The Role of MEK in Leukemogenesis

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Immunology in the Graduate School
of Duke University

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ABSTRACT

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Abstract

Hematopoiesis is the continual process of blood cell generation that primarily occurs in the bone marrow of adult animals. Hematologic neoplasms can also occur in the bone marrow and often result from dysregulation of signal transduction pathways. One example is the activation of the Ras oncogene, which has been linked to a variety of different cancers, including hematologic neoplasms. Ras is located proximal to the cell membrane and can activate many downstream effector pathways, thus it is difficult to determine which downstream pathway is mediating oncogenic Ras function. My thesis work focused on the effect of inappropriate activation of MEK/ERK, a downstream Ras effector pathway, in the hematopoietic system.

Using a retroviral transduction system, we expressed a constitutively active form of MEK1 in hematopoietic stem cells (HSCs). Mice transplanted with HSCs expressing active MEK developed a lethal myelodysplastic syndrome/myeloproliferative disease (MDS/MPN) characterized by the expansion of granulocytes/macrophages (G/M) at the expense of lymphoid cell development. Transplantation of active MEK-induced MDS/MPNs into naïve mice did not result in further disease, suggesting that the MDS/MPN is not a frank leukemia.

Bcl-2 is an anti-apoptotic molecule that has been shown to play a role in leukemia development and maintenance. Coupling expression of active MEK and Bcl-2 resulted

in MDS/MPNs that were phenotypically identical and had very similar disease onset compared to active MEK-induced MDS/MPNs. However, transplantation of Bcl-2/active MEK-induced MDS/MPNs did not result in a myeloid disease; rather, it resulted in the development of T-acute lymphoblastic leukemia (T-ALL) that was marked by activated Notch signaling.

These results led us to conclude that activation of MEK/ERK was sufficient to cause a pre-leukemic myeloid disease; however, additional oncogenic factors, such as Bcl-2 and Notch, were necessary for frank leukemia development. Moreover, additional oncogenic factors can alter the disease phenotype and disease course. Future analysis of the interplay between oncogenic factors will help shed light on disease development and aid in the development of more effective cancer treatments.

Dedication

To my family- the eternal source of stability, love, and happiness in my life.

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List of Abbreviations

7-AAD: 7-aminoactinomycin D

aCML: atypical chronic myeloid leukmia

AML: acute myeloid leukemia

B-ALL: B cell acute lymphoblastic leukemia

βc: common β chain

Bcl-2: B-cell lymphoma 2

BSA: bovine serum albumin

CSC: cancer stem cell

CLP: common lymphoid progenitor

CML: chronic myelogenous leukemia

CMML: chronic myelomonocytic leukemia

CMP: common myeloid progenitor

CNL: chronic neutrophilic leukemia

CR: conserved region

DC: dendritic cell

DN: double negative

DP: double positive

ERK: extracellular signal-related kinases

EPO: erythropoietin

ET: essential thrombocytopenia

FACS: fluorescent-activated cell sorting

FBS: fetal bovine serum

G-CSF: granulocyte-colony stimulating factor

GDP: guanosine diphosphate

GEF: guanine nucleotide exchange factor

GFP: green fluorescent protein

G/M: granulocyte/macrophage

GM-CSF: granulocyte/macrophage-colony stimulating factor

GTP: guanosine triphosphate

GMP: granulocyte/macrophage progenitor

HD: homodimerization domain

HSC: hematopoietic stem cell

ICN: intracellular notch

IL: interluekin

IRES: internal ribosome entry site

JAK: Janus kinase

JMML: juvenile myelomonocytic leukemia

JNK: c-Jun N-terminal kinase

kDa: kilodalton

LT-HSC: long-term hematopoietic stem cell

MAML1: mastermind-like protein 1

MAPK: mitogen-activated protein kinase

M-CSF: macrophage-colony stimulating factor

MDS: myelodysplastic syndrome

MegE: megakarocyte/erythrocyte

MEK: mitogen-activated protein kinase/extracellular kinase

MEP: megakaryoctye/erythrocyte progenitor

MHC: major histocompatibility complex

MPN: myeloproliferative neoplasm

MPP: multipotent progenitor

MSCV: murine stem cell virus

NK cell: natural killer cells

PDK: 3-phosphoinositide-dependent kinase

PH: pleckstrin homology

PI3K: phosphatidylinositol 3-kinase

PKC: protein kinase C

PMF: primary myelofibrosis

polyI:C: polyinosinic:polycytidylic acid

PTEN: phosphatase and tensin homolog

PV: polycythemia vera

RAG: recombination-activating gene

RBD: Ras binding domain

SCF: stem cell factor

SP: single positive

ST-HSC: short-term hematopoietic stem cell

STAT: signal transducer and activator of transcription

T-ALL: T cell acute lymphoblastic leukemia

TPO: thrombopoietin

WHO: World Health Organization

WT: wild-type

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1. Introduction

1.1 Hematopoiesis and lineage commitment

Hematopoietic stem cells (HSCs) are a rare population of progenitor cells found in the bone marrow that have the unique ability to self-renew, or to undergo multiple rounds of cellular division while remaining in an undifferentiated state. HSCs are also pluripotent, meaning they can give rise to all classes of blood cells. HSCs can be sub-divided into two groups based on their self-renewal capacity; long-term (LT-) HSCs, which have life-long self-renewal capacity and short-term (ST-) HSCs, which have limited self-renewal capacity (Kondo, Wagers et al. 2003).

Progenitor populations follow a specific developmental sequence where self-renewal capacity is diminished and lineage differentiation ability is gradually restricted. Accordingly, LT-HSCs give rise to ST-HSCs, which give rise to multipotent progenitors (MPPs) (Figure 1). MPPs have lost the ability to undergo self-renewal but retain full differentiation potential and are the major branching point for the myeloid and lymphoid lineages. Additional analysis suggests that MPPs can be further subdivided into three subsets that gradually undergo lineage restriction based on their expression of Flt3 and VCAM-1. Flt3^{lo}VCAM-1^{+} MPPs are the most primitive subpopulation and can give rise to bipotent lymphoid and granulocyte/macrophage (G/M) MPP populations

along with the common myeloid progenitor (CMP). In turn, CMPs can give rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEP). Flt3^{hi}VCAM-1^{+}MPPs can give rise to GMPs but can no longer give rise to MEPs, thus marking the loss of megakaryocyte/erythroid lineage potential. Flt3^{hi}VCAM-1^{-} MPPs have lost all myeloid cell potential but retain full lymphoid lineage potential as they can give rise to the common lymphoid progenitor (CLP). CLPs can subsequently give rise to terminally differentiated T, B, natural killer (NK) cells, and some classes of dendritic cells (DCs) (Lai, Lin et al. 2005; Lai and Kondo 2006; Hsu and Kondo 2007).

It is important to note that hematopoiesis is a hierarchical process; that is, cell differentiation is unidirectional and irreversible. While some committed progenitor populations retain some degree of differentiation plasticity, mature cells do not dedifferentiate or revert to a previous progenitor state and reacquire self-renewal capacity or the ability to differentiate into other lineages (Kondo, Scherer et al. 2000; Hsu and Kondo 2007).

The mechanisms that govern lineage fate decisions have been under intense study but are still unclear. One theory posits that lineage fate decisions are intrinsically determined in a stochastic manner. An opposing theory posits that extrinsic cues provided by soluble factors, such as growth factors and cytokines, may also impact cell fate decisions (de Groot, Coffer et al. 1998; Enver, Heyworth et al. 1998; Kaushansky

2006). More recent studies seem to support both models, leading to the creation of a "hybrid" model. This model suggests that promiscuous expression of lineage-specific genes occurs at a low level in multipotent progenitors and can be modified by extracellular cues (Enver, Heyworth et al. 1998). Regardless of the mechanism, lineage commitment is promoted through the expression of lineage-specific genes, which are controlled in part by transcription factors (Orkin 2000; Laslo, Pongubala et al. 2008). These transcription factors also help maintain lineage fidelity by actively suppressing alternative lineage programs (Orkin 2000; Laslo, Pongubala et al. 2008).

1.1.1 Myeloid cell development

Akashi and colleagues were the first to identify and purify committed myeloid lineage progenitors. Using CD34 and FcγR expression, they were able to isolate CMPs ($Fc\gamma R^{lo}CD34^{+}$), GMPs ($Fc\gamma R^{hi}CD34^{+}$), and MEPs ($Fc\gamma R^{lo}CD34^{-}$) from adult murine bone marrow (Akashi, Traver et al. 2000).

Whether cytokine receptor signaling plays an instructive or permissive role during myeloid cell development is still under intense debate; however, it is clear that they are important for myeloid cell development. For example, erythropoietin (EPO)

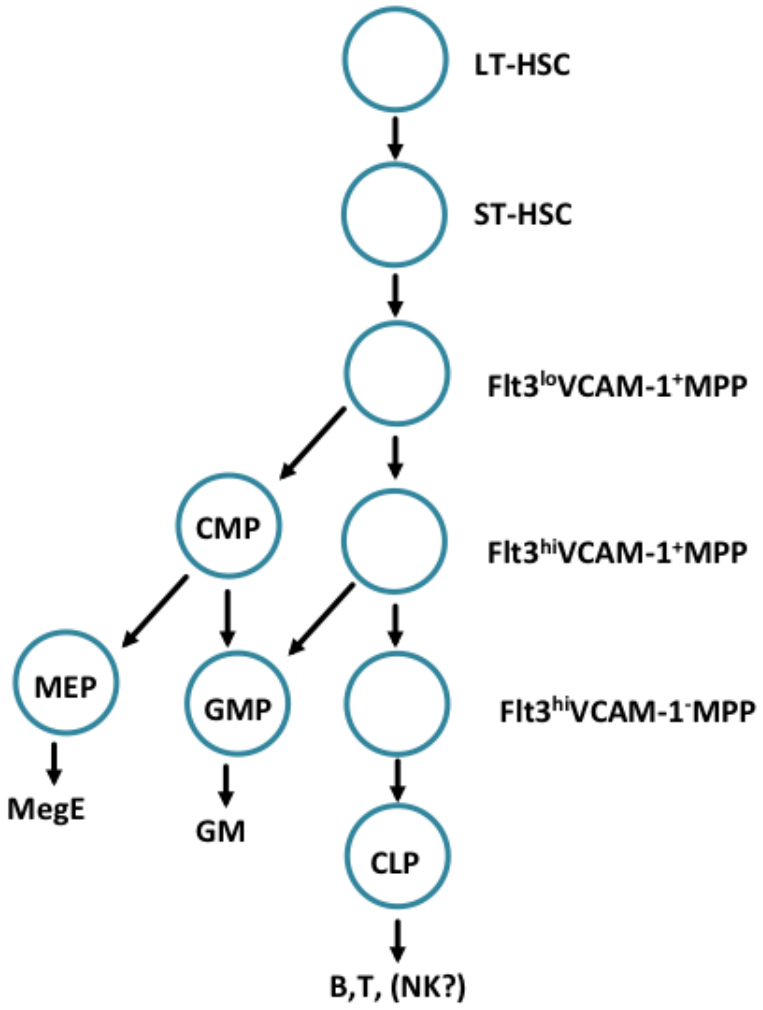


Figure 1: Working model of hematopoiesis.

The classic model of hematopoiesis has been refined by new knowledge of progenitor population and their progenies. Our current model of hematopoiesis suggests that the loss of self-renewal occurs concomitantly with restricted differentiation potential. Moreover, the model showcases how MPPs lose erythroid potential first, followed by GM, and then lymphoid. Adapted from (Lai and Kondo 2006).

plays a critical role in erythropoiesis as evidenced by studies showing that targeted deletion of EPO or erythropoietin receptor completely halt erythrocyte production and result in early embryonic death (Wu, Liu et al. 1995). Granulocyte-colony stimulating factor- (G-CSF) deficient mice have severely reduced populations of granulocyte and macrophage progenitors in the bone marrow. Mature granulocyte numbers in the periphery are not completely ablated but are decreased by half, suggesting that there is another mechanism that can compensate for G-CSF signaling during granulocyte maturation (Lieschke, Grail et al. 1994). Ablation of granulocyte-macrophage-colony stimulating factor (GM-CSF) does not affect steady-state hematopoiesis, but the loss of GM-CSF has a significant impact on emergency hematopoiesis following infection (Stanley, Lieschke et al. 1994; Hamilton and Anderson 2004).

Cytokine receptors can activate a variety of different pathways simultaneously to promote differentiation, proliferation, and cell survival (Watowich, Wu et al. 1996; Kaushansky 2006). These pathways include Ras/Raf/MEK/ERK, JAK/STAT, phosphatidylinositol 3-kinase (PI3K)/Akt, protein kinase C (PKC), and Smads. Once activated, these pathways can activate myeloid transcription factors that drive the differentiation process (Miranda and Johnson 2007).

A network of transcription factors is responsible for regulating myeloid cell development. PU.1 and CEBPα can cooperate to regulate a variety of granulocyte-

specific genes including myeloperoxidase, G-CSF receptor, and GM-CSF receptor (Friedman 2002). They can also cooperate to regulate a variety of macrophage/monocyte-specific genes, such as macrophage colony stimulating factor (M-CSF) receptor and lysozyme. The relative expression levels of CEBPα and PU.1 greatly impacts cellular differentiation; while high CEBPα levels lead to neutrophil production, high PU.1 levels initiate monocyte production (Friedman 2002). Lack of CEBPα expression does not affect megakaryocyte/erythrocyte (MegE) development, but the absence of PU.1 leads to slightly increased MegE numbers (Iwasaki and Akashi 2007). This is expected in light of the antagonist relationship between PU.1 and GATA-1, a transcription factor critical for MegE differentiation (Iwasaki, Mizuno et al. 2003). GATA-1 expression has been found to upregulate MegE-specific genes, such as EPO receptor and β-globulin (Iwasaki, Mizuno et al. 2003; Iwasaki and Akashi 2007). GATA-1 also contributes to maintaining lineage fidelity by suppressing lymphoid-related genes, such as IL-7Rα, Notch-1, EBF, and Pax5 (Iwasaki and Akashi 2007).

