang and Lodish, 2004). Inhibitors of Raf and MEK may therefore prove useful not only for treatment of the myeloid malignancy, but also for correcting anemia.

There are two broad categories of strategies that we have considered to investigate the contribution of individual effectors. The "addition" strategy consists of attempting to recapitulate the oncogenic Ras phenotype by expressing activated forms of effector proteins in cells with wild-type (WT) Ras. Effectors that can confer part or all of the oncogenic Ras phenotype are presumably the most important for Ras-induced transformation. Initial studies with B-Raf^{V600E} showed that it could constitutively activate the MAPK pathway and induce transformation mostly in the absence of *RAS* mutations, thereby implicating its importance in carcinogenesis (Davies *et al.*, 2002; Wan *et al.*, 2004). The disadvantage of this approach stems from the ability of Ras to activate multiple pathways simultaneously, which raises the possibility that many different effector proteins and their isoforms are required for transformation. For example, B-Raf^{V600E} has 50-fold lower transforming activity than oncogenic H-Ras (Davies *et al.*, 2002). Also, inherent problems using over-expression systems to study signaling in primary cells and difficulties in modulating the signaling strength of mutant effectors to the same level achieved by oncogenic Ras makes it difficult to compare the biologic effects of these mutations.

In the "subtraction" strategy, the activation of single effectors or subsets of effectors are abrogated to investigate the effects on Ras-induced transformation. One way to do this is to engineer strains of mice in which a particular effector is knocked out and either use chemical metagenesis to induce *Ras* mutations or intercross them with *Ras* mutant mice. Studies utilizing dimethylbenzanthracene (DBMA) and TPA to induce *Hras*

mutations (Quintanilla *et al.*, 1986) in the skin of *RalGDS* knockout mice showed that RalGDS plays a substantial role in skin tumor formation (Gonzalez-Garcia *et al.*, 2005). However, it is unclear whether the effects of these deletions are specific for Ras or just chemical-induced carcinogenesis in general. Also, this method is only feasible to study *Hras*, not *Kras* or *Nras*. Crossing different oncogenic Ras mice with mice deficient for certain effectors can allow for the investigation of multiple Ras isoforms in a more genetically defined system. However, this strategy is labor-intensive. In addition, such approaches are limited by the early lethality of mice deleted for important Ras effectors such as p110α, p110β, B-Raf, and Raf-1 (Vanhaesebroeck *et al.*, 2005; Wellbrock *et al.*, 2004).

Another strategy for investigating the role of specific effectors in Ras-induced tumor formation and maintenance is to use pharmacologic inhibitors or siRNA to inhibit a particular pathway in cells with oncogenic Ras. The advantage of this general approach is the ability to quickly assay the effects of effector inhibition on Ras-mediated transformation *in vitro* and then translate that to *in vivo* studies. If a pharmacologic inhibitor is promising, then it can easily be scaled up to clinical application. For example, use of a Raf inhibitor blocked proliferation and xenograft tumor formation by cell lines with *KRAS* mutations, showing the importance of the MAPK pathway for Ras-mediated transformation (Wilhelm *et al.*, 2004). However, one caveat is the difficulty in finding a siRNA or inhibitor that has very specific inhibition of the effector in question without off-target effects. In the case of siRNA it necessitates creation of many different vectors and with inhibitors it necessitates scaling the dose in a manner such that phenotypic effects arise from target effector inhibition and not inhibition of other substrates.

Additionally, unlike the genetic knockout where there is loss of the effector protein, each siRNA or inhibitor has varying degrees of inhibition and therefore definitive interpretation of the results can be more difficult.

A final approach is to use second site mutants, which are partial loss-of-function mutant Ras proteins that can bind and activate certain subsets of effectors, but not others. A distinct advantage of this strategy is that the second site mutation is on a specific Ras isoform and does not alter the function of normal Ras proteins in the cell. By contrast, inhibiting effectors through other methods affect the normal Ras signaling as well as signaling through Ras-related proteins. Another advantage is that second site mutant Ras proteins are processed, targeted to subcellular compartments, and activated in the same temporal and spatial manner as oncogenic Ras. They also have similar output signal strength to effectors that are unaffected by the second site substitution. One drawback of this approach is that these secondary mutations may not have "all-or-none" effects. Effectors that retain the ability to bind may have diminished signal and others may bind weakly while still contributing to the overall Ras signal. However, because of the advantages that second site mutants offered in investigating Ras-specific activation of effectors, we decided to use this approach to dissect apart downstream effectors necessary for Ras-mediated transformation.

Second Site Mutants

Second site mutants were initially isolated from a yeast-two hybrid screen designed to identify mutations in H-Ras that could separate its ability to interact with Raf-1 from its ability to interact with byr2, a putative serine/threonine protein kinase. H-

Ras was randomly mutated by PCR, fused to a GAL4 DNA-binding domain and then screened for interactions with either a byr2-GAL4 activating domain (GAD) or Raf-1-GAD. Mutants that failed to bind were then screened for those that retained ability to interact with the other GAD. Of these, H-Ras (T35S) retained ability to interact with Raf1 but not byr2 while H-Ras (E37G) interacted with byr2 but not Raf-1. After the G12V mutation was introduced into these mutant proteins, they were then used to investigate the function of these different effector pathways in the context of oncogenic Ras in different cell types (White *et al.*, 1995). Soon after, work from Julian Downward's lab validated and expanded the number of second site mutants. They isolated GST-fusion proteins of full-length PI3K, and the Ras-binding sites of Raf-1 and RalGDS and screened different H-Ras^{G12V} second site mutants for their ability to interact with these downstream effectors. While many retained the ability to interact with all three effector pathways or none at all, some were able to preferentially bind to one effector while failing to bind to the other two. As in yeast, T35S could bind Raf-1 but not the RalGDS or PI3K. E37G was also isolated in this screen and shown to interact solely with RalGDS. In addition, two other mutations D38E and Y40C were shown to interact with solely Raf-1 or PI3K respectively (Rodriguez-Viciana *et al.*, 1997).

Co-crystallization of Ras with Raf, PI3K and RalGDS has led to a model describing how second site mutants retain the capacity to bind to one effector while preventing interaction with other effectors. Although all three Ras effectors utilize similar Ras-RBD intermolecular β-sheet interactions, Ras can discriminate between effectors by rotating with respect to the RBD of a given effector (**Fig. 6**) (Pacold *et al.*, 2000). Therefore, different residues within the effector binding domain carry varying degrees of

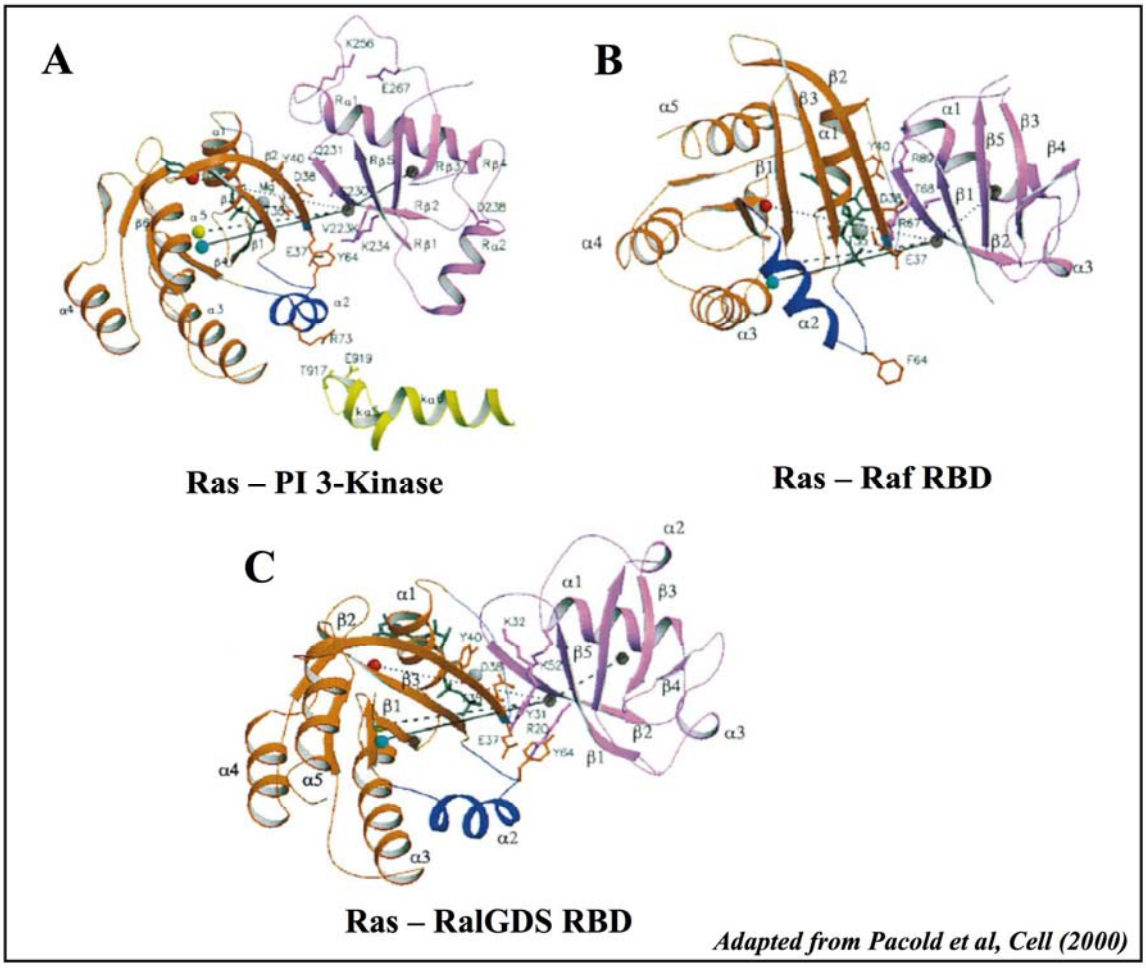

Figure 6 – Comparisons of Ras Orientations in complex with PI3K, Raf, and RalGDS. *Ribbon plots showing how Ras-GTP interacts with different effectors. Lines are traced from the centroid of the RBD to Cα of Threonine-232 in PI3K and its equivalent in other RBDs, then to centroid of Ras bound to* ***(A)*** *PI3K (cyan sphere, solid line),* ***(B)*** *Raf (red sphere, dotted line), and* ***(C)*** *RalGDS (yellow sphere, dashed line). Ras bound to PI3K is rotated 35^{o} relative to the centroid of Ras-Raf and 17^{o} relative to Ras-RalGDS.*

importance for each individual effector interaction. Second site mutants exploit this by allowing for selective binding of certain effectors while preventing binding of others. For example, Tyrosine-40 of Ras helps stabilize the RBD of Raf-1 and RalGDS to form a critical salt bridge with D38. However, it does not form any meaningful contact with PI3K. As a result, changing this Tyrosine-40 to cysteine in the Y40C mutation disrupts binding to Raf and RalGDS but not PI3K (Pacold *et al.*, 2000; Vetter *et al.*, 1999).

Since the second site mutations are within the effector binding domain, it is possible that some effectors bind with reduced affinity. In fact, use of scintillation proximity assay to determine binding strength between second site mutants and their effectors has shown that binding strength is somewhat diminished compared with the binding of H-Ras^{G12V} to effectors (Rodriguez-Viciana *et al.*, 1997). However, quantification of downstream effector activation levels has shown that second site mutants activate them to levels comparable to H-Ras^{G12V} (Oliva *et al.*, 2004) indicating that oncogenic Ras signaling is still transduced with similar strength.

Data from these second site mutants have helped elucidate the functions of specific effectors in Ras-driven transformation of cultured cell lines and primary human fibroblasts. These studies have shown that, in general, second site mutants alone are unable to cause transformation in 3T3 cells as assayed by focus forming assays. However, complementation of these mutants with other second site mutants or complementary effector pathways restored the ability to form foci; showing that the effects of oncogenic Ras are likely mediated by all three effector pathways (Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1996; Yang *et al.*, 1998). The Counter laboratory also made similar observations when they isolated tumorigenic human

cells immortalized with viral oncogenes and the catalytic subunit of telomerase, transduced with second site mutants, and implanted them into immunodeficient mice. All three effector pathways were required for tumor initiation while only PI3K was important for tumor maintenance (Lim and Counter, 2005). Interestingly, the importance of different effectors in transformation appears to be cell-type specific. In NIH 3T3 cells, the ability of T35S to induce some anchorage-independent growth indicated the possible importance of Raf-1 for Ras-driven transformation in murine cells. In contrast it was found that E37G, but not T35S, could transform immortalized human cells, thereby implicating the role of RalGDS in Ras-driven transformation of human cells (Hamad *et al.*, 2002; Rangarajan *et al.*, 2004).

While these studies provided new insights into the importance of the major effectors for Ras-driven tumorigenesis, they have been limited by the cell types, constructs and assays used. First, there is the uncertain relevance of immortalized cells as experimental models. Many of the studies were done in immortalized cells lines that have acquired mutations while in culture. Those that were performed in human primary cells required immortalization through co-transfection of T-Ag, t-Ag, and hTERT. With these underlying lesions, it is difficult to draw conclusions about how Ras and its effectors themselves initiate and drive tumorigenesis. Second, as most Ras-driven diseases are found in epithelial and hematopoietic cells, it is unclear how relevant fibroblast data is to human oncogenesis. For example, in 3T3 cells, oncogenic Ras transformation of breast epithelial cells results in destruction of adherens junctions and appearance of stress fibers which contrasts the loss of stress fibers seen in Ras-transformed fibroblasts (Shields *et al.*, 2000). Even within different 3T3 cells, there is variation in which effectors are

important for Ras-driven tumorigenesis (Hamad *et al.*, 2002; Khosravi-Far *et al.*, 1996). Therefore, the mechanism and degree to which Ras causes transformation may be very species and tissue specific. Third, many of the studies utilize oncogenic H-Ras second site mutant proteins. However, *HRAS* mutations are relatively uncommon in human cancer relative to *KRAS* and *NRAS* mutations. In addition, comparisons between the oncogenic mutations of the four isoforms show distinct differences in their ability to form foci, induce anchorage independent growth, and even to activate different effectors (Oliva *et al.*, 2004; Voice *et al.*, 1999). Therefore, it is not clear if the results of studies of H-Ras can be applied to other Ras proteins. Lastly, focus forming and tumor formation assays are relatively stringent assays that may not detect subtle or intermediate biologic effects.

In summary, though second site mutants have proven useful for examining the role of effectors in Ras oncogenesis, much of the work has been done using H-Ras and in immortalized cells or cells that are not commonly transformed with oncogenic Ras in human cancers. Additionally, the readout of each effector's ability to contribute to Ras-driven disease has commonly been in stringent focus forming or tumor formation assays. In order to gain a more complete picture of the role of effectors in oncogenic Ras transformation, we decided to study the K-Ras isoform in physiologically relevant cell types that model human Ras-driven disease and with assays that are sensitive to subtler phenotypes.

Role of Ras Effectors in Hematopoietic Malignancy

Observations in a strain of mice in which oncogenic *Kras*^{G12D} is expressed from its endogenous promoter provided a starting point for the work in this thesis. In this system, injecting *Mx1-Cre, LSL-Kras*^{G12D} mice with pIpC induces expression of the *Mx1-Cre* transgene in hematopoietic cells, which results in Cre recombinase expression, excision of an inhibitory lox-stop-lox (LSL) cassette, and *Kras*^{G12D} expression. *Mx1-Cre*, *LSL-Kras*^{G12D} mice that are injected with pIpC developed a rapidly fatal MPD with leukocytosis, hepatosplenomegaly, and anemia. When bone marrow cells were plated on methylcellulose and assayed for formation of colony-forming units granulocyte-macrophage (CFU-GM) in myeloid progenitors in response to increasing doses of granulocyte macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3), it was found that K-Ras^{G12D} conferred cytokine-independent growth and hypersensitivity to growth factor (Braun *et al.*, 2004). The growth factor hypersensitivity, leukocytosis, anemia and nonhematopoietic tissue infiltration are very reminiscient of what is seen in human juvenile myelomonocytic leukemia (JMML) (Emanuel, 2008).

JMML is a myeloid malignancy in young children characterized by overproduction of infiltrative myeloid lineage cells (Lauchle *et al.*, 2006). Mutational analysis of JMML has uncovered Ras pathway mutations in over 75% of cases and a significant number of *NRAS* and *KRAS* mutations (Emanuel, 2004; Flotho *et al.*, 1999). The median survival is less than one year without transplant and up to 30% of patients progress to AML (Lauchle *et al.*, 2006). The only curative treatment is allogeneic stem cell transplant, which is accompanied by an unacceptably high 35-40% relapse rate (Emanuel, 2008). In order to develop more effective treatments, it is important to

understand how Ras and its downstream effectors are driving malignant growth and the role that each effector plays in leukemogenesis.

The striking difference between the growth of WT and $Kras^{G12D}$ myeloid progenitors in methylcellulose cultures containing GM-CSF provides a robust system for scoring intermediate effects of second site mutations in primary cells. In addition, the use of a range of growth factor concentrations and the ability to look qualitatively at the character of each colony makes this a sensitive assay to look for subtle effects of abrogating each effector pathway. In Chapter 2 we describe the transduction of primary hematopoietic cells with $Kras^{G12D}$ vectors encoding a variety of second site mutations, and compare each substitution with both WT *Kras* and $Kras^{G12D}$. We show that two mutants, $Kras^{G12D,E37G}$ and $Kras^{G12D,Y64G}$ maintain the growth factor hypersensitivity phenotype of $Kras^{G12D}$ colonies but do not recapitulate cytokine independent growth. Biochemical analysis of the second site mutants showed that both K-$Ras^{G12D,E37G}$ (E37G) and K-$Ras^{G12D,Y64G}$ (Y64G) activate two of the three major effector pathways, RalGDS/PI3K and Raf/RalGDS respectively, whereas other second site mutations that lack activity in this assay only activate one pathway. This implies that it takes activation of at least two pathways to maintain part of Ras' oncogenic phenotype.

In Chapter 3 we used adoptive transfer experiments to investigate whether these second site mutants retained sufficient transformation potential to cause hematologic disease *in vivo*. With both E37G and Y64G, we saw evolution of a transplantable, fatal T-ALL that originated from the thymus. All the malignant cells were GFP-positive indicating that it was the presence of these constructs that was driving disease. However, even though E37G and Y64G activate different pathways, the T-ALL that arose from

them were indistinguishable from each other. In Chapter 4, we further investigate the clonality and signaling within the different tumors. We found that both second site mutants typically induce monoclonal disease that has acquired multiple secondary clonal integrations. All of the T-ALLs show increased levels of cleaved Notch, which is mutated in many human T-ALLs. Interestingly, whereas most of the E37G tumors upregulate Ras protein levels, this is not consistently associated with activation of MAP kinase signaling. By contrast, the Y64G tumors show a marked reduction in PTEN and elevated levels of phosphorylated Akt, which is consistent with an essential role of this pathway in T lineage leukemogenesis. An important conclusion of these experiments that has therapeutic implications is that abrogating a single major effector pathway downstream of oncogenic Ras is unlikely to completely reverse the growth advantage conferred by oncogenic Ras.

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Chapter 2: Effects of Second Site Mutations on Myeloid Progenitor Colony Growth

Introduction

Somatic *RAS* mutations are common in myeloid malignancies (Downward, 2003). *NRAS* and *KRAS2* mutations are prevalent in chronic myelomonocytic leukemia (CMML) (~40% of cases) (Onida *et al.*, 2002), juvenile myelomonocytic leukemia (JMML) and acute myeloid leukemia (AML) (~25% of cases) (Kalra *et al.*, 1994; Miyauchi *et al.*, 1994). *NRAS* mutations account for ~70% of *RAS* mutations found in myeloid malignancies with *KRAS* mutations found in the other 30%. The most frequent point mutation found in myeloid malignancies with *RAS* mutations is a glycine to asparatic acid (G12D) substitution at codon 12 (Bacher *et al.*, 2006).

To investigate the effects of oncogenic *Ras* expression *in vivo,* our laboratory mated mice with a conditional *LSL-Kras*^{G12D} allele to *Mx1-Cre* transgenic mice and injected the progeny with pIpC to induce Cre recombinase production and excise the LoxP-STOP-LoxP element. This results in *Kras*^{G12D} expression from the endogenous locus in the hematopoietic compartment. Mice expressing *Kras*^{G12D} exhibited leukocytosis, anemia, hepatosplenomegaly and ultimately died from a fatal myeloproliferative disease (MPD) that models JMML and CMML. Bone marrow cells that were plated in methylcellulose media containing increasing concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF) formed colony-forming units granulocyte-macrophage (CFU-GM) in a factor dependent and hypersensitive manner similar to what was seen in the human disease (Braun *et al.*, 2004; Chan *et al.*, 2004). This model provides a platform for interrogating pathogenesis of MPD and for testing new therapeutic strategies. Understanding the roles of different Ras effector pathways in the disease pathogenesis is an important question that has therapeutic

implications. Here we describe how we constructed second site mutants and investigated their biochemical and functional properties. We also present the results of complementary experiments in which we studied activated Ras effector proteins.

Results

Biochemical characterization of second site mutants. *In vitro* binding assays were utilized previously to characterize the ability of second site H-Ras mutant proteins to engage downstream effector pathways [described in (Rodriguez-Viciana *et al.*, 1997)]. A potential limitation of these studies is that they assessed binding to specific effector isoforms, but did not measure the degree or duration of downstream pathway activation. In addition, much of the past work was done using H-Ras^{G12V} whereas we decided to focus on K-Ras^{G12D}, a more common cancer-associated mutant protein. It is therefore possible that differences in H-Ras and K-Ras processing and sub-cellular localization could affect the biologic properties of oncogenic and second site mutant proteins.

Based on these considerations, we first employed an over-expression system to examine effector pathway activation by various K-Ras second site mutant proteins. We first cloned *Kras^{G12D}* and *wt Kras* cDNAs from mouse embryo fibroblasts (MEFs) harvested from *wt Kras* and *Kras^{G12D}* E14.5 embryos into pENTR/D-TOPO vectors. Second site mutants were created by making the specified point mutation off of the *Kras^{G12D}* backbone. We initially created four second site mutants that were previously described to bind and activate only one of the major downstream effector pathways: K-Ras^{G12D,T35S} (T35S) and K-Ras^{G12D,D38E} (D38E) described to bind mainly to Raf-1, K-

Ras^{G12D,E37G} (E37G) mainly to Ral-GDS, and K-Ras^{G12D,Y40C} (Y40C) mainly to p110α (Rodriguez-Viciana *et al.*, 1997).

We transiently transfected *wt Kras*, *Kras^{G12D}*, and each second site mutant into COS-7 monkey kidney cells and assayed the levels of phosphorylated effectors. The level of protein phosphorylation in the starved state was the most informative in differentiating the biochemical effects of WT K-Ras and K-Ras^{G12D}. Interestingly, whereas expression of *Kras^{G12D}* was associated with markedly increased levels of pERK and pMEK in COS-7 cells that were deprived of serum, this was not entirely true of either T35S or D38E, which had been previously reported to selectively activate Raf/MEK/ERK signaling (**Fig. 1a**). Although pMEK levels were higher in cells expressing either second site mutant protein, they were significantly lower than the levels in cells expressing K-Ras^{G12D}. Thus, K-Ras^{G12D,T35S} and K-Ras^{G12D,D38E} are hypomorphic alleles and initial studies have indicated that they do not bind or activate Raf at the same levels as K-Ras^{G12D} (Rodriguez-Viciana *et al.*, 1997; Webb *et al.*, 1998).

E37G was originally shown to only activate the RalGDS pathway (Rodriguez-Viciana *et al.*, 1997). However, serum starved COS-7 cells expressing this protein showed elevated levels of pAkt in addition to elevated RalA-GTP, with no evidence of pMEK activation (**Fig. 1a,c**). This is consistent with another report that the E37G second site mutant enhances both PI3K/Akt and RalGDS signaling through an interaction with the gamma isoform of p110 as opposed to the alpha form which was used in earlier binding assays (Lim and Counter, 2005; Pacold *et al.*, 2000). Y40C activated pAkt to

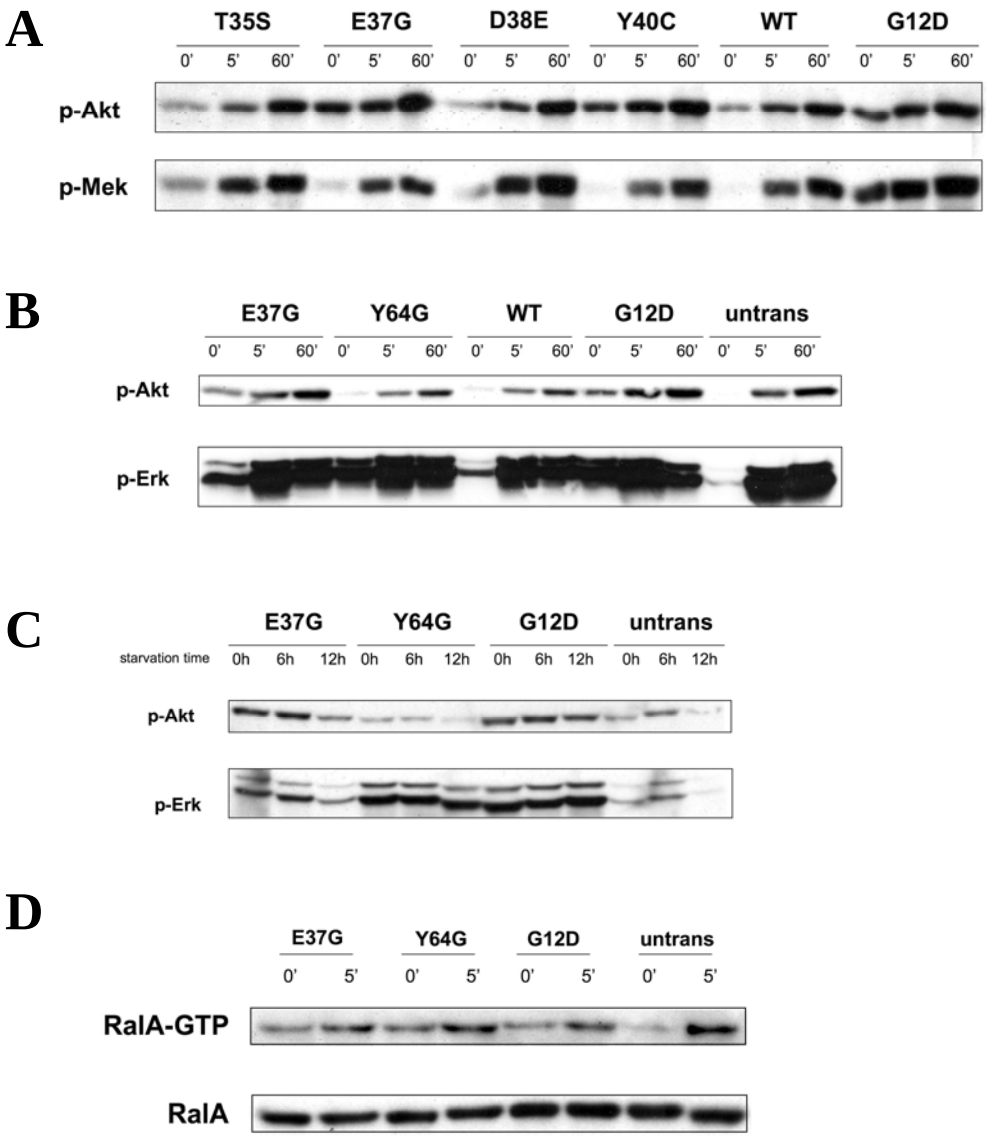


Figure 1 – Biochemical characterization of second site mutants. (A,B) *Activation of Akt and MEK/ERK in COS-7 cells transiently transfected with second site mutants, wild-type Kras or Kras^{G12D}. Cells were starved in 0.1% serum and stimulated with epidermal growth factor (EGF) for 5 or 60 minutes.* ***(C)*** *Maintenance of activated Akt and MEK/ERK activation during starvation. COS-7 cells were transfected and starved as above and then harvested at basal, 6h, and 12h post-starvation.* ***(D)*** *Assay for Ral activation in E37G, Y64G, wild-type or G12D Kras-transfected COS-7 cells. Cells were starved and stimulated as above, pulled down with GST-RalBP1, and blotted with anti-RalA.*

levels similar to that of K-Ras^{G12D} while having no effect on pMEK – consistent with what had been previously described.

With signaling in COS-7 cells showing the ability of E37G to activate the PI3K and RalGDS pathways to a similar extent as K-Ras^{G12D}, we wanted to create a second site mutant that could activate a different combination of two downstream effector pathways. Structural analysis of the p110 component of PI3K co-crystallized with Ras revealed a necessary PI3K binding site within the switch II domain of Ras (Pacold *et al.*, 2000) – something that Raf and RalGDS do not have (Moodie *et al.*, 1995; Pacold *et al.*, 2000). Tyrosine-64 of Ras is an important contact residue that mediates this interaction with PI3K. We synthesized K-Ras$^{G12D, Y64G}$ (Y64G) by changing this tyrosine to glycine. Expressing Y64G in COS-7 cells resulted in normal pAkt levels in COS-7 cells, but pERK and RalA-GTP were elevated (**Fig. 1b,d**). When signaling in COS-7 cells was assayed after 6 hours and 12 hours starvation, the E37G mutant maintained high pAkt levels that were similar to those in G12D-expressing cells whereas pERK levels declined over time. By contrast, the Y64G mutant protein maintained high levels of pERK while pAkt decreased (**Fig. 1c**).

Second site mutants that activate two of three effector pathways confer growth factor hypersensitivity in CFU-GM colony assays. Human JMML cells and bone marrow progenitors from *Mx1-Cre, Kras^{G12D}* mice with MPD myeloid cells show a characteristic *in vitro* phenotype of growth factor independent formation of CFU-GM colonies and hypersensitivity to growth factor when plated in methylcellulose media. Importantly, murine hematopoietic cells transduced with a murine stem cell virus (MSCV)-IRES-

Kras^{G12D}-GFP retroviral vector retain this striking phenotype. The pronounced differences in CFU-GM growth between cells expressing WT *Kras* and *Kras*^{G12D} provides a tractable system for asking if second site mutants maintain any of the abnormal properties of *Kras*^{G12D}. Indeed, this provided a sensitive system to assess the relative potency of germline *KRAS* mutations that underlie human developmental disorders (Schubbert *et al.*, 2006). We infected E14.5 fetal liver cells with MSCV-IRES-GFP retroviruses encoding each mutant K-Ras protein. GFP-positive cells were isolated by sorting and plated in methylcellulose medium over a range of GM-CSF concentrations to assay for growth of CFU-GM.

Whereas the *T35S*, *D38E* and *Y40C* second site mutations all reverted CFU-GM colony growth to wild type levels (**Fig. 2a**), cells expressing *E37G* and *Y64G* showed an intermediate growth phenotype with modest hypersensitivity compared to WT K-Ras, but no cytokine-independent colony growth (**Fig. 2b**). At low concentrations of GM-CSF, fetal liver cells transduced with *E37G* not only formed increased numbers of CFU-GM but also formed larger colonies than wild-type cells at the same concentration of GM-CSF. In the case of Y64G there were more colonies as well, but they were not enlarged when compared with wild type (**Fig. 2c**). Thus, both K-Ras second site mutant proteins that can activate two of three effector pathways are activated relative to WT K-Ras but attenuated compared to K-Ras^{G12D}; while maintaining qualitatively different aspects of CFU-GM formation depending on which effector is activated.

Another assay for characterizing the properties of these mutants is to enumerate burst forming units-erythroid (BFU-E) colony growth at differing concentrations of erythropoietin (EPO). In this system, WT cells require EPO as well as a source of burst-

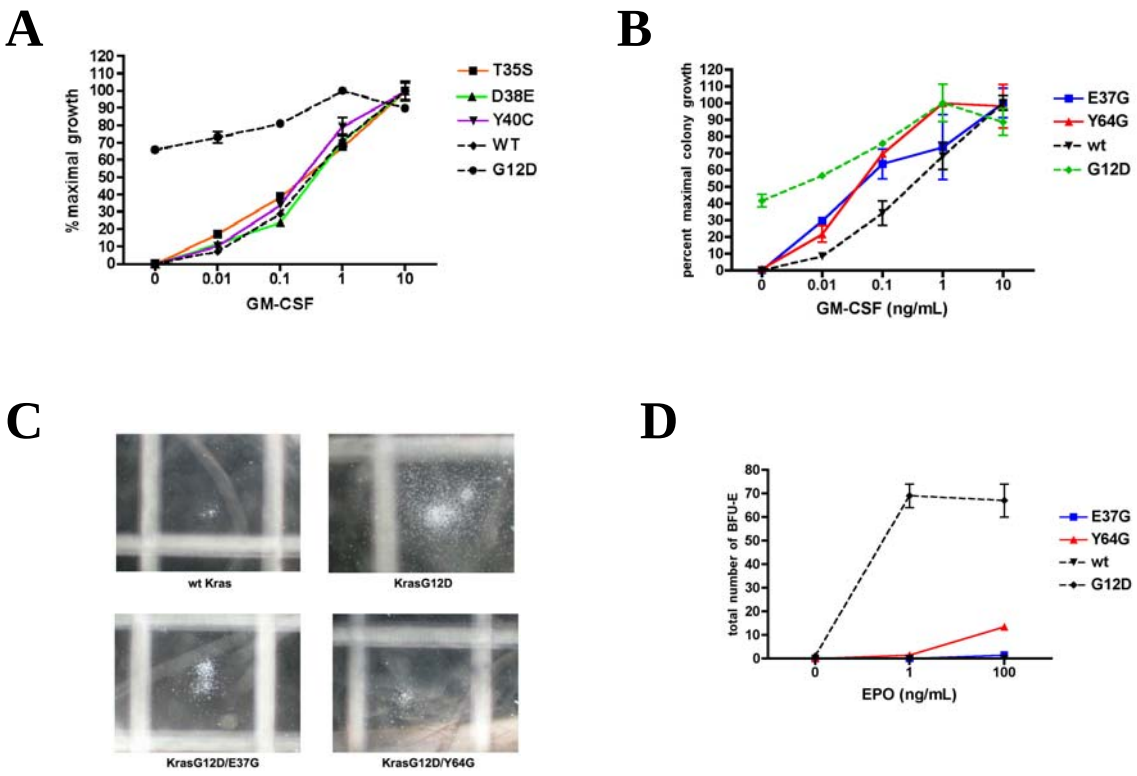


Figure 2 – E37G and Y64G display colony hypersensitivity to GM-CSF.
(A) *Percent maximal CFU-GM colony growth of fetal liver expressing wild-type Kras, T35S, D38E, Y40C or G12D over a range of GM-CSF concentrations. These mutants only activate one of the major effector pathways.* ***(B)*** *Percent maximal CFU-GM colony growth of fetal liver expressing wild-type Kras, E37G, Y64G or G12D over a range of GM-CSF concentrations. These mutants activate two of the three major effector pathways and display hypersensitivity to GM-CSF. Percent maximal growth was used because absolute colonies were similar at saturating GM-CSF.* ***(C)*** *Photomicrographs (original magnification x40) of wild-type, E37G, Y64G and G12D K-Ras myeloid progenitor colonies grown in low dose GM-CSF (0.01 ng/mL). E37G shows increased in colony size with low GM-CSF concentrations.* ***(D)*** *BFU-E colony formation from fetal liver cells expressing wild-type Kras, E37G, Y64G or G12D over a range of EPO concentrations. Addition of EPO increases number of BFU-E colonies in Y64G. (other mutants not shown, but they look like wild type Kras).*

promoting activity (BPA) to form BFU-E colonies. BPA is important in early erythroid progenitor formation and is usually supplied by IL-3 or SCF (Kobayashi *et al.*, 1995). Without a source of BPA, wild-type cells are unable to form BFU-E at any concentration of EPO whereas $Kras^{G12D}$ cells are able to form increasing numbers of BFU-E at increasing concentrations of EPO (Braun *et al.*, 2006). When *E37G* and *Y64G*-transduced fetal liver cells were plated in methylcellulose over differing concentrations of EPO, Y64G cells formed BFU-E at high concentrations of EPO, although not to levels of $Kras^{G12D}$, while E37G did not (**Fig. 2d**). This is consistent with the known role of activated Raf in formation of CFU-E (Zhang and Lodish, 2004).

Signaling of second site mutants in 3T3s and primary myeloid cells. Since we hypothesized that E37G and Y64G confer growth factor hypersensitivity because they retained the ability to activate two of the major Ras downstream effector pathways, we wanted to characterize their signaling in other contexts. We first infected 3T3 fibroblasts with MIG retroviral vectors encoding *wt Kras*, $Kras^{G12D}$, and second site mutant, grew the cells out, and assayed signaling after serum starvation. Consistent with what was seen in the COS-7 cells, 3T cells transduced with *E37G* showed maintenance of pAkt at levels similar to G12D while pErk levels were unelevated. *Y64G*-transduced 3T3s showed maintenance of high pErk levels during starvation while pAkt remained similar to wild-type (**Fig. 3a**). In addition, 3T3 cells transduced with *Y64G* and *G12D* showed morphological changes consistent with Raf/MEK/ERK activation (Yeh *et al.*, 2008) while *E37G*-transduced 3T3 cells were morphologically similar to untransduced 3T3s because of their inability to hyperactivate the Raf/MEK/ERK pathway (**Fig. 3b**).

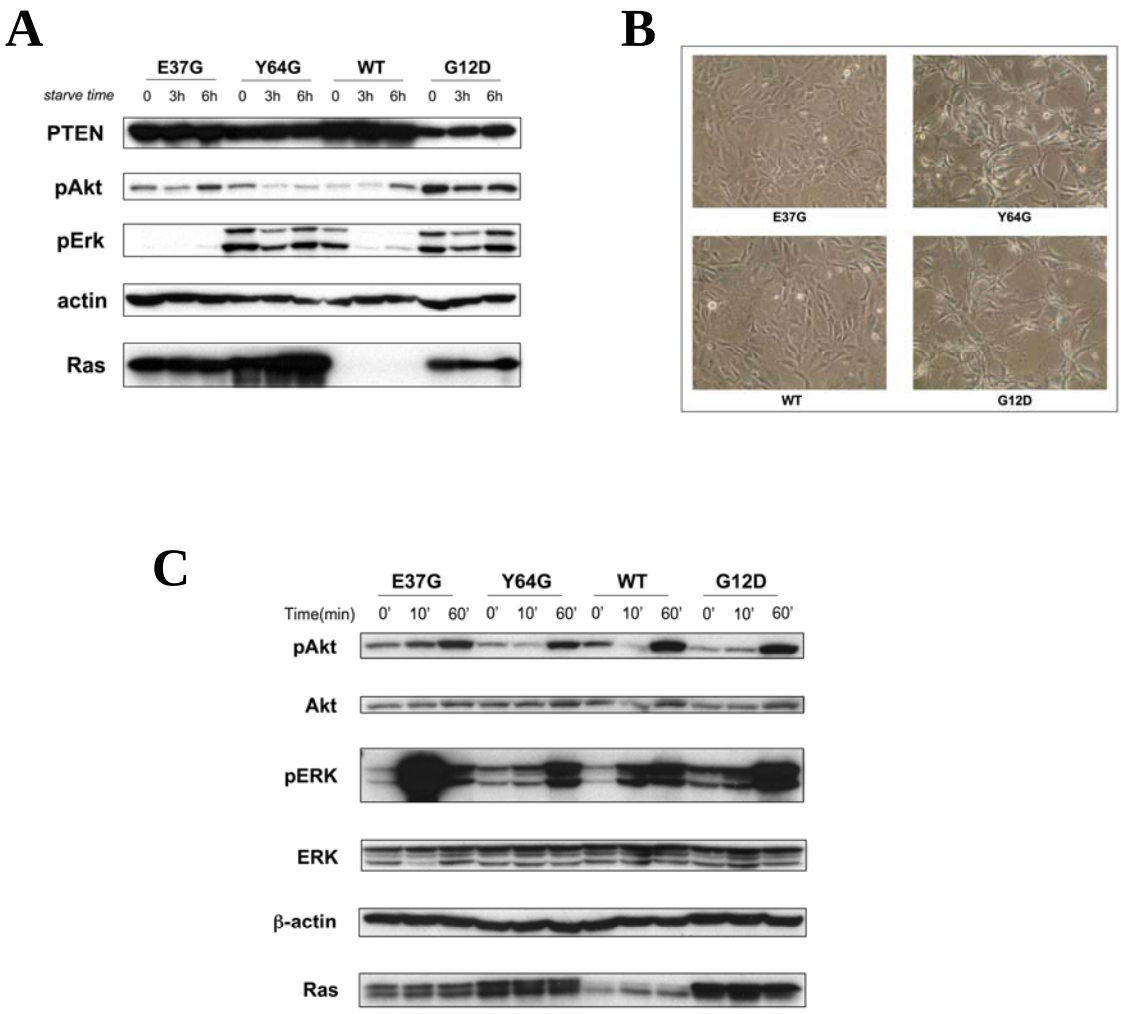


Figure 3 – Second site mutants maintain signaling profiles in 3T3 and primary macrophage progenitors. (A) *3T3 cells were transduced with E37G, Y64G, wt Kras, and G12D using a high titer MIG-virus. After growing out, 3T3 cells were starved for 0, 3, and 6 hours and then lysed to look at activation status of Akt and ERK. E37G-transduced 3T3s maintained increased pAkt levels during starvation and Y64G-transduced 3T3s maintained increased pERK levels similar to that of G12D.* ***(B)*** *Y64G and G12D 3T3 cells exhibit aberrant morphology (magnification – 100x).* ***(C)*** *Macrophage progenitors derived from sorted GFP-positive transduced E14.5 fetal liver cells were starved and stimulated with GM-CSF. Similar to G12D and what was seen before, E37G showed increased pAkt while Y64G showed increased pERK.*

Since much of the signaling characterization of the second site mutants was done in cell lines where other alterations are present, we wanted to see if they maintained their distinct signaling profiles within the context of primary hematopoietic cells. We transduced E14.5 fetal liver cells with *E37G*, *Y64G*, *wt Kras*, and *G12D* and then sorted for GFP. Instead of plating in methylcellulose, we placed the cells in media containing M-CSF and allowed them to differentiate into macrophage progenitors. After starving the cells for 24 hours, we stimulated them with GM-CSF and observed similar effects as in COS-7 and 3T3 cells (**Fig. 3c**). *Y64G*-transduced macrophage progenitors had hyperactivated pERK at levels similar to G12D whereas pERK was not activated in *E37G*-transduced macrophage progenitors. When looking at pAkt levels, the opposite was generally true with E37G showing hyperactivated pAkt but Y64G only with wild-type pAkt levels (**Fig. 3c**). These studies in 3T3 cells and in macrophage progenitors confirm our observations regarding the biochemical properties of the *E37G* and *Y64G* second site substitutions.

Activated effectors alone do not display growth factor hypersensitivity. A complementary method of assessing the contribution of different effectors is to add activated forms of each effector onto a wild-type background. We used B-raf^{V600E}, p110α-CAAX, and RalGDS-CAAX as activated effectors to mimic oncogenic Ras activation of specific pathways. B-Raf^{V600E}, increases downstream ERK kinase activity by 5-fold over wild-type B-Raf but at levels equal to that of H-Ras^{G12V}. Based on signaling and proliferation data, this mutant is thought to mediate its tumorigenic effect in

	Downstream Effector Pathway				
Kras mutants	**MEK/ERK**	**Akt**	**Ral-GDS**	**CFU-GM**	**BFU-E**
none (wt)	+	+	+	+	-
G12D	++++	++++	++++	++++	++++
G12D, T35S	++	+		+	
G12D, E37G	+/++	++++	+++	++/+++	-
G12D, D38E	++	+		+	
G12D, Y40C	+	++++		+	
G12D, Y64G	+++	+	+++	++/+++	++

"-" indicates no activation/growth

"+" indicates level of activation/growth ranging from "+" designating minimal activation and "++++" designating full activation

Table 1 – Summary of Second Site Mutant Data. *Data from second site mutant protein signaling in COS7 cells as well as in vitro hematopoietic colony assays is summarized using wt Kras as the baseline and Kras^{G12D} as the upper limit. Grey boxes indicate that data was not determined.*

a manner similar to oncogenic Ras (Mercer *et al.*, 2005; Wan *et al.*, 2004). p110a-CAAX constitutively targets PI3K to the membrane and leads to activation of Akt to levels similar to that of H-Ras^{G12V} (Bader *et al.*, 2005; Klippel *et al.*, 1996). RalGDS-CAAX was shown to activate RalA at levels similar to that of K-Ras^{G12V} (Matsubara *et al.*, 1999).

We verified signaling of the activated effectors in COS-7 cells. COS-7 cells transfected with *wt Braf* had slightly increased p-ERK signaling, which is most likely due to overexpression of wt B-Raf protein. This is consistent with what has been reported in *wt Braf*-transfected fibroblasts (Rodriguez-Viciana *et al.*, 2006). B-Raf^{V600E} activated pERK to levels similar to that of K-Ras^{G12D} while leaving pAKT activation unchanged. p110-CAAX had elevated levels of pAKT, while pERK levels remained similar to that of wild-type K-Ras (**Fig. 4a**). Lastly, Ral-GDS-CAAX did not elevate levels of either pAkt or pERK, but showed increased levels of RalA-GTP (**Fig. 4b**).

The activated effectors were transduced into E14.5 fetal liver, cells sorted for GFP positivity and plated in CFU-GM colony assays. Each activated effector was transduced separately in order to corroborate what was shown with the second site mutants that activation of only one of the three major effector pathways downstream of K-Ras^{G12D} was insufficient to cause hypersensitive growth. Wild-type B-Raf, p110α-CAAX and Ral-GDS-CAAX responded to increasing GM-CSF in a similar manner as wild-type K-Ras (**Fig. 4c**). This shows that either activated RalGDS or p110α alone does not perturb growth of CFU-GM. Interestingly, B-Raf^{V600E} colonies were hypersensitive at low concentrations of GM-CSF (**Fig. 4c**), but leveled off such that even at saturating

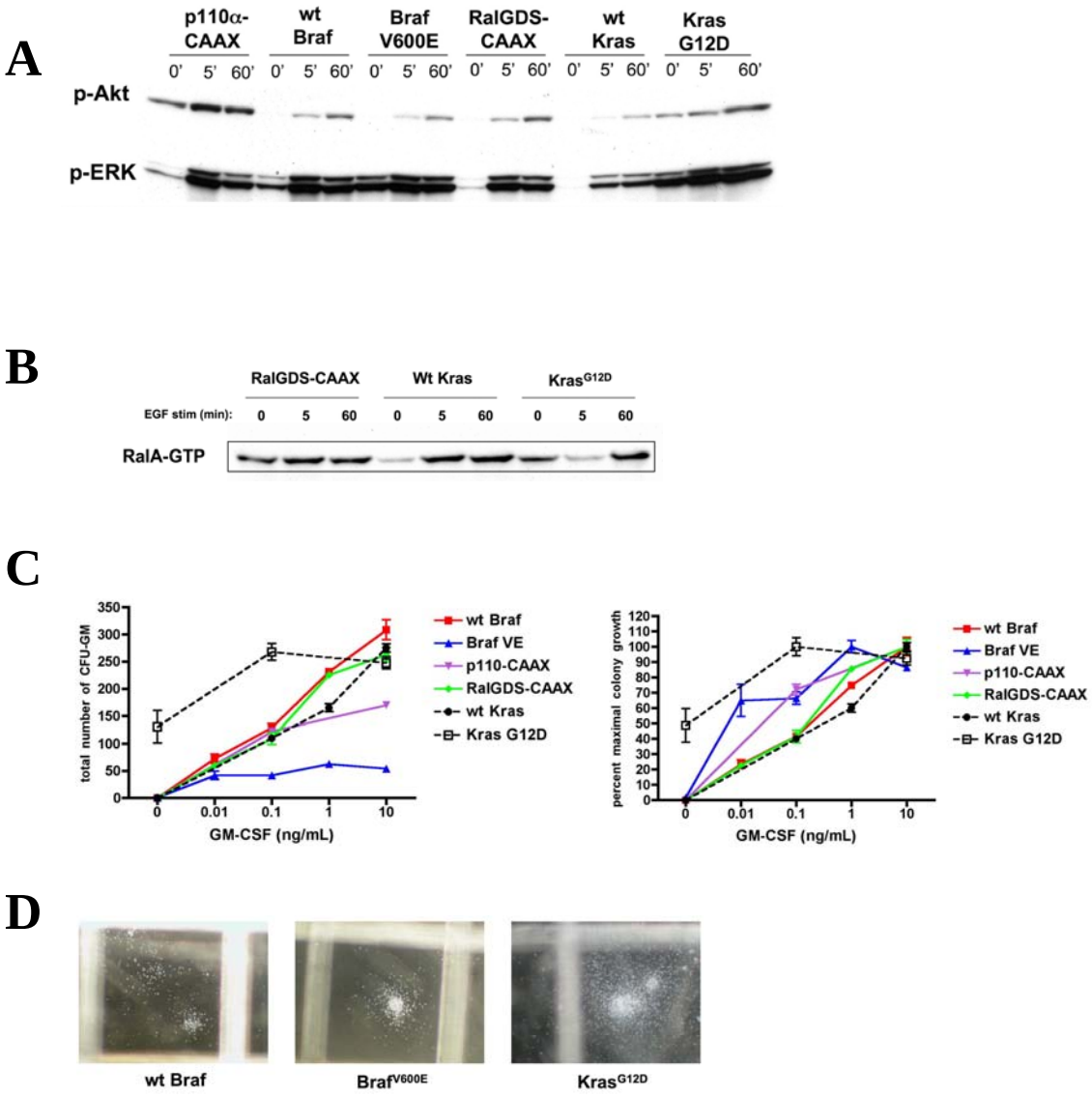


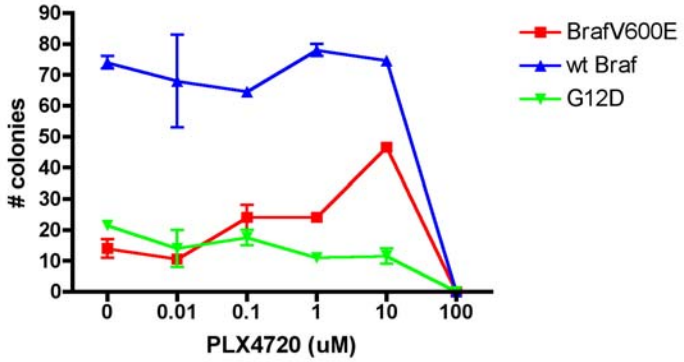
Figure 4 –Activated Effectors alone do not confer hypersensitivity to GM-CSF. ***(A)*** *Activation of Akt and ERK in COS-7 cells transiently transfected with p110α, wild type Braf, Braf^{V600E}, RalGDS-CAAX, wild-type Kras or Kras^{G12D}. Cells were starved and stimulated as described.* ***(B)*** *Activation of Ral pathway in cells transfected with RalGDS-CAAX compared with wt Kras and Kras^{G12D}.* ***(C)*** *Total number and percent max of CFU-GM in fetal liver cells expressing activated effectors over a range of GM-CSF concentrations. Most effectors alone behave similar to wild type. Surprisingly, Braf^{V600E} -expressing fetal liver cells do not form many colonies in response to growth factor.* ***(D)*** *Photomicrographs of wt Braf, Braf^{V600E}, and Kras^{G12D} colonies at 0.01ng/mL GM-CSF. Braf^{V600E} colonies are larger in size than wt Braf although not as large as Kras^{G12D} colonies.*

concentrations of GM-CSF, there was no significant change in the number of colonies (**Fig. 4c**). The colonies themselves were slightly larger, but the changes in CFU-GM morphology seen at higher doses of GM-CSF were not as dramatic as seen in the wt *Kras* fetal liver (data not shown). In sum, none of the activated effectors individually were able to recapitulate any aspect of the K-Ras^{G12D} phenotype.

Additional Studies Addressing the Effects of Braf^{V600E} Expression on CFU-GM Growth. B-Raf^{V600E} demonstrates high basal kinase activity and expressing this protein in cultured cells results in elevated levels of pERK and pMEK (Wan *et al.*, 2004). Since *BRAF^{V600E}* is a potent oncogene, we expected that it would either behave like the other effector mutants and not alter myeloid progenitor growth or perhaps induce a hypersensitive pattern of CFU-GM growth. Instead, we unexpectedly observed a few large CFU-GM colonies at low concentrations of GM-CSF (**Fig. 4d**), but no additional growth at higher concentrations (**Fig. 4c**). Thus, *Braf^{V600E}* confers a proliferative signal that increases the size that of individual CFU-GM at low doses of GM-CSF, but also blocks the normal increase in colony numbers seen at higher cytokine concentrations.

If B-Raf^{V600E} dominantly interferes with the ability of CFU-GM to respond to GM-CSF due to elevated kinase activity, we reasoned that B-Raf inhibitors might partially restore cytokine responsiveness. To test this hypothesis, we used PLX-4720, a B-Raf inhibitor developed by Plexxikon. This inhibitor is relatively specific for B-Raf^{V600E} compared with WT Braf (biochemical IC50 of 13nM versus 160nM). In *in vitro* growth assays, PLX-4720 is ten to a hundred-fold more potent in inhibiting B-Raf^{V600E}-driven tumor lines than mutant Ras-driven lines (Tsai *et al.*, 2008). We transduced fetal

A



B

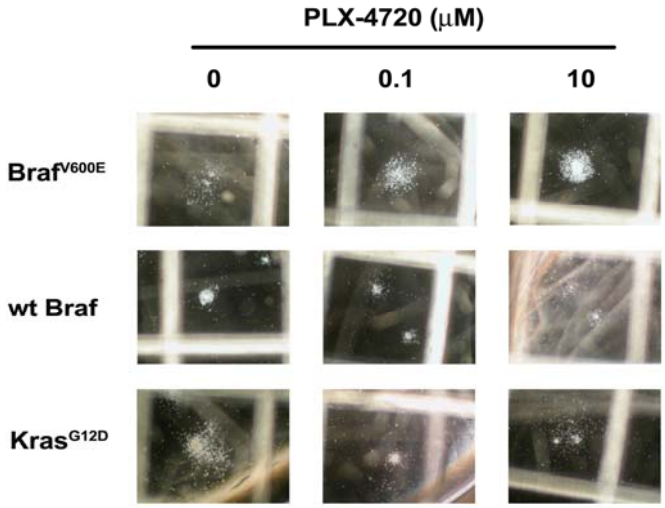


Figure 5 – Addition of Raf inhibitor leads to increased CFU-GM formation in Braf^{V600E}-transduced fetal liver cells. (A) *Total number of CFU-GM in fetal liver cells expressing Braf^{V600E}, wt Braf, and Kras^{G12D} in various concentrations of PLX-4270. Sorted cells were plated at 5ng/mL of GM-CSF with PLX-4270 doses ranging from DMSO only to 100μM. Treatment with PLX-4270 leads to increased numbers of CFU-GM in Braf^{V600E} cells. Wild-type B-Raf and K-Ras^{G12D} cells maintain or decrease in their ability to form CFU-GM with increasing PLX-4270 concentration.* ***(B)*** *Photomicrographs of CFU-GM at select doses of PLX-4270. Addition of PLX-4270 paradoxically causes B-Raf^{V600E} CFU-GM to increase in size. Size of both wild-type Braf and K-Ras^{G12D} CFU-GM decreases in presence of increased inhibitor.*

liver cells with MSCV-IRES-GFP vectors encoding WT B-Raf, B-Raf^{V600E} or K-Ras^{G12D}, and plated GFP^{+} cells in methylcellulose medium containing increasing concentrations of PLX-4720 and a saturating dose of GM-CSF. Under these conditions, cells expressing B-Raf^{V600E} that were exposed to 10 μM of PLX-4720 selectively formed more CFU-GM colonies. Interestingly, not only did the number of colonies increase, but also the size and cellularity of each colony increased as well (**Fig. 5b**). At the 100 μM concentration, cells transduced with all three constructs failed to form colonies probably due to global inhibition of MEK/ERK signaling (**Fig. 5a**).