1.1.2 T Cell Development

The earliest thymic progenitor cells destined to be T cells exit the bone marrow and home to the thymus in an adhesion molecule-dependent manner (Sambandam, Maillard et al. 2005). The most immature thymocyte population does not express the

mature T cell markers CD4 or CD8; hence, they are termed double negative 1 (DN1) cells. The DN1 subset contains the most primitive intrathymic progenitors that can give rise to αβ and γδ lineage T cells (Porritt, Rumfelt et al. 2004). Additionally, DN1 and DN2 cells retain limited DC and NK differentiation potential (Schmitt, Ciofani et al. 2004). Irreversible commitment to the T cell lineage occurs at the DN3 stage when recombination-activating genes (RAG) 1 and 2 are expressed and mediate rearrangement of the TCRβ, TCRγ, and TCRδ loci (Rothenberg and Taghon 2005; Ciofani and Zúñiga-Pflücker 2007). A functionally rearranged TCRβ chain can associate with the invariant pre-TCRα chain and CD3 signaling molecules to form the pre-TCR complex (Rothenberg and Taghon 2005; Ciofani and Zúñiga-Pflücker 2007). Signaling through the pre-TCR complex marks successful passage through the β-selection checkpoint (Ciofani and Zúñiga-Pflücker 2007). The thymocytes can then progress to the double positive (DP) stage where RAG1/2 proteins are re-expressed, allowing the α locus to rearrange (Rothenberg and Taghon 2005; Ciofani and Zúñiga-Pflücker 2007). Cells expressing a mature αβ TCR undergo positive selection and then mature into single positive (SP) $CD4^+$ or $CD8^+$ cells (Rothenberg and Taghon 2005; Ciofani and Zúñiga-Pflücker 2007). These mature thymocytes can then exit the thymus and circulate throughout the periphery (Ciofani and Zúñiga-Pflücker 2007).

Several transcription factors are necessary to promote mature T cell development. Expression of E proteins, such as E2A, HEB, and Id3, are necessary for T cell maturation, but do not appear to play a role in early T cell development (Rothenberg and Taghon 2005; Laiosa, Stadtfeld et al. 2006). On the other hand, ablation of GATA-3 or Notch1 expression results in the complete lack of T cells, suggesting that they play a key role in early thymocyte development (Rothenberg and Taghon 2005; Laiosa, Stadtfeld et al. 2006). Indeed, Notch signaling has been shown to be required for the generation of the most primitive T cell progenitors in the thymus and has also been shown to play a crucial role in the decision between T and B lineage fates from bipotent lymphoid progenitors (Pui, Allman et al. 1999; Radtke, Wilson et al. 1999; Wilson, MacDonald et al. 2001; Sambandam, Maillard et al. 2005). Notch signaling further supports T cell development by suppressing other lineage fates. For example, interruption of Notch1 signaling prior to the DN2 stage of thymocyte development resulted in the development of NK cells, macrophages-granuloctyes, and DCs (Schmitt, Ciofani et al. 2004). Additionally, Notch signaling has been shown to suppress myeloid development in DN thymocytes (Zhou, Li et al. 2008).

However, the requirement for Notch signaling does not abate when progenitors specify the T cell fate. Notch signaling has been found to be crucial at the DN3 stage of T cell development (Laiosa, Stadtfeld et al. 2006; Yuan, Kousis et al. 2010). Specifically,

signaling through the pre-TCR complex and Notch1 leads to cell survival and rapid proliferation of DN3 cells (Laiosa, Stadtfeld et al. 2006; Aster, Pear et al. 2008). Notch signaling may also play a role in the upregulation of nutrient receptors by supporting or enhancing signaling through the PI3K pathway (Laiosa, Stadtfeld et al. 2006; Yuan, Kousis et al. 2010). However, Notch signaling must be tightly regulated during the transition from the DP to SP stage because prolonged Notch signaling can halt this transition (Laiosa, Stadtfeld et al. 2006; Yuan, Kousis et al. 2010).

1.2 Overview of Oncogenesis

Cancer can be viewed as the sum of its mutated genes that confer certain qualities necessary to establish, perpetuate, and maintain its malignant phenotype. These oncogenic qualities can be separated into six distinct categories that are acquired at various time points during disease according to the cancer hallmarks described by Hanahan and Weinberg (Figure 2)(Hanahan and Weinberg 2000). Acquired capabilities such as self-sufficiency in growth signals and insensitivity to anti-growth signals are oncogenic properties generally acquired early during the transformation process (Hanahan and Weinberg 2000). Other properties such as evasion of apoptosis and limitless replicative potential can be acquired early, but are often thought to be acquired after the initial oncogenic "hit" (Hanahan and Weinberg 2000). Finally, sustained

angiogenesis and tissue invasion and metastasis are among the last of the acquired capabilities observed in cancer cells because they are the most deadly cancer hallmarks (Hanahan and Weinberg 2000). Acquisition of all cancer hallmarks is not necessary to establish malignancy and certain mutations can confer multiple oncogenic properties, such as p53 deletions (Hanahan and Weinberg 2000; Hahn and Weinberg 2002). Studies using rodent cell lines showed that at least two genetic changes were necessary in order for cells to become tumorigenic (Land, Parada et al. 1983). However, human cells were found to be much more difficult to transform, requiring between four and seven genetic alterations before the appearance of a diagnosable tumor (Hahn and Weinberg 2002).

1.2.1 Hematological Neoplasms

Hematological neoplasms are cancers of or relating to the blood or bone marrow. They include diseases such as lymphomas, leukemia, and pre-leukemic conditions, examples of which are myelodysplastic syndrome (MDS) and myeloproliferative neoplasm (MPN). Leukemia is caused by the malignant transformation of hematopoietic cells by inappropriate activation or inactivation of molecules induced by genetic mutations or chromosomal translocations (Chen, Odenike et al. 2010). Studies using leukemia models are particularly useful in the study of cancer biology because the hematopoietic tree is well-defined; therefore, blocks in cellular differentiation can be

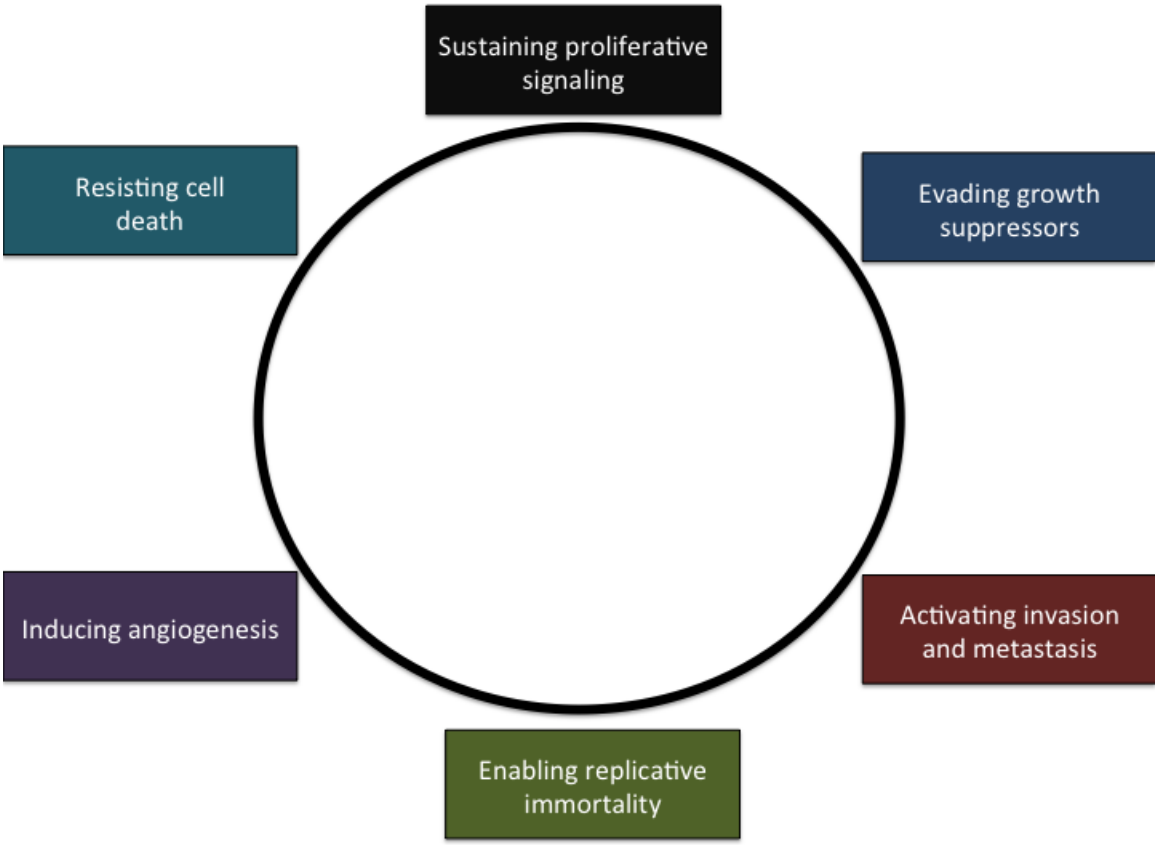


Figure 2: Hallmarks of cancer

List of the six original acquired capabilities observed in cancer proposed by Hanahan and Weinberg. Adapted from (Hanahan and Weinberg 2000).

clearly pinpointed so that isolation of a particular progenitor population can be carried out with relative ease (Kennedy and Barabe 2008). Also, development of hematopoietic tumors is less complex than solid tumors, which require angiogenesis along with a variety of physiological changes in order to breach tissue barriers and metastasize to foreign sites (Hanahan and Weinberg 2000; Kennedy and Barabe 2008). Because angiogenesis and metastasis occur during the later stages of solid tumor development, studying leukemia models can focus efforts on early oncogenic hits that result in aberrant cell growth and resistance to apoptotic signals (Hanahan and Weinberg 2000; Kennedy and Barabe 2008).

Hematopoietic neoplasms are classified by cell lineage and developmental maturity. Leukemias can arise from cells of the myeloid lineage as well as the lymphoid lineage (Harris, Jaffe et al. 1994; Vardiman 2010). Acute leukemias are marked by a tremendous increase in immature blast cells in the bone marrow and blood (Harris, Jaffe et al. 1994; Vardiman, Thiele et al. 2009). Although there is an appreciable increase in cells in the bone marrow, these cells cannot differentiate and therefore are non-functional. These non-functional cells eventually out-compete functional cells for space in the bone marrow, which often leads to bone marrow failure (Vardiman, Thiele et al. 2009). In acute cases, malignant cells accumulate very quickly, spurring rapid disease progression. On the other hand, chronic leukemias are generally caused by the over-

production of malignant cells with a mature phenotype (Harris, Jaffe et al. 1994; Vardiman, Thiele et al. 2009). The expansion ability of malignant cells in chronic diseases is not as great as those found in acute leukemias, thus the accumulation of malignant cells requires more time, resulting in longer disease progression (Harris, Jaffe et al. 1994; Vardiman, Thiele et al. 2009).

Ubiquitously expressed tumor suppressors, such as p53, and oncogenes that regulate cell death or proliferation, such as c-Myc, have been shown to affect hematologic neoplasm development and maintenance (Hunter 1991). However, mutations in pathways involved in lineage decisions, such as Ras/Raf/MEK/ERK, PI3K/Akt, and Notch, and mutations in transcription factors, such as CEBPα, have also been found (Downward 2003; Steelman, Pohnert et al. 2004; Aster, Pear et al. 2008; Koschmieder, Halmos et al. 2009). These observations suggest that signaling pathways involved in maintaining homeostatic hematopoiesis can be dysregulated, thus giving rise to hematologic neoplasms (Hunter 1991; Hanahan and Weinberg 2000; Chen, Odenike et al. 2010).

1.2.2 Myelodysplastic Syndrome (MDS) and Myeloproliferative Neoplasm (MPN)

Myelodysplastic syndrome (MDS) is a collection of diseases that display myeloid dysplasia, marked by abnormal development in erythroid cells, granulocytes, or

megakaryocytes (Tefferi, Skoda et al. 2009; Vardiman, Thiele et al. 2009). Cells undergo maturation during MDS, but often have dysplastic morphology and undergo premature apoptosis, resulting in ineffective hematopoiesis that causes decreased cell numbers, or cytopenia (Tefferi, Skoda et al. 2009; Vardiman, Thiele et al. 2009). Various types of refractory cytopenias and anemias fall into the MDS category (Tefferi and Gilliland 2007; Vardiman, Thiele et al. 2009).

MPNs are clonal diseases that originate in HSCs (Tefferi and Gilliland 2007). All classes of myeloid cells undergo full and effective maturation, but the cells are hyperproliferative, leading to hepatosplenomegaly and hypercellular bone marrow. MPNs are a heterogenous group of diseases that are regarded as pre-leukemic because subsequent genetic lesions often lead to progressive disease development (Vardiman, Thiele et al. 2009). While MPNs share some common phenotypic traits, they are also very diverse in nature, suggesting that no single genetic event is responsible for disease development. For example, the Philadelphia (Ph) chromosome, t(9;22), is the result of a translocation event where the N-terminus of the BCR protein is fused with the tyrosine kinase catalytic domain of the ABL oncogene (Tefferi and Gilliland 2007). The Ph chromosome has been found to be the causative cytogenic event responsible for chronic myelogenous leukemia (CML), but not other types of MPNs (Tefferi and Gilliland 2007; Vardiman, Thiele et al. 2009). Other examples of MPNs include: chronic neutrophilic

leukemia (CNL), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) (Tefferi and Gilliland 2007; Vardiman, Thiele et al. 2009). Similarly, gain-of-function JAK2 mutations have been linked to virtually all PV and half of those with ET or PMF, but not with other MPNs, such as CNL and CML (Tefferi and Gilliland 2007).

Chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML) have always been classified as MPNs, even though CMMLs often exhibit mixed MDS and MPN phenotypes (Emanuel 2008; Vardiman, Thiele et al. 2009). Revision of the World Health Organization (WHO) classification scheme of myeloid neoplasms in 2001 created a combined MDS/MPN category to better suit diseases such as CMML, JMML, and atypical CML, which often display proliferative as well as dysplastic features (Vardiman, Thiele et al. 2009).

JMMLs are characterized by the absence of the Ph chromosome or BCR/ABL fusion protein, low blast counts in the peripheral blood and bone marrow, moderate leukocytosis, monocytosis, thrombocytopenia, occasional immature neutrophils, and few nucleated red blood cells (Emanuel 2008; Tefferi, Skoda et al. 2009; Vardiman, Thiele et al. 2009). Hepatosplenomegly is also observed, along with infiltration into non-lymphoid organs such as liver, skin, and lungs (Emanuel 2008; Vardiman, Thiele et al. 2009). Most JMML patients have a normal karyotype. However, activation of the Ras

pathway has also been linked to JMML (Tefferi and Gilliland 2007). Activation of the Ras pathway can occur by point mutations that result in an active form of Ras or by inhibiting the function of negative regulators of Ras signaling, such as Nf1 and SHP-2 (Tefferi and Gilliland 2007).

As evidenced by the difficulty in its classification, CMMLs are a collection of heterogenous diseases (Tefferi and Gilliland 2007; Emanuel 2008; Vardiman, Thiele et al. 2009). Similar to JMMLs, all CMMLs lack expression of the Ph chromosome and BCR-ABL fusion protein and persistent monocytosis; however, a wide variation exists among hematologic parameters (Emanuel 2008; Vardiman, Thiele et al. 2009). Some human patients exhibit neutropenia, while others exhibit neutrophilia and whole bone marrow cell counts can range from average to leukopenic levels (Vardiman, Thiele et al. 2009). Also, blast percentages in the bone marrow and blood can vary greatly from patient to patient (Tefferi and Gilliland 2007; Vardiman, Thiele et al. 2009).