Feedback inhibition by downstream proteins in response to high B-Raf^{V600E} kinase activity could underlie the failure of myeloid progenitors to respond to increasing concentrations of GM-CSF. In studies of Raf-1, it was found that phosphorylation at S43, S233, and S259 by protein kinase A (PKA) inhibited Raf-1 kinase activity and decreased pERK levels (Dhillon *et al.*, 2002; Dumaz and Marais, 2003). More recently, Dougherty et al identified five additional serine phosphorylation sites that contribute to down-regulation of Raf-1 (S29, S289, S296, S301, S642). These sites are direct targets of activated ERK and phosphorylation of these sites is dependent on MEK signaling, indicating an ERK-driven negative feedback loop of Raf-1 (Dougherty *et al.*, 2005). To investigate if an ERK or PKA-driven negative feedback loop is responsible for B-Raf^{V600E}-dependent colony growth inhibition, we aligned the amino acid sequences of B-Raf and Raf-1 to ask if B-Raf contains serine residues that are homologous to the inhibitory serines in Raf-1. One serine, S750 in B-Raf, aligns with S642 of Raf-1 and is the only ERK phosphorylation site that is shared by both two proteins. Two serines in B-

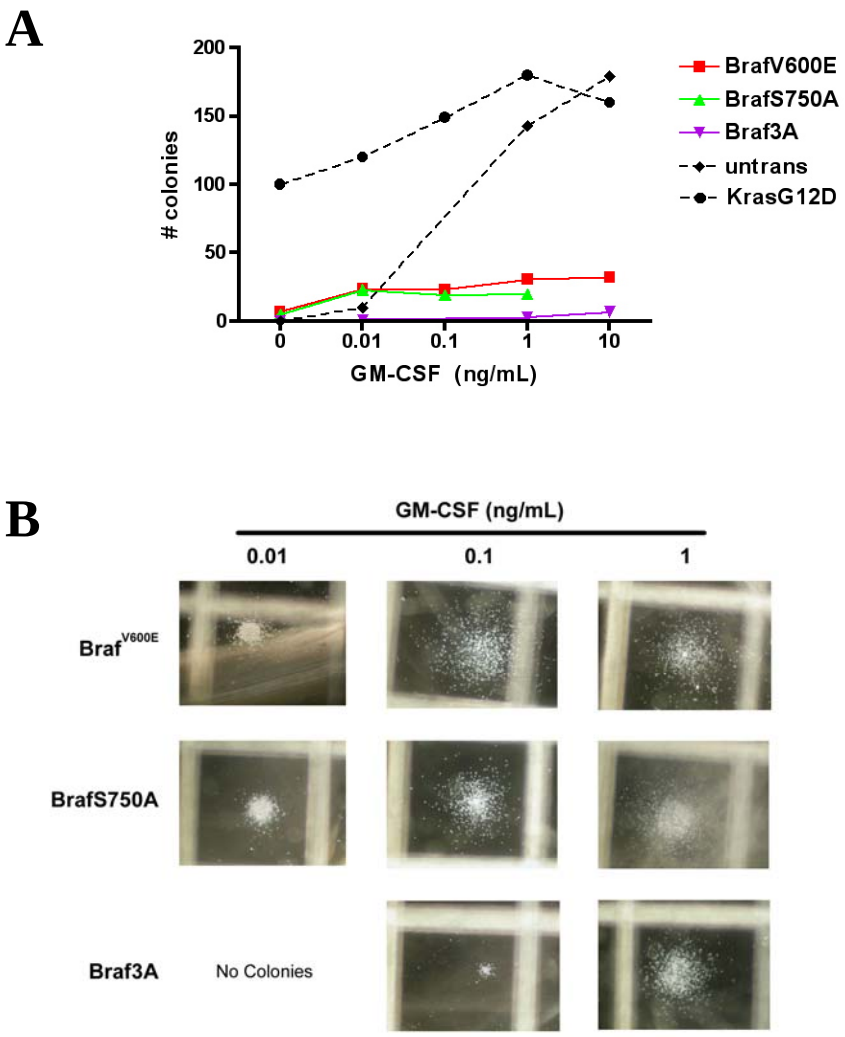


Figure 6 – Phosphorylation mutants are unable to form increased numbers of CFU-GM in response to increasing concentrations of GM-CSF. (A) *Total number of CFU-GM in fetal liver cells expressing* $Braf^{V600E}$*,* $Braf^{V600E}$ *mutants, and* $Kras^{G12D}$ *over a range of GM-CSF. BrafS750A has a mutation at an ERK inhibitory phosphorylation site on top of the* $Braf^{V600E}$ *mutation. Braf3A carries the same mutations as BrafS750A along with additional S339A and S365A point mutations that change PKA inhibitory phosphorylation sites. BrafS750A has similar pattern of CFU-GM formation to* $Braf^{V600E}$*, while Braf3A inhibits CFU-GM formation to a greater extent than* $Braf^{V600E}$*.* ***(B)*** *Photomicrograph of B-*Raf^{V600E} *and B-*Raf^{V600E} *mutant progenitor colonies grown in different concentrations of GM-CSF. BrafS750A colonies are large even at low concentrations of GM-CSF but are unable to form additional colonies. Cells transduced with Braf3A are unable to even form CFU-GM until 0.1ng/mL GM-CSF.*

Raf - S339 and S365 - correspond with the PKA phosphorylation sites S233 and S259 in Raf-1. We created *Braf*^{V600E,S750A} (BrafS750A) and *Braf*$^{V600E, S750A, S339A, S365A}$ (Braf3A) mutants to test if loss of either the ERK-dependent or both the ERK- and PKA-dependent inhibitory phosphorylation sites would relieve colony inhibition. The BrafS750A mutant did not affect the number of colonies formed or size of the colonies when compared with B-Raf^{V600E}. The Braf3A mutant was unable to form colonies at low doses of GM-CSF and only a few colonies were present at high doses (**Fig. 6a,b**).

Discussion

To understand the contributions of different effectors to the oncogenic activity of K-Ras^{G12D}, we generated and characterized a panel of second site mutants that activate either one or two of the major signaling pathways downstream of Ras. Second site mutants that activated only one of the three major effector cascades had no effect on myeloid progenitor colony growth in response to GM-CSF (**Fig. 3a**). This result contrasts what was found in mouse fibroblasts where T35S, which activates the Raf/MEK/ERK pathway, was shown to be transforming in soft agar assays (Hamad *et al.*, 2002; White *et al.*, 1995). The difference appears to be cell-type specific as studies expressing *T35S* in hematopoietic cell lines show no increase in proliferation compared with wild-type (Matsuguchi and Kraft, 1998; Omidvar *et al.*, 2006). Even in different mouse fibroblast lines, T35S is not always transforming (Khosravi-Far *et al.*, 1996; Yang *et al.*, 1998). This underscores the importance of studying Ras effector functions in the context of disease-relevant primary cells.

The fact that *T35S*–and *D38E*– transduced myeloid progenitors do not display hypersensitive colony formation in response to GM-CSF does not preclude the Raf/MEK/ERK pathway from being important in hematopoietic cell transformation. As seen in Figure 1a, although these mutants activate MEK at increased levels compared with wild-type, their level of MEK activation does not match G12D. In scintillation proximity assays and immunoprecipitation studies, T35S and D38E displayed significantly reduced Ras-Raf association as well as diminished MEK activation when compared with oncogenic Ras (Rodriguez-Viciana *et al.*, 1997; Webb *et al.*, 1998). In an attempt to address whether higher activation of the Raf/MEK/ERK pathway can lead to hematopoietic cell deregulation, we investigated the $Braf^{V600E}$ allele, which activates the MAPK pathway to similar levels as oncogenic Ras (**Fig. 4a**) (Wan *et al.*, 2004). However, $Braf^{V600E}$ expression resulted in aberrant colony growth (discussed further below) that made it difficult to discern the effects of increased Raf activation. Creating additional second site mutants or use of other constitutively active Raf constructs might further clarify the role of Raf. The other mutant, Y40C, activates the PI3K pathway to similar levels as oncogenic Ras (**Fig. 1a**) (Oliva *et al.*, 2004) but does not show any hypersensitive colony growth. Therefore, the PI3K pathway alone is unable to drive deregulated myeloid progenitor colony formation. This is consistent with studies showing that Y40C may play a role in tumor survival, but not in initiation and progression (Lim and Counter, 2005; Matsuguchi and Kraft, 1998).

Unlike the mutants above, second site mutants that activated two of the three effector pathways displayed a pattern of *in vitro* hypersensitivity to GM-CSF. When we were characterizing the signaling of E37G, we were surprised to see robust activation of

the PI3K pathway at levels comparable with G12D. Initial binding assays demonstrated that E37G only maintained interaction with RalGDS and ablated interaction with PI3K (Rodriguez-Viciana *et al.*, 1997). However, those assays were done using recombinant p110α. Although E37G does not appear to bind to p110α, Pacold et al used anisotropic analysis to show that E37G binds to p110γ at levels higher than that of oncogenic Ras. H-Ras^{G12V} interacts with p110γ to accelerate formation of PIP_3 20-fold (Pacold *et al.*, 2000) and can also activate Akt through p110γ (Vanhaesebroeck *et al.*, 2005). Therefore, interaction with p110γ is a likely the mechanism behind increased PI3K pathway activation. Additionally, p110γ is expressed mainly in leukocytes and therefore is relevant in our hematopoietic assays (Vanhaesebroeck *et al.*, 2005).

We created the Y64G mutant based on mutational and crystallographic studies that highlighted essential binding interactions between PI3K and the switch II region of Ras (Moodie *et al.*, 1995; Pacold *et al.*, 2000). Analysis of signaling in COS-7 cells showed that Y64G does not activate the PI3K pathway, but maintains both Raf and RalGDS activation to levels similar to G12D. Incidentally, this Y64G mutant is complementary to a p110α mutant described by Gupta et al that can no longer bind to Ras. Cells with the mutant p110α show decreased proliferation and greatly diminished signaling through Akt. Mice expressing mutated p110α have defective lymphatic vasculature and show increased perinatal lethality. Importantly, the surviving mice were resistant to $Kras^{G12D}$-induced tumorigenesis (Gupta *et al.*, 2007). Whereas these data support an important role of PI3K for Ras-driven disease, our Y64G studies show that myeloid cells still display aberrant growth even with loss of PI3K signaling. The data of Gupta et al is confounded by the perinatal lethality and poor health of the mutated p110α

mice. Further investigation using Y64G will serve to shed further light on the importance of PI3K in Ras-driven malignancy.

E37G– and *Y64G*–trasduced myeloid progenitor cells displayed hyperactive CFU-GM formation in response to growth factor showing that they were able to recapitulate part of the $Kras^{G12D}$ *in vitro* colony phenotype. Interestingly, the pattern of CFU-GM formation differed between E37G and Y64G. At low concentrations of GM-CSF, not only did *E37G*-transduced progenitors form increased numbers of CFU-GM, but each CFU-GM was enlarged when compared with Y64G and wild-type (**Fig. 2c**). This data is consistent with the role of the PI3K pathway in optimal generation of myeloid progenitors and their subsequent differentiation into CFU-GM (Bone and Welham, 2007). Myeloid cell lines that activate PI3K were also shown to increase survival in response to growth factor withdrawal (Matsuguchi and Kraft, 1998). So, the increase in colony size of E37G as compared with Y64G reflects the differential activation of the PI3K pathway.

On the other hand, Y64G cells show increased BFU-E formation with increasing concentrations of EPO while E37G cells show no BFU-E growth, even at saturating doses of EPO (**Fig. 2d**). Increased numbers of BFU-E without addition of BPA-stimulating cytokines, is an indicator of deregulated erythroid growth. In $Kras^{G12D}$ mice, erythroid progenitor cells were expanded as a result of inefficient terminal erythroid differentiation and the size and numbers of BFU-E is increased in response to EPO (Braun *et al.*, 2006). This was hypothesized to be due to hyperactivation of Ras downstream effectors in a cytokine-dependent manner (Zhang *et al.*, 2007). The observation that Y64G drives BFU-E formation while E37G does not shows that

Raf/MEK/ERK pathway is a critical effector pathway for increased BFU-E formation and concomitant erythroid defect seen in the presence of oncogenic Ras. This is also consistent with the observation that constitutively active MEK induces an erythroid differentiation block and results in Epo-independent proliferation (Zhang and Lodish, 2004). Thus, the use of second site mutants that activate different subsets of effector pathways allows us to tease apart which effectors are uniquely responsible for different facets of the complex $Kras^{G12D}$ phenotype.

Cells expressing either E37G or Y64G second site mutants were unable to form CFU-GM colonies in the absence of growth factor. Interestingly, bone marrow cells from *Nf1-/-* mice where hyperactive Ras signaling results from a loss of neurofibromin GAP activity, also do not display factor independent growth (Le *et al.*, 2004). Therefore, factor-independent colony formation is a higher bar to overcome and may require the activation of all three effector pathways to levels even higher than those achieved with loss of *Nf1*.

Since both E37G and Y64G were shown to activate RalGDS, it is possible that their hypersensitivity to growth factor is not due to activation of two signaling pathways, but simply the activation of RalGDS. It was shown that out of the three major effectors, the Ral pathway plays the most important role in transformation of human fibroblasts and human embryonic kidney cells (Hamad *et al.*, 2002; Rangarajan *et al.*, 2004). Fibroblasts with activated RalGDS showed more aggressive disease and increased metastases when transplanted compared with the other effector pathways (Ward *et al.*, 2001). In FDCP-mix myeloid cell lines, RalGDS was shown to be responsible for Ras-driven proliferation and inhibition of myeloid terminal differentiation. FDCP cells with RalGDS-CAAX

showed increased proliferation in response to GM-CSF (Omidvar *et al.*, 2006). These results seem to suggest that RalGDS alone may be responsible for much of the oncogenic Ras phenotype seen in different cells and in our system as well.

However, when we expressed RalGDS-CAAX in primary hematopoietic stem cells, there was no difference in progenitor growth when compared with wild-type (**Fig. 4c,d**). When we expressed $Kras^{G12D}$ in RalGDS-/- fetal liver cells, we saw no change in CFU-GM growth in response to GM-CSF (data not shown). These data infer that RalGDS alone is insufficient to recapitulate the phenotype of G12D nor is it alone necessary for Ras-driven transformation. The apparent discrepancy lies in the use of different cell types. FDCP-mix cells are cloned from long-term bone marrow cultures infected with src-MoMuLV and have not retained the ability to form spleen colonies nor to reconstitute the hematopoietic system in lethally irradiated recipients. Although they are non-leukemic and are mostly diploid, with 10% polypoloidy, they have acquired other changes over time (Spooncer *et al.*, 1984; Spooncer *et al.*, 1986). Therefore, they may be more permissive and become biased towards dependence on the RalGDS pathway in the process of immortalization. In contrast, we use primary hematopoietic stem cells that do not contain genetic changes and have the potential to differentiate into any lineage. In this setting, activation of RalGDS needs to act in concert with either PI3K or Raf to recapitulate the leukemogenic properties of $K\text{-}Ras^{G12D}$. Further studies utilizing second site mutants that activate Raf and PI3K but not RalGDS and use of dominant negative Ral constructs can be used to further clarify the role of RalGDS.

Finally, progenitor colony assays are an excellent system *in vitro* for scoring the relative strength of germline and somatic mutations that result in hyperactive Ras.

Germline *KRAS* and *PTPN11* mutations that cause developmental disorders demonstrate attenuated CFU-GM phenotypes compared to somatic leukemia-associated mutations. Interestingly, whereas infants with germline *KRAS* and *PTPN11* mutations frequently develop hematologic abnormalities and sometimes manifest overt MPD, these disorders are usually self-limited. Differences in *in vitro* growth factor independent colony formation and hypersensitivity to growth factor between $Kras^{G12D}$ and *Nf1-/-* bone marrow cells are reflected in the severity of MPD seen in these mice (Braun *et al.*, 2004; Le *et al.*, 2004). The *E37G* and *Y64G* mutations are associated with intermediate CFU-GM phenotypes that most closely resemble the effects of germline *KRAS* mutations (mild to moderate hypersensitivity to GM-CSF with normal colony morphology even in the presence of saturating doses of cytokine). *Nf1-/-* bone marrow cells also do not form cytokine-independent CFU-GM colonies and show a similar degree of hypersensitivity at low concentrations of GM-CSF. However, *Nf1-/-* mutant progenitors form very large monocytic colonies at high concentrations of GM-CSF. We therefore wished to determine if hematopoietic cells engineered to express E37G or Y64G would induce hematologic disease *in vivo.* Since these mutant proteins only activate a subset of downstream effector pathways, we questioned whether they will get the same disease or if the disease will differ in spectrum or potency. The colony assay data seems to suggest that the character of the disease may vary. The results of these experiments are presented in Chapter 3.

One provocative result that stemmed from the transduction of activated effectors into fetal liver was the aberrant colony formation pattern of $Braf^{V600E}$-transduced cells. Since $Braf^{V600E}$ is a potent oncogene, we hypothesized that it might cause CFU-GM

formation with a hypersensitivity pattern similar to K-Ras^{G12D} or at the very least similar to that of wt K-Ras. Instead, we observed large CFU-GM at low concentrations of GM-CSF (**Fig. 4e**), but upon increasing concentrations of GM-CSF, there was no additional formation of colonies (**Fig. 4d**). Therefore, B-Raf^{V600E} confers a proliferative signal that increases the size that of each individual CFU-GM but also gives a colony-inhibitory effect that prevents further colonies from forming upon addition of GM-CSF. When we treated the cells with a B-Raf^{V600E}-specific inhibitor, we found that these cells paradoxically formed increased number of colonies with increasing doses of PLX-4270 and the colonies also grew in size. This was opposite of what was seen in the K-Ras^{G12D} and WT Braf cells where increasing doses of inhibitor led to decreased formation of CFU-GM and diminishment in size of colonies (**Fig. 5a**). The most likely explanation for this result is that overexpression of B-Raf^{V600E} through the retroviral promoter is driving a negative feedback loop that is relieved when the signaling downstream of B-Raf^{V600E} is decreased by inhibitor.

Since Raf-1 was known to be negatively regulated by increased pERK levels through phosphorylation of specific serine residues (Dougherty *et al.*, 2005), we identified corresponding residues in B-Raf and mutated them in the context of *Braf*^{V600E}. We hypothesized that at low levels of GM-CSF, *Braf*^{V600E} cells form larger colonies than wild-type because of the increase in proliferation signals. At these low levels ERK may not be activated enough to trigger the negative feedback loop and cause loss of colony formation. Upon addition of higher concentrations of GM-CSF, there is increased signaling down the Raf/MEK/ERK pathway because of growth factor receptor activation, which may subsequently lead to ERK-driven feedback inhibition of B-Raf^{V600E} and

inability of additional colonies to form. However, rather than relieving inhibitory effect of B-Raf^{V600E}, these serine-to-alanine mutants either had no effect or negatively affected growth (**Fig. 6a**). In the case of BrafS750A where we mutated the only serine in Braf that corresponded to a negatively regulated Raf-1 serine, there was no change in formation of CFU-GM. Therefore, either B-Raf is not regulated in the same ERK-dependent manner as Raf-1 or it may have different residues that play a role in negative feedback. It seems that both may be partially true as there are also several unique ERK-mediated negative feedback phosphorylation sites on the C-terminus of B-Raf that do not have corresponding residues in Raf-1. Mutation of these residues to negatively charged amino acids that mimic constitutive phosphorylation led to decreased B-Raf activity and diminished differentiation of PC-12 cells, a B-Raf-dependent process (Brummer *et al.*, 2003). Further investigation using these mutants or looking at the effects of a MEK inhibitor on growth will give insights into the mechanism of B-Raf^{V600E} regulation.

When the Braf3A mutant was transduced into fetal liver, these cells were only able to form a handful of colonies at the highest dose of GM-CSF whereas Braf^{V600E} and BrafS750A still maintained ability to form colonies at low doses of GM-CSF. Normally, as seen with the other second site mutants, if the mutant is non-functional, the pattern of colony formation in response to GM-CSF mirrors that of WT Ras (**Fig. 2a**) because of the presence of both *wt Ras* alleles. The fact that Braf3A causes such a dramatic decrease in colony formation to levels even below Braf^{V600E} suggests that it may be acting as a dominant negative protein that sequesters Ras-GTP or MEK and prevents WT B-Raf from activating the MEK/ERK cascade. This is similar to the effects of N17 Ras which preferentially binds GDP and inhibits endogenous Ras activation by sequestering Ras-

GEFs (Stewart and Guan, 2000). Failure to sufficiently activate the MAPK system can lead to very inefficient CFU-GM formation (Bugarski *et al.*, 2007), which likely accounts for the lack of Braf3A colonies.

Another possible mechanism that we have not yet investigated is oncogene-induced senescence. This was first described by Serrano et al when they demonstrated that ectopically expressed $Hras^{G12V}$ induced a permanent G1 cell cycle arrest in primary murine embryonic fibroblasts (MEFs) and human-derived fibroblasts (HDFs) (Serrano *et al.*, 1997). Since inhibition of the MEK/ERK pathway has been shown to prevent H-Ras^{G12V}-induced senescence, it is not surprising that B-Raf^{V600E} induces senescence in HDFs and primary human melanocytes (Davies *et al.*, 2002; Michaloglou *et al.*, 2005; Zhu *et al.*, 1998). It is possible that ectopic overexpression of B-Raf^{V600E} under a retroviral promoter causes the primary fetal liver cells to undergo senescence and thereby limit the outgrowth of colonies. At very low concentrations of GM-CSF, B-Raf^{V600E} induces a proliferative signal that leads to an increase in colony size. But the signal is not yet high enough to cross the senescence threshold and so colonies can still form. Upon addition of increasing amounts of GM-CSF, there might be increased signaling down the Raf/MEK/ERK pathway due to activation of growth factor receptors that triggers senescence and prevents formation of CFU-GM. The few CFU-GM that actually grow out are presumably those whose $Braf^{V600E}$ expression is lower such that senescence is not induced.

Although these B-Raf^{V600E} results were intriguing to us, they were not a main thrust of my work and many questions still remain. By investigating other potential negative phosphorylation sites, assaying for senescence, and treating with MEK

inhibitors, further insight may be gained into the nature of the negative effects of B-Raf^{V600E} on primary hematopoietic cell growth.

Materials and Methods

Isolation of Kras^{G12D} cDNA. *LSL-Kras^{G12D}* 129Sv/J and *Mx1-Cre* C57Bl/6 mice were mated and checked for plugs. Pregnant females were killed by CO_2 inhalation at E14.5 and fetal bodies were isolated in PBS. Mouse embryonic fibroblasts (MEFs) were isolated by placing bodies in 0.05% trypsin, mincing with scalpel, and filtering through 70μm strainer. Cells were resuspended in MEF media containing Dulbecco's Modified Eagle Medium High Glucose (Cell Culture Facility, UCSF), 10% Fetal Bovine Serum (FBS) (Hyclone Laboratories, Logan, UT), L-glutamine, non-essential amino acids, penicillin/streptomycin, 100μM beta mercaptoethanol. The lox-stop-lox cassette of *Mx1-Cre LSL-Kras^{G12D}* MEFs was excised through *in vitro* administration of 800 units of β-interferon to allow for expression of the recombined *Kras^{G12D}* allele. RNA was isolated from MEFs using RNeasy Mini Kit (Qiagen). Superscript First-Strand Synthesis System (Invitrogen) was used to form cDNA from 2μg of RNA. cDNA was also isolated from wild-type MEFs through similar methods.

Kras and Activated Effector Expression Constructs. Oligonucleotide primers containing a CACC sequence on the 5' end of the 5' primer for use with Gateway cloning technology (Invitrogen) as well as murine *Kras* gene specific sequences were used to

amplify cDNA sequences from the ATG start codon to nucleotide 595. Then, 40 cycles of amplification using the Platinum PCR Supermix (Invitrogen) with the *Kras*-specific primers was used to generate a PCR product to clone into a Gateway entry clone using the pENTR Directional TOPO Cloning Kit (Invitrogen). A *wt Kras* PCR product was generated from amplification of wild type MEF cDNA while a $Kras^{G12D}$ PCR product was amplified from the recombined $Kras^{G12D}$ MEF cDNA and confirmed through *HindIII* and *XbaI* restriction enzyme digest. Next, using the LR enzyme mix from Gateway technology, *wt Kras* and $Kras^{G12D}$ were cloned into either a MSCV-IRES-GFP retroviral vector (Hawley *et al.*, 1994) that was modified for use with the Gateway system or a pDEST12.2 vector. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate second site mutations on top of the $Kras^{G12D}$ backbone. Mutations were verified through sequencing. *p110a-CAAX*, $Braf^{V600E}$, *wt Braf*, and *Ral-CAAX* were generously donated by A. Swarbrick (Univ of California, San Francisco).

Transient Transfection of COS-7 cells. COS-7 cells were transiently transfected using Lipofectamine2000 (Invitrogen) with 10μg of pDEST12.2 constructs encoding WT, G12D, G12D/T35S, G12D/E37G, G12D/D38E, G12D/Y40C, and G12D/Y64G K-Ras mutant proteins. The media was changed on cells 24 hours after transfection to DME-H21 containing 0.1% FBS and starved for 24 hours. Cells were stimulated for 5 or 60 minutes with 50μl of 10μg/mL EGF in 10mM acetic acid and 0.1% BSA and collected on ice. COS-7 cells were lysed with an NP-40 lysis buffer. Signaling of activated effectors was analyzed in the same way. The antibodies used for immunoblotting included anti-

Pan-Ras (Ab-3) (Calbiochem, San Diego, CA), anti-phospho-MEK1/2 (Ser217/221), anti-phospho-Akt (Ser473), anti-β-actin, and anti-phospho-ERK1/2 (Thr202/Tyr204) (all from Cell Signaling Technology, Beverly, MA).

Ral Activation Assay. COS-7 cells were transfected, starved, and stimulated as above. Cells were lysed on the plate with Ral Assay Lysis Buffer [1% NP-40, 10% glycerol, 50mM Tris-HCl (pH=7.4), 200mM NaCl, 2.5mM $MgCl_2$] supplemented with PMSF, leupeptin, aprotinin, sodium orthovanadate, and Complete Protease Inhibitor cocktail (Roche). Protein lysates were precleared with GST-beads and then 325μg of protein was incubated with 10μl of GST-Ral-BP1 (Upstate, Lake Placid, NY) beads for 1 hour at 4^{o}C. Beads were washed twice and boiled with 5xsample buffer to release RalA-GTP. Activated RalA was detected by Western blotting using anti-RalA (BD Transduction Laboratories). Total RalA levels were determined by Western blot analysis of an aliquot of the lysates removed before immunoprecipitation.

Hematopoietic Cell Isolation and Retroviral Transduction. E14.5 embryos were harvested from pregnant wild-type C57Bl/6 and fetal livers isolated and homogenized as described (Birnbaum *et al.*, 2000). Fetal liver cells were cultured in stimulation media containing StemSpan SFEM (StemCell Technologies, Vancouver, BC), 15% FBS, 100ng/mL stem cell factor (SCF) (Peprotech, Rocky Hill, NJ), 50ng/mL FLT-3 ligand (Peprotech), 100ng/mL interleukin 11 (R&D Technologies, Minneapolis, MN), and 50ng/mL IL-6 (Peprotech*). MSCV-Kras-IRES-GFP* plasmids engineered to express wild-

type, G12D, second site mutant K-Ras proteins or activated effectors were co-transfected with plasmids encoding retroviral gag-pol and env proteins into Phoenix cells using Lipofectamine2000 (Invitrogen). Viral supernatants from the Phoenix cells were used to transduced fetal liver cells 24-48 hours after harvest.