1.2.3 T-Acute Lymphoblastic Leukemia (T-ALL)

T-acute lymphoblastic leukemia (T-ALL) is a malignant condition involving lymphoblasts committed to the T cell lineage. T-ALLs are a group of heterogenous diseases that have different immunophenotypes, cytogenetics, molecular genetic abnormalities, and clinical features (Graux, Cools et al. 2006).

Approximately half of all T-ALL cases exhibit chromosomal translocations, gene fusions, and cryptic deletions (Graux, Cools et al. 2006). Pre-malignant cells often display chromosomal instability that can lead to breakage events within the chromosome (Graux, Cools et al. 2006). There are several ways that breaks can be resolved that promote malignancy. Chromosomal translocation events often involve promoter swaps where transcription factor genes are placed under the control of strong promoter and enhancer elements of the TCR genes (Graux, Cools et al. 2006; Aifantis, Raetz et al. 2008). One example of this is the t(7;9) chromosomal translocation, which provided the earliest piece of evidence that linked Notch signaling to oncogenesis (Ellisen, Bird et al. 1991). This translocation event in humans juxtaposes Notch1 and the TCRβ enhancer, resulting in a constitutively active form of Notch1 that has been linked to T-ALL development (Ellisen, Bird et al. 1991). Subsequent animal studies have also shown that the t(7;9) translocation event causes T-ALL (Pear, Aster et al. 1996).

Gene fusion can also resolve chromosomal breaks by fusing two breakpoints in frame, leading to the generation of a new chimeric protein that has oncogenic properties (Graux, Cools et al. 2006). SIL-TAL1 is an example of a fusion gene that is found in 9-30% of childhood T-ALL cases (Graux, Cools et al. 2006). Cryptic deletions are chromosomal deletions that cannot be detected with standard cytogenetic analysis techniques and often result in the deletion of negative regulators of cell cycle or cell

death (Graux, Cools et al. 2006). For example, the INK4/ARK locus is often deleted in many malignancies, including T-ALL, leading to the loss of G1 control of the cell cycle (Graux, Cools et al. 2006; Aifantis, Raetz et al. 2008).

In cases where the patient karotype is normal, other genetic aberrations are observed. Mutations that result in constitutive activation of signaling molecules such as Notch or N-Ras have also been shown to contribute to T-ALL development (Graux, Cools et al. 2006; Aster, Pear et al. 2008). Similarly, mutations that result in inactivation of tumor suppressors, such as phosphatase and tensin homolog (PTEN), can also lead to disease development (Graux, Cools et al. 2006). These genetic and chromosomal abnormalities are not mutually exclusive. In fact, inappropriate activation of Notch signaling can be the consequence of either a translocation event or a mutation (Graux, Cools et al. 2006; Aster, Pear et al. 2008).

Notch is a cellular membrane protein and can ligate members of the Delta-Serrate-Lag2 (DSL) family (Figure 3) (Grabher, von Boehmer et al. 2006). Interaction with its ligand induces a conformational change within the homodimerization (HD) domain of Notch1 that allows the ADAM family of metalloproteinases to cleave the extracellular portion of Notch (Grabher, von Boehmer et al. 2006). This is followed by a second cleavage event at the transmembrane domain that is mediated by the γ-secretase

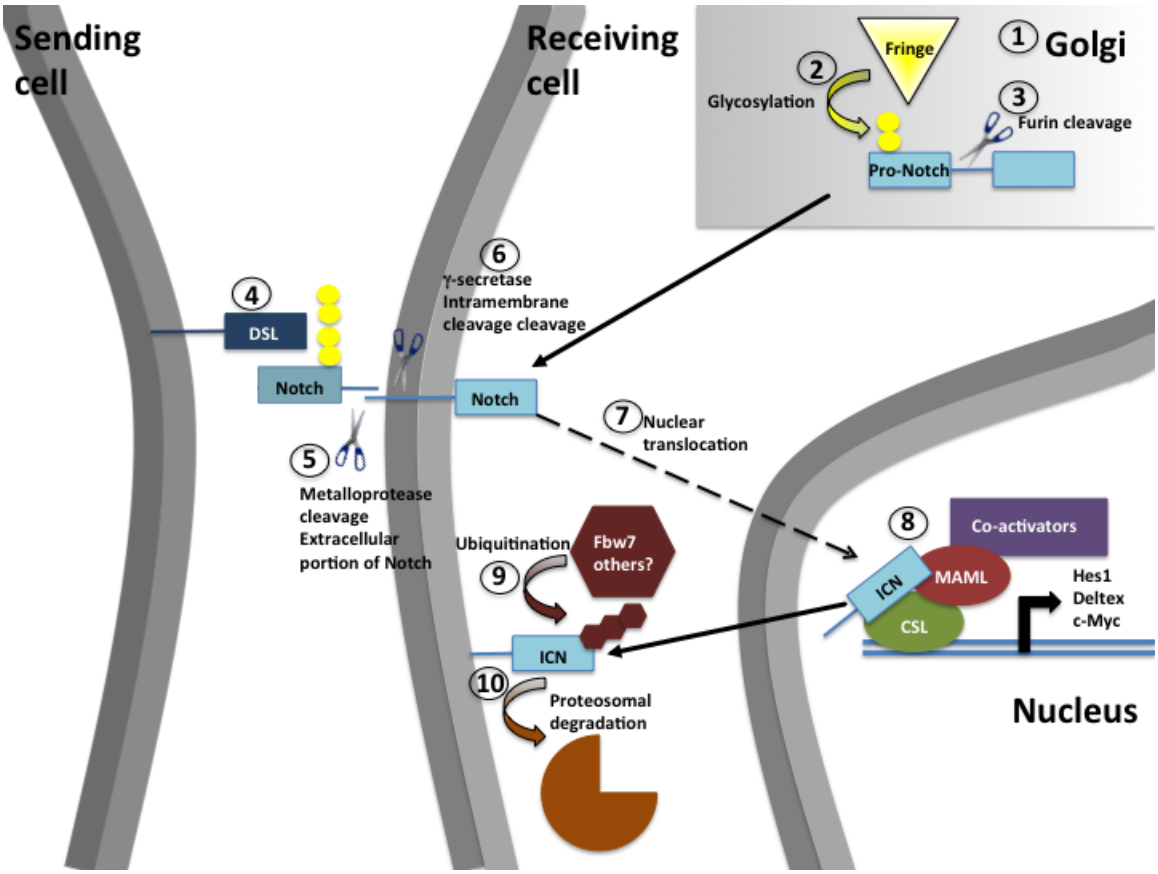


Figure 3: Overview of the canonical Notch signaling pathway.

(1) The intracellular and extracellular domains of the Notch receptor are synthesized as a single protein (pro-Notch) and are processed in the Golgi. (2) Here, glycosylation of Pro-Notch, which is important for ligand and Notch receptor interaction, is mediated by Fringe family members. (3) A Furin-like convertase cleaves pro-Notch into the extracellular and intracellular domains, resulting in a heterodimeric receptor that is transported to the plasma membrane. (4) Activation of the Notch receptor occurs when Notch receptors bind to the Delta-Serrate-Lag2 (DSL) ligands. (5) The first cleavage event occurs when ADAM-type-metalloproteases (MP) cleave the extracellular portion of Notch. (6) This is followed by another cleavage event mediated by γ-secretases at the transmembrane domain. (7) This releases the intracellular domain (ICN) of Notch, allowing it to translocate to the nucleus. (8) ICN can then bind to CSL/RBP-Jκ,

Mastermind-like (MAML) family members, and other co-activators to drive transcription of downstream Notch target genes. (9) Notch signaling is terminated when phosphorylated ICN is ubiquinated by E3 ligases such as Fbw7 and (10) degraded by proteasomes. Adapted from (Aster, Pear et al. 2008).

complex, which releases the intracellular domain (ICN), allowing it to translocate to the nucleus (Grabher, von Boehmer et al. 2006; Aster, Pear et al. 2008). The C-terminal region of ICN contains a domain rich in proline (P), glutamic acid (E), serine (S), and theronine (T). Ubiquitination of this PEST domain controls the stability of ICN (Grabher, von Boehmer et al. 2006; Aster, Pear et al. 2008). Once in the nucleus, ICN converts CSL/RBP-Jκ from a transcriptional repressor to a transcriptional activator and recruits co-activator proteins, such as mastermind-like 1 (MAML1) and histone acetylase p300, resulting in the transcription of Notch-target genes. These genes include *hes1, pre-TCRα, deltex, nrarp, cdkn1a,* and *cd25* (Grabher, von Boehmer et al. 2006).

Notch signaling has been found to be critically important for normal T cell development and its importance in malignancy is underscored by the fact that somatic activating Notch mutations were observed in 55% of human T-ALL cases (Weng, Ferrando et al. 2004). Analysis of T-ALL patients shows that 27% of cases harbor mutations in the HD domain, 15% of cases harbor mutations solely in the PEST domain, and 16% harbor mutations in both domains (Weng, Ferrando et al. 2004). The HD domain is required to stabilize the association of the extracellular and intracellular portions of the Notch1 receptor and prevents ligand-independent signaling (Aster, Pear et al. 2008). Therefore, mutations in the HD domain may result in ligand-hypersensitivity or aberrant signaling in the absence of ligand (Grabher, von Boehmer et

al. 2006; Aster, Pear et al. 2008). The ubiquitination of the PEST domain results in proteasome-dependent degradation of ICN1, thus ceasing Notch signaling (Grabher, von Boehmer et al. 2006; Aster, Pear et al. 2008). Truncation of the PEST domain ablates these ubiquitin sites, leading to increased ICN1 half-life in the nucleus (Grabher, von Boehmer et al. 2006).

1.2.4 Cancer Stem Cell Hypothesis

Tumors exhibit heterogeneous morphology and function. There are two possible explanations to explain this observation. The stochastic model argues that tumors are biologically homogenous and the behavior of tumor cells is influenced by intrinsic factors, such as transcription factor expression levels or dysregulation of signaling pathways, or extrinsic factors, such as immune responses or microenvironment (Dick 2008). Because these influences act randomly and cannot be controlled, extracellular marker expression, cell cycle status, and tumor initiation capacity will vary. Moreover, these intrinsic and extrinsic factors do not result in permanent change so the properties of tumor cells are transient (Dick 2008).

Alternatively, the hierarchy model views tumors as malignant organs (Dalerba, Cho et al. 2007; Dick 2008). Akin to normal tissues such as blood, skin, and colon, tumors are also organized into hierarchies maintained by stem cells (Dalerba, Cho et al.

2007; Dick 2008). According to this model, cellular heterogeneity is a result of this organizational structure, in which primitive cell populations with self-renewal capacity give rise to mature cell populations with heterogeneous function that lack self-renewal capacity (Dalerba, Cho et al. 2007; Dick 2008).

Because changes in the stochastic model are not permanent, the fate of the cell is constantly in flux. Each cell has the potential to give rise to disease, but only under permissive conditions. On the other hand, the hierarchy model supposes that a rare and distinct subset of cells, also known as cancer stem cells (CSC), is capable of propagating a tumor (Reya, Morrison et al. 2001; Dick 2008; Lane and Gilliland 2010). Similar to normal stem cells, CSCs have extensive proliferative and self-renewal potential, as evidenced by their ability to give rise to new abnormal tissues (Kondo, Wagers et al. 2003; Dick 2008). Additionally, CSCs are pluripotent. Tumors are composed of a heterogeneous collection of cells that have been found to have different proliferation rates and varying abilities to propagate new tumors (Dick 2008). According to the hierarchy model, fractionation of the CSC population is possible because cells capable of propagating a tumor are phenotypically distinct from cells with limited self-renewal capacity (Dick 2008). In contrast, the stochastic model posits that all tumor cells have the same potential to give rise to disease so sub-fractionation of the tumor population is not possible (Dick 2008; Lane and Gilliland 2010).

The longevity of HSCs is crucial because they can persist throughout the entire lifespan of an adult animal, thus allowing them to accumulate multiple mutations (Hanahan and Weinberg 2000; Reya, Morrison et al. 2001). Pathways that control self-renewal are already activated in HSCs; therefore, maintaining activation of these pathways may require fewer mutations than de novo activation. Indeed, dysregulation of pathways involved in HSC self-renewal, such as Bmi1, Notch, PTEN, Wnt, and sonic hedgehog, have been found in several different types of cancers (Pardal, Clarke et al. 2003). Although more mature progenitor populations have shorter lifespans, it is possible that mutations that activate certain self-renewal pathways help to extend the lifespan of those progenitors, allowing additional mutations to accumulate until disease onset. Indeed, several cases of transformed progenitor populations leading to disease have been observed (Dash and Gilliland 2001). However, these are very specific instances requiring very precise translocation events, such as MOZ-TIF, MLL-AF9, and MLL-ENL, which all lead to AML, and are not applicable to all types of cancers (Dash and Gilliland 2001). These data suggest that progenitor populations can be targets of transformation; however, HSCs are most likely the main target of transformation events due to their unique properties (Dash and Gilliland 2001; Reya, Morrison et al. 2001).

Many causal relationships between pathogens and infectious diseases have been established since Koch's postulate was originally proposed. Progress on a cellular

version of Koch's postulate establishing a causal relationship between cancer-initiating cells and malignancy has moved forward for some types of cancer. The CSC population was first identified in human AML and defined as $CD34^{+}CD38^{-}$ cells (Lapidot, Sirard et al. 1994; Bonnet and Dick 1997). Not only were these cells phenotypically identical to normal progenitor populations, they also displayed differentiation potential and the ability to repopulate a NOD-SCID immunodeficient mouse following xenotransplantation (Lapidot, Sirard et al. 1994; Bonnet and Dick 1997). Since then, CSCs have been identified in an increasingly long list of solid tumors, including brain, breast, and colon (Dick 2008). However, the notion that CSCs are a rare population has been challenged by more recent work that utilizes NOD-SCID $Il2r\gamma^{-/-}$ mice as recipients. These mice are more immunocompromised than recipients used in the original study, suggesting that the reason for low CSC numbers is due to the host immune response. Regardless of the rarity of the CSC population, their ability to propagate tumors remains (Quintana, Shackleton et al. 2008).