3T3 Starvation Assay. 3T3 cells were plated at a density of 10^6 cells per 10cm plate day before infection. 2mL of viral supernatant was overlaid on media the next day and incubated overnight. Cells were grown out and then analyzed by FACS to confirm that all 3T3 cells were GFP-positive. 3T3 cells were starved in DME-H21 with 0.1% FBS for 0, 3, and 6 hours, scraped off the plate, and then lysed for Western analysis.

Hematopoietic Progenitor Assays. After transduction, fetal liver cells were sorted for GFP on a FACSAria. GFP-positive fetal liver cells (1×10^5) were suspended in methylcellulose medium (M3231, Stem Cell Technologies) containing recombinant murine GM-CSF (Peprotech) at varying concentrations and plated in 1-mL duplicates. CFU-GM colonies were enumerated on day 8 by indirect microscopy. For erythroid progenitor assays, cells were seeded in methylcellulose medium containing transferrin (M3234, Stem Cell Technologies) with varying amounts of recombinant murine EPO (R&D Technologies). BFU-E colonies were enumerated on day 7. To grow out macrophage progenitors, sorted GFP-positive fetal liver cells were plated on 6cm plates in macrophage media consisting of IMDM, 20% bovine growth serum, penicillin/strepto mycin, L-gluamine, and 50ng/mL M-CSF. After 6-8 days, macrophages were starved in IMDM for 24 hours and then stimulated with 10ng/mL GM-CSF for 10 or 60 minutes.

Cells were scraped off the plate with cell dissociation buffer and then harvested for Western blot analysis.

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Chapter 3: Second Site Mutants drive formation of $CD4^{+}CD8^{+}$ T-ALL in a bone marrow transduction-transplantation model

Introduction

Second site mutants have been developed as a way of addressing the contributions of different effectors to transformation by oncogenic Ras. Since many of the previous studies with second site mutant proteins had been done using stringent growth assays in cell types that were not normally found to contain Ras mutations, we utilized methylcellulose colony assays with increasing doses of GM-CSF to study the effects of Ras downstream effector activation in the hematopoietic system. We identified two second site mutant proteins that maintain activation of two of the three major downstream effector pathways and retained some hypersensitive myeloid progenitor growth in response to GM-CSF. However, neither of these second site mutants, E37G and Y64G, which activated the PI3K/Ral and Raf/Ral pathways respectively, fully recapitulates the phenotype seen in $Kras^{G12D}$-transduced fetal liver cells. Previous studies investigating the role of second site mutants in tumor formation experiments showed that although second site mutants showed some features of transformation *in vitro*, they were unable to form tumors (Lim and Counter, 2005; White *et al.*, 1995). On the other hand, cells from *Nf1-/-* bone marrow have colony growth phenotype that shares some similarity with E37G and Y64G and were able to drive development of a fatal myeloproliferative disorder (MPD) with latency of 7.5 months (Le *et al.*, 2004). The milder *in vitro* hypersensitivity growth pattern of bone marrow cells in these mice correlated with a more indolent MPD when compared with $Kras^{G12D}$ (Braun *et al.*, 2004).

Many studies have examined the leukemogenic potential of oncogenic Ras. Transduction and transplantation of oncogenic *Nras* initiated MPD with prolonged latency and incomplete penetrance which infers the need for cooperating mutations

(MacKenzie *et al.*, 1999). Expression of different oncogenic Ras isoforms in transduced bone marrow led to a diverse spectrum of AMLs (Parikh *et al.*, 2007). These data infer a role of oncogenic Ras in driving myeloid disease. Others have shown that oncogenic Ras can also initiate lymphoid leukemia. Transgenic mice expressing mutant *Nras* under an Eμ promoter developed sporadic T-cell lymphomas (Dunbar *et al.*, 1991). Transduction and transplantation of bone marrow cells with oncogenic *Hras* also leads to efficient formation of T-cell lymphomas (Haupt *et al.*, 1992; Hawley *et al.*, 1995). Given that Ras contributes to the pathogenesis of both myeloid and lymphoid diseases, it is important to understand what role downstream effector proteins play in disease and whether all of them are important for leukemogenesis.

In studies presented here, we investigated whether second site mutants that displayed colony hypersensitivity *in vitro* and activate two of the three downstream effector pathways could initiate malignant hematologic disease *in vivo*. In addition, we sought to distinguish which subsets of downstream effector pathways were most important in disease evolution. We find that both E37G and Y64G initiate development of a $GFP^{+}CD4^{+}CD8+$ T-cell acute lymphoblastic leukemia (T-ALL) that arises initially from the thymus and then invades systemically into other tissues.

Results

$Kras^{G12D,E37G}$ and $Kras^{G12D,Y64G}$ are oncogenic in vivo. *In vitro* colony forming assays of fetal liver cells transduced with second site mutants showed that expression of $Kras^{G12D,E37G}$ (E37G) and $Kras^{G12D,Y64G}$ (Y64G), which retained the ability to activate two of the three major downstream effector pathways, confer a pattern of hypersensitive

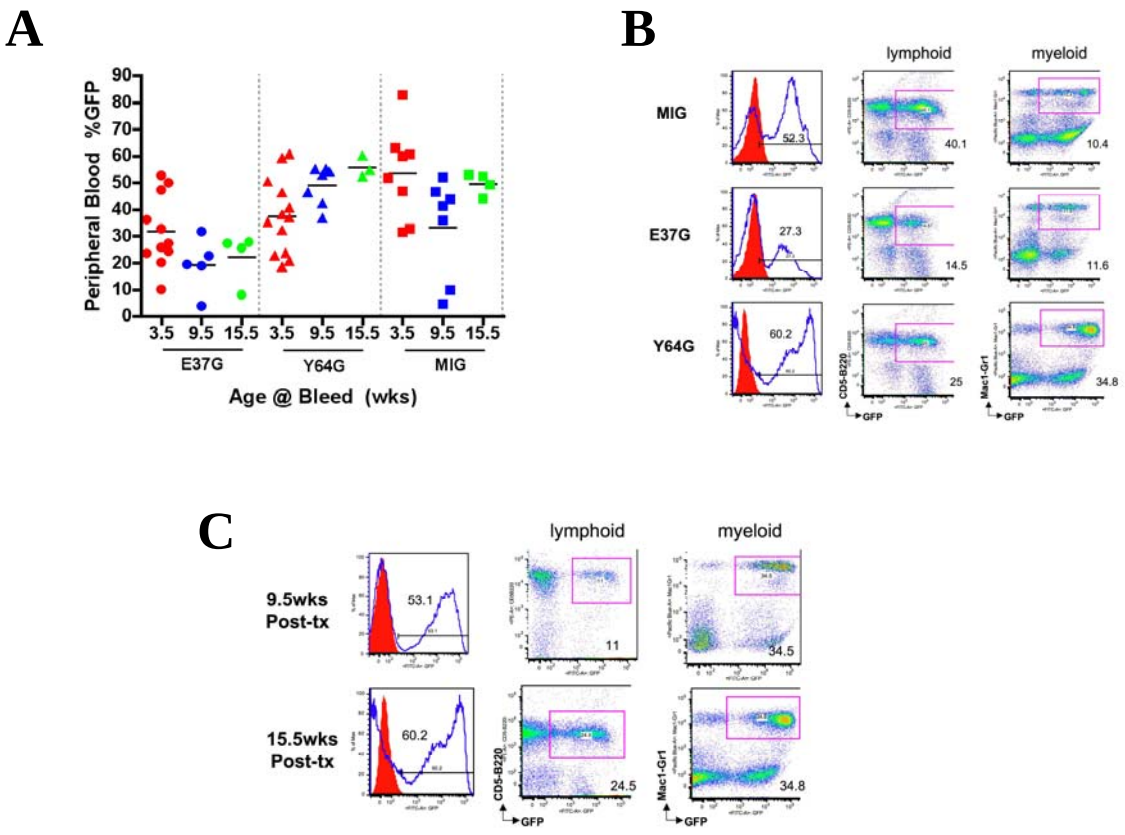


Figure 1 – Transduced hematopoietic stem cells engraft and contribute equally to lymphoid and myeloid populations. ***(A)*** *Mice were tail-vein bled every 6 weeks starting at 3.5 weeks post-transplant. RBCs were lysed and peripheral mononuclear cells were analyzed for expression of GFP. Continual contribution of GFP-positive cells to peripheral blood indicates stable engraftment.* ***(B)*** *Mononuclear cells from the peripheral blood were labeled with PE-CD5, PE-B220 and PacBlue-Mac1, PacBlue-Gr1 to determine percentage contribution of GFP-positive cells to lymphoid and myeloid compartments respectively. Proportion is similar between genotypes (representative plots).* ***(C)*** *Over time, GFP-positive stem cells continue to contribute to lymphoid and myeloid lineages without skewing. Plot is of a Y64G mouse peripheral blood but is representative of all genotypes*

myeloid progenitor growth in response to GM-CSF. Thus, although these mutants are attenuated compared to *Kras*^{G12D}, they retain biologic activity. To investigate the consequence of expressing these alleles *in vivo*, 5-FU treated bone marrow cells from Balb/c mice (Symons *et al.*, 2002) were transduced with *pMIG*, *pMIG-wt Kras*, *pMIG-KrasG12D*, *pMIG-Kras*^{G12D,E37G}, or *pMIG-Kras*^{G12D,Y64G} constructs and transplanted into lethally irradiated recipients. Beginning 3.5 weeks post-transplant, we bled the recipients every 6 weeks to measure leukocyte counts and monitor levels of GFP in the peripheral blood. There was stable contribution of GFP in the peripheral blood mononuclear cells at 15.5 weeks and beyond, indicating that transduced stem cells had engrafted in the bone marrow and were contributing to different lineages in the peripheral circulation. The percentage of GFP-positive cells was varied due to different transduction efficiencies and transplantation rates (**Fig. 1a**).

The percentage of GFP^{+} leukocytes in the peripheral blood was similar between E37G and Y64G mice. Since E37G and Y64G activate different subsets of effector pathways, it's possible that they may bias the contribution of the transduced hematopoietic stem cells to different lineages. High levels of Akt promote neutrophil and monocyte development while reduction of Akt activity induced optimal eosinophil differentiation (Buitenhuis *et al.*, 2008). Activation of the MAPK pathway was also described to play a role in myeloid commitment (Hsu *et al.*, 2007). To investigate this, peripheral mononuclear cells were stained with antibodies to cell surface antigens present on myeloid cells (Mac1 and Gr1), T-cells (CD5) and B-cells (B220). Comparing representative MIG, E37G and Y64G peripheral blood, there was significant contribution of GFP-positive cells to both lineages (**Fig. 1b**). Moreover, the percent of GFP-positive

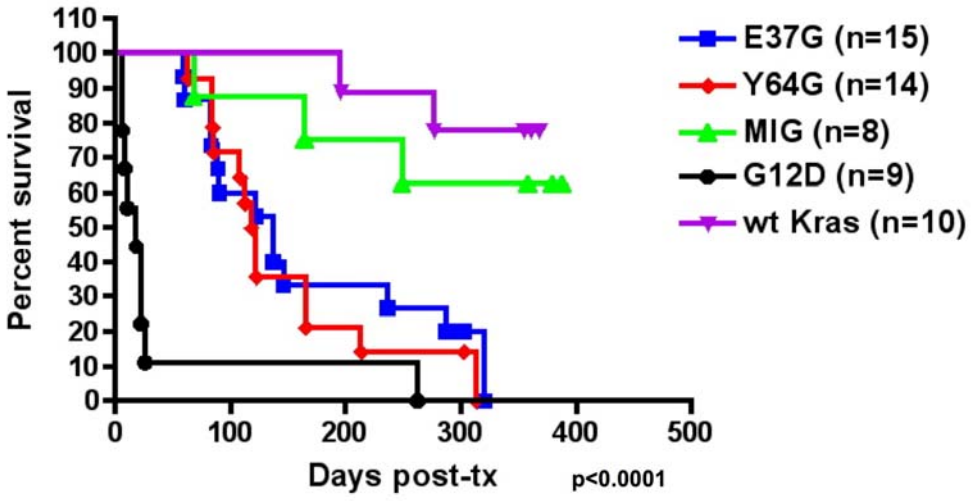


Figure 2 – E37G and Y64G mice show significantly diminished survival. *Mice from both E37G and Y64G began to die at 60 days post-transplant with a median survival time of 137 days (p = 0.016) and 120 days (p = 0.007) respectively. G12D mice died of engraftment failure. Majority of MIG and wt Kras mice survive over a year without signs of disease.*

cells in both the lymphoid and myeloid compartments remained stable over time (**Fig. 1c**).

Mice transplanted with either E37G or Y64G began to die around 60 days after adoptive transfer with median survival of 137 days ($p = 0.016$) and 120 days ($p = 0.007$) respectively (**Fig. 2**). Moribund E37G and Y64G mice showed sudden respiratory distress, moderately elevated blood leukocyte counts, and thymic masses. The lymph nodes, spleen and liver were not enlarged compared with MIG control mice (**Fig. 3a,b**). Blast cells were visible in the peripheral blood (**Fig. 3c**), and FACS analysis revealed a large percentage of GFP^+ peripheral blood mononuclear cells with a characteristic forward scatter-side scatter pattern (**Fig. 3d**).

The disease was highly invasive with gross enlargement of the thymus and microscopic infiltrates of blast cells identified in many tissues. The normal thymic architecture was effaced by large numbers of blasts. Pathologic analysis of the sternum showed invasion of blasts from the marrow to muscle tissue. Blasts were also found in the lungs, perivascular areas of the liver and the spleen showed distortion of the normal splenic architecture (**Fig. 4**). To further characterize the disease-causing population, cells in the thymus, bone marrow, spleen, and peripheral blood were stained for surface markers expressed on myeloid and lymphoid lineage cells. GFP^+ cells were found in every compartment, including the spleen even though it was not enlarged. A majority of the GFP^+ cells expressed the CD4 and CD8 antigens (double positive) and were negative for CD3, B220, Mac1, and Gr1. This immunophenotype is characteristic of T-cell acute lymphoblastic leukemia (T-ALL) (**Fig. 5**).

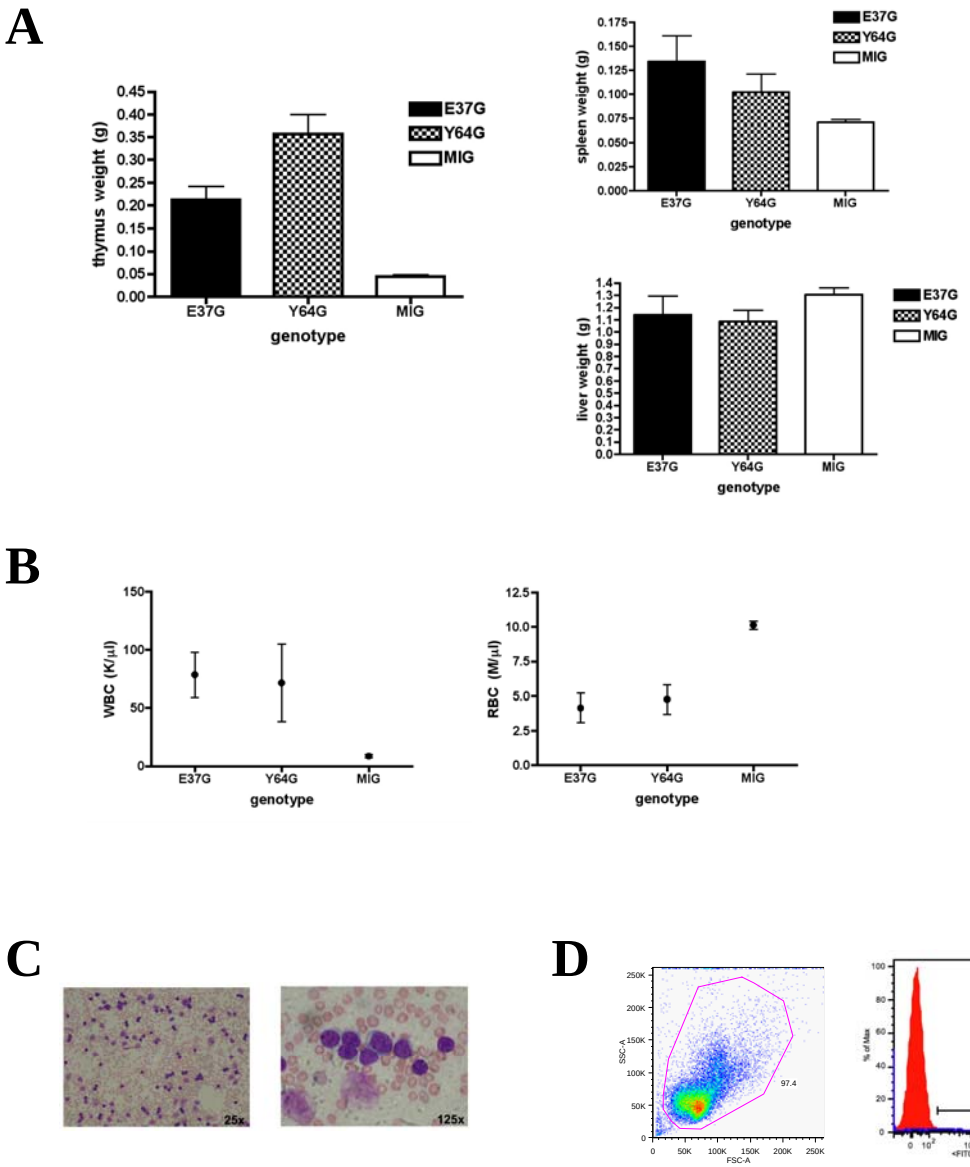


Figure 3 – E37G and Y64G mice die of a GFP-positive T-ALL. (A) *Sacrificed mice showed enlarged thymi (p = 0.0063) without significant enlargement of the spleen or liver.* ***(B)*** *White count was moderately elevated and mice were anemic when compared with MIG control mice.* ***(C)*** *Peripheral blood smear shows presence of blasts.* ***(D)*** *Flow cytometric analysis shows significant accumulation of GFP-positive cells in the peripheral blood. These cells have a characteristic lymphoid-like FSC-SSC pattern.*

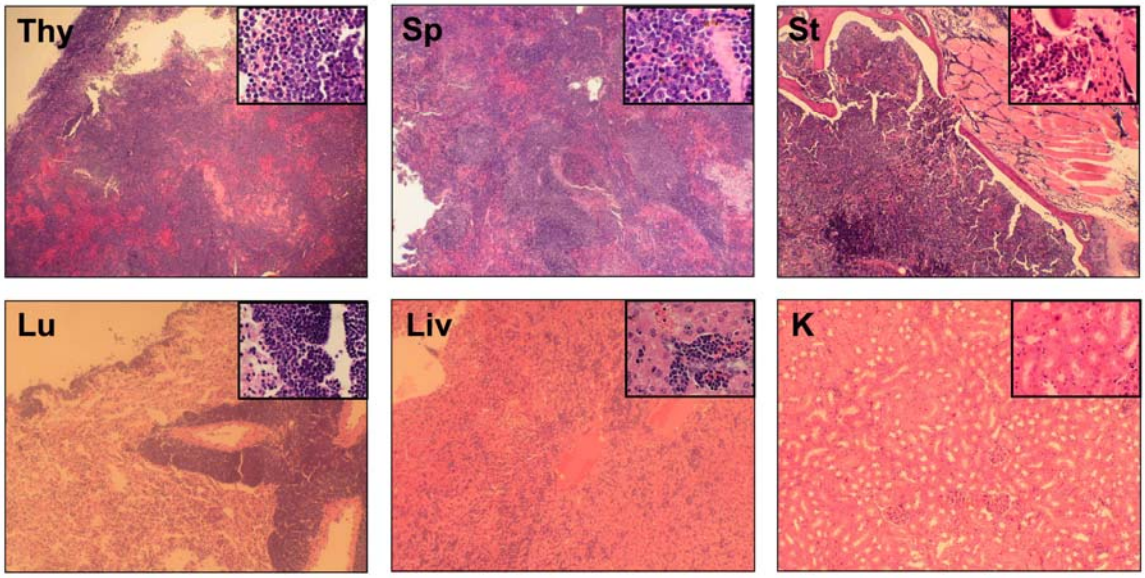


Figure 4 – Second site mutation-initiated disease is highly invasive into different tissues. *Tissues shown at low magnification and high magnification (inset) demonstrate infiltration of cells into extrathymic tissues. Kidney was unaffected. (Thy, thymus; Sp, spleen; St. sternum; Lu, lung; Liv, liver; K, kidney)*

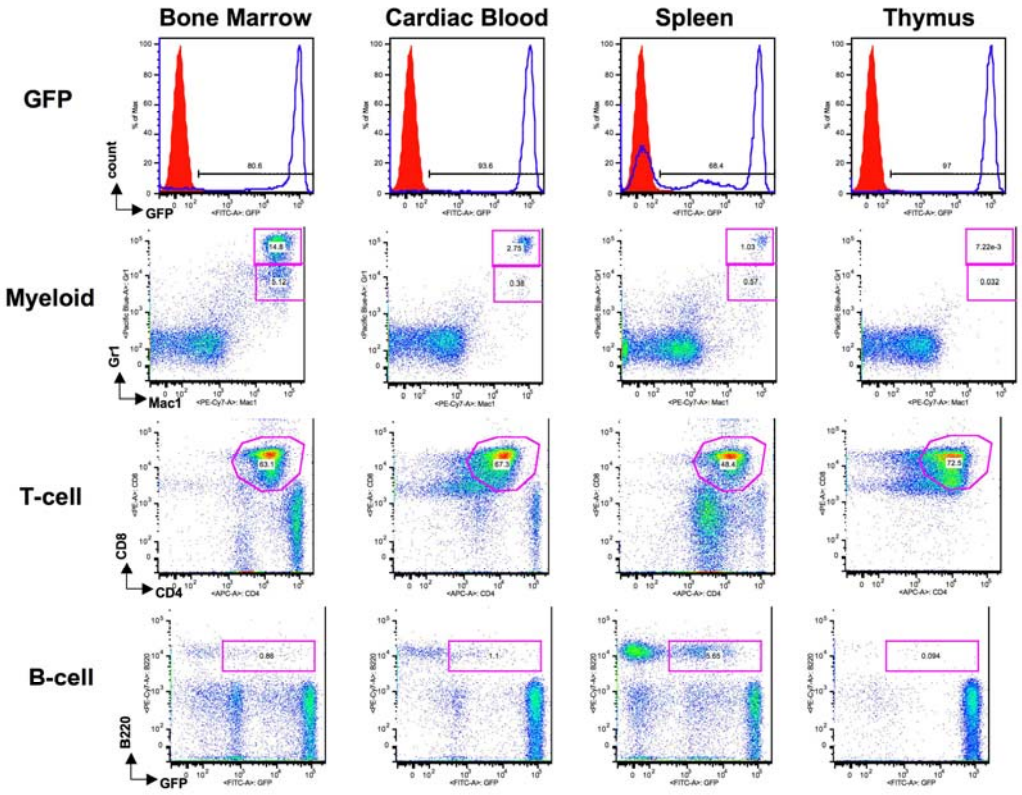


Figure 5 – Analysis of cell populations in different tissues of primary mouse. *Tissues were homogenized and then labeled with surface marker antibodies to distinguish between the myeloid (Mac1,Gr1), T-cell (CD4,CD8), and B-cell (B220) lineages. Disease-causing cells are Mac1-Gr1-B220-CD4+CD8+GFP+ and make up the majority population in bone marrow, cardiac blood, spleen and thymus. Cells are also CD3-negative (data not shown).*

Importantly, the morphology, immunophenotype, and pattern of tissue involvement were similar in recipients of cells expressing E37G and Y64G that developed T-ALL. There was no significant difference in survival in recipients transplanted with cells expressing the E37G or Y64G mutant proteins ($p = 0.53$). The average thymic size was somewhat greater in the Y64G recipients (**Fig. 3a**), although this difference was not statistically significant. Leukocyte and red blood cell counts were also similar and the peripheral blood smears were indistinguishable. Overall, of the 15 mice transplanted with E37G-infected bone marrow, 6 succumbed with T-ALL and 2 were presumed to have died from T-ALL because of the speed and sudden onset of death (59 and 122 days post-transplant). Four other E37G mice presented with severe anemia at death but without evidence of leukemia. Of the 14 mice transplanted with Y64G cells, 8 succumbed to T-ALL and 4 were presumed dead of T-ALL (median survival of 103 days).

Since it is possible that random integration of the retroviral construct alone can cause disease (Kim *et al.*, 2003), we also transplanted cells that were transduced with a MIG vector. None of these control mice developed a lymphoid malignancy, and most survived for over a year. Control mice that died had no signs of hematopoietic abnormalities or any disease that caused the outgrowth of a GFP^+ population. It is also possible that increased expression of a *wt Kras* gene might increase the risk of hematologic disease. However, none of the mice transplanted with *wt Kras*-transduced cells died of lymphoid malignancy. Like the MIG mice, only a few within a year of transplantation and none of these had signs of hematopoietic malignancy or outgrowth of a GFP^+ population. One potential caveat is that the percentage of GFP^+ cells in the

peripheral blood of these mice was two- to three-fold lower than those of MIG, E37G and Y64G indicating the *wt Kras* virus was not as efficient in infecting the stem cell compartment. The intensity of GFP was lower as well, indicating fewer retroviral integrations per cell. We also transduced bone marrow cells with $Kras^{G12D}$ and transplanted them into lethally irradiated recipients. Eight of 9 mice from four different experiments died of severe anemia and pancytopenia within two weeks of transplantation due to engraftment failure (**Fig. 2**). These data support the idea that $Kras^{G12D}$-transduced cells are unable to either home or engraft into the bone marrow niche, and they further underscore functional differences between G12D and both second site mutants.

T-ALLs are transplantable but with different organ tropism. To test if the T-ALLs that arose in primary recipients of bone marrow cells transduced with *E37G* and *Y64G* were transplantable, cells from different tissues were injected into sublethally irradiated recipients. Using cells from different tissues of the same primary transplant mice, the secondary transplants all succumbed to T-ALL with an average latency of 26.6 days post-transplant (ranging from 13-45 days) (**Table 1**). The T-ALL that arose was indistinguishable from the primary malignancy and independent of the tissue used to transplant.