1.3 Overview of the Ras/Raf/MEK/ERK Pathway

The mitogen-activated protein kinase (MAPK) pathway can transmit extracellular cues into the cell resulting in a variety of biological functions (Figure 4)(Steelman, Pohnert et al. 2004). This pathway can be activated by cellular stress, such

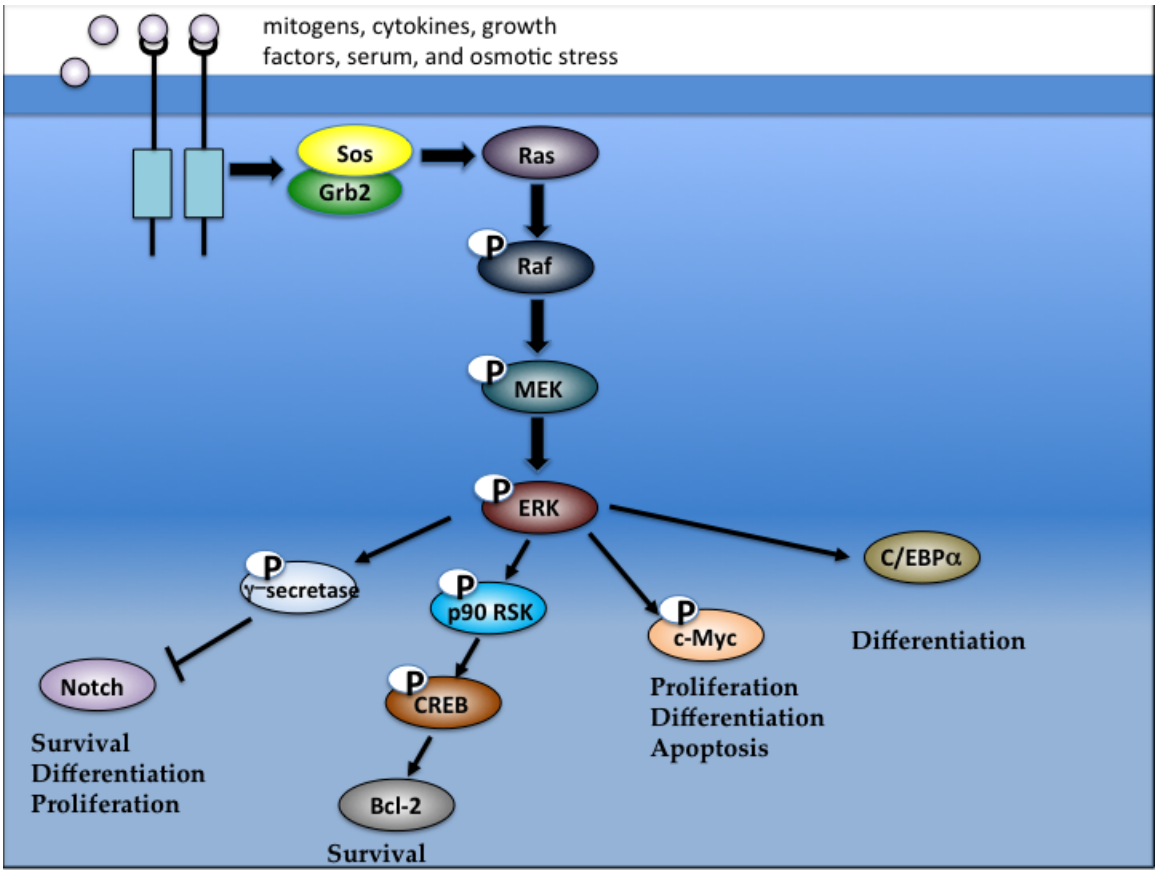


Figure 4: The MAP kinase signaling cascade.

Stimulation by mitogens, cytokines, growth factors, serum, and osmotic stress can trigger a signal cascade that leads to signaling through the MAP kinase pathway. Activation of receptor tyrosine kinases result in the recruitment of Grb2, an adaptor protein, and Sos, a guanine exchange factor (GEF), to the plasma membrane. This brings the proteins in close proximity with Ras, thus facilitating activation. Activated Ras can then recruit Raf to the plasma membrane where it can be activated. Activated Raf can then go on to phosphorylate and activate MEK, which can then phosphorylate and active ERK. Activated ERK can phosphorylate a large number of cytoplasmic and nuclear proteins, some of which are shown here. ERK has been shown to increase transcription of C/EBPα, a transcription factor important for differentiation. ERK phosphorylation of c-Myc stabilizes the protein by protecting it from proteasomal degradation. c-Myc functions to drive a variety of cellular functions including proliferation, differentiation, and apoptosis. Phosphorylation of p90 RSK activates the protein so it can phosphorylate CREB, which leads to transcription of pro-survival genes, including Bcl-2. Nicastrin is one of four components that form the γ-secretase complex responsible for cleaving the Notch receptor, which releases the intracellular domain of Notch and propogates signaling. ERK phosphorylation of nicastrin decreases its ability to cleave and release the intracellular portion of Notch, thus inhibiting downstream Notch signaling. Adapted from (Roberts and Der 2007).

as osmotic shock and ionizing radiation, cytokine stimulation, and growth factors (Figure 4) (Steelman, Pohnert et al. 2004). Following stimulation, signals are relayed into the cell via a series of protein modifications, namely protein phosphorylation (Steelman, Pohnert et al. 2004). These biochemical signals result in physiological cellular responses, such as cellular proliferation, cellular senescence, cell survival, and differentiation. There are four major groups of MAPK pathways: the p38 Map kinase family, the extracellular signal-regulated kinase family (ERK), and the c-Jun NH2-terminal kinase (JNK) kinase family, and the ERK5 kinase family (Steelman, Pohnert et al. 2000). Here, we focus on ERK1/2 and its upstream components.

1.3.1 Biochemical properties of proteins

Viral homologues of the Ras (Rat Sarcoma Virus) gene were first identified in the 1960's and subsequent research in the 1970's showed that they were responsible for the oncogenic activity of Harvey and Kirsten rat sarcoma viruses (Malumbres and Barbacid 2003). There are three Ras genes that encode four highly homologous 21 kDa proteins: *H-ras, N-ras, K-ras4A, and K-ras4B*. Alternative splicing of the C-terminus results in the two different K-Ras isoforms (Schubbert, Shannon et al. 2007).

Stimulation with mitogens, cytokines, and growth factors has been shown to activate Ras, a member of the low-molecular-weight GTP (guanine triphosphate)-

binding family of proteins (Neal, J.F. et al. 1988). In the inactive form, Ras is bound to guanine diphosphate (GDP) and is localized to the plasma membrane (Neal, J.F. et al. 1988). Following ligand binding, upstream guanine exchange factors (GEFs) are activated, bind to Ras, disrupt nucleotide binding and triggers the release of GDP (Neal, J.F. et al. 1988; Boriack-Sjodin, Margarit et al. 1998). GTP, which is present at higher concentrations in the cell than GDP, binds more readily to Ras (Neal, J.F. et al. 1988). GTP binding triggers dissociation of GEFs and a conformational change occurs which increases the affinity of Ras for effector targets such as Raf (Gibbs, Sigal et al. 1984; John, Frech et al. 1988; Neal, J.F. et al. 1988; Boriack-Sjodin, Margarit et al. 1998; Schubbert, Shannon et al. 2007).

Several mutational hotspots have been identified for Ras: Glycine 12, Glycine 13, and Glutamine 61(Krengel, Schlichting et al. 1990). Substitution of any other amino acid for glycine at position 12 results in steric hindrance and decreased GTP hydrolysis to GDP. Conversion to Ras-GDP is necessary to turn off signaling since Ras remains active when bound to GTP. Also, mutation of Glycine 61 decreases the stability of the transition state, thus significantly decreasing GTPase activity (Krengel, Schlichting et al. 1990; Tong, de Vos et al. 1991; Scheffzek, Ahmadian et al. 1997).

There are three main isoforms of the Raf (Rapidly Accelerated Fibrosarcoma) protein: A-Raf, B-Raf, and Raf-1 (c-Raf). Each isoform contains three conserved regions:

CR1, CR2, and CR3. CR1 contains the Ras binding domain (RBD) and a cysteine rich domain which forms a zinc finger structure (Mercer and Pritchard 2003). The cysteine rich domain can interact with a lipid moiety on Ras, thereby stabilizing the protein association and allowing Raf to be recruited to the cellular membrane (Mercer and Pritchard 2003). The CR2 region contains the regulatory binding site for 14-3-3 proteins, which are a class of scaffolding and adaptor proteins (Mercer and Pritchard 2003; Leicht, Balan et al. 2007). The kinase domain along with a second 14-3-3 protein binding site is located in the CR3 region (Mercer and Pritchard 2003; Leicht, Balan et al. 2007). In the absence of stimulation, the catalytic kinase domain is folded and bound to the amino terminus, which serves as a negative regulatory domain. The entire interaction is stabilized by the binding of phosphorylated serine residues on Raf to a 14-3-3 dimer (Mercer and Pritchard 2003; Leicht, Balan et al. 2007). Following activation, Ras-GTP binding to the RBD and the cysteine rich domain disrupts 14-3-3 dimer binding to Raf, thus causing a conformational change (Leicht, Balan et al. 2007). This conformational change causes the release of the catalytic domain, priming Raf for activation via a variety of different events such as phosphorylation (Mercer and Pritchard 2003; Leicht, Balan et al. 2007). It should be noted that Ras binding to Raf is not sufficient to activate the catalytic domain on Raf; however, it does recruit Raf to the membrane, thus increasing the likelihood it can become activated by other effector proteins (Mercer and Pritchard

2003; Leicht, Balan et al. 2007). Also, the conformational change may be important in revealing a docking site for the Raf substrate MEK (Pearson, Robinson et al. 2001; Mercer and Pritchard 2003; Sebolt-Leopold and Herrera 2004; Leicht, Balan et al. 2007).

There are five genes in the MEK (Mitogen-activated protein kinase/ERK Kinase) family: *MEK1, MEK2, MEK3, MEK4*, and *MEK5*. All five are dual specificity kinases, meaning they are able to phosphorylate serine/threonine residues along with tyrosine residues (Pearson, Robinson et al. 2001). Of those, MEK1 and MEK2 are activated downstream of Ras and Raf by phosphorylation of two serine residues located within the activation domain. Although MEK1 and MEK2 have 90% amino acid sequence similarity, differences in nucleotide sequence are found throughout the aligned cDNAs suggesting that they are distinct gene products (Wu, Harrison et al. 1993; Bonni, Brunet et al. 1999).

ERK1/2 (Extracellular Signal-Related Kinases) is the only known substrate for MEK1/2. There are four members of the ERK family: ERK1/2, JNK1/2/3, p38 MAPK (p38 α/β/γ/δ) and ERK5. ERK1/2 are the only known targets of MEK1/2 phosphorylation (Zheng and Guan 1993; Pearson, Robinson et al. 2001). ERK1 and ERK2 are co-expressed in virtually every type of tissue and are encoded by distinct genes, sharing 83% amino acid homology (Boulton, Yancopoulos et al. 1990; Boulton, Nye et al. 1991). Both ERKs recognize the same substrates, which are estimated to be in excess of 160 and

include a variety of membrane, cytoplasmic, and nuclear substrates (Gonzalez, Raden et al. 1991; Pearson, Robinson et al. 2001). Despite all these similiarities, knockdown studies suggest that ERK1 and ERK2 do not share overlapping functions. One potential explanation for their opposing roles may lie in the N-terminal domain (Marchi, D'Antoni et al. 2008). Marchi and colleagues found that ERK1 traffics at a much slower rate than ERK2, which in turn results in decreased nuclear phosphorylation and severely hinders the ability of ERK1 to transmit signals to the nucleus (Marchi, D'Antoni et al. 2008).

1.3.2 Differential signaling requirements of the Ras/Raf/MEK/ERK pathways

Since proteins in the Ras/Raf/MEK/ERK pathway are ubiquitously expressed, multiple layers of regulation are needed to ensure the proper signal is transmitted at the appropriate time. This system also lends itself to great diversity since each member of the pathway has multiple isoforms. Differential expression and activation of each isoform can have important biological consequences (Chang, Steelman et al. 2003; Moon 2006). Each member of the Ras/Raf/MEK/ERK pathway has multiple isoforms capable of activating a number of downstream isoforms. The greatest biological activity occurs when preferred isoforms are paired together. While sub-optimal isoform pairings will also transduce a signal, weaker biological outcomes are generally observed (Figure 5) (Chang, Steelman et al. 2003; Moon 2006).

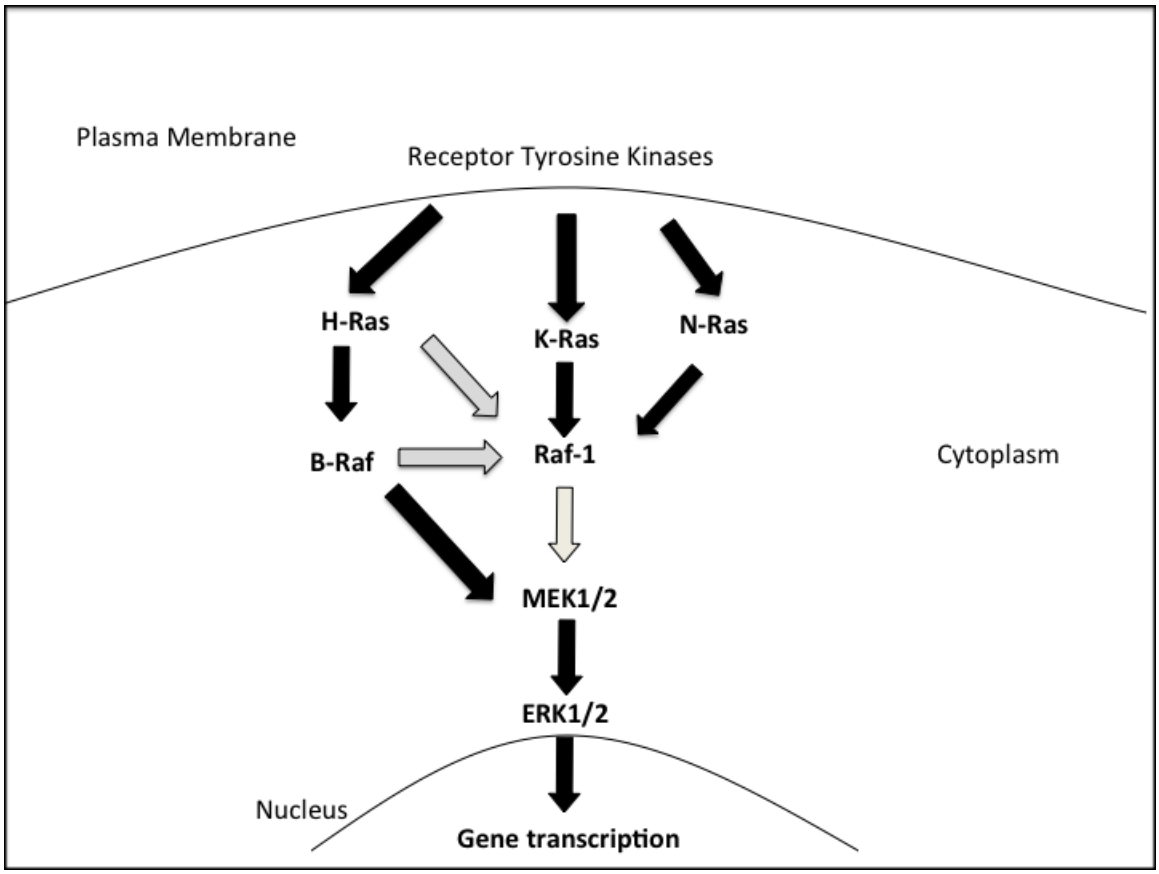


Figure 5: MAP kinase protein isoforms and their targets.