Interestingly, although a majority of the thymic population was GFP-positive in secondary recipients, the size and weight of the thymus did not change. Instead, all the mice had hepatosplenomegaly and complete involvement of the bone marrow by leukemic blasts (**Fig. 6a**). Pathological analysis revealed dense infiltration of T-ALL blasts into the sternum, lungs, kidneys, liver, spleen and lymph nodes (**Fig. 6b**). Flow

Parental	Genotype	Tissue Transplanted	#recipient mice	Median survival (d)	Diagnosis	physical exam
1301	E37G	BM, Thy	5	32	T-ALL/L	inc SP, Liv
1302	E37G	BM, Thy	4	29.5	T-ALL/L	inc SP, Liv
2102	E37G	BM, LN	4	26.5	T-ALL/L	inc SP, Liv, LN
3300	E37G	Thy	2	38	T-ALL/L	inc SP, Liv, LN
3303	E37G	BM, LN	2	14.5	T-ALL/L	inc SP, Liv
1303	Y64G	BM, Thy	5	23	T-ALL/L	inc SP, Liv
1304	Y64G	BM, Thy	4	30	T-ALL/L	inc SP, Liv, LN
1011	Y64G	BM, Thy	4	45.5	T-ALL/L	inc SP, Liv, LN
2104	Y64G	BM, Thy	4	13.5	T-ALL/L	inc SP, Liv
2204	Y64G	BM, Thy	4	22	T-ALL/L	inc SP, Liv
1103	Y64G	Thy	2	29.5	T-ALL/L	inc SP, Liv, LN
2203	Y64G	Thy	2	31	T-ALL/L	inc SP, Liv, LN
3306	Y64G	Thy	2	19	T-ALL/L	inc SP, Liv, LN

Table 1 – Summary table of transplanted T-ALLs from primary tumors. *Tissues were taken from E37G and Y64G mice that had succumbed to T-ALL and were transplanted into sublethally-irradiated recipients. Disease was transplantable from thymic, bone marrow or lymph node cells. Upon physical exam, spleen and liver were enlarged in all recipients with a significant number of them also showing enlarged lymph nodes. (BM, bone marrow; SP, spleen; Liv, liver, LN, lymph node; Thy, thymus)*

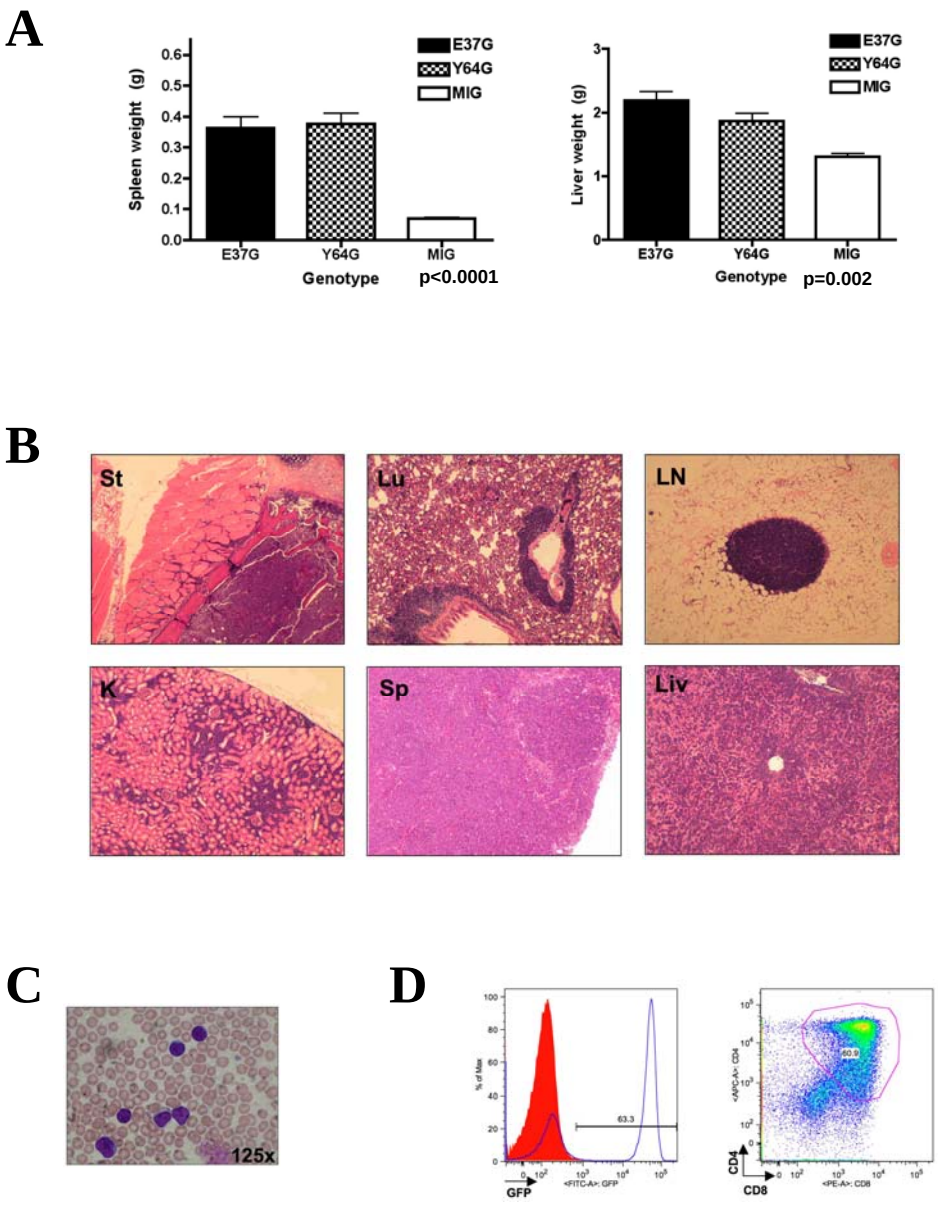


Figure 6 – Primary T-ALL is transplantable into sublethally irradiated recipients. ***(A)*** *Primary T-ALL thymic, bone marrow or spleen cells were transplanted into sublethally irradiated mice. At sacrifice, recipient mice showed enlarged spleens and livers, but thymus size remained unchanged.* ***(B)*** *Infiltration of blast cells into multiple extrahematopoietic organs. (St, sternum; Lu, lung; LN, lymph node, K, kidney; Sp, spleen; Liv, liver;)* ***(C)*** *Peripheral blood smear shows presence of blasts.* ***(D)*** *Presence of characteristic GFP+CD4+CD8+ population in the bone marrow. Same population also contributes to a majority of cells in the thymus, spleen and peripheral blood (data not shown).*

cytometric analysis demonstrated presence of the characteristic $CD4^+CD8^+$ population in different tissues (**Fig. 6c**). As in the primary tumors, there was no difference in latency or spectrum of disease between recipients of cells expressing E37G or Y64G.

Mice transplanted with second site mutants exhibit mild hypersensitivity to growth factor in the bone marrow and an expanded population of GFP^+ cells in the thymus.

Analysis of primary recipients that succumbed to T-ALL suggested that this disease probably arose in the thymus and then progressed to overtake other compartments. When we isolated and examined the GFP^+ population of mice at sacrifice, those without very advanced disease still had a significant Mac1-Gr1 myeloid population in the bone marrow. However, as the disease progressed, this population was reduced; and in the most advanced cases, there was loss of the Mac1-Gr1 population and almost a complete replacement by T-ALL cells (**Fig. 7**). To investigate disease pathogenesis in greater detail, we injected transduced bone marrow cells into sublethally irradiated mice and sacrificed these recipients at 45 days and 90 days post-transplant and examined both the myeloid and lymphoid compartments. Since most mice died anywhere from 90-130 days, we selected these time points so that we could capture pre-leukemic cells and early evolution of disease. At sacrifice, bone marrow cells were sorted for GFP and then plated in colony assays to screen for GM-CSF hypersensitivity colony growth. Thymi were also assayed for increased size and then analyzed further if thymi were enlarged.

At 45 days, there was no significant hypersensitive colony growth in response to GM-CSF (**Fig. 8**) and thymi in all mice were not enlarged (data not shown). At 90 days post-transplant, *E37G*-transduced bone marrow cells formed colonies with some

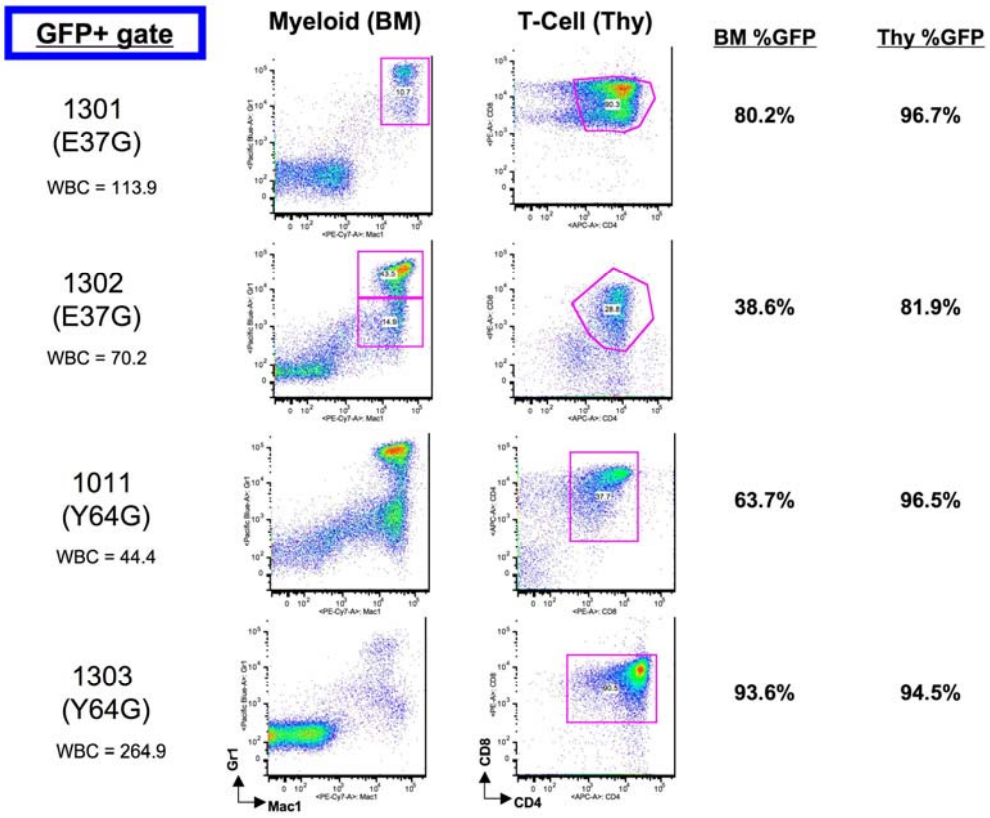
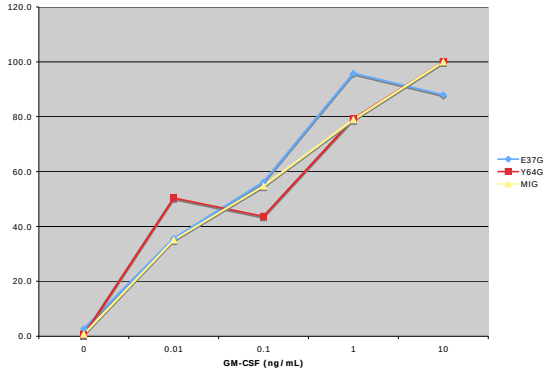


Figure 7 – E37G and Y64G myeloid cells in the bone marrow are overwhelmed by GFP$^+$CD4$^+$CD8$^+$ T-cells. *Cells were gated for GFP-positivity and then further subdivided into the Mac1-Gr1 population in the bone marrow (BM) and CD4-CD8 population in the thymus (Thy). Increasing WBC and percentage GFP correlate with increasing disease severity. As disease progresses, the myeloid GFP-positive cells in the bone marrow become replaced by the CD4-CD8 double positive population that is seen in the thymus. Therefore, although the myeloid cells express the hyperactive E37G and Y64G second site mutants, they are unable to out compete the malignant T-ALL cells.*

A



B

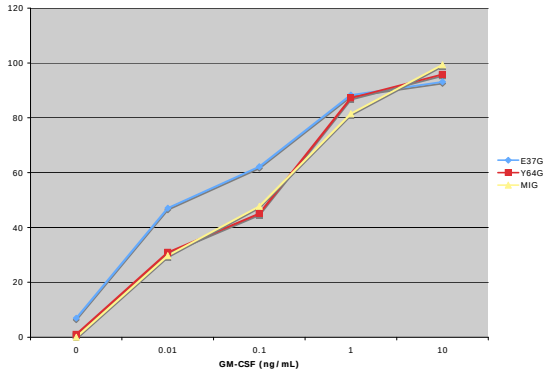


Figure 8 – GFP$^+$ bone marrow cells are only nominally hypersensitive to GM-CSF. *Mice were sacrificed at 45 and 90 days post-transplant, bone marrow cells were sorted for GFP, and cells were plated on M3231 in the presence of increasing concentrations of GM-CSF. CFU-GM colonies were enumerated 7 days after plating.* ***(A)*** *At 45 days, there was no noticeable difference between E37G, Y64G and MIG-transduced bone marrow (summation of three experiments).* ***(B)*** *At 90 days, E37G displays some colony hypersensitivity to growth factor (summation of two experiments).*

hypersensitivity to GM-CSF whereas *Y64G*-transduced cells and *MIG*-transduced cells showed a similar pattern of growth (**Fig. 8**). The fact that there were colonies from the sorted GFP^+ population indicates that a fraction of stem and myeloid progenitors had integrated the mutant constructs and were able to form CFU-GM. Even though the transduced bone marrow cells were unable to show as robust a hypersensitivity to growth factor phenotype as the fetal liver, at 90 days, they began to show some hypersensitivity to GM-CSF, suggesting that they have the same potential for malignant outgrowth.

Also at 90 days, recipients of *E37G* and *Y64G*-transduced cells exhibited enlarged thymi compared with recipients of MIG-transduced cells, although still smaller than mice with overt T-ALL. When the composition of the GFP-positive population of the different thymi was examined, the MIG thymus had normal distribution of single-positive and double-positive thymocytes. However, in the E37G and Y64G thymi, there was accumulation of double-positive thymocytes as well as a $CD4^+CD8^-$ population. In addition, 80-95% of the cells were GFP-positive compared with only 40% in the MIG thymus (**Fig. 9**).

In contrast to mice that died with advanced T-ALL, there was no $CD4^+CD8^+$ GFP^+ population in the bone marrow, spleen or peripheral blood of the E37G and Y64G mice that were euthanized at 90 days. When the GFP-positive population in these tissues was compared with the MIG mice, the distribution of cell types was identical. In the bone marrow, there was a similar proportion of the $Mac1^+Gr1^+$ myeloid population (**Fig. 9**, left panel). Spleen and peripheral blood populations were also identical between all three genotypes. Moreover, while the percentage of GFP-positive cells is comparable between all the different tissues of the MIG mice, the thymi of E37G and Y64G had a four to five-

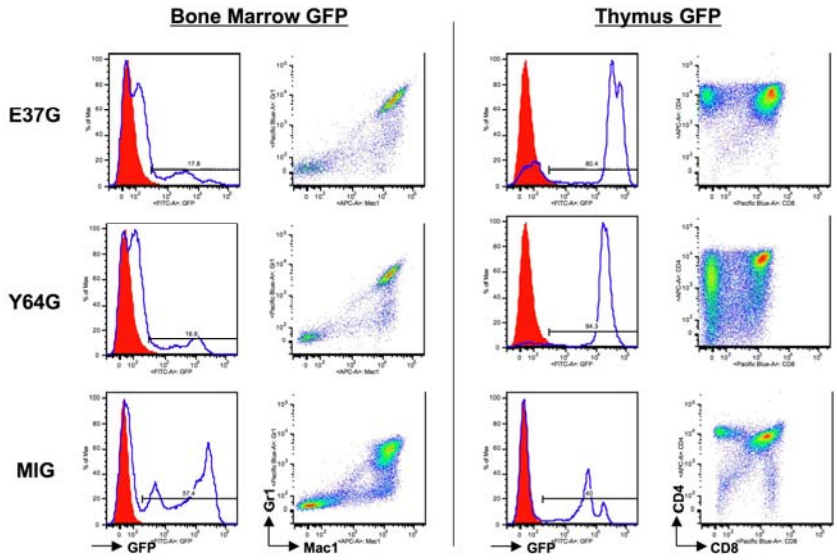


Figure 9 – GFP^{+}CD4^{+}CD8^{+} cells aberrantly accumulate in the thymus. *Mice were sacrificed at 90 days post-transplant. Bone marrow and thymic GFP-positive cells were examined for composition of myeloid and T-cells respectively. Distribution of Mac1-Gr1 myeloid cells in the bone marrow is similar between all three genotype and is identical to wild-type bone marrow (left panel). The MIG thymus has a normal distribution of CD4 and CD8 single and double positive cells. However, E37G and Y64G thymi show accumulation of a double-positive population and CD4+ single-positive population with concomitant loss of CD8-positive and double-negative cells (right panel). E37G and Y64G also show much greater proportion of GFP-positive cells in their thymi than in their bone marrow while in MIG they are comparable.*

fold higher proportion of GFP-positive cells compared with other tissues indicating selective outgrowth of a GFP-positive population in the thymic microenvironment (**Fig. 9**, right panel). So, at 90 days, there is evidence of disease evolution in the thymus of E37G and Y64G mice that is not seen in the MIG mouse.

T-ALLs originate in the thymus. To confirm that the T-ALLs were arising from the thymus, DNA isolated from the different tissues of mice that were sacrificed at 45 days and 90 days were digested with restriction enzymes and analyzed by Southern blotting with a GFP-specific probe. In tissues without a disease or clonal population of cells, no specific band is detected despite many GFP-positive cells because each cell has a different pattern of retroviral integrations. At 45 days, there was no detectable population in either the bone marrow or spleen. At 90 days, there was still no detectable population in either of these tissues. However, in the thymi of E37G and Y64G mice, the presence of distinct bands provided evidence that a clonal population had emerged. In the MIG mouse, even though the percentage of GFP was high in the thymus, no clonal population could be ascertained (**Fig. 10a**). This demonstrates, again, that the disease arises in the thymus and it is dependent upon the presence of E37G and Y64G. Selection of a GFP^+ population is not solely determined by integration into the genome and random selection by the thymic environment.

Since the aberrant clones were only present in the thymus of presymptomatic mice, it is possible that they may not be able to exit and cause widespread T-ALL. In normal T-cells, egress from the thymus is dependent upon upregulation of S1P1. In double-positive cells, S1P1 mRNA is poorly expressed and therefore these cells remain

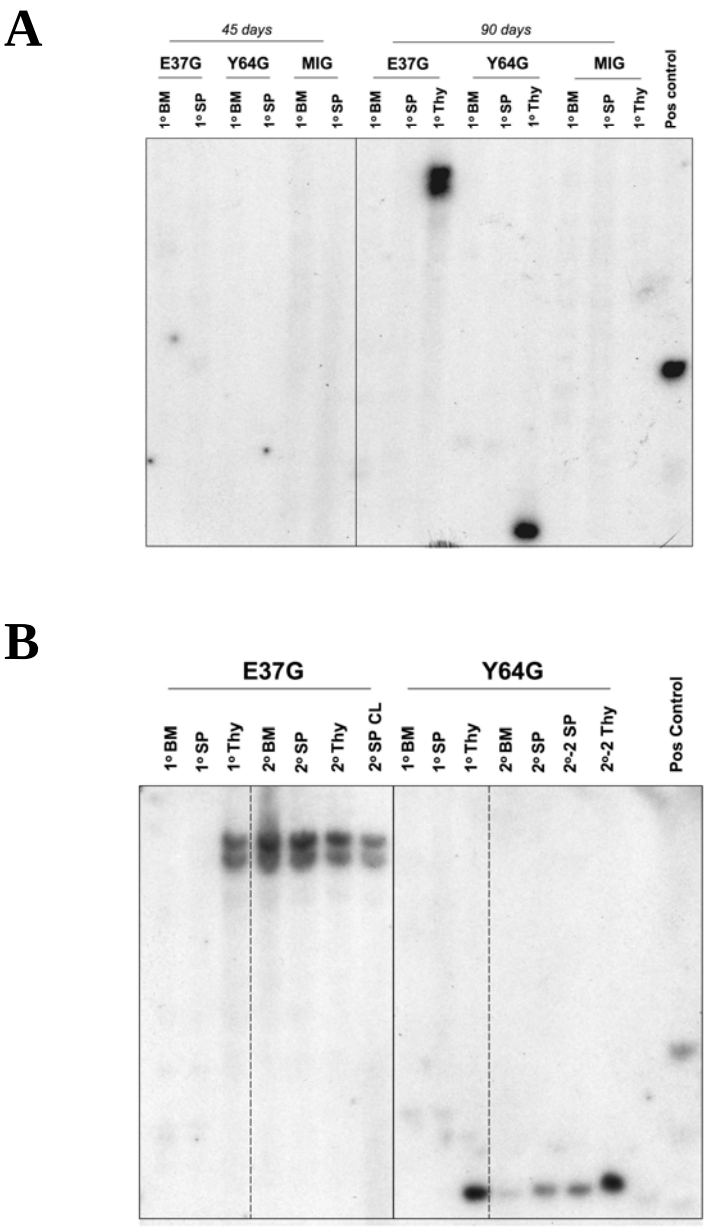


Figure 10 – T-ALL arises as a clonal population from the thymus. (A) *E37G, Y64G, and MIG mice were sacrificed 45 days and 90 days post-transplant. Genomic DNA was isolated from bone marrow, spleen, and thymic cells. Southern blot was performed on Eco-RI digested genomic DNA using an alpha-dCTP radiolabelled 0.7kb-GFP probe. At 90 days, there is an outgrowth of a clonal population in the thymus of both E37G and Y64G but not MIG. In addition, the clonal population is not yet present in the bone marrow or spleen. Thymic DNA was not isolated from the 45 day-old mice because there was no sign of involvement.* ***(B)*** *E37G and Y64G thymic cells were transplanted into secondary recipients. Southern was done using the same radiolabelled GFP probe on tissues from both primary and secondary mice. In one case, DNA was isolated from a cell-line derived from splenic cells of an E37G secondary recipient. All secondary recipient tissues have the same integration pattern as the primary thymic cells. 2^{o}-2 indicates DNA from another secondary recipient.*

sequestered within the thymus. But upon single-positive maturation, S1P1 becomes upregulated and allows for subsequent egress of $CD4^+$ or $CD8^+$ lymphocytes (Cyster, 2005; Matloubian *et al.*, 2004). Since the leukemia in these mice are CD4-CD8 double positive, they may not have the ability to exit the thymus. The ability to leave the thymus and infiltrate other tissues may be a late event that the mice with advanced disease progression into multiple tissues have acquired. To test this, thymic cells from E37G and Y64G mice were transplanted into sublethally irradiated recipients to ask if they could confer disease. In both cases, recipient mice succumbed to an immunophenotypically identical disease that showed infiltration of GFP-positive leukemic cells into the spleen, liver, lymph nodes and thymus (data not shown). Like previous secondary recipients, the primary manifestation of disease was hepatosplenomegaly as opposed to enlarged thymi. When DNA was subjected to Southern blot analysis and probed for GFP, all the recipients had T-ALL with an identical retroviral integration pattern as the presymptomatic thymus (**Fig. 10b**). These data demonstrate that the induction of T-ALL by bone marrow cells transduced with E37G or Y64G involves clonal expansion in the thymus.

Discussion

Although oncogenic Ras plays an important role in many hematopoietic malignancies, it is unknown which downstream effectors are important and whether Ras requires all of its major downstream effector pathways to induce disease. We show that HSCs that are transduced with second site mutants *E37G* and *Y64G*, which are defective for activation of Ras effectors, Raf/MEK/ERK and PI3K/Akt, respectively, cause

transplantable $CD4^+CD8^+$ T-ALL that initially arises in the thymus and then progresses to involve many other tissues. This demonstrates that loss of activated signaling down one of the downstream effector pathways is insufficient to abrogate the oncogenic potential of Ras.

The evolution of a malignancy from *E37G*- and *Y64G*-transduced cells is interesting given that second site mutants alone were previously shown to be insufficient for transformation and tumor formation (Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1995; Yang *et al.*, 1998). It has been known that infection with a retrovirus into mice increases the incidence of lymphomas. Sequencing of these retroviral integrations has shown that many of these lymphomas are driven by integration of the viral promoter elements upstream of proto-oncogenes (Kim *et al.*, 2003; Suzuki *et al.*, 2006; Suzuki *et al.*, 2002). These data raised the possibility that the T-ALLs that arose in our mice were due to random retroviral integration upstream of proto-oncogenes that pushed the progenitor cells to develop a lymphoid malignancy. High GFP expression in the peripheral blood and other tissues indicate stable integration of the retroviral construct into the cellular DNA that could act as an impetus for disease (**Fig. 1**). However, when we transplanted bone marrow cells transduced with MIG, the mice survived significantly longer than E37G and Y64G-transplanted mice (**Fig. 2**) and none of the recipients developed hematologic disease. In addition, the recipients that died showed no signs of clonally elevated GFP levels, demonstrating that mortality was due to MIG-independent effects.

In some tumors, one of the oncogenic events is amplification of *wt Ras* (Bos, 1988; Hoa *et al.*, 2002; Salhab *et al.*, 1989). This amplified expression is thought to

increase the pool of Ras proteins so that there is a net gain in activated forms of Ras (Liu *et al.*, 1998). Since our constructs are in MSCV vectors with LTR promoters that increase expression about five-fold over endogenous levels, it is possible that an increase in total K-Ras protein is important for lymphomagenesis. As retroviral integration has been shown to trigger clonal expansion through expression of self-renewal and survival genes (Kustikova *et al.*, 2005), it is also possible that the overexpression of *Kras* coupled with the random integration of the LTR promoter in front of self-renewal genes can cooperate to drive disease. However, when *wt Kras*-transduced cells were transplanted, like the MIG-transplanted mice, the recipients survived longer and did not develop hematologic disease (**Fig. 2**). Therefore, the T-ALLs in transplanted mice arose specifically because of construct-dependent hyperactivation of the Ral and PI3K pathways by E37G and of the Ral and Raf/MEK/ERK pathways by Y64G.

When cells transduced with $Kras^{G12D}$ were transplanted into lethally-irradiated recipients, almost all of the mice died of engraftment failure (**Fig. 2**), implying that the overexpression of oncogenic Ras in HSCs caused a defect in homing or engraftment. It is known that hematopoietic stem cells need to be quiescent in order to home and engraft efficiently (Cheshier *et al.*, 1999; Quesenberry *et al.*, 2003). Since $Kras^{G12D}$ activates proliferation signals and push cells out of quiescence into G1 through increased cyclin D1, cells with this oncogene may have difficulty engrafting (Winston *et al.*, 1996). In fact, cells with endogenous $Kras^{G12D}$ or *Nf1-/-* are unable to be transplanted into sublethally-irradiated recipients indicating an inability to outcompete wild type niche cells (Braun *et al.*, 2004; Le *et al.*, 2004). In our cells, with $Kras^{G12D}$ being expressed at high levels in the transduced bone marrow cells, it is likely that the proliferation signal is

too strong and very few of these cells are able to home and engraft into the niche – thereby leading to ineffective hematopoiesis.

However, it is not clear why untransduced stem cells do not reconstitute the hematopoietic system. When the transduced bone marrow cells were transplanted, GFP-positive cells were not sorted. As a result, the transplanted population included both transduced and untransduced stem cells. Therefore, even if *Kras*^{G12D} is detrimental to the stem cell, the untransduced stem cells should be able to reconstitute the hematopoietic system; unless enough of the stem cells were transduced with the construct and rendered ineffective such that there are not enough wild-type stem cells left. This seems to be the case with our mice. In fetal liver cells, the *pMIG-Kras*^{G12D} construct consistently has a 25-30% transduction efficiency. In a *Kras*^{G12D}-transduced mouse that was bled three weeks post-transplant right before dying, 35% of the cells in the peripheral blood were GFP-positive (data not shown). At three weeks, GFP^{+} peripheral blood cells are not derived from GFP^{+} stem cells but from more differentiated progenitors that were derived from the original pre-transplantation sample. It is an indicator of the efficiency of transduction rather than engraftment of a transduced stem cell. Also to support this, the only mouse that survived had a poor transduction efficiency of its stem cells demonstrated by a very low percentage of GFP^{+} cells in the peripheral blood – 5% at 3.5 weeks and 2% at 9.5 weeks post-transplant (data not shown). Since only a small proportion of stem cells were infected by the *Kras*^{G12D} vector, there were enough remaining untransduced cells to reconstitute the hematopoietic system and allow the mouse to survive. This supports the hypothesis that *Kras*^{G12D} overexpression has a detrimental effect on homing and engraftment.

Another interesting question that this observation raises is why do the *Kras*^{G12D} second site mutants survive? These cells, transduced with either E37G or Y64G, have no difficulties engrafting and subsequently causing disease even though they activate two major effector pathways to levels similar to that of G12D. It is possible that G12D's detrimental effect on homing and engraftment requires the hyperactivation of all three major effector pathways and that the second site mutants cripple the protein enough to pass under the threshold. Even when cells expressing endogenous levels of K-Ras^{G12D} were transplanted into lethally irradiated recipients, half of the recipients died of engraftment failure (Braun *et al.*, 2004). This result has implications on potential adverse effects of incomplete inhibition of one pathway in the setting of oncogenic Ras-driven malignancy as it may increase the likelihood of engraftment or seeding of different niches. It would be interesting to see if expression of the missing pathway could re-induce the engraftment defect.