Protein isoforms display a preference in the activation of downstream targets. The level of activation is modulated by additional factors such as binding affinity and the requirement for additional activating co-factors. Black arrows indicate robust activation while grey arrows indicate more moderate activation. Adapted from (Chung and Kondo 2011).

Cell line data suggests that H-RasG12R (glycine=> arginine mutation) signals are principally mediated by B-Raf and not Raf-1 or A-Raf, which are the preferred targets of oncogenic Src (Khosravi-Far, White et al. 1996; Marais, Light et al. 1997). Also, H-Ras mutants were shown to be able to transform fibroblasts but were incapable of binding Raf-1 (Khosravi-Far, White et al. 1996). Moreover, B-Raf can be activated upon Ras binding whereas Raf-1 and A-Raf require additional signals (Khosravi-Far, White et al. 1996; Marais, Light et al. 1997). Another method of controlling specificity is through binding affinity. For example, H-RasG12V (glycine=> valine mutation) has been shown to have a higher binding affinity for Raf-1 than A-Raf (Khosravi-Far, White et al. 1996; Weber, Slupsky et al. 2000). On the other hand, N-Ras has been found to co-immunoprecipate with Raf-1, but not H-Ras (Hamilton and Wolfman 1998). This suggests that Raf-1 has a higher binding affinity for N-Ras than H-Ras.

Studies show that H-Ras can activate B-Raf *in vitro* and has a higher binding affinity for B-Raf than Raf-1 (Yan, Roy et al. 1998). However, H-Ras is a more potent activator of the PI3K pathway *in vivo* (Rodriguez-Viciana, Warne et al. 1996). Also, PI3K has been shown to be activated downstream of K-Ras (Rodriguez-Viciana, Warne et al. 1996). However, K-Ras has been found to be the most potent activator of the MAPK pathway through its efficient recruitment of Raf-1 and subsequent activation (Yan, Roy et al. 1998). These data are further supported by biochemical evidence showing that

Raf-1 has the highest affinity for K-Ras (Rodriguez-Viciana, Warne et al. 1996; Yan, Roy et al. 1998).

Studies in cell lines show that all three Raf isoforms are able to phosphorylate and activate MEK1/2 (Pritchard, Samuels et al. 1995). However, B-Raf has been found to have the highest affinity for MEK1/2 (Papin, Denouel-Galy et al. 1998). B-Raf is able to specifically recognize and activate MEK1 and MEK2 because of a proline-rich region found in both proteins that are not found in other MAPKKs (Catling, Schaeffer et al. 1995). Also, ablation of A-Raf expression resulted in a concomitant increase in B-Raf and Raf-1 kinase activity (Mercer, Chiloeches et al. 2002). These data indicate that A-Raf has virtually no unique role in MEK/ERK activation (Catling, Schaeffer et al. 1995; Pritchard, Samuels et al. 1995; Papin, Denouel-Galy et al. 1996; Papin, Denouel-Galy et al. 1998; Mercer, Chiloeches et al. 2002).

Another layer of regulation for Raf signaling is through combinatorial effects. For example, B-Raf and Raf-1 have been shown to form heterodimers. It is believed that once B-Raf is activated, it will assume the "active" conformation, which will allow Raf-1 to bind in a 14-3-3 protein-dependent manner and become activated by B-Raf (Farrar, Alberola-lla et al. 1996; Luo, Tzivion et al. 1996; Garnett, Rana et al. 2005; Rushworth, Hindley et al. 2006). The resulting heterodimers have increased kinase activity, as evidenced by higher phospho-MEK levels (Rushworth, Hindley et al. 2006). Moreover,

heterodimer formation between a WT Raf and a kinase-dead Raf still yielded higher phospho-MEK than B-Raf alone (Rushworth, Hindley et al. 2006). These data suggest that heterodimerization of Raf proteins confer more control over downstream targets perhaps by augmenting signal intensity or duration (Farrar, Alberola-lla et al. 1996; Luo, Tzivion et al. 1996; Mizutani, Inouye et al. 2001; Wan, Garnett et al. 2004; Garnett, Rana et al. 2005; Rushworth, Hindley et al. 2006).

Interestingly, downstream components of PI3K signaling such as Akt have been found to negatively regulate Raf-1 and B-Raf (Rommel, Clarke et al. 1999). Phosphorylated and activated Akt is able to phosphorylate multiple residues within the amino-terminal regulatory domain, thus inhibiting B-Raf function (Zimmermann and Moelling 1999). This inhibits MEK/ERK activation resulting in cell cycle arrest (Rommel, Clarke et al. 1999; Zimmermann and Moelling 1999). Another factor that can negatively regulate B-Raf is serum and glucocorticoid-inducible kinase (SGK). The catalytic domain of SGK has a high degree of sequence identity with the catalytic domain of Akt (Zhang, Tang et al. 2001). Unlike Akt, SGK only phosphorylates B-Raf at a single sight that is also found in Raf-1 (Zhang, Tang et al. 2001). When compared side by side, SGK was found to have a much stronger inhibitory effect that Akt (Zhang, Tang et al. 2001). Taken together, the data suggest that signal specificity is most likely regulated by ligand

stimulation and signal strength (Guan, Figueroa et al. 2000; Zhang, Tang et al. 2001; Moelling, Schad et al. 2002).

While MEK1 and MEK2 are very similar, there are some marked differences between them. For example, biochemical studies show that the Ras/Raf-1 complex is able to pull down MEK1, but not MEK2 (Jelinek, Catling et al. 1994). The proline-rich region of MEK1 is believed to contain a phosphorylation site that greatly facilitates signal complex formation. Furthermore, MEK1 activity was found to be significantly higher than MEK2 (Jelinek, Catling et al. 1994). This suggests that Ras signaling preferentially activates MEK1 and not MEK2 (Jelinek, Catling et al. 1994).

1.3.3 Protein expression pattern and homeostatic protein function

K-Ras is expressed at high levels in the gut and thymus and low levels in the skin and skeletal muscles (Lowy and Willumsen 1993). The function of K-Ras is unique and does not share overlapping functions with either N-Ras or H-Ras in regards to mouse growth and development (Esteban, Vicario-Abejon et al. 2001). Unlike H-Ras$^{-/-}$N-Ras$^{-/-}$ mice, which are viable and fertile, K-Ras$^{-/-}$ mice die between E12-E14. Further analysis suggests that the cause of death is most likely due to a defect in the fetal liver microenvironment rather than dysfunctional hematopoietic progenitors (Umanoff, Edelmann et al. 1995; Johnson, Greenbaum et al. 1997; Esteban, Vicario-Abejon et al.

2001). N-Ras expression is highest in the testis and thymus and lowest in the liver and kidney (Lowy and Willumsen 1993). Similar to the other Ras isoforms, H-Ras expression is ubiquitous, but is the highest in the skin and skeletal muscle and lowest in the liver (Lowy and Willumsen 1993).

The phenotype of A-Raf$^{-/-}$ mice is highly dependent on genetic background. On a C57B6 inbred background, mice were normal size at birth, but growth slowed significantly by 2-3 days post-birth and mice died within 1-3 weeks of birth. A-Raf is most highly expressed in urogenital tissue; therefore, it is not surprising that A-Raf$^{-/-}$ mice developed megacolon. Mice maintained on a 129/OLA background exhibited a 50% survival rate. While these animals were severely runted and have some neurological defects, they were fertile and did not show any signs of intestinal abnormalities (Pritchard, Bolin et al. 1996; Pearson, Robinson et al. 2001).

Intrauterine death of B-Raf$^{-/-}$ mice is observed between E10.5-E12.5. These mice have enlarged blood vessels and increased apoptosis in the vascular endothelium (Wojnowski, Zimmer et al. 1997). B-Raf expression is observed in the testis and spleen but is most highly expressed in neuronal tissue and is required for primary neuron survival. Using purified primary neurons from knockout mice, Raf-1 expression was shown to be unable to compensate for the loss of B-Raf in neurons suggesting distinct functions for each isoform (Wiese, Pei et al. 2001).

Raf-1 is ubiquitously expressed and has been the most studied Raf isoform (Leicht, Balan et al. 2007). Raf-1$^{-/-}$ mice were embryonic lethal. These mice also had underdeveloped placentas and embryonic organs, specifically the liver and hematopoietic cells. Additionally, these mice did not have any proliferation defects. On the other hand, fibroblasts cultured *in vitro* were more sensitive to apoptotic stimuli and increased apoptosis was observed *in vivo* in the liver (Huser, Luckett et al. 2001; Mikula, Schreiber et al. 2001).

The pathway(s) that mediate(s) cell proliferation and cell survival downstream of Raf-1 are not completely clear. For example, ERK activation by mitogen stimulation was normal in Raf-1$^{-/-}$. In order to further investigate the ability of B-Raf to compensate for the loss of Raf-1 expression, a B-Raf$^{-/-}$Raf-1$^{-/-}$ double knockout mouse was generated. These double knockouts had an even more severe defect and could not develop past the blastocyst stage. This suggests that Raf-1 and B-Raf have non-overlapping functions in very early embryonic development (Wojnowski, Stancato et al. 2000). Another possible explanation is that Raf-1 can directly or indirectly interact with anti-apoptotic proteins such as Bcl-2 and pro-apoptotic proteins such as Bad. However, the exact mechanism behind these interactions is still under investigation (Niault and Baccarini 2010).

Both MEK1 and MEK2 proteins are ubiquitously expressed; however, they do not share the same expression pattern or function (Brott, Alessandrini et al. 1993). While

MEK2 is highly expressed in both adult and embryonic tissue, MEK1 is only highly expressed in adult tissue (Alessandrini, Brott et al. 1997). Regardless of low expression in embryonic tissue, MEK1 function is required for proper vascularization of the placenta and MEK1$^{-/-}$ mice die at E10.5 (Giroux, Tremblay et al. 1999; Bissonauth, Roy et al. 2006). On the other hand, MEK2$^{-/-}$ mice are normal, viable, and fertile (Belanger, Roy et al. 2003). Not surprisingly, MEK1$^{-/-}$MEK2$^{-/-}$ mice were unresponsive to Raf-1 signaling while introduction of activated ERK2 rescued the MEK1$^{-/-}$MEK2$^{-/-}$ phenotype and restored cellular proliferation and inhibited cell death (Scholl, Dumesic et al. 2007).

While ERK1 and ERK2 are widely expressed throughout early-stage embryos, only ERK2$^{-/-}$ mice die in utero due to abnormalities in the placenta. ERK1$^{-/-}$ mice on the other hand are viable, normal size, and fertile (Pages, Guerin et al. 1999; Hatano, Mori et al. 2003; Saba-El-Leil, Vella et al. 2003). Knockdown studies where ERK1 expression is ablated in fibroblasts indicates that ERK2-dependent signaling along with cellular proliferation are increased. Accordingly, knockdown of ERK2 completely abrogates cellular proliferation (Vantaggiato, Formentini et al. 2006). Moreover, introduction of ERK1 can mitigate some of the effects of oncogenic Ras in certain cell types and ERK1$^{-/-}$ mice were shown to have less frequent, smaller, and delayed onset of skin papillomas. These studies suggest that the primary function of ERK2 is to promote cellular proliferation while ERK1 functions as a negative regulator of ERK2 (Bourcier, Jacquel et

al. 2006; Lloyd 2006; Vantaggiato, Formentini et al. 2006). However, a recent study challenges these previous assumptions with genetic models that show ERK1 and ERK2 have redundant roles with regards to proliferation (Voisin, Saba-El-Leil et al. 2010).

1.3.4 Role in physiological hematopoiesis

A fine balance between self-renewal, proliferation, and differentiation is critical for hematopoietic homeostasis. Stimulation of cord-blood derived HSCs with stem cell factor (SCF) resulted in MEK/ERK-dependent proliferation, but did not confer self-renewal activity (Oostendorp, Gilfillan et al. 2008). On the other hand, coupling SCF signaling with oncostatin M, a growth factor, resulted in decreased ERK phosphorylation along with increased HSC repopulating ability (Oostendorp, Gilfillan et al. 2008). These data suggest that modulating MEK/ERK signaling is important in achieving the correct balance between proliferation and self-renewal in HSCs.

One extrinsic factor that is important in determining lineage fate is cytokines. Cytokines are small proteins that are secreted by a wide variety of cells capable of delivering messages to target cells, thus affecting their physiological outcome. Growth factors can also work in cooperation with cytokines to achieve the desired effect (Watowich, Wu et al. 1996). MAPK pathway activation can occur downstream of many cytokine receptors, especially those involved in myelopoiesis resulting in cellular

differentiation (de Groot, Coffer et al. 1998). Using a system where ectopic cytokine receptors were expressed in committed lymphoid progenitors, Kondo and colleagues were able to show the instructive function of cytokines during "lineage conversion", a term used to describe the ability of lymphoid lineage-committed cells to give rise to mature myeloid cells (Kondo, Scherer et al. 2000). Follow-up work by Hsu and colleagues found that MEK/ERK signaling is responsible for lineage conversion (Hsu, Kikuchi et al. 2007). Moreover, introduction of an active form of MEK1 specifically skews HSCs toward the granulocyte/macrophage lineage. Specifically, MEK appears to be particularly important during the myeloid lineage commitment stage because differentiation from CMPs is not affected by MEK inhibitors (Kondo, Scherer et al. 2000; Hsu, Kikuchi et al. 2007).

Myelopoiesis is controlled by cytokine receptor signaling downstream of interleukin (IL)-3, G-CSF, and GM-CSF. Accordingly, expression of these cytokine receptors can be found on granulocytes, macrophages, erythrocytes, megakaryocyte, and early hematopoietic progenitors (de Groot, Coffer et al. 1998). Several studies using cell lines have found that components of the Ras/Raf/MEK/ERK pathway can be activated downstream of IL-3 and GM-CSF signaling leading to proliferation (de Groot, Coffer et al. 1998). IL-3 and GM-CSF receptors are heterodimers composed of a unique α chain (IL-3Rα and GM-CSFRα) complexed to the common β chain (βc). To determine

whether MAPK signaling was required for functions such as proliferation and prevention of apoptosis, mutant βc receptors were generated and expressed in cell lines. The mutant βc receptor featured deletion of the entire cytoplasmic domain so these mutants were unable to activate the Ras/Raf/MEK/ERK pathway. Cells expressing the mutant βc receptor did not proliferate and underwent apoptosis following cytokine stimulation (Kinoshita, Yokota et al. 1995). However, introduction of activated Ras and Raf proteins into cell lines expressing the truncated βc receptor resulted in proliferation and cell survival, suggesting a role for Ras and Raf in these crucial cellular functions (de Groot, Coffer et al. 1998).

The role of MEK/ERK signaling downstream of cytokine receptors in differentiation was examined using a variety of myeloid cell lines that could differentiate following cytokine stimulation. The 32D cell line is an IL-3-dependent murine myeloid progenitor cell line that is capable of differentiation into granulocytes following IL-3 withdrawal (Greenberger, Sakakeeny et al. 1983). G-CSF stimulation of 32D cells in the absence of IL-3 resulted in rapid and sustained MEK and ERK activation (Miranda, Xu et al. 2005) . However, simultaneous stimulation with IL-3 and G-CSF greatly inhibited MEK phosphorylation and granulocyte differentiation (Miranda, Xu et al. 2005). Studies with human $CD34^+$ progenitors show that MEK promotes cell survival and plays a role in driving proliferation of neutrophil progenitors but not their

maturation (Geest, Buitenhuis et al. 2009). Studies using TF-1a an erythroleukemic cell line also supports the role of MEK/ERK in cellular differentiation. Stimulation with GM-CSF resulted in increased MEK and ERK phosphorylation and cell proliferation. Inhibition of the MEK/ERK pathway abrogated macrophage differentiation, suggesting that MEK/ERK activation by GM-CSF is important in macrophage proliferation as well as differentiation (Hu, Moscinski et al. 2000).

Thrombopoietin (TPO) is a growth factor that is necessary for survival and proliferation of HSCs, as well as proliferation and differentiation of megakaryocytes into platelets (Watowich, Wu et al. 1996). MEK/ERK signaling is activated downstream of TPO signaling in megakaryoblastic cell lines, primary murine cells, and human progenitor cells (Rouyez, Boucheron et al. 1997; Rojnuckarin, Drachman et al. 1999; Miyazaki, Ogata et al. 2001). Moreover, MEK/ERK has specifically been shown to be important for the maturation of human $CD34^{+}$ progenitors into megakaryocytes (Fichelson, Freyssinier et al. 1999). MEK inhibitors were found to interfere with normal polyploidization, downregulation of immature progenitor markers, and proplatelet formation (Rojnuckarin, Drachman et al. 1999). Reports using bone marrow cells isolated from a Raf-$1^{-/-}$ backcrossed to an outbred mouse line demonstrated that Raf-1 was dispensable for megakaryocyte development. Lack of Raf-1 expression did not alter phospho-ERK levels (Kamata, Pritchard et al. 2004). This can be explained by similar, if

not identical to WT levels of A-Raf and B-Raf which could potentially compensate for the lack of Raf-1 expression (Kamata, Pritchard et al. 2004). Indeed, B-Raf$^{-/-}$ mice have defects in myeloid development *in vitro* (Kamata, Pritchard et al. 2004). Unlike cells lacking Raf-1, cells lacking B-Raf have a defect in megakarocytosis most likely due to the inability to respond to proliferation signals downstream of TPO signaling (Rouyez, Boucheron et al. 1997; Sui, Krantz et al. 1998; Fichelson, Freyssinier et al. 1999; Rojnuckarin, Drachman et al. 1999; Miyazaki, Ogata et al. 2001; Kamata, Pritchard et al. 2004; Kamata, Kang et al. 2005; Mazharian, Watson et al. 2009; SÉVerin, Ghevaert et al. 2010).

Under steady state conditions, megakaryocyte development is balanced with erythrocyte development (Zhang and Lodish 2004). Although the Ras/Raf/MEK/ERK pathway is not a direct target of EPO signaling, augmented signaling greatly affects erythropoiesis. Introduction of an activated form of H-Ras into primary fetal liver TER119^{-} erythroid progenitors promoted EPO-independent proliferation and a block in terminal differentiation. This was specifically mediated by the MEK/ERK pathway (Zhang and Lodish 2004). However, coupling a constitutively active form of MEK with Akt caused a synergistic effect, such that levels of Epo-independent proliferation were even greater than with active H-Ras alone or constitutively active MEK. The role of Akt in this system is unclear but it appears to involve promoting proliferation and possibly

cell survival (Zhang and Lodish 2004). Similar studies using fetal liver cells from K-Ras$^{-/-}$ mice show that K-Ras functions mainly to promote cell survival by activating the Akt pathway (Zhang and Lodish 2005). Also, mutant N-Ras can promote erythropoietin-independent proliferation of primitive human CD34^{+} erythroid progenitors. However, increased Ras signaling makes cells refractory to EPO signaling, resulting in inefficient production of erythrocytes (Darley, Pearn et al. 2002).

MEK/ERK signaling appears to play a negative regulatory role in erythroid differentiation following TPO signaling in HSCs obtained from cord blood (Miyazaki, Ogata et al. 2001). On the other hand, ERK1 has been found to play a negative regulatory role in splenic erythropoiesis. Active erythropoiesis occurs in the bone marrow with the spleen serving as a back-up site during homeostasis. However, stress conditions such as bone marrow damage could shift more of the erythroid production to the spleen (Guihard, Clay et al. 2010). While ERK1$^{-/-}$ mice did not show any perturbations in the bone marrow and did not have signs of anemia, they displayed increased splenic erythropoiesis. Additionally, there were excessive numbers of erythroid progenitors in the spleen with enhanced survival ability mediated by decreased Fas and FasL levels (Guihard, Clay et al. 2010). Expression and activation of ERK2 was normal suggesting that ERK1 plays a unique role in erythropoiesis (Guihard, Clay et al. 2010).

With regards to the lymphoid lineage, H-Ras and MEK1 have been shown to be important for the transition from DN to DP thymoctyes (Crompton, Gilmour et al. 1996; Swat, Shinkai et al. 1996). While H-Ras does not appear to have a role in negative selection, active H-Ras can inhibit positive selection (Alberola-lla, Forbush et al. 1995; Swan, Alberola-lla et al. 1995; Alberola-lla, Hogquist et al. 1996; Crompton, Gilmour et al. 1996; Swat, Shinkai et al. 1996).

On the other hand, MEK2 was found to be dispensable for thymocyte development. Also, T cells were able to proliferate normally following mitogen stimulation and cell death was not altered in these mice suggesting that MEK2 is dispensable for activated TCR signaling (Belanger, Roy et al. 2003). Development of γδ T cells was also unaffected (Alberola-lla, Hogquist et al. 1996).

Both $ERK1^{-/-}$ and $ERK2^{-/-}$ mice have a defect in positive selection with $ERK1^{-/-}$ $ERK2^{-/-}$ mice having a more severe phenotype (Fischer, Katayama et al. 2005). The precise role of ERK1/2 in negative selection is more controversial with some groups finding evidence to support a role while others do not (Alberola-lla, Forbush et al. 1995; Sugawara, Moriguchi et al. 1998; Bommhardt, Scheuring et al. 2000; Mariathasan, Ho et al. 2000). ERK2 has been found to be required for proper development of mature CD4+ T cells; however, neither of the ERKs are required for CD8 development (Sharp, Schwarz

et al. 1997; Fischer, Katayama et al. 2005). These results suggest that modulation of ERK signaling can impact CD4 versus CD8 lineage choice.

1.3.5 Role in hematopoietic malignancy

Ras oncogenes were among the first oncogenes to be discovered and activating Ras mutations have been observed in approximately 30% of all cancers (Bos 1989). However, studies investigating the specific role of Ras in disease initiation and maintenance yielded conflicting results. Early studies on oncogenes suggested that mutated Ras was insufficient to give rise to cancer and additional oncogenes had to be mutated for a transformation event to occur (Land, Parada et al. 1983; Ruley 1983; Tanaka, Ishihara et al. 1994; Kamijo, Zindy et al. 1997; Serrano, Lin et al. 1997; Zindy, Eischen et al. 1998). Some groups found that introduction of active H-Ras into fibroblasts caused "premature cellular senescence" which mimics many aspects of cellular replicative senescence (Serrano, Lin et al. 1997). Additionally, other groups have also found that hyperstimulation of the MAPK pathway leads to premature senescence (Lin, Barradas et al. 1998; Zhu, Woods et al. 1998). These results suggest cellular senescence is one mechanism used by tumor suppressors to prevent disease development and that in order to achieve full transformation tumor suppressors that

negatively regulate cell cycle or factors that promote cell cycle progression must be mutated.

However, many of those early studies relied on methods that resulted in supraphysiological levels of Ras expression that may not accurately reflect endogenous Ras signaling (Ruley 1983; Kamijo, Zindy et al. 1997; Serrano, Lin et al. 1997; Lin, Barradas et al. 1998). On the other hand, models where K-RasG12D (glycine=> aspartic acid mutation) is expressed from the endogenous locus show that K-Ras mutation is sufficient to cause a transformation event even in the presence of functional cell cycle regulators such as p19ARF and p53 (Guerra, Mijimolle et al. 2003; Tuveson, Shaw et al. 2004). Moreover, cells expressing active K-RasG12D exhibit attenuated MAPK pathway activation, which may explain why cells do not undergo premature cellular senescence (Tuveson, Shaw et al. 2004). Taken together, these studies suggest that tumor development as a result of oncogenic Ras expression is cell-type dependent and also may be isotype dependent.

K-Ras is of extreme interest because it is the most frequently activated ras oncogene in human tumors. For example, K-Ras oncogenes have been found in 90% of pancreatic cancers, 50% of colon cancers, 25% of lung adenocarcinomas, 10-15% of AMLs, but have not been found in prostate and breast tumors, suggesting that tumor induction is dependent on cell type (Bos 1989; Guerra, Mijimolle et al. 2003). In order to

develop a better understanding of the role of activated K-Ras in cancer development, researchers turned to animal models. Johnson and colleagues were the first to design a mouse model where an oncogenic version of K-Ras was expressed from the endogenous locus (Johnson, Mercer et al. 2001). This model is particularly elegant in that a mutated version of the K-Ras gene commonly found in human cancers is expressed at random following a spontaneous recombination event that occurs every 10^3-10^7division. They reasoned that this is a better reflection of the *in vivo* situation because the gene is expressed at endogenous levels (Johnson, Mercer et al. 2001). Moreover, mutant cells in this model would be surrounded by normal cells that could suppress their growth. On the other hand, mouse models where oncogene expression is driven by a ubiquitous promoter would result in a "field" of mutant cells that would specifically promote mutant cell growth (Berns 2001; Johnson, Mercer et al. 2001). Mice where oncogenic K-Ras expression is driven by the endogenous locus had a high incidence of lung cancers along with some thymic lymphomas and skin papillomas (Johnson, Mercer et al. 2001).

One major caveat of the animal model generated by Johnson and colleagues is that expression of the oncogene of interest cannot be controlled in a tissue-specific manner (Johnson, Mercer et al. 2001). To solve this problem, while accurately representing the situation *in vivo*, Braun and colleagues generated an inducible mouse model where expression of active K-Ras could be controlled by turning on tissue-specific

promoters (Braun, Tuveson et al. 2004). Several groups used the Mx promoter, which is responsive to type I interferon signaling, to activate Cre recombinase that selectively deletes a floxed stop cassette immediately upstream of the mutant K-RasG12D allele, which encodes for an activated mutant form of K-Ras (Van Meter, Diaz-Flores et al. 2007; Sabnis, Cheung et al. 2009). Following poly-IC injections, mice expressed the mutant form of K-Ras and developed MPNs that closely resembled CMML and JMML in humans (Braun, Tuveson et al. 2004; Chan, Kutok et al. 2004). The MPNs were marked by a massive expansion of myeloid cells, which was most likely caused by increased proliferative capacity of myeloid progenitors in the bone marrow and increased numbers and proliferative capacity of HSCs and myeloid progenitors in the spleen (Braun, Tuveson et al. 2004; Chan, Kutok et al. 2004; Van Meter, Diaz-Flores et al. 2007).

However, this inducible system also has its flaws. Type I interferons can be produced by somatic cells following stimulation; therefore Cre is constitutively expressed at a low level. Moreover, cells other than hematopoietic cells can respond to interferon stimulation (Mattei, Schiavoni et al. 2010). In order to eliminate any potential contribution from somatic cells that may be able to respond to interferon signaling, Braun's group harvested bone marrow cells from mice previously treated with polyinosinic:polycytidylic acid (poly I:C) and injected them into untreated recipients.

Similar to results obtained with non-transplanted mice, all the transplanted mice developed MPN (Van Meter, Diaz-Flores et al. 2007; Sabnis, Cheung et al. 2009). Zhang and colleagues also used the same transplant system and found that transplanted mice also developed a JMML-like disease, but at a much lower frequency. More strikingly, approximately 60% of the transplanted mice developed a thymic T-cell lymphoma or T-cell leukemia (Zhang, Wang et al. 2009).

While Braun's group did not observe T-ALL development following primary transplant of K-RasG12D HSCs, T-ALL development was observed following secondary transplant of thymocytes, but not bone marrow cells. Moreover, T-ALLs contained activating Notch mutations (Sabnis, Cheung et al. 2009). Zhang's group found chromosomal abnormalities, such as trisomy at chromosome 14 and 15 and a gain of a fused chromosome, in cells isolated from thymic lymphoma T cells (Zhang, Wang et al. 2009). On the other hand, myeloid cells isolated from mice with JMML had a normal karyotype (Zhang, Wang et al. 2009). Both groups hypothesize that HSCs are the primary target of oncogenic K-Ras; however, MPNs generated in Braun's model were not recapitulated following transplantation into a naïve mouse (Sabnis, Cheung et al. 2009; Zhang, Wang et al. 2009). Thus, these MPNs are not frank leukemias and require additional oncogenic "hits" (Sabnis, Cheung et al. 2009). The karyotype data from Zhang's group strongly suggest that additional oncogenic "hits" most likely occur in

lineage-specific progenitor populations (Braun, Tuveson et al. 2004; Sabnis, Cheung et al. 2009; Zhang, Wang et al. 2009).

It should be noted that the myeloid cells observed in the aforementioned MPN models were composed of mature cells of the granulocyte/macrophage lineage. Also, the number of MEPs was elevated in bone marrow and spleen; however, mice had decreased platelet counts and showed signs of anemia (Van Meter, Diaz-Flores et al. 2007; Sabnis, Cheung et al. 2009; Zhang, Wang et al. 2009). Further investigation showed that oncogenic K-Ras expression resulted in mild hyperproliferation of erythroid progenitors, blocked erythroid differentiation at the proerythroblast stage, and lead to increased sensitivity to signaling pathways dependent on erythropoietin, a growth factor required for proper differentiation of erythrocytes (Braun, Archard et al. 2006; Zhang, Liu et al. 2007). All these factors contributed significantly to dyserythropoiesis, which accounts for the anemia observed in these mice (Braun, Archard et al. 2006; Zhang, Liu et al. 2007; Zhang and Lodish 2007).