In the endogenous *Kras*^{G12D} model, upon injection of pIpC, mice develop a uniformly fatal MPD (Braun *et al.*, 2004). The malignant cells display a characteristic deregulated colony growth *in vitro* which was reproduced when we used *Kras*^{G12D} expressed under a retroviral promoter. Since colony assays are a good *in vitro* surrogate for the *in vivo* disease, we hypothesized that any second site mutant that displayed hypersensitive myeloid progenitor growth in response to growth factor should also display a MPD phenotype *in vivo*. Previous studies in bone marrow transduction/ transplant models of oncogenic Ras have shown that retrovirally driven oncogenic Ras can induce myeloproliferative disorders, acute myeloid leukemia (AML) – or chronic myelomonocytic leukemia (CMML) – like diseases (MacKenzie *et al.*, 1999; Parikh *et*

al., 2006, 2007). In addition, *Nf1-/-* mice, which have an *in vitro* colony phenotype very similar to our second site mutants, succumbed to a more mild MPD in comparison with $Kras^{G12D}$ around 6 months of age (Le *et al.*, 2004). Therefore, when we transplanted cells transduced with *E37G* or *Y64G* into lethally irradiated recipients, we hypothesized that we might observe an MPD similar to *Nf1-/-* mice.

To our surprise, in all the mice that were transplanted with *E37G*- or *Y64G*-transduced stem cells, we only saw evolution to T-ALL (**Fig. 3,5**). The difference may stem from the retroviral construct used to express the oncogenic *Ras* alleles (Fiordalisi *et al.*, 2001). Even in the two different studies that observed myeloid disease after retroviral transduction of $Nras^{G12D}$, the disease spectrum varied significantly. The only main difference between the two studies was the construction of the $Nras^{G12D}$ retroviral vector. MacKenzie et al utilized a Moloney murine leukemia virus (Mo-MuLV) construct while Parikh et al utilized murine stem cell virus (MSCV)-IRES-GFP (MacKenzie *et al.*, 1999; Parikh *et al.*, 2006). Transcription of Mo-MuLV is suppressed in early hematopoietic cells and therefore can account for its incomplete penetrance of the myeloid disease (Halene *et al.*, 1999). Like Parikh et al, we utilized a MSCV-IRES-GFP construct but with a significant difference. We cloned $Kras^{G12D}$ and second site mutants upstream of the IRES-GFP (MSCV-Ras-IRES-GFP) while they cloned oncogenic Ras under control of the IRES and downstream of the GFP (MSCV-GFP-IRES-Ras), which has been shown to decrease expression levels. When Hawley et al expressed oncogenic Ras directly under the control of the MSCV LTR in a bone marrow transplantation model, none of the recipients presented with myeloid neoplasms. Instead, they developed pre-T-cell thymic lymphomas and/or pre-B-cell lymphomas (Hawley *et al.*, 1995). In another study,

utilizing a similar method, a majority of mice developed fatal thymic lymphomas without presence of myeloid disease (Dunbar *et al.*, 1991). Therefore, it is most likely that our mice develop T-ALLs because of the $Kras^{G12D}$ second site mutant expression driven directly under the MSCV LTR promoter.

Nevertheless, that does not fully reconcile why there is an *in vitro* myeloid phenotype but there is no clear sign of myeloid hyperproliferation *in vivo*. Stable production of myeloid and lymphoid GFP^+ cells in the peripheral blood demonstrates that the second site mutant constructs are being expressed in HSCs as well as progenitor cells. When we did timed sacrifices of the mice and sorted for GFP^+ bone marrow cells, they did not show hypersensitivity to growth factor at 45 days even though each cell had the second site mutant construct as indicated by GFP-positivity. At 90 days there was some hypersensitive CFU-GM growth but not to the extent seen in the fetal liver assays. One possibility is that the bone marrow microenvironment provides extrinsic signals that blunt hyperproliferation or even selects for lower expressing mutants.

Another possibility is that there is an intrinsic difference between expressing these second site mutants in fetal liver cells as opposed to bone marrow. Fetal liver hematopoietic stem cells are found in a $Mac1^+$ population as opposed to bone marrow HSCs which are found in $Mac1^-$ populations. Also, a high proportion of FL HSCs are in cycle compared with BM HSCs (40% versus 4%) (Fleming *et al.*, 1993; Morrison and Weissman, 1994). FL HSCs repopulate lethally irradiated recipients more efficiently and HSCs are almost seven times more frequent in FL than in adult BM (Morrison *et al.*, 1995). Separation of the progenitor populations of FL into common myeloid progenitor (CMP), granulocyte/monocyte myeloid progenitor (GMP), and megakaryocyte/erythroid

myeloid progenitor (MEP) demonstrated that the proportion of each myeloid progenitor population was significantly higher in FL when compared with BM (Akashi *et al.*, 2000; Traver *et al.*, 2001). In fetal liver, the greater proportion of HSCs and progenitors along with the increased numbers of HSCs in cycle leads to efficient transduction of constructs and increased colony formation. So, using FL HSCs offer a more sensitive readout of the effects of expressing these second site mutants and show a more robust phenotype than in transduced bone marrow. Therefore, it is possible that there is a myeloid phenotype in our mice that is not as easy to distinguish and characterize before being overcome by the malignant thymocytes.

Analysis of human lymphoid malignancies for presence of Ras mutations shows that mutations are present in B-lineage disease (~15%) and in T-lineage disease (~2%) (Neri *et al.*, 1988; Paulsson *et al.*, 2008; Perentesis *et al.*, 2004). In addition, transplantation of murine bone marrow cells transduced with oncogenic Ras leads to development of fatal lymphoid disease (Dunbar *et al.*, 1991; Hawley *et al.*, 1995). Also, transgenic mice expressing O6-alkylguanine-DNA alkyltransferase, a repair protein that corrects G to A transitions, are protected from development of thymic lymphomas induced by N-methyl-N-nitrosurea, which causes Ras mutations by G to A transitions (Dumenco *et al.*, 1993; Newcomb *et al.*, 1995). In mice with latent $Kras^{G12D}$ that becomes activated upon homologous recombination, 30% of mice develop thymic lymphomas (Johnson *et al.*, 2001). This along with our data showing development of T-ALL with expression of second site mutants supports a role for oncogenic Ras in lymphoid disease.

However, it is unclear what role Ras plays in driving lymphoid disease. The development of lymphocytes in the thymus is a complex process that is initiated with migration of hematopoietic progenitors from the bone marrow into the thymus. Initially cells are double negative (DN) and undergo T-cell receptor β rearrangement, proliferate rapidly and express a pre-TCR complex. At the next stage, cells undergo TCRα-chain rearrangement and express both CD4 and CD8 (termed double positive – DP). These DP cells are subjected to positive and negative selection and finally result in mature CD4 or CD8 single positive (SP) T-lymphocytes that exit into the periphery (Bhandoola *et al.*, 2007; Cantrell, 2002). During thymocyte development, Ras has several important functions. In the DN stage, Ras induces pre-T-cell proliferation and differentiation (Iritani *et al.*, 1999). Expression of *N17Ras*, a dominant negative Ras allele, under the Lck promoter demonstrated that Ras plays an important role in positive selection of lymphocytes to progress from DP to SP but has no effect on negative selection (Swan *et al.*, 1995). In addition, it has been observed that thymi from *Nf1-/-* mice were enlarged and showed expansion of thymocytes along each stage of differentiation (Ingram *et al.*, 2002). Taken together, these results indicate that increased levels of activated Ras in thymocytes can cause hyperproliferation and impaired differentiation, leading to lymphoid malignancy. This can account for the outgrowth of thymocytes that we saw in each of our T-ALLs.

Even though oncogenic Ras can increase thymocyte growth and proliferation, thymocytes with an inability to generate a pre-TCR complex cannot progress to the DP stage and either become growth-arrested or apoptose. The formation of the pre-TCR complex requires not only proper β-chain rearrangement, but also association with CD3

(Goldsby *et al.*, 2000). Since our T-ALLs are CD3-negative, they would be expected to remain in the DN stage. However the T-ALLs were still able to progress to the DP stage and were not growth-arrested. In studies with Rag-deficient mice where cells are arrested in the DN stage, expression of oncogenic Ras restored formation of DP cells with equal efficiency as expression of rearranged TCRβ. In addition, the DP thymocytes were unable to differentiate further into SP cells (Swat *et al.*, 1996). Overexpression of *RasGRP1* in transgenic Rag-deficient mice, leading to increased levels of activated Ras, drives maturation of DN cells into DP cell in the absence of a pre-TCR signal (Klinger *et al.*, 2005; Norment *et al.*, 2003). Studies utilizing *Raf-CAAX* in Rag-deficient mice demonstrated that the ability of activated Ras to drive progression to the DP stage was due to its activation of the MAPK pathway (Iritani *et al.*, 1999). Recently, overexpression of *Akt* in Rag-deficient mice was also able to increase proliferation and rescue the DN-to-DP transition (Mao *et al.*, 2007). This could explain why we get this particular immunophenotype in both *E37G*- and *Y64G*-transduced cells that activate PI3K/Akt and Raf/MAPK to similar levels as K-Ras^{G12D}. It is also possible that other mutations may happen concurrently to increase the efficiency of this transition. For example, expression of constitutively active Notch1 was able to rescue the DN to DP transition in Rag-deficient mice (Michie *et al.*, 2007). Further analysis of the signaling pathways involved and insertion sites will be useful in determining potential cooperating factors in the formation of this Ras-driven T-ALL.

When the primary T-ALL was transplanted into secondary recipients, the primary organ of infiltration shifted from the thymus to the spleen, liver, and in some cases lymph nodes. It may be the case that the primary disease initiates in the thymus and then through

the acquisition of late events is then able to leave the thymus and infiltrate other organs. Therefore, when the mature T-ALL is transplanted, it has gained the ability to home to any organ rather than just the thymus; with the spleen being most efficiently engraftable (Szilvassy *et al.*, 1999). The results from our timed sacrifice experiments showing that thymus-restricted T-ALLs are transplantable into sublethal recipients (**Fig. 10**) seems to indicate that this may be the case. However, sacrificing and re-transplanting thymocytes at more time points is needed to make a clearer conclusion. It also may be the case that the thymic environment provides an environment that is more permissive for development of disease so that there is evolution of T-ALL before any other hematopoietic malignancy. Studies in thymoectomized mice and transplantation of thymic stroma have shown that the thymic environment is important for susceptibility to lymphocytic malignancies (Cooper *et al.*, 1967; Davey *et al.*, 1996). With both the *E37G*- and *Y64G*-transduced cells, evolution of T-ALL seems to initiate in the thymus and once a clonal population forms, it is readily transplantable. Similar to the first hypothesis, tropism changes because the spleen provides a permissive and engraftable environment for the systemically transplanted T-ALL cells. It may also be possible that although the clonal population arises in the thymus, the initiating cell may originate from the bone marrow and then seed the thymus. Investigating disease initiation in athymic mice will be useful in understanding this.

The evolution of a transplantable $CD4^{+}CD8^{+}$ T-ALL from second site mutants raises the possibility that crippling oncogenic Ras by inhibiting one of its effector pathways may be insufficient to inhibit disease. On the heels of the successes of imatinib in chronic myelogenous leukemia (Druker *et al.*, 2001a; Druker *et al.*, 2001b; Kantarjian

et al., 2002) and gefitinib in non-small cell lung cancer with EGFR mutations (Paez *et al.*, 2004; Sordella *et al.*, 2004), many studies have been focused on finding a single target in a pathway whose inhibition would lead to selective loss of the malignant cells. In cancers with oncogenic Ras, work has focused on inhibiting individual effector pathways because of the difficulty of targeting oncogenic Ras itself. This has led to the development of Raf and PI3K pathway inhibitors which have had limited success to date (Perl and Carroll, 2007). Our findings suggest that inhibition of hematopoietic malignancies driven by oncogenic Ras will require inhibiting more than a single effector pathway and therefore stresses the need for combination therapies. Further work on discovering mutations or second hits that cooperate with these second site mutants in driving malignancy will be helpful in identifying other relevant targets for inhibition.

Materials and Methods

Bone Marrow Isolation and Retroviral Transduction. Six to eight-week old wild type Balb/c female mice were injected intraperitoneally with a single dose of 5-fluorouracil (150mg/kg) 4 days prior to sacrifice. Tibias, femurs, humeri, and hips were isolated and cleaned of all tissue. Bones were placed in mortar with Iscove's Modified Dulbecco's Medium (IMDM) (Cell Culture Facility, UCSF) supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and crushed with a pestle. Media was filtered through a 70μM filter and then the whole process was repeated. Bone marrow cells were isolated through addition of Ficoll, centrifugation, and then removal of the interface layer into IMDM+20%. After centrifugation to pellet the cells, bone marrow cells were cultured in a stimulation media containing StemSpan SFEM (Stem Cell

Technologies, Vancouver, BC), 15% FBS, 100ng/mL IL-11 (R&D Technologies), 100ng/mL SCF, 50 ng/mL Flt3, 50ng/mL IL-6 and 10ng/mL IL-3 (Peprotech). MSCV-IRES-GFP (MIG) vector alone as well MIG vectors containing *wt Kras*, *Kras*^{G12D}, *E37G* and *Y64G* were transfected with plasmids encoding retroviral gag-po and env proteins into Phoenix cells using Lipofectamine 2000. Supernatants from transfected cells were used to transduce bone marrow cells 24 - 72 hours after harvest. Cells 24 hours post-transduction were used for transplantation.

Adoptive Transfer. 8-10 week old male wild-type Balb/c mice were lethally irradiated with a single dose of 850 cGy. Retrovirally transduced hematopoietic stem cells were injected 2-3 hours post-radiation through retro-orbital injection. Recipients received prophylactic antibiotic water containing polymyxin sulfate and neomycin sulfate for 2 weeks. CBCs were measured every 6 weeks from 3.5 weeks until 45 weeks. For sublethal transplants, recipient mice were irradiated with a single 500 cGy dose. Recipients were retro-orbitally transplanted with thawed bone marrow, splenic or thymic cells from leukemic mice.

Peripheral Blood Screening. Starting from 3.5 weeks post-transplant, mice were monitored by nicking their tail and collecting 100-200μl of blood for CBC, blood smear, and FACS analysis. Peripheral blood was incubated with RBC lysis buffer (0.16 M NH4Cl, 0.1 M KHCO3, 0.1 mM EDTA [ethylenediaminetetraacetic acid]) for 10 minutes on ice and washed with IMDM+20%. Cells were resuspended in HBSS+3%FBS, blocked with Fc block (1:100), and then stained for surface markers on ice. Antibodies used were

phycoerythrin(PE)-conjugated CD5 and B220 and PacificBlue(PacBlue)-conjugated Mac1 and Gr1 (BD Pharmingen).

Pathologic analysis and flow cytometry. Blood smears were stained with Wright Giemsa (Sigma). Thymi, spleens, livers, lymph nodes, kidneys, lungs, and sternums were collected and sent for sectioning to the Mouse Pathology Shared Resource at the UCSF Comprehensive Cancer Center. CBCs were measured in peripheral blood collected through cardiac puncture. Flow cytometric analysis was performed on single cell suspensions of peripheral blood, bone marrow, splenocytes and thymocytes that had been treated with red blood cell lysis buffer. After cells were washed with IMDM+20%, they were resuspended in HBSS+3%FBS, blocked with Fc block, divided into aliquots, and placed in tubes. Antibodies were added on ice for 30 minutes in the dark. Different conjugated surface antibodies were used to stain for different populations. For myeloid/erythroid – PE-Cy7-Mac1, PacBlue-Gr1, PE-CD71, APC-Ter119. For T-cell – PE-Cy7-CD3, PE-CD8, APC-CD4. For B-cell/stem cell markers – PE-Cy7-B220, PacBlue-CD19, PE-Sca1, APC-ckit. (all antibodies from Pharmingen). Analysis was performed using FlowJo (Tree Star, San Carlos, CA), and data were collected using CellQuest software (Becton Dickinson, San Jose, CA)

DNA Purification and Southern Analysis. Cells from peripheral blood, bone marrow, spleen, enlarged lymph nodes, and thymus were collected and lysed in DNA lysis solution (100mM Tris-HCl pH8.5, 5mM EDTA pH8.0, 200mM NaCl, 0.2% SDS). Genomic DNA was isolated using isopropanol precipitation followed by 70% EtOH wash

and resuspension in ddH20. Following restriction digestion with EcoRI, DNA fragments were separated by electrophoresis and immobilized onto a Nitran membrane. A probe of GFP was isolated from a sequence-verified MSCV-IRES-GFP vector and labeled with radioactive alpha-dCTP using Rediprime II Random Prime Labeling System (Amersham). DNA was hybridized with the radio-labeled probe in a solution (7%SDS, 0.5M NaP pH7.2, 1mM EDTA and 1% BSA) at 60^{o}C overnight, membrane was washed three times and autoradiograph was performed.

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Chapter 4: Mechanisms of Transformation by Second Site K-Ras Mutant Proteins

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a malignancy originating from deregulated thymocytes and characterized by a high peripheral blood cell count, increased number of blasts, invasion of leukemic blasts into the CNS, and large mediastinal masses derived from thymic tissue that restrict respiration. It develops mainly in children and adolescents, but also arises in adults. Historically, patients with T-ALL have had poor prognoses because the speed of blast dissemination from the thymus to other tissues leads to rapid death. However, 80% of children and 40% for adults that are treated by aggressive multi-agent chemotherapeutic regimens are now cured (Pui *et al.*, 2004). Efforts to improve the outcome of T-ALL through bone marrow transplant has only led to minor improvements, indicating that intensification of current regimens will primarily lead to increased morbidity without significant effects on survival. Therefore, focus has now shifted to understanding the pathogenesis of T-ALL and mechanisms of chemoresistance in order to search for molecular drug targets and design targeted therapies (Aifantis *et al.*, 2008; Grabher *et al.*, 2006; Pui *et al.*, 2004).

The incidence of *RAS* mutations in T-ALL ranges from 5-10% with a majority of mutations found in *NRAS* (De Keersmaecker *et al.*, 2005; Kawamura *et al.*, 1999; Yokota *et al.*, 1998). Additionally, mutations in *NF1* have been found in a small percentage of T-ALLs (Balgobind *et al.*, 2008). Although these Ras pathway mutations are less common in T-ALL than in myeloid disease, ~50% of T-ALL cases show biochemical evidence of Ras pathway activation, which supports an important role of hyperactive Ras in leukemogenesis (von Lintig *et al.*, 2000). When murine HSCs are transduced with oncogenic *Ras* genes and transplanted into lethally irradiated mice, these recipients

develop T-cell lymphomas and T-ALL with high efficiency (Dunbar *et al.*, 1991; Hawley *et al.*, 1995). To investigate which Ras effector pathways were important for the initiation of hematopoietic malignancies, we harvested bone marrow from 5-fluorouracil-treated mice, transduced these cells with retroviral vectors encoding second site mutant proteins, and injected these cells into lethally irradiated recipients. We found that the mice still developed T-ALL even though the Raf (E37G) or PI3K (Y64G) pathway was no longer able to be activated by oncogenic Ras. Importantly, the immunophenotype and latency of T-ALL was similar between E37G and Y64G even though they activated different subsets of Ras effector pathways.

One potential explanation for these findings is the acquisition of cooperating mutations that complement aberrant signaling through Ras effector pathways. Like other cancers, T-ALL is thought to occur through a multistep process that involves aberrant expression of proto-oncogenes and chromosomal translocations creating fusion genes that activate kinases or deregulate transcription factors (Grabher *et al.*, 2006). Together these changes alter self-renewal, increase proliferation, block differentiation, and promote resistance to apoptosis. These transformation events occur at crucial steps in thymocyte development and have been shown to cause developmental arrest at different stages. Cooperation of all these events drives leukemogenic transformation of thymocytes (Pui *et al.*, 2004). Two such events found in many T-ALLs are *Notch1* and *PTEN* mutations.

Notch1 is a transmembrane receptor that undergoes two consecutive proteolytic cleavages first by an ADAM metalloprotease followed by a γ-secretase complex. This final cleavage releases an activated intracellular form of Notch (ICN1) that can translocate into the nucleus and activate target genes. Notch signaling is important at

multiple stages of T-cell development. In early hematopoiesis, Notch1 is required for activation of the T-cell program and subsequent commitment to the T-cell lineage. Later in development, Notch1 is important for progression through DN2 to DN3, regulation of T-cell receptor rearrangement, and maturation of double positive thymocytes (Grabher *et al.*, 2006; Palomero *et al.*, 2008; Rothenberg *et al.*, 2008). Based on its central role in T-cell development, it was hypothesized that deregulated Notch1 activity might contribute to the development of T-ALL where malignant thymocytes were able to survive and proliferate in the midst of the normally restrictive thymic environment. In human T-ALLs, over 50% of cases acquire a *NOTCH1* mutation (Weng *et al.*, 2004) and there is evidence that *NOTCH1* mutations can act as initiating lesions or early events in T-ALL (Eguchi-Ishimae *et al.*, 2008). Other studies revealed somatic *Notch1* mutations in some murine models of T-ALL (Lin *et al.*, 2006; O'Neil *et al.*, 2006). In addition, mice reconstituted with hematopoietic cells transduced with constitutively active *Notch1* developed T-cell tumors (Pear *et al.*, 1996).

Phosphate and Tensin homolog (PTEN) is a negative regulator of the PI3K/Akt pathway. Ligand binding to many growth factor receptors leads to activation and localization of PI3K to the membrane where it converts PIP2 to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The subsequent accumulation of PIP3 on the membrane leads to recruitment of Akt and induces its phosphorylation and activation by PDK1 and mTOR-Rictor. PTEN is a lipid phosphatase tumor suppressor that recognizes PIP3 and removes the D3 phosphate to convert it to PIP2 and thereby inhibits activation of Akt (Cully *et al.*, 2006; Palomero *et al.*, 2008; Sulis and Parsons, 2003). Given the important role of the PI3K-Akt pathway in cell growth, cell cycle progression, and survival, it is not

surprising that *PTEN* functions as a tumor suppressor gene that is down-regulated or mutated in many different cancers (Li *et al.*, 1997). Recently, *PTEN* was found to contribute to the pathogenesis of human T-ALL with loss of PTEN protein expression in 17% of primary T-ALL samples, and mutations in *PTEN* in 8%, at diagnosis (Palomero *et al.*, 2007). In addition, analysis of diagnostic and relapse samples showed that loss of *PTEN* occurs as a secondary event during tumor progression. Concommitent *Notch1* mutations and *PTEN* loss were also found in multiple samples indicating that they represent independent events in pathogenesis of T-ALL (Maser *et al.*, 2007; Palomero *et al.*, 2007).

We found that K-Ras^{G12D} second site mutants can initiate formation of monoclonal double-positive T-ALLs even though they can only activate certain subsets of effectors. Chapter 4 presents data showing the acquisition of cooperating events in these leukemias, which include *Notch1* mutations and diminished *PTEN* expression.

Results

Possible model of T-ALL evolution. Although E37G and Y64G activate different downstream effectors of Ras and had somewhat different *in vitro* phenotypes, both second site mutants initiated T-ALLs with similar latency, invasiveness, and immunophenotypic features in a bone marrow transduction/transplantation system. We considered two general explanations as to why different initiating lesions would lead to the same disease (**Fig. 1**). One simple explanation could be that aberrant activation of two of the three major effector pathways is necessary and sufficient to cause T-ALL without any additional events. In a study of childhood ALL, it was found that *BRAF* and *NRAS*

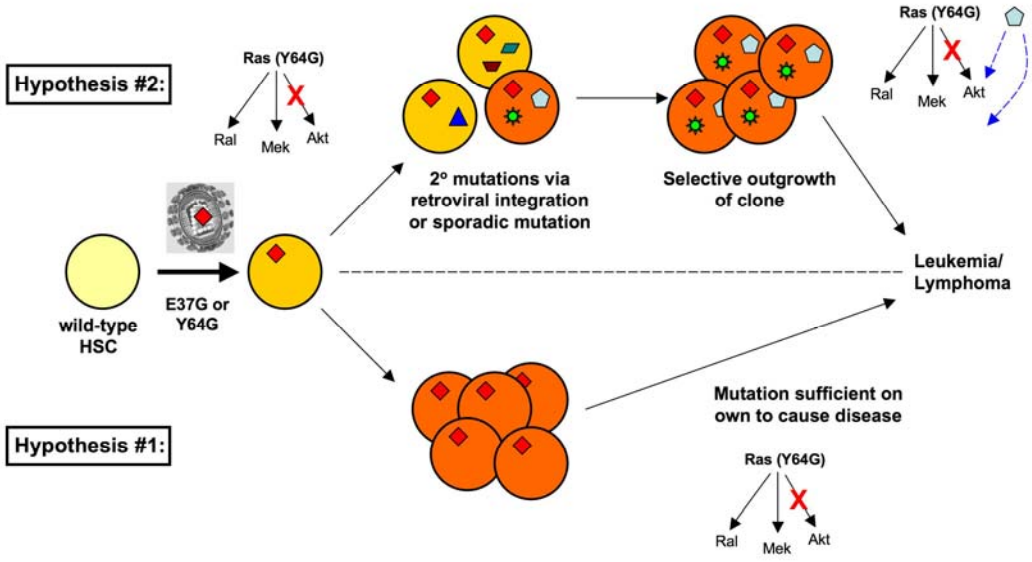

Figure 1 – Possible models of T-ALL formation from E37G- or Y64G-transduced HSCs. *Incorporation of E37G or Y64G into wild-type HSCs and the subsequent expression of these second site mutants constitutes the initial lesion (red diamond) and leads to increased signaling down subsets of Ras effectors. It may be the case that this increased signaling down the Ral-GDS and Akt pathway or Ral-GDS and MAPK respectively is sufficient enough to transform the cell and lead to evolution of T-ALL (hypothesis 1). However, it is more probable that although these are initiating lesions, they are insufficient to drive towards malignancy and require the presence of secondary mutations (illustrated by appearance of other shapes within the cell). These mutations can arise by integration of the virus or acquisition of mutations. Secondary mutations that acquire a proliferation or survival advantage form a malignant clone (orange cells) that gives rise to the T-ALL. In this case, the secondary mutations may affect signaling by incorporating into the missing Ras effector pathway or a parallel pathway that "makes up" for the defect (blue pentagon).*

mutations were generally mutually exclusive, indicating that hyperactivation of a downstream effector of Ras may have similar ability to transform as Ras (Gustafsson *et al.*, 2005). This model infers that immature T-cell precursors are more permissive for transformation than other lineages due to their active DNA recombination and/or undergoing positive and negative selection during development. If this hypothesis is correct, we anticipate obtaining T-ALLs that are polyclonal because many cells that receive the construct should be able to transform. Also, these cells are expected to maintain the initial signaling differences that we see between E37G and Y64G.

A second more plausible explanation is that both E37G and Y64G initiate T-ALL growth by conferring a proliferative or survival advantage in a susceptible leukemia-initiating cell, but that secondary mutations are required for malignant transformation. The latency of the disease with average survival of 4-5 months for both E37G and Y64G supports this idea. Retroviral integrations that deregulate signaling in the effector cascade that is not constitutively active or alter parallel growth control pathways are a likely mechanism for generating cooperating mutations. In unpublished data from our lab, when endogenously expressed $Kras^{G12D}$ HSCs were transplanted into lethally irradiated recipients, they gave rise to a T-ALL with a double-positive immunophenotype with a latency of 3-4 months. Since this is very similar to the phenotype that arises with second site mutant-transduced HSCs, it argues for reactivation of the missing effector pathway such that the disease from these crippled mutants now phenocopies that of the $Kras^{G12D}$. In the case of Y64G when we clone integration sites, we might see integrations that upregulate *Akt* or disrupts *PTEN* to "make up" for that missing pathway and vice versa for E37G.

Another source of cooperating mutations could come from retroviral integrations into promoters of pro-oncogenic sequences or mutations that arise during development of the T-cell in the thymus. In mouse models it has been shown that retroviral infection can lead to development of T-ALL by insertion of viral components into promoter sequences of transcription factors, cyclins, and Ras family members (Kim *et al.*, 2003; Suzuki *et al.*, 2002). This mechanism is also relevant in human cells as demonstrated in a human gene therapy trial where children with severe combined immunodeficiency received HSCs transduced with human *IL2Rγc* using a defective retroviral vector similar to MSCV. Over time, they showed uncontrolled exponential proliferation of T-cells and developed a T-cell acute leukemia-like syndrome due to retroviral insertion-induced overexpression of LMO2, a known oncogene (Hacein-Bey-Abina *et al.*, 2003; McCormack and Rabbitts, 2004). In addition, during development of thymocytes, mutations arising in genes such as *Notch1*, *MYC*, *TAL1* and more recently *PTEN* have been shown to contribute to formation of T-ALL (Armstrong and Look, 2005; Palomero *et al.*, 2007).