While N-Ras has been shown to have redundant functions during normal mouse development, activating mutations have often times been linked to a broad range of hematological cancers. N-Ras mutations have been identified in 70% of lymphoid and myeloid malignancies (Bos 1989). The frequency of N-Ras mutations in AML is approximately 20-25% and 30% in JMML (Janssen, Steenvoorden et al. 1987; Miyauchi,

Asada et al. 1994). Approximately 10% of T-lymphoma and T-leukemias have N-Ras or K-Ras mutations (von Lintig, Huvar et al. 2000). However, 50% of these diseases have increased Ras signaling, suggesting that Ras signaling plays a very important role in disease (Bos, Verlaan-de Vries et al. 1987; Janssen, Steenvoorden et al. 1987; Miyauchi, Asada et al. 1994; von Lintig, Huvar et al. 2000).

Animal models involving retroviral transduction of an activated form of N-RasG12D into bone marrow cells have been used to study AML and CMML (Parikh, Subrahmanyam et al. 2006). While retroviral transduction systems are very useful in studying disease initiation and progression, it is difficult to control the level of oncogene expression. Parikh and colleagues found that bone marrow cells transduced with high viral titers generally resulted in AML whereas low viral titers yielded CMML suggesting differential N-Ras expression may affect disease phenotype (Parikh, Subrahmanyam et al. 2007). The authors also noticed that N-Ras expression appeared to be higher in some, but not all, AML samples (Parikh, Subrahmanyam et al. 2007). These studies show that N-Ras can initiate myeloid malignancies and mouse models expressing oncogenic N-Ras mimic the human form of the disease (MacKenzie, Dolnikov et al. 1999; Parikh, Subrahmanyam et al. 2006).

Data generated from cell lines and animal models have suggested a link between activated N-Ras and leukemia. However, studies using AML patient samples yielded

some unexpected results: ERK, a downstream N-Ras target, was only activated in 9% of samples that harbored N-Ras mutations (Iida, Towatari et al. 1999). The mouse retroviral model yielded similar results in that MEK1/2 and ERK1/2 activation varied among samples and did not show any clear correlation with N-RasG12D expression (Parikh, Subrahmanyam et al. 2006). Moreover, phospho-S6 ribosomal protein, but not phospho-Akt, was elevated in the mice, suggesting that oncogenic N-Ras proliferation signaling proceeds through channels other than the canonical MAPK pathway (Iida, Towatari et al. 1999; Parikh, Subrahmanyam et al. 2006).

Although activating K-Ras and N-Ras mutations are relatively prevalent in hematopoietic cancers, activating H-Ras mutations are more rare (Janssen, Steenvoorden et al. 1987). Moreover, germline H-Ras mutations in humans did not increase leukemia incidence (Aoki, Niihori et al. 2005). However, introduction of an activated form of H-Ras into murine HSCs resulted in AML (Parikh, Subrahmanyam et al. 2007). These data suggest that H-Ras has the potential to transform hematopoietic cells but that potential is never actualized. This could be due to several reasons. A germline activating mutation may not be sufficient to lead to transformation because the physiological expression of the oncogene is still too low (Aoki, Niihori et al. 2005; Parikh, Subrahmanyam et al. 2007). Integration of the retrovirus cannot be controlled and could result in extremely high levels of oncogene expression that would never be observed

under physiological conditions (Parikh, Subrahmanyam et al. 2007). Also, H-Ras expression is the lowest of all the other Ras isoforms in the hematopoietic system suggesting that its expression is tightly regulated (Aoki, Niihori et al. 2005; Parikh, Subrahmanyam et al. 2007). Although the AML observed in the mouse model closely resembles that of the human disease, it is also possible that H-Ras mutants do not impact human cells in the same manner as mouse cells (Aoki, Niihori et al. 2005; Parikh, Subrahmanyam et al. 2007).

It is also worth noting that some tumors display "addiction" to oncogenic Ras and its downstream effector pathways (Weinstein and Joe 2008; Kim, Matise et al. 2009). Oncogene addiction occurs when a tumor with multiple genetic, epigenetic, or chromosomal abnormalities develops a physiological dependence on one or two oncogenes for its survival and maintenance (Weinstein 2002). Inactivation of the oncogene often results in tumor regression and increased survival, which are marked by decreased proliferation, apoptosis, and differentiation (Felsher and Bishop 1999; Weinstein 2002). Interestingly in some animal models, reactivation of the oncogene does not restore the malignant phenotype (Jain, Arvanitis et al. 2002). This may reflect rewiring of intracellular circuitry or epigenetic changes caused by the oncogene. These changes could make cells insensitive to malignant transformation because greater levels of oncogene expression are necessary to promote cell survival (Weinstein and Joe 2008).

Ras can activate a number of different downstream effector pathways (Figure 6), such as Raf/MEK/ERK, PI3K/Akt, and RalGDS that can promote cellular functions important for oncogenesis, such as proliferation and cell survival. Moreover, expression of oncogenic Ras has been shown to be sufficient to initiate disease development (Johnson, Mercer et al. 2001; Downward 2003). However, downstream Ras targets, rather than Ras itself, may be responsible for disease maintenance. Specifically, the Raf/MEK/ERK and RalGDS pathways were shown to have a role in tumor maintenance, but the PI3K/Akt pathway was the only pathway shown to be required for tumor maintenance (Lim and Counter 2005).

These data suggest that oncogenes capable of initiating disease are not necessarily the same oncogenes that tumors develop an addiction to during tumor maintenance. Also, mutations acquired in other downstream effector pathways may not play a prominent role in tumor maintenance since the tumor is not addicted to that oncogene. Targeted therapies against these unique oncogenes are being explored, but it is necessary to be mindful of other related pathways that contain possible oncogenes since oncogene addiction can be altered, resulting in drug-resistance.

Raf isoforms appear to have limited transformation potential. Studies using 3T3 cells have shown a constitutively active form of A-Raf could push quiescent cells into cell cycle entry (Pritchard, Samuels et al. 1995). Although expression of activated A-Raf

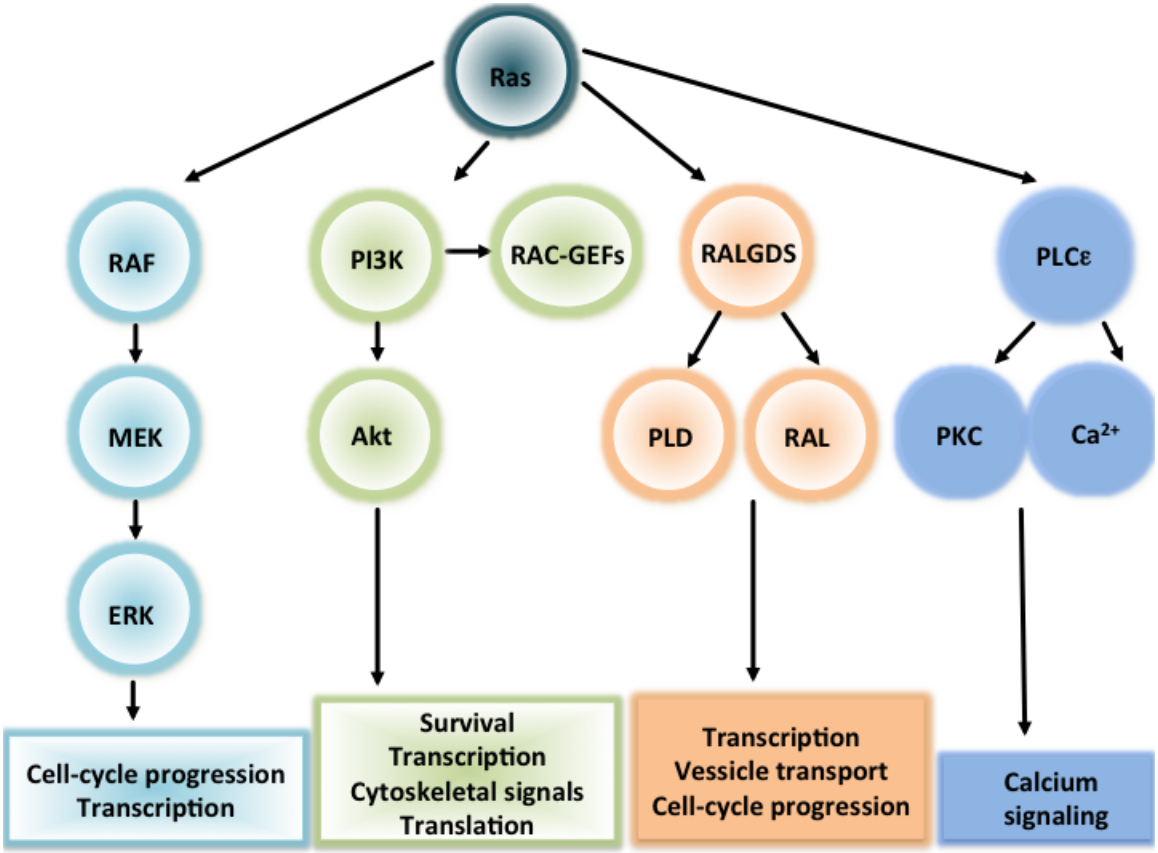


Figure 6: Effector pathways downstream of Ras signaling.

Ras is a membrane proximal protein that is able to activate several different pathways. Ras can activate Raf kinases, which are then able to activate MEK and ERK. There are many ERK targets located in the cytoplasm and nucleus that can mediate a variety of different functions including cell-cycle progression and transcription. Activation of the PI3K/Akt pathway is mainly associated with the promotion of cell survival, but this pathway can also activate targets involved in transcription, cytoskeletal signals, and translation. RALGDS proteins serve as guanine nucleotide exchange factors (GEFs) for the Ras-related protein, Ral. The RALGDS signaling pathway is involved in transcription, vesicle transport, and cell-cycle progression. Activation of the phospholipase Cε (PLCε) pathway results in the release of calcium from intracellular stores. Adapted from (Downward, 2003).

did not lead to transformation, cells expressing activated A-Raf had the greatest ability to abrogate cytokine dependency. These data show that active A-Raf displays characteristics of an oncogene *in vitro*, but its transformation potential is never realized *in vivo* (McCubrey, Steelman et al. 1998; Hoyle, Moye et al. 2000).

Similarly, Raf-1 involvement in cancer development appears to be slim with less than 1% of tested cancer cell lines harboring Raf-1 mutations (Bos 1989). Introduction of mutated forms of Raf-1 into fibroblasts failed to cause morphological changes. Growth in soft agar was substantially slower than cells expressing v-Ras or v-Raf, although the mutant conferred a higher tolerance to apoptotic stimuli (Zebisch, Staber et al. 2006). Other cell line studies also suggest that Raf-1 has intrinsically low transformation potential. While the exact mechanism is unknown, one theory is that the lack of transformation potential is due to the neutral charge found in the N-region. Accordingly, neutralizing the charge of a mutated form of Raf-1 resulted in decreased kinase activity and transformation potential (Emuss, Garnett et al. 2005).

Recently, a direct link between B-Raf mutations and cancer has been identified. A large sequencing screen of cancer cell lines and primary tumor samples found that the *B-Raf* gene was mutated in 70% of malignant melanomas and 15% of colorectal cancers (Davies, Bignell et al. 2002). The activating mutation appears to enhance B-Raf activity because the mutation mimics phosphorylation of the activation loop (Leicht, Balan et al.

2007). B-Raf only has two Ras-GTP dependent phosphorylation sites required for maximal activation, compared to four sites in Raf-1, which may explain why B-Raf is mutated at a higher frequency. Moreover, B-Raf has a higher basal kinase activity compared to the other Raf isoforms. B-Raf mutations have not been identified in hematopoietic cancers; however, somatic expression of B-RafV600G results in bone marrow failure and a non-lymphoid neoplasia (Mercer and Pritchard 2003; Mercer, Giblett et al. 2005).

The more upstream components of the MAPK pathway such as Ras and Raf, specifically K-Ras and B-Raf, have received a lot of attention for their role in cancer development; however, MEK and ERK also has an important role in cancer development that has only been recently explored. While MEK mutations have not yet been observed in human cancers, evidence is mounting to suggest that it may have a role in cellular transformation. One model often used to study transformation ability is cytokine-dependent cell lines. Since these cell lines require growth factors to provide proliferative and anti-apoptotic signals, transformation candidates can be introduced in lieu of growth factors to determine if they can provide the required signals. Blalock and colleagues used this approach to determine what effect introduction of activated MEK would have on various cytokine-dependent hematopoietic progenitor cell lines. Expression of active MEK in these cells allowed them to proliferate and prevented

apoptosis in the absence of cytokines. Moreover, some cell types acquired the ability to autonomously produce growth factors which further perpetuated cell growth (Blalock, Pearce et al. 2000; Blalock, Pearce et al. 2001). Fibroblasts expressing active MEK were shown to have great tumorigenic properties when injected into nude mice (Mansour, Matten et al. 1994). Taken together, these studies strongly suggest that activated MEK can initiate and contribute to oncogenesis.

Several studies show that activated ERK, the only known substrate for MEK phosphorylation, has been found in 51-83% of human AML cases (Towatari, Iida et al. 1997; Ricciardi, McQueen et al. 2005). Of these, one study showed that Ras mutations were lacking in patient AML samples that had activated ERK2, suggesting that activation of a molecule further downstream in the MAPK pathway was driving disease development (Towatari, Iida et al. 1997). ERK1/2 has also been shown to be an important prognostic indicator for B acute lymphocytic leukemia (B-ALL) and T-ALL patients (Gregorj, Ricciardi et al. 2007). In this study, ERK1/2 activation was found to be correlated with higher white blood cell counts and decreased likelihood of complete remission. While the study did not examine the activation status of more upstream MAPK components, high phospho-ERK1/2 levels were observed in patients regardless of the presence or absence of BCR/ABL and t(4;11) translocation events (Gregorj,

Ricciardi et al. 2007). These data suggests that multiple signaling pathways can converge upon ERK1/2 activation.

1.4 Function of Bcl-2 under physiological and malignant conditions

Apoptosis, or programmed cell death, is necessary for embryogenesis, tissue homeostasis, elimination of damaged or aged cells, and defense against pathogens (Cory and Adams 2002; Borner 2003; Cory, Huang et al. 2003). Decreased sensitivity to apoptotic signals can lead to autoimmunity or neoplasia due to the persistence of autoreactive or mutated cells (Cory, Huang et al. 2003). On the other hand, increased sensitivity to apoptotic signals can lead to acute diseases, such as infection by certain microorganisms, infarction, immunodeficiency, or chronic diseases, such as neurogenerative and neuromuscuclar diseases (Cory and Adams 2002; Borner 2003; Cory, Huang et al. 2003).