If the E37G and Y64G second site mutants induce T-ALL through a mechanism that involves the acquisition of cooperating mutations, we expected that the disease would be monoclonal in most cases. In addition, the existence of a spectrum of genetic lesions might also result in a diverse pattern of signaling abnormalities.

Most of the T-ALLs Initiated by E37G and Y64G Are Monoclonal. In order to determine the clonality of T-ALLs induced by E37G and Y64G, bone marrow, thymic, or splenic cells were transplanted from primary mice with T-ALL into sublethally irradiated recipients. All secondary recipients succumbed to T-ALL. Southern blot analysis of the

retroviral integration patterns between the primary mice and secondary recipients using a GFP-specific probe showed that 12 of 13 T-ALLs (data not shown) that we analyzed were monoclonal – independent of whether E37G or Y64G was the initiating lesion (**Fig. 2a**). Based on the number of independent restriction fragments see on Souther blots, each T-ALL contains 1-5 retroviral insertions.

Some tumors had a mixture of bands where there was one clear major population as indicated by a strong integration pattern but a smaller clonal population was also present. Interestingly, when these T-ALLs were transplanted, the minor population transplanted as well. However, neither population seemed to have a proliferative advantage over the other because after transplantation, the intensity of the bands remained similar to the parental (**Fig. 2b**). This suggests the presence of more than one T-ALL clone with similar aggressivity.

We identified one T-ALL where the transplanted population was different depending on which tissue was used to transplant. When 1304 bone marrow cells were transplanted into recipient mice T3100 and T3101, the resulting T-ALL carried a completely different integration site pattern than when 1304 thymic cells were transplanted into T3102 and T3103 (**Fig. 2c**). Interestingly, the pattern of insertions in the 1304 thymus did not match its transplants T3102 and T3103. Instead, it matched the pattern seen in the bone marrow transplants T3100 and T3101. One possible explanation for the unique 1304 pattern is that there are two T-ALL clones in the original mouse. There is one that has spread from the thymus into the other tissues. However, there is a secondary population within the thymus that is not visible in the primary sample, but gains a survival advantage upon transplant and then becomes the dominant clonal T-ALL

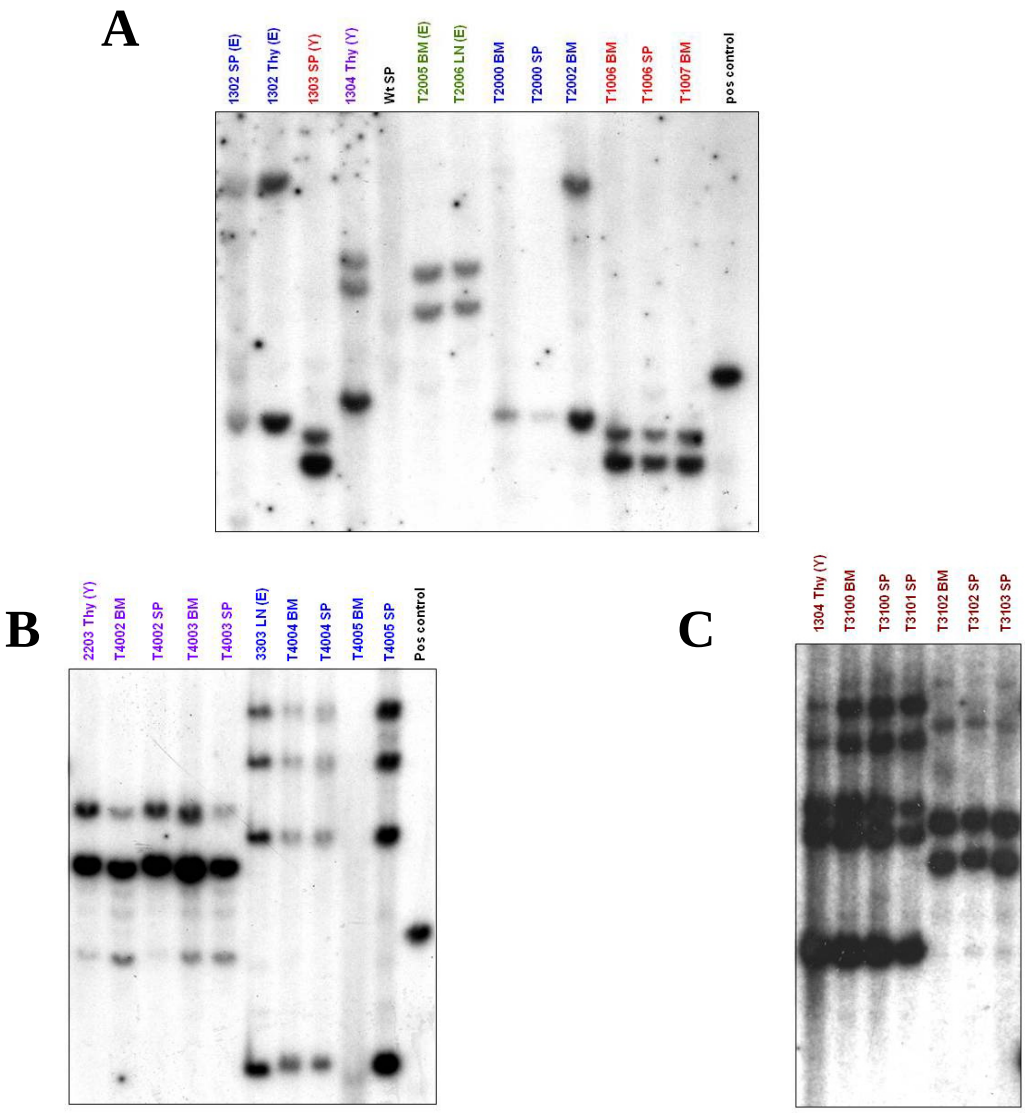

Figure 2 – T-ALLs are mostly monoclonal. *Southern blot was performed on Eco-RI digested genomic DNA using an alpha-dCTP radiolabelled 0.7kb-GFP probe. E=E37G tumors and Y=Y64G tumors. Secondary transplants are indicated with a "T" followed by a number and share the same color as their parental tissues.* ***(A)*** *Parental and transplant cells show the same integration pattern. T2000 was transplanted with 1302 bone marrow cells while T2002 was transplanted with 1302 thymocytes. T1006 was transplanted with 1303 bone marrow cells and T1007 was transplanted with 1303 thymocytes. Even though different tissues were used for transplantation, the resulting T-ALLs all carried the same set of integrations indicating that there was only one clone present in the original T-ALL.* ***(B)*** *Both T4002 and T4003 were transplanted with 2203 thymocytes. There are three major bands of relatively equal intensity indicating a dominant clone. Also present are fainter bands that are maintained in the secondary recipient but do not grow out post-transplantation.* ***(C)*** *In one tumor, 1304, transplantation of different tissues led to identification of a different T-ALL clone. Transplantation of 1304 bone marrow cells into T3100 and T3101 showed same pattern as 1304 thymus. However, transplantation of 1304 thymocytes into T3102 and T3103 gave a completely different pattern of integrations.*

in the thymus-transplanted mice.

Signaling within primary T-ALLs. Since the diseases were mostly monoclonal in nature, we wondered if the signaling within each tumor would remain consistent with the initiating lesion or whether the advent of secondary mutations would affect the activation of downstream effector pathways. Spleen or bone marrow cells from secondary recipients were lysed immediately after harvest and probed to measure the levels of phosphorylated (active) and total Akt and Erk (Fig 3a). T-ALLs that developed in cells expressing E37G showed elevated levels of pAkt. Consistent with PI3K pathway activation, levels of pS6 were also elevated. Interestingly, levels of pERK were also elevated in these tumors. For example, in T3001 SP, pERK was hyperactivated when compared with T1002 SP and T2002 SP.

The pattern of signaling was more complex in Y64G tumors. Although this second site mutant retains the ability to constitutively activate the Raf/MEK/ERK cascade, many T-ALLs from recipients that were transplanted with cells expressing the Y64G second site mutant did not show elevated pERK levels. Interestingly, T3104 showed hyperactive pAkt levels even though Y64G itself does not hyperactivate the PI3K pathway (**Fig. 3a**). Tumors that did not have increased pERK or pAkt levels showed increased activation of pS6 possibly through some other mechanism. These signaling changes suggest the acquisition of cooperating mutations that remodel the signaling within T-ALL during clonal evolution.

Somatic Notch1 Mutations. To investigate potential cooperating mutations in the

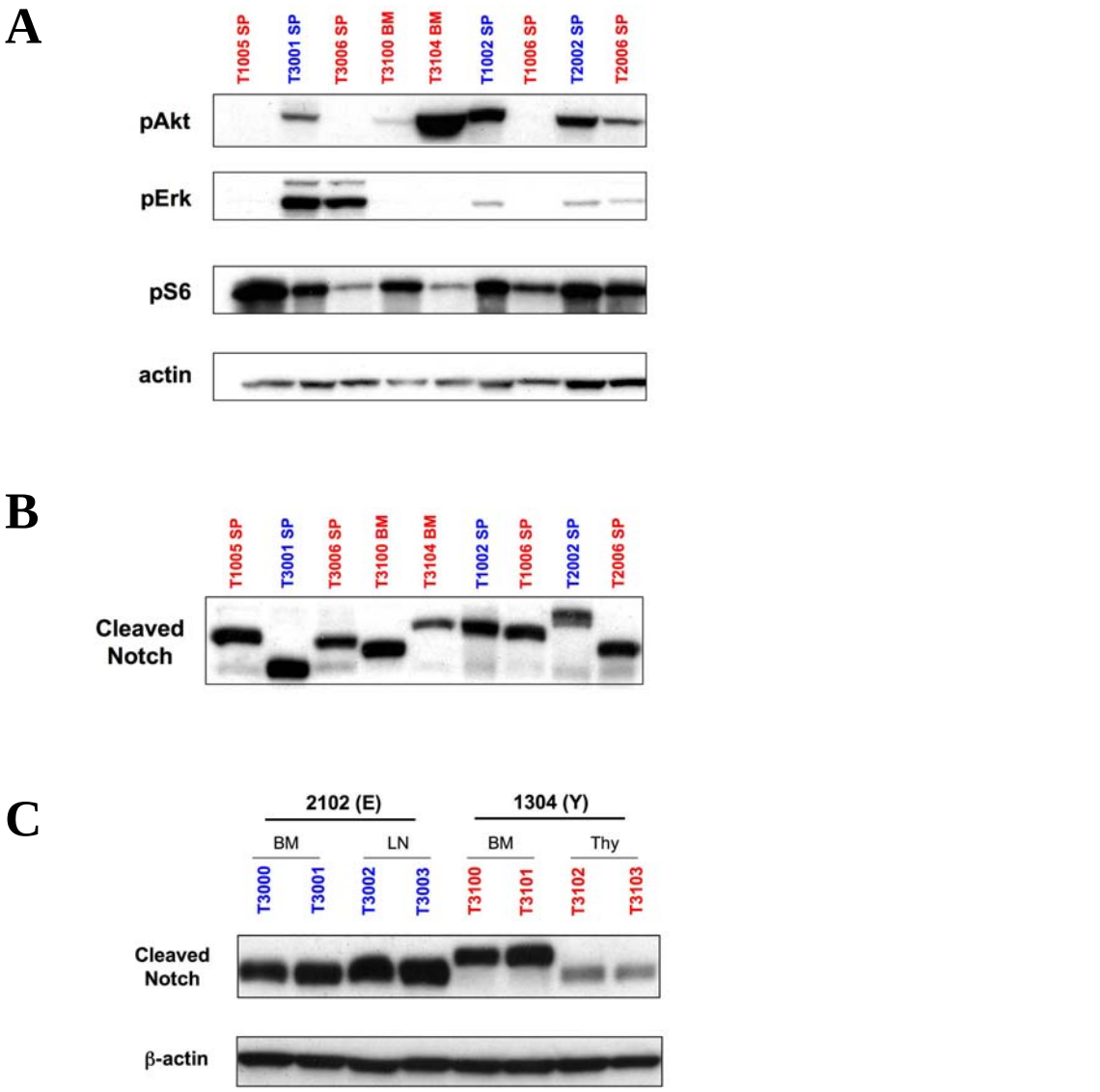

Figure 3 – T-ALLs show increased levels of activated Notch1*. Immediately after sacrifice, cells were lysed and protein lysates were probed for different antibodies. E37G cells are in blue, Y64G cells are in red.* ***(A)*** *All of the E37G T-ALLs have increased levels of pAkt and pS6, but variable levels of pERK activation. Y64G T-ALLs have rewired their signaling such that only a subset continue to hyperactivate pERK and some have activated pAkt.* ***(B)*** *Blotting with an antibody specific for activated Notch1 shows increased levels of activated Notch1 in all T-ALLs. Bands range in size from 80-120 kDa.* ***(C)*** *When 2102 bone marrow and lymph nodes were transplanted, all secondary recipient T-ALLs carried the same size activated Notch1 band indicating that it was present in the original sample. Consistent with Southern data, 1304 secondary transplants show two different sizes of activated Notch1 depending on which tissue was transplanted.*

development of this unique CD4, CD8 double positive T-ALL, we examined the activation status of Notch1. Somatic *NOTCH1* mutations are identified in ~50% of human T-ALLs (Weng *et al.*, 2004). To screen for increased levels of activated Notch1, we used an antibody to cleaved Notch1 at Val1744 that recognizes Notch1 only after it has been cleaved between Gly1743 and Val1744 by the γ-secretase complex (Schroeter *et al.*, 1998). When we probed for cleaved Notch1 in the same secondary recipient T-ALL cells as above (**Fig. 3b**), all of the samples had high levels of cleaved Notch1 that were much higher than wild-type thymi (data not shown). Not only did they show increased levels of activated Notch1, but the sizes of the bands were different. Expected band size is 110 kDa in wild-type cells but the bands from the different tumors ranged from 75-110 kDa suggesting that there were deletions, insertions or missense mutations present in the *Notch1* gene.

In addition, secondary recipient T-ALLs that were transplanted with cells from the same primary T-ALL had identically-sized activated Notch1 bands (**Fig. 3c**) independent of which cells were used for transplantation. The one exception to this was 1304 which showed two different Notch1 sizes depending on what tissue was used for transplantation (**Fig. 3c**). However, this was consistent with the Southern data in Figure 2c that showed evolution of clonal T-ALLs with completely different integration patterns depending on whether 1304 bone marrow or thymus was used for transplantation. These data indicate that each T-ALL harbors clonal *Notch1* mutations that lead to increased levels of cleaved Notch.

To investigate this further, we sequenced *Notch1* from genomic DNA. Previously in human T-ALL, Notch1 had only been known to be activated in less than 1% of T-

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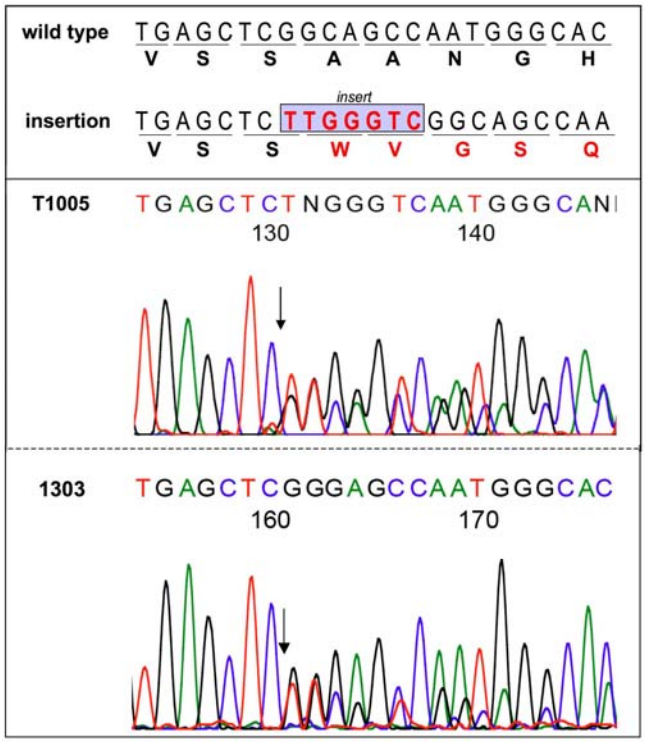

B

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      2501
Notch1    PEHPFLTPSPESPDQWSSSSPHSNISDWSEGISSPPTTMPSQITHIPEAFK* 2551

T1005     ARAPLPHPIP* 2510
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Figure 4 – T-ALLs have Notch1 PEST domain mutations. *Exon 34 of Notch1, containing the PEST domain, was amplified from genomic DNA and then sequenced. T1005 is a secondary recipient of 1303, a Y64G-initated T-ALL.* ***(A)*** *Insertion of 7 base pairs at nucleotide 7273 of Notch1 leads to a frameshift mutation, which leads to a* ***(B)*** *premature stop codon in the PEST domain that results in expression of a truncated Notch1 (shorter by 40 amino acids). The same Notch1 mutation is present in both parental and transplant.*

Parental/Transplant	Genotype	mutation (location)	consequence
1301/T1002	E37G	Deletion GA (7231)	premature stop
1302/T2002	E37G	none	none
3300/T4201	E37G	Insertion CCTC (7160)	premature stop
2102/T3002	E37G	none	none
3303/T4203	E37G	Insertion CCCTT (7160)	premature stop
1303/T1005	Y64G	Insertion TTGGGTC 7273)	premature stop
2204/T3006	Y64G	Insertion TT (7339)	premature stop
1304/T3101	Y64G	Insertion GG (7275)	premature stop
1011/T2006	Y64G	none	none
2104	Y64G	none	none
3306/T4100	Y64G	Insertion CC (7160)	premature stop
3308	MIG	none	none

Table 1 – Summary of Notch1 mutations in PEST domain of T-ALLs. *Exon 34 of Notch1, containing the PEST domain, was sequenced in 5 E37G tumors, 6 Y64G tumors, and one MIG sample as well as their secondary transplants. Mutations were present in both the parental and transplant DNA. Highlighted rows indicate presence of insertion or deletions that led to a premature stop codon in the PEST domain.*

ALLs through a (7;9) translocation. However, recently, sequencing of *NOTCH1* in patients with T-ALL revealed activated mutations of *NOTCH1* that contribute to over 50% of human T-ALL (Weng *et al.*, 2004). Sequencing revealed missense mutations in the heterodimerization (HD) domain that enhanced cleavage by γ-secretase and short insertions or deletions in the negative regulatory PEST domain that are thought to increase the half-life of Notch1 (Weng *et al.*, 2004). Since a majority of mutations found in mice are in the PEST domain (O'Neil *et al.*, 2006) and truncated forms of activated Notch1 were expressed in the T-ALLs, we sequenced the PEST domain. We found that over 60% of second site mutant-driven T-ALLs (7 out of 11) had insertions or deletions in *Notch1* that led to a premature stop codon in the PEST domain (**Fig. 4, Table 1**). There was no difference in the prevalence of PEST domain mutations in E37G tumors compared with Y64G tumors – 60% and 66% respectively. For each T-ALL, both parental and secondary transplant samples carried identical *Notch1* mutations. DNA from MIG-transduced cells did not contain any PEST domain mutations (**Table 1**).

Drug sensitivity of T-ALLs. In order to further study the characteristics of the different T-ALLs, we created cell lines from the tissue of secondary recipients. Cells from either the bone marrow or spleen were grown in media supplemented with IL-2 and IL-7 to support outgrowth of the T-ALL cells. When the cell lines were analyzed by flow cytometry, the cells were GFP-positive (**Table 2**). Southern analysis was done to confirm that there was no outgrowth of a minor clone as they grew in culture and most of the cell lines had exactly the same integration pattern as the original T-ALL with the exception of T2006 from which a different clone grew out in culture (**Fig. 5**).

Parental	Genotype	Survival of recipient(d)	Recipient/ Cell Line	Cell Line GFP (%)
1301	E37G	28	T1000 SP	99.4
1302	E37G	27	T2002 SP	97.5
2102	E37G	30	F1002 SP	99
3300	E37G	13	T4203 SP	99.5
1011	Y64G	21	T2006 SP	*ND*
1303	Y64G	43	F1006 Thy	99.5
1304	Y64G	20	F1007 SP	98.9
2104	Y64G	13	T3104 BM	99.4
2204	Y64G	22	T3006 SP	98.9
3306	Y64G	19	T4100 SP	99

Table 2 – Cell lines were generated from both E37G and Y64G T-ALLs. *Cells from the parental T-ALL were transplanted into secondary recipients and then grown out in lymphocyte media. Length of survival of recipients, tissue harvested from recipient for the cell line, and name of cell line are indicated above. Since the original T-ALL cells were GFP-positive, FACS analysis was done to confirm that the cell lines remained GFP-positive.*

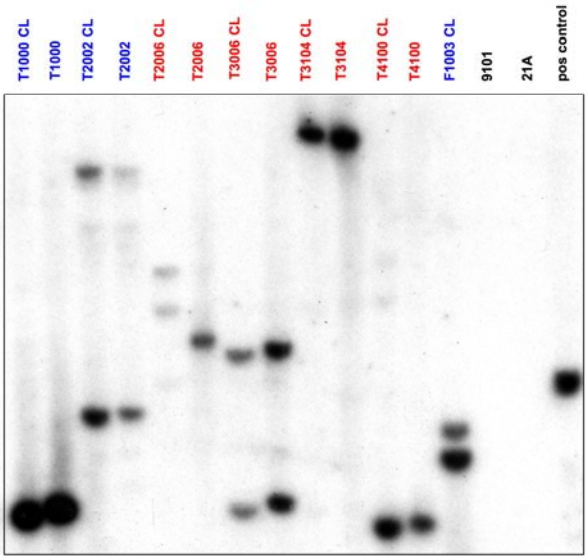

Figure 5 – Cell Lines Maintain Similar Integration Pattern as Parental. *Southern blot analysis comparing cell line DNA, labeled "CL", with the parental DNA from which the cell line was derived. Most of the cell lines contain identical integration patterns, indicating that there was no outgrowth of a minor clone.*

Using these cells lines, we wanted to test whether the T-ALL cells still depended upon the hyperactive signaling of their initial mutation. If we inhibited the Raf/MEK/ERK pathway, would it preferentially affect Y64G more than E37G because Y64G has hyperactivation of the MAPK pathway? And conversely, if we inhibited the PI3K pathway, would E37G be more sensitive than Y64G? We grew E37G and Y64G cell lines in the presence of PD0325901, a MEK inhibitor (Sebolt-Leopold and Herrera, 2004), and found that theY64G cell lines, T2006 and T3104, showed resistance to the drug whereas the E37G cell lines T1000 and T2002 were sensitive (**Fig. 6a**, left panel). 21A is a cell line derived from a G12D-initiated T-ALL that is sensitive to PD0325901. 9101 is a T-ALL cell line derived from infection of wild-type cells with a Moloney replication competent retrovirus and is resistant to 901. When the same cell lines were exposed to PI-103, a dual PI3K and mTOR inhibitor (Fan *et al.*, 2006; Knight *et al.*, 2006), all of the cell lines were equally sensitive (**Fig. 6a**, right panel).

We hypothesized that resistance of the Y64G cell lines to PD0325901 could be due to the inability of this inhibitor to completely shut down MEK/ERK signaling in the Y64G cells. Since Y64G cells have increased Raf/MEK/ERK signaling, they may be better able to withstand drug inhibition whereas E37G only has a small amount of Raf/MEK/ERK signaling upon which it may have become dependent. With E37G, loss of MEK/ERK signaling at low concentrations of inhibitor may then lead to inhibition of growth. However, when we probed for pERK levels in response to drug, both E37G and Y64G cell lines were inhibited at 0.1μM of PD0325901 (**Fig. 6b**). Even though the Y64G cells had higher levels of pERK, by 0.1μM they were equally inhibited when compared

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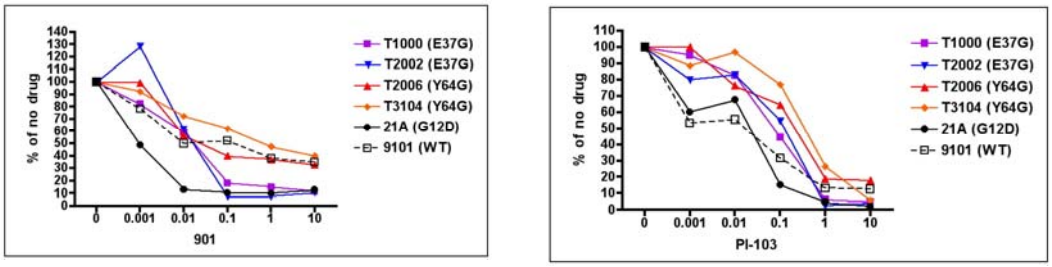

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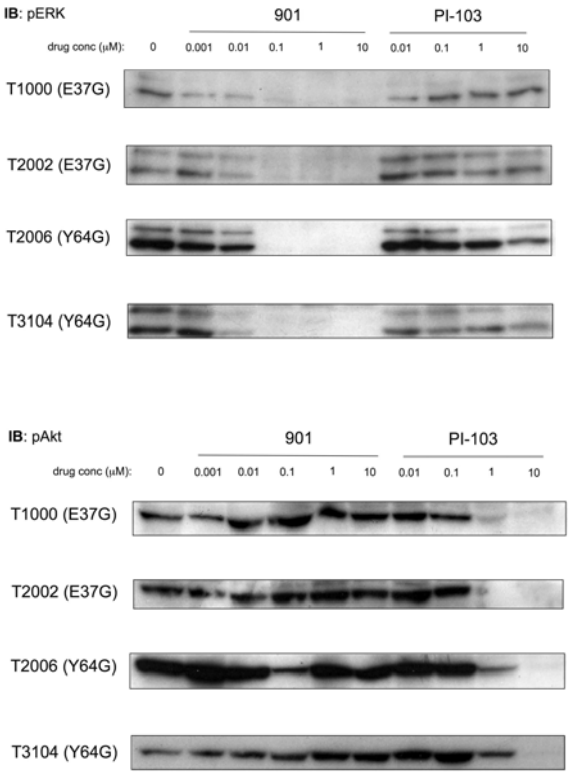

Figure 6 – Y64G cell lines are resistant to MEK inhibitor. (A) *E37G and Y64G T-ALL lines along with 9101 (wt Kras T-ALL line) and 21A (G12D T-ALL line) were grown in the presence of increasing concentrations of inhibitor. Cells were counted after 4 days and then normalized to number of cells that had grown in presence of no drug. T-ALL lines were exposed to PD0325901, a MEK inhibitor (left panel) and PI-103, a PI3K and mTOR inhibitor (right panel).* ***(B)*** *Western blot analysis was done to assay the effects of inhibitors on pERK (top panel) and pAkt (bottom panel). Inhibitors were specific for either the MEK or PI3K pathway. E37G and Y64G lines showed pERK and pAkt inhibition at similar concentrations of inhibitor.*

with E37G cells. PD0325901 treatment did not affect levels of pAkt, and PI-103 did not affect levels of pERK but reduced pAkt in each cell line at 10μM (**Fig. 6b**).

Signaling in the T-ALL cell lines. Since the resistance to PD0325901 in the Y64G cell lines was not due to differential sensitivity to drug, we interrogated the signaling pathways to see if up-regulation of other pathways could account for the increased survival. When the cell lines were starved for 6 and 24 hours, both Y64G cell lines, T2006 and T3104, had elevated levels of pAkt as well as elevated pERK. Levels of pAkt did not diminish significantly throughout the course of starvation (**Fig. 7a**, left). In the E37G cell lines, T1000 and T2002, we expected the pAkt levels to be just as high if not higher than the Y64G cell lines because of the initial lesion. Surprisingly, the levels of pAkt were diminished. Activation of pERK was also minimal. However, the levels of total Ras were elevated in the E37G cell lines compared with the Y64G cell lines (**Fig. 7a**). In sum, the Y64G cell lines had increased levels of both pERK and pAkt whereas E37G cell lines had increased levels of Ras but minimal activation of pERK and pAkt.

These data could account for our cell line proliferation data as the Y64G cell lines might have survived pERK inhibition because of high pAkt levels that protect against apoptosis. On the other hand, the E37G cell lines did not have elevated pAkt levels and therefore were more sensitive to inhibition by PD0325901. To see if this pattern of activation between E37G and Y64G cell lines was consistent, we investigated signaling in two additional Y64G cell lines and one additional E37G cell line. In these cell lines we saw the same pattern, with both Y64G cell lines showing elevated pAkt and pERK levels

with low levels of Ras expression and the E37G cell line showing minimal pAkt and pERK levels but high expression of Ras (**Fig. 7a**, right).

Since the Ras antibody probes for total Ras, it was unclear if the elevated levels of Ras in the E37G cell lines was due to a general increase in Ras levels secondary to retroviral integration or if they were an indication of amplification of the E37G second site mutation construct. Since the E37G and Y64G constructs both contain the G12D mutation, we did a pulldown of Ras using an anti-Hras agarose conjugate and then probed with a G12D-specific antibody to see if the second site mutants were being overexpressed. We found that in E37G, most of the increased Ras levels was due to elevated K-Ras$^{G12D, E37G}$ protein levels (**Fig. 7b**).

However, with the increased levels of E37G protein, we wondered why we did not see increased levels of pAkt in the E37G cell lines. In addition, what was the mechanism behind the increase in pAkt that was seen in the Y64G cell lines? One major regulator of pAkt levels is PTEN. Since *PTEN* mutations have been found to play a role in T-ALL (Maser *et al.*, 2007; Palomero *et al.*, 2008), inactivation or loss of *PTEN* may be responsible for the differences in pAkt regulation in the T-ALL cell lines.

One way PTEN is regulated is through phosphorylation in its C-terminal domain by protein kinase C that leads to its subsequent degradation (Leslie and Downes, 2002; Miller *et al.*, 2002; Torres and Pulido, 2001). It could be that there is increased PTEN phosphorylation in the cell lines with increased pAkt activity. However, when we probed with the pPTEN antibody, all the cell lines had similar levels of constitutively phosphorylated PTEN (**Fig. 7c**). As discussed above, total PTEN levels can also be diminished through mutations or loss of expression. When we probed for total PTEN, we

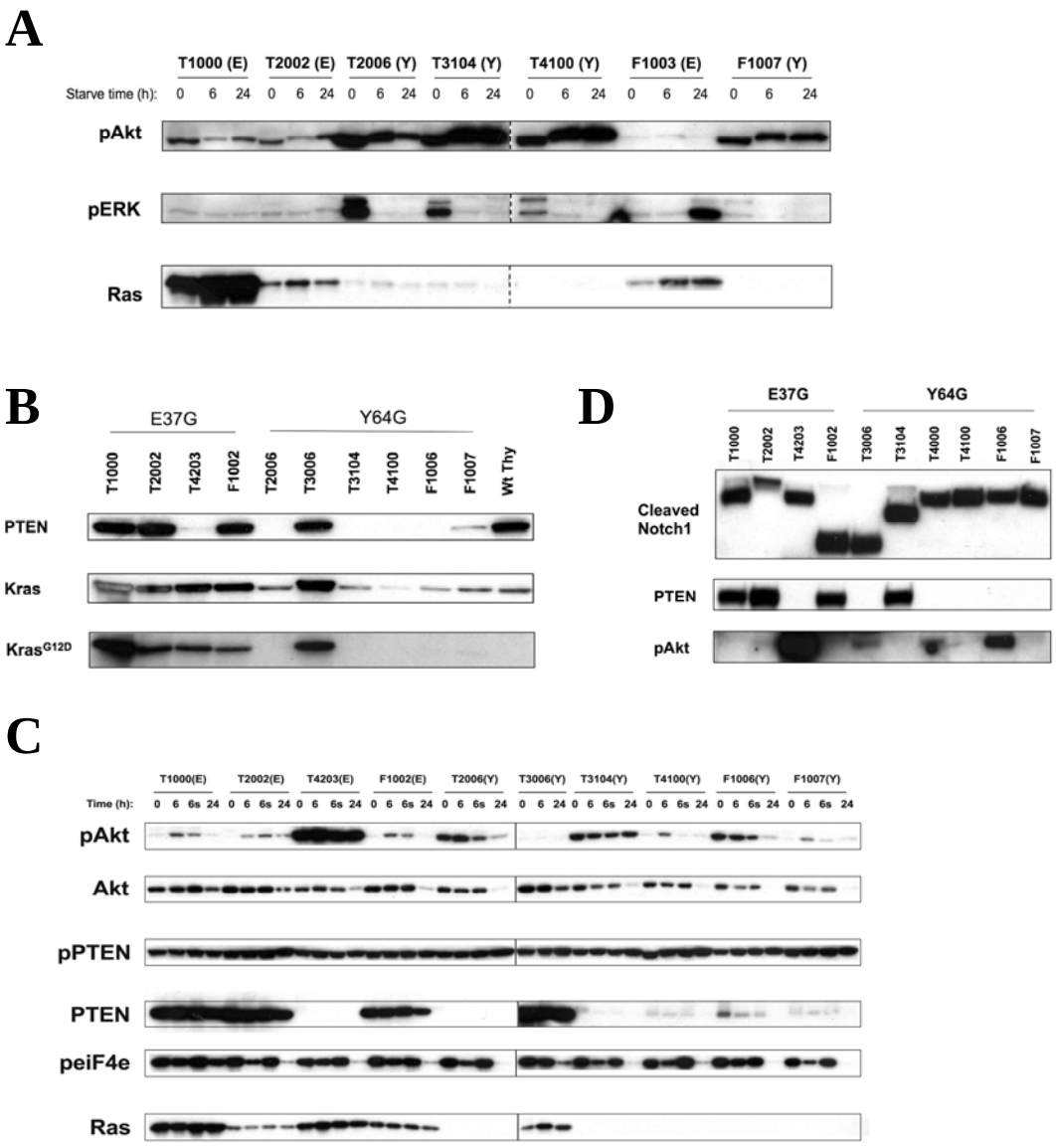

Figure 7 – Modulation of pAkt levels in T-ALL lines associated with changes in expression of PTEN. (A) *Cell lines were starved in media without IL-2, IL-7 and FBS for 0, 6, and 24 hours and then lysed. Blot in the left half was loaded with 40µg protein while the right half was loaded with 25µg. E37G cells show increased levels of total Ras while Y64G cells had increased pERK and constitutive pAkt signaling but no elevation of Ras levels.* ***(B)*** *Cell line lysates were immunoprecipitated with H-Ras agarose-conjugate and then probed with a Ras^{G12D}-specific antibody. Elevated total Kras levels in E37G cells are due to increased expression of the E37G second site mutant protein.* ***(C)*** *A panel of T-ALL lines were starved for 0, 6, 24 hours. At 6 hours, they were also stimulated with PMA for 10 minutes. Increased levels of pAkt were associated with decreased PTEN expression and vice versa.* ***(D)*** *Activated Notch1 levels are elevated in all the cells lines and were not correlated with PTEN expression.*

found that all the cell lines with elevated pAkt had significantly decreased levels of PTEN expression. Those that had decreased levels of pAkt showed a significant upregulation of PTEN. Interestingly, with the exception of T4203, cell lines that had increased expression of *Ras* also show increased levels of PTEN (**Fig. 7c**).

Lastly, since it has been described that activated Notch1 can modulate pAkt levels through regulation of PTEN (Chappell *et al.*, 2005; Palomero *et al.*, 2008), we wanted to see if there were differences in activated Notch1 between the cell lines that had increased PTEN versus those that did not show elevated PTEN. However, increased levels of Notch1 were present in all samples independent of *PTEN* expression (**Fig. 7d**).

Discussion

We found that transplanting bone marrow cells that had been transduced with $Kras^{G12D}$ second site mutants led to formation of a predominantly monoclonal, double-positive T-ALL. These T-ALLs transplanted efficiently into secondary sublethally irradiated recipients and appeared immunophenotypically similar to the parental tumors. However, their signaling profiles were heterogeneous. These results are intriguing as each T-ALL had started with either E37G or Y64G as the initiating lesion. Therefore, one might expect that tumors arising from these constructs would maintain the same signaling profile. As we observed in Figure 3a, this was not the case. Whereas the E37G T-ALLs maintained pAkt activation, they differed in their Raf/MEK/ERK and pS6 activation. Additionally, many of the Y64G T-ALLs did not show elevated pERK levels and even seemed to downregulate pERK (**Fig. 3a**). The signaling heterogeneity seen in T-ALLs that arose from the same initiating lesion argues that secondary mutations arise during the

disease evolution to modulate the output of Ras-dependent signaling networks such that many of them no longer carry their original signaling profile. These cooperating mutations can arise through retroviral insertional mutagenesis, random point mutations, or aberrant gene expression due to chromosomal translocations acquired during T-cell development (Armstrong and Look, 2005). Our data also suggest that there are multiple paths to T-ALL from an initiating Ras lesion. If there was only a restricted set of mutations or pathway changes that could lead to T-ALL, we would expect that the signaling from each of the tumors would have looked very similar.

After investigating signaling within each individual T-ALL, we showed that acquired changes in *Notch1*, *PTEN*, and *Ras* expression are associated with tumorigenesis. In most of the T-ALLs, levels of activated Notch1 were much higher than in wild-type thymi. In addition, we found mutations that introduced premature stop codons into the PEST domain of *Notch1* and are hypothesized to increase stability of intracellular Notch1 protein. The *Notch1* mutations arose in the parental T-ALL and then were maintained in the secondary transplants. So, it is clear that Notch1 is playing a cooperating role in driving the second site mutant-initiated T-ALLs. But what is that role? Notch1 plays a significant role in every stage of thymocyte development from HSCs to the double positive stage. Inactivation of *Notch1* leads to significant decreases in double and single-positive thymocytes and accumulation of double negative thymocytes in the most immature stage and a huge shift towards accumulation of B-cells (Radtke *et al.*, 1999). Reciprocally, overexpression of activated *Notch1* leads to production of $CD4^{+}CD8^{+}$ T-cells from bone marrow and a decrease in B-cells (Izon *et al.*, 2001; Pui *et al.*, 1999). Therefore, it is possible that Notch1 directs the development of second site

mutant HSCs down the thymocyte maturation pathway and allows them to survive negative selection processes.

Thymocyte selection occurs as a result of T-cell receptor interactions with self-peptides and MHC molecules as displayed on antigen-presenting cells. If the strength of the interaction is too weak, thymocytes undergo death by neglect. If the signal is too strong, cells are directed to negative selection pathways and undergo apoptosis. Progression to mature T-cell lineages (positive selection) only happens when the signal is between the extremes. Daniels et al found that the decision to undergo negative selection is mediated by Ras localization on the plasma membrane as opposed to the Golgi and increased activation of Raf/MEK/ERK (Daniels *et al.*, 2006). With our second site mutant Y64G, which hyperactivates the Raf/MEK/ERK pathway, thymocytes could potentially be driven down the negative selection pathway and therefore would require increased Notch1 levels to rescue them from apoptosis. It is not known how signaling down PI3K affects negative selection, but E37G may also be undergoing a similar process.

Alternatively, Notch1 may be playing a role as a secondary driver of disease independent of its role in T-cell development. After Notch1 is cleaved, intracellular Notch1 (ICN) forms a nuclear-transcription complex with CSL and MAML and induces transcription of target genes. Some pathways activated by cleaved Notch1 are cellular metabolism, self-renewal and transformation, cell survival, and angiogenesis. In T-ALL, p53, c-Myc, and PI3K/mTOR have all been shown to be activated by Notch1 (Aster *et al.*, 2008; Roy *et al.*, 2007). In fact, in some studies, Notch1 levels have been correlated with increased PTEN levels (Calzavara *et al.*, 2008; Chappell *et al.*, 2005). Although in

some cases (Maser *et al.*, 2007), including ours, Notch1 does not always correlate with *PTEN* expression, it is nevertheless possible that Notch1 activates other proteins that then drive T-ALL formation. It has also been shown that Notch1 signaling is important for maintenance of the neoplastic phenotype in different Ras-transformed cells (Weijzen *et al.*, 2002) and so activated Notch1 may also play a role in cooperating with the attenuated second site mutants to drive and maintain T-ALL.

When we examined pERK and pAkt levels in the T-ALL cell lines, E37G cell lines had diminished levels of pAkt and pERK, but had a very prominent increase in Ras levels due to overexpression of the second site mutant protein. On the other hand, cell lines derived from Y64G T-ALLs showed elevated pAkt levels but much lower levels of Ras. We found that the levels of pAkt were correlated with PTEN expression. One possible explanation for this is that in order to fully transform thymocytes, these second site mutants need to "rescue" the missing effector pathway. With E37G, instead of re-activating the ERK pathway, there is overexpression of Ras which can compensate for the defect in Raf/MEK/ERK signaling. In Y64G, we observed loss of PTEN expression, which leads to hyperactivation of the missing PI3K pathway. Alternatively, T-ALL could be driven by a total Ras effect that is independent of which individual effectors are activated. Or in other words, it is the sum total of Ras pathway outputs that drives disease once it reaches a certain threshold. In E37G T-ALLs, the tumors achieve this through overexpression of the second site mutant construct. In Y64G T-ALLs, they increase signal down the PI3K pathway by a mechanism that is likely to involve mutation or down-regulation of *PTEN*.

These results also point to the importance of the PI3K pathway in driving T-ALL formation. In the case of E37G, instead of seeing increased signaling in the Raf/MEK/ERK pathway, we see elevated levels of Ras. Using a Ras^{G12D}-specific antibody, we found that almost all of the increased Ras was due to increased levels of the E37G protein and not wild-type Ras. Since E37G hyperactives PI3K and RalGDS pathways, E37G T-ALLs are able to continue to activate PI3K in spite of high PTEN expression. In the Y64G-driven T-ALLs, increased expression of Y64G would have no effect on the PI3K pathway, so we do not see increased expression of the second site mutant. Instead, these T-ALLs hyperactivate pAkt by decreasing expression of PTEN presumably either through mutation followed by loss of heterozygosity or haploinsufficiency (Sulis and Parsons, 2003). This is significant because PI3K pathway inhibitors may be useful in targeting T-ALLs. In our cell lines, treatment with PI-103 was potent against all the cell lines independent of the initiating lesion. It could be that all of the T-ALL cell lines have a dependence upon PI3K and therefore are equally sensitive. Also, since it is known that T-ALLs driven by Notch1 upregulate PI3K and mTOR, (Chan *et al.*, 2007; Palomero *et al.*, 2007) it may be that the dual specificity of PI-103 to both PI3K and mTOR (Fan *et al.*, 2006) are too potent for the cells and use of a PI3K-specific compound may lead to selective inhibition of growth in the second site mutant cell lines. Use of different PI3K inhibitors and comparisons with other T-ALL cell lines will help clarify the role of the PI3K pathway in T-ALL.

Even though most of the E37G and Y64G T-ALL cell lines have genotype-specific signaling patterns, both T4203 (E37G) and T3006 (Y64G) did not follow this pattern. T4203 has both increased expression of E37G and loss of PTEN expression,

leading to very high levels of pAkt. It is unclear why this T-ALL acquired a second defect in the PI3K pathway. However, the fact that the PI3K pathway is required for IL-7-mediated growth, proliferation, viability and metabolic activation of T-ALL cells (Barata *et al.*, 2004) point towards elevated pAkt levels as potentially making this particular T-ALL more potent. Parenthetically, this T-ALL is very aggressive and kills recipient mice within 14 days compared with the average latency of 26.6 days (**Table 1**, data not shown).

T3006 shows a pattern very similar to the E37G tumors with increased levels of Ras and a concomitant increase in PTEN levels that is associated with downregulation of pAkt. Additionally, some of the Y64G tumors do not activate pAkt very highly in spite of low PTEN levels. It is possible that there may be other mutations present, especially due to random integration of the retrovirus. From Southern, each T-ALL has anywhere from 1-5 integration sites that are maintained through transplantation and are present in the cell lines as well. We are currently sequencing these insertion sites to determine if integration into certain signaling pathways is being selected for and contributing to outgrowth of T-ALL.

In sum, we show that K-Ras^{G12D} second site mutants drive formation of T-ALL in cooperation with deregulation of other pathways such as Notch1 and PTEN. Whereas E37G tumors upregulate Ras, Y64G cells tumors show a marked reduction in PTEN levels and elevated pAkt, which suggests an essential role for this pathway in T-cell leukemogenesis. Importantly, these results argue that inhibition of one pathway downstream of Ras is unlikely to completely reverse the oncogenic phenotype of Ras and that treatment of Ras-driven diseases necessitates multiple targeted therapies. Further

investigation of these mutants in a model in which mutant Ras is expressed from the endogenous promoter as well as other Ras-driven malignancies will help determine whether this is a T-cell specific effect or universally applicable to all Ras-driven diseases.

Materials and Methods

DNA Purification and Southern Analysis. Cells from peripheral blood, bone marrow, spleen, enlarged lymph nodes, and thymus were collected and lysed in DNA lysis solution (100mM Tris-HCl pH8.5, 5mM EDTA pH=8.0, 200mM NaCl, 0.2% SDS). Genomic DNA was isolated using isopropanol precipitation followed by 70% EtOH wash and resuspension in ddH20. Following restriction digestion with EcoRI, DNA fragments were separated by electrophoresis and immobilized onto a Nitran membrane. A probe of GFP was isolated from a sequence-verified MSCV-IRES-GFP vector and labeled with radioactive alpha-dCTP using Rediprime II Random Prime Labelling System (Amersham). DNA was hybridized with the radio-labeled probe in a solution (7%SDS, 0.5M NaP pH7.2, 1mM EDTA and 1% BSA) at 60^{o}C overnight, membrane was washed three times and autoradiograph was performed.

Western blot analysis. Single-cell suspensions of tissue or cell lines were pelleted and then lysed with a RIPA lysis buffer to look at basal levels of signaling. Cell lines were also starved in lymphocyte media without IL-2, IL-7, and FBS and then harvested at set time points. After 6 hours of starvation, cell lines were stimulated with 20μg/mL PMA. The antibodies used for immunoblotting included anti-Pan-Ras (Ab-3) (Calbiochem, San

Diego, CA), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-β-actin, anti-PTEN, anti-cleaved Notch1 (Val1744) (Cell Signaling Technology, Beverly, MA), anti-Akt, anti-pAkt (Ser473), anti-phospho-PTEN (Ser380/Thr382/Ser385), anti-phospho-eIF4e (Ser209) (Invitrogen). (pERK, pAkt, PTEN, pPTEN, Notch1, pEIF4e, Ras, actin)

Specific immunoblotting for Ras^{G12D}. Cells were lysed in Ras^{G12D} IP lysis buffer [50mM Tris-HCl (pH=8.0), 150mM NaCl, 5mM MgCl2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS] and 500µg of protein lysate were incubated with 5µl H-Ras (sc-34) agarose conjugate (Santa Cruz Biotechnology) overnight. Beads were washed 3 times with lysis buffer, run on gel, and then probed with a rabbit pan-*ras*$^{Asp-12}$(Ab-1) polyclonal antibody.

Growing primary T-ALL cell lines. Single-cell suspensions from bone marrow, thymus, or spleen of sick mice were resuspended in IMDM+20%FBS. 7.5x10^6 cells were aliquoted into a 24-well plated in duplicate and resuspended in lymphocyte media [DME-H21, 20% FBS, 10mM Hepes, penicillin/streptomycin, L-glutamine, 50µM β-mercaptoethanol, non-essential amino acids, pyruvate, 10ng/mL IL-2, 10ng/mL IL-7]. Cells were passaged into larger flasks as they became confluent and then stocks were frozen down in FBS+10%DMSO.

T-ALL Cell Line Proliferation Assay. 5x10^5 cells were plated per well of a 24-well plate in lymphocyte media. For each cell line, 12 wells were used (6 per drug). Both 901 and PI-103 were added at increasing concentrations ranging from 0 to 10µM with dilutions in

DMSO. After 6 days, a 50µl aliquot was removed from each well and counted on a Vi-Cell XR (Beckman-Coulter). Cell counts were normalized to number of cells in well without drug.

Notch1 Mutation Detection. Genomic DNA was isolated from different tissues as described in Southern analysis protocol. Exon 34 was amplified using the following primers: mPEST-forward, 5'-ATAGCATGATGGGGCCACTA-3'; mPEST-reverse, 5'-GCCTCTGGAATGTGGGTGAT-3'. Products were directly sequenced with an ABI 3100 DNA Sequencer (Foster City, CA) and results were analyzed utilizing FinchTV (Seattle, WA). To confirm mutations, PCR products were cloned using Zero Blunt Cloning Kit (Invitrogen) or pGEM-T Easy Vector System (Promega) and then re-sequenced.

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Chapter 5: Conclusions and Future Directions

Concluding Remarks

Use of second site mutants has allowed us to gain insight into the necessity of downstream effectors for Ras-driven hematologic malignancies. Loss of two of the three major effector pathways abrogates oncogenic potential whereas mutant K-Ras proteins that can engage two downstream effector pathways retain enough activity to initiate transformation. Further analysis of second site mutant-driven T-ALLs shows that the PI3K pathway may be important for development or maintenance of disease. Additionally, the observation that both E37G and Y64G can initiate leukemogenesis despite not fully activating all of the effector pathways suggests that blocking just one of the Ras effector pathways may not be enough to fully reverse the transformation potential of oncogenic Ras. Therefore, in treating Ras-driven hematologic malignancies, it will likely be more effective to simultaneously inhibit multiple downstream effector pathways.

Future Directions

In our work we have described use of 5 second site mutants. T35S and D38E solely activate Raf/MEK/ERK at moderate levels, Y40C activates only the PI3K pathway, E37G activates both RalGDS and PI3K and Y64G activates both RalGDS and Raf/MEK/ERK. These reagents allowed us to investigate the effect of different pathways on tumorigenesis. However, to gain a more complete picture of which effectors were responsible for different aspects of disease, it would be advantageous to have second site mutants that activate other combinations of effectors. For example, a RalGDS-only mutant or a strong Raf-only mutant or a Raf-PI3K combination that no longer activates

RalGDS. While these have not been described in literature, with increased knowledge of the structure of Ras-effector interactions, it is now possible to make *in silico* predictions regarding additional second site mutants that hypothetically selectively interact with the desired effector pathways.

In collaboration with the Serrano lab, we were given a list of ten hypothetical second site mutants that were derived from Ras-effector crystal structures and were predicted to show one of the three following activation patterns: Raf and PI3K, only Raf, and only RalGDS. After constructing these second site mutants, we did initial biochemical analyses on some of these mutants to determine their *in vitro* downstream effector activities. Unfortunately, many of the predicted mutants were not specific for the hypothesized effector pathways. A majority continued to activate all three pathways. Interestingly, even though the intensity of activation varied, as long as all three pathways were activated, the mutants showed cytokine-independent growth as well as hypersensitivity to GM-CSF. One mutant, Y64V, activated the Raf/MEK/ERK pathway to the same levels as G12D while not activating PI3K. In methylcellulose, Y64V had the same pattern of colony growth as WT. Development of a more complete second site mutant allele series will help refine the importance of these individual effector pathways in K-Ras^{G12D}-driven malignancy.

One interesting observation from T-ALL cell lines was the reacquisition of hyperactive signaling in the PI3K pathway of Y64G-driven T-ALLs, which was associated with absent or decreased PTEN expression. At this time, it is unclear the mechanism through which these tumors lose PTEN expression. *PTEN* may undergo inactivation due to somatic frameshift mutations as described elsewhere (Li *et al.*, 1997).

Alternatively, insertion of the retrovirus or point mutations may disrupt promoter or methylation sequences and therefore lead to diminished expression of the *PTEN* gene. We are currently determining whether PTEN loss is due to an mRNA expression or translation defect. Also, the increased expression of Ras in E37G cells and reactivation of PI3K pathway in Y64G cells implies an important role of the PI3K pathway in T-lineage leukemogenesis. It would be interesting to see the effects of inhibiting the PI3K pathway in these cell lines either pharmacologically or by siRNA to see if these cells are still dependent upon the PI3K pathway for survival. Additionally, we could co-transduce HSCs with a second site mutant and a *PTEN* siRNA to see if there is acceleration of T-ALL development.

We have identified somatic *Notch1* mutations and loss of PTEN expression as secondary alterations that are likely to contribute to the development of T-ALL in this system. However, it is likely that additional changes may be present as well. One important source of genetic alteration is the insertion sites of our MSCV-IRES-GFP constructs. From Southern blot analysis, each leukemia contains anywhere from 1-5 integration sites. Insertion of viral sequences into the genome can potentially have several phenotypic consequences. They can cause disruption of tumor suppressor genes leading to haploinsufficiency. Or they can integrate upstream of proto-oncogenes and drive overexpression of these genes. Even if they integrate into intronic sequences, they may have consequences on mRNA expression or methylation patterns. Together, these lesions can cooperate in the evolution of disease. Currently we are sequencing different tumors to identify the insertion sites. Confirming aberrant expression of these genes via RT-PCR and then investigating the effects of expressing these candidate genes in the context of

second-site mutants will give us increased insight into how these T-ALLs form and the second hits necessary for disease evolution.

In our *in vitro* and *in vivo* studies, we demonstrate that in cells containing E37G or Y64G, two of the three major Ras effector pathways remain activated, and that these constructs can initiate malignant growth *in vivo*. While this has been very informative in terms of what role different effectors play in tumorigenesis, one weakness of our approach has been the inability to express these second site mutants at physiologic levels. The MSCV construct contains an LTR promoter that drives expression of genes 3-5 times higher than physiological levels. For us, over-expression was an efficient strategy that allowed us to look at the effects of strongly activating Ras effector pathways. However, over-expression may have had unintended consequences, as seen with the $Braf^{V600E}$ mutation as well as what has been well-characterized in oncogenic Ras, and may not accurately reflect what happens in human disease. To more accurately model the physiological effects of these second site mutant proteins, we are currently collaborating with the lab of David Tuveson to create lox-stop-lox $Kras^{G12D,E37G}$ and $Kras^{G12D,Y64G}$ mice where the different *Kras* constructs are driven by the endogenous promoter.

In addition to expressing these alleles at endogenous levels, these mice will allow for temporal and spatial control of second site mutant expression. After crossing these mice with *Mx1-Cre* mice, we can use pIpC to initiate recombination of the allele at different time points within the hematopoietic cell compartment. It will be interesting to see if these second site mutants develop T-ALL like we observed in the transduction-transplantation models or whether they develop an attenuated form of the MPD found in *LSL-*$Kras^{G12D}$ mice (Braun *et al.*, 2004; Chan *et al.*, 2004).

Moreover, these mice can be used to study the role of downstream effectors in other *Kras*^{G12D} models. Use of inhaled aerosolized adeno-Cre in *LSL-Kras*^{G12D} mice drove formation of lung adenocarcinomas with high penetrance (Jackson *et al.*, 2001). Crossing *LSL-Kras*^{G12D} mice with *Fabp1-Cre* mice led to formation of colon adenocarcinoma (Haigis *et al.*, 2008). Expression of *Kras*^{G12D} using *Pdx-Cre* or *P48-Cre* led to formation of pancreatic ductal adenocarcinoma (Hingorani *et al.*, 2003; Hingorani *et al.*, 2005). Crossing these different tissue-specific *Cre* mice with the LSL-second site mutant mice will shed insight into how these cancers are initiated and whether the MAPK or PI3K pathways are necessary for tumor formation and maintenance. Overall, through continuing to investigate in more detail the role of each effector pathway in Ras-driven malignancy, we hope to understand the limitations of current therapeutic regimens as well as open the avenue for development of more specific and potent inhibitors.

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