B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic protein and the founding member of the Bcl-2 protein family, which contains both anti-and pro-apoptotic proteins (Figure 7)(Cory and Adams 2002; Cory, Huang et al. 2003). Bcl-XL, Bcl-w, Mcl-1, and A1, along with Bcl-2, make up the group of Bcl-2-like survival factors (Cory and Adams 2002; Cory, Huang et al. 2003). Pro-apoptotic proteins of the Bcl-2 family include Bax-like death factors, such as Bax, Bak, and Bok, and BH3-only death factors, which include

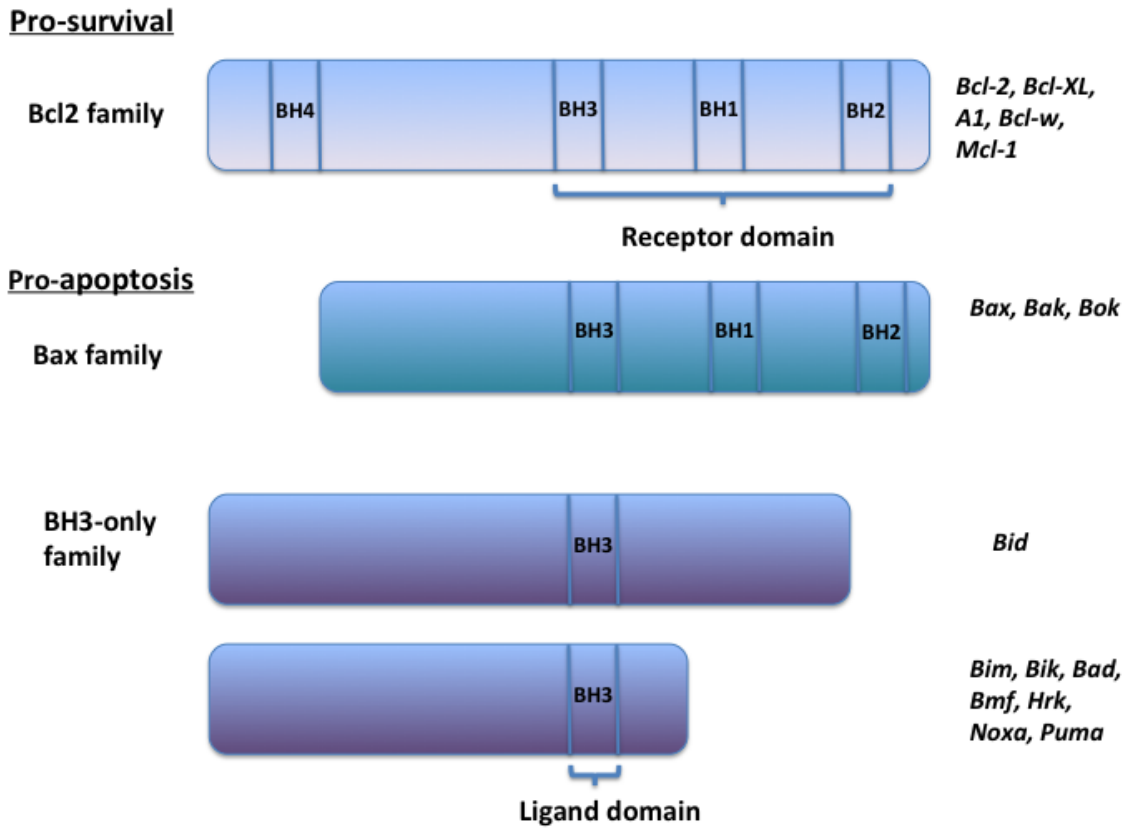


Figure 7: Bcl-2-related protein subfamilies.

The Bcl-2 family of proteins contains both pro- and anti-apoptotic proteins. Protein regions, such as the Bcl-2 homology (BH) region, are denoted in the diagram. Pro-survival proteins all contain BH1-BH4 domains while pro-apoptotic proteins all contain the BH3 domain. Examples of each subfamily are listed on the right-hand side. Adapted from (Cory and Adams 2002).

Bim, Bad, Bid, Noxa, and Puma (Cory and Adams 2002; Cory, Huang et al. 2003). All Bcl-2 family proteins contain a Bcl-2 homology region (Borner 2003). All the anti-apoptotic proteins have BH1-BH4 domains, while the pro-apoptotic proteins all contain the BH3 domain with varying inclusion of other BH domains (Borner 2003). While the BH domains do not possess any enzymatic activity, they facilitate interaction between pro-and anti-apoptotic proteins. For example, the BH3 domain found on pro-apoptotic proteins can ligate a hydrophobic pocket formed by BH1-BH3 domains found on anti-apoptotic proteins (Borner 2003).

The balance and interaction between survival and death factors determine mitochondrial membrane integrity and, consequently, cell fate (Chonghaile and Letai 2009; Giam, Huang et al. 2009). Withdrawal of tropic factors causes transcription-independent and –dependent changes that disrupt this balance between pro- and anti-apoptotic Bcl-2 family members (Chonghaile and Letai 2009; Giam, Huang et al. 2009). There are two prevailing models to explain how Bcl-2 may mediate cell survival (Figure 8). In the direct model, Bcl-2 can bind activator BH3 proteins such as Bim and Bid, thus inhibiting their ability to interact and activate the effector proteins Bax and Bak (Chonghaile and Letai 2009; Giam, Huang et al. 2009). Bax and Bak oligomers lead to the release of apoptogenic proteins from the mitochondria and cell death. Alternatively, the indirect model postulates that the main role of Bcl-2 is to directly bind effector

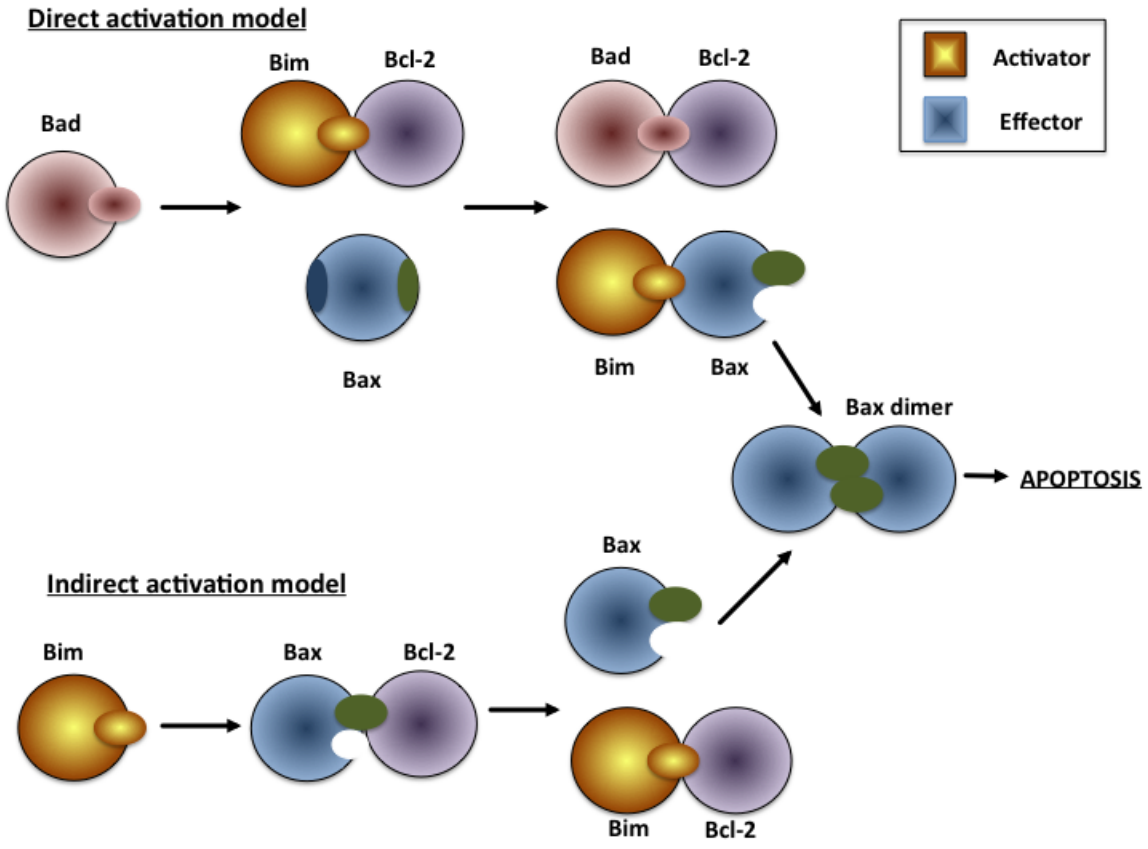


Figure 8: Competing models of apoptosis induced by Bax dimerization.

It is clear that insertion of Bax dimers into the mitochondrial membrane permeabilizes the membrane, leading to apoptosis. However, the events leading up to Bax dimerization are unclear. The direct activation model suggests that activator proteins such as Bim and Bid are bound to Bcl-2. Bad, a BH3 protein can displace Bim binding to Bcl-2, allowing Bim to directly interact with and activate Bax, leading to apoptosis. In this model, Bcl-2 binding to activator proteins inhibits activation of effector proteins, leading to cell survival. The indirect activation model suggests that Bim does not directly interact with Bax. Rather, Bim can displace Bax binding to Bcl-2, which frees Bax, allowing it to form homodimers, leading to apoptosis. Here, Bcl-2 can directly bind effector proteins, inhibiting their ability to form dimers and trigger apoptosis. Adapted from (Giam, Huang et al. 2009).

proteins, thus inhibiting their ability to form oligomers and protecting the integrity of the mitochondrial membrane, which ultimately results in cell survival (Chonghaile and Letai 2009; Giam, Huang et al. 2009).

Bcl-2 was originally discovered in human B-cell lymphomas at the chromosomal breakpoint t(14;18) and was the first oncogene shown to enhance cell survival rather than cell proliferation (Vaux, Cory et al. 1988). Enforced expression of Bcl-2 promotes survival of cytokine-dependent cell lines in the absence of growth factor for a limited amount of time, but does not give rise to tumors when injected into mice (Vaux, Cory et al. 1988). Moreover, in animal models of follicular lymphoma, the expression of the t(14:18) mini-gene gives rise to indolent tumors. Following a long latency period, the animals progress to a malignant diffuse large-cell lymphoma that also harbors a rearranged c-myc gene (Strasser, Harris et al. 1990; McDonnell and Korsmeyer 1991).

Similarly, co-expression of Bcl-2 and active c-myc results in the development of lymphoid tumors at a much faster rate. One important function of the c-myc oncogene is to promote cell cycle progression. However, overexpression of c-myc can also lead to apoptosis following growth factor withdrawal (Askew, Ashmun et al. 1991; Evan, Wyllie et al. 1992). These observations lead to the "two signal" model, which postulates that c-myc is able to provide the initial signal to proliferate or to undergo apoptosis and the second signal, presumably mediated by the presence or absence of growth factors,

provides the accompanying execution signal (Bissonnette, Echeverri et al. 1992; Wagner, Small et al. 1993). Cell line studies suggest that the anti-apoptotic signals provided by Bcl-2 can inhibit c-myc-mediated apoptosis, suggesting that Bcl-2 can aid in the persistence of pre-neoplastic cells and cooperate with other oncogenes (Bissonnette, Echeverri et al. 1992; Wagner, Small et al. 1993).

As mentioned previously, MEK/ERK signaling promotes cell survival through several different methods, including regulation of Bcl-2 expression (Scholl, Dumesic et al. 2007). ERK, the only known target of MEK, can phosphorylate $p90^{RSK}$ (Zheng and Guan 1993; Shimamura, Ballif et al. 2000; Pearson, Robinson et al. 2001). Once activated RSK can phosphorylate CREB, which can bind to CRE sites within the Bcl-2 promoter, causing transcription of the Bcl-2 gene (Wilson, Mochon et al. 1996; Xing, Kornhauser et al. 1998; Bonni, Brunet et al. 1999). RSK also promotes cell survival by phosphorylating Bad, a BH3-only pro-apoptotic protein (Bonni, Brunet et al. 1999; Shimamura, Ballif et al. 2000; Cory and Adams 2002). Phosphorylated Bad is sequestered in the cytoplasm by 14-3-3 proteins, which protect the integrity of the mitochondria, leading to cell survival (Zha, Harada et al. 1996; Adams 2003).

However, activation of MEK of alone is insufficient to promote cell survival. For example, cytokine-dependent cell lines become growth factor-independent only in the presence of both MEK and Bcl-2 (Blalock, Moye et al. 2000). Cells expressing active

MEK did not display elevated Bcl-2 expression and had poor cell viability following growth factor withdrawal (Perkins, Marshall et al. 1996; Blalock, Pearce et al. 2000). These data suggest that MEK may not be the only pathway that promotes cell survival downstream of growth factor stimulation and signaling through MEK may not provide the most robust survival signal.

The inability of a cell to die is believed to be a critical step in cancer development. Apoptosis is triggered by exposure to cytotoxic drugs, growth factor deprivation, and a variety of different pathways, including those that control DNA replication and cell cycle progression (Strasser, O'Connor et al. 2000). Apoptosis is also the main mechanism used to purge damaged or mutated cells from the body (Strasser, O'Connor et al. 2000). However, when cells become refractory to apoptosis, they gain a distinct survival advantage (Strasser, O'Connor et al. 2000; Cory, Huang et al. 2003). These pre-neoplastic cells can persist in environments where untransformed cells would readily undergo apoptosis. Moreover, pre-neoplastic cells can become insensitive to surrounding normal tissue and metastasize to foreign sites (Cory, Huang et al. 2003). While impaired apoptosis by itself is not sufficient to cause malignant transformation, it affords damaged cells more opportunities to acquire additional mutations necessary for transformation (Cory, Huang et al. 2003).

1.5 Overview of the PI3K/Akt pathway role in homeostasis and malignancy

Phosphatidylinositaol 3-kinases (PI3Ks) are a family of intracellular lipid kinases, which are classified based on structure and substrate specificity into three groups (class I, II, and II) composed of multiple subunits and isoforms (Vivanco and Sawyers 2002). Class I PI3Ks are further subdivided according to their mode of activation. Class IA PI3Ks are activated by growth factor receptor tyrosine kinases, whereas class IB PI3Ks are activated by G protein-coupled receptors (Vivanco and Sawyers 2002). Here, I focus exclusively on class IA PI3Ks due to their role in oncogenesis (Vivanco and Sawyers 2002).

PI3Ks are heterodimers composed of separate catalytic (p110 α, p110 β, p110 γ) and regulatory (p85 α, p85 β, p55 γ) subunits (Vivanco and Sawyers 2002). Following growth factor stimulation, the p85-p110 complex is recruited to the cell membrane and activated (Figure 9) (Vivanco and Sawyers 2002). Additionally, PI3Ks can be activated by Ras, which binds and activates the p110 subunit (Vivanco and Sawyers 2002). Activated PI3Ks can initiate the conversion of phosphatidylinosital (4,5)-phosphate [PI(4,5)P_2] into phosphatidylinositol (3,4,5)-phosphate [PI(3,4,5)P_3]. PIP_3 acts as a secondary messenger, recruiting downstream targets, such as 3-phosphoinositide-dependent kinase (PDK1) and Akt, to the plasma membrane by binding pleckstrin homology (PH) domains (Datta, Brunet et al. 1999; Vivanco and Sawyers 2002). PDK1 and the rapamycin-insensitive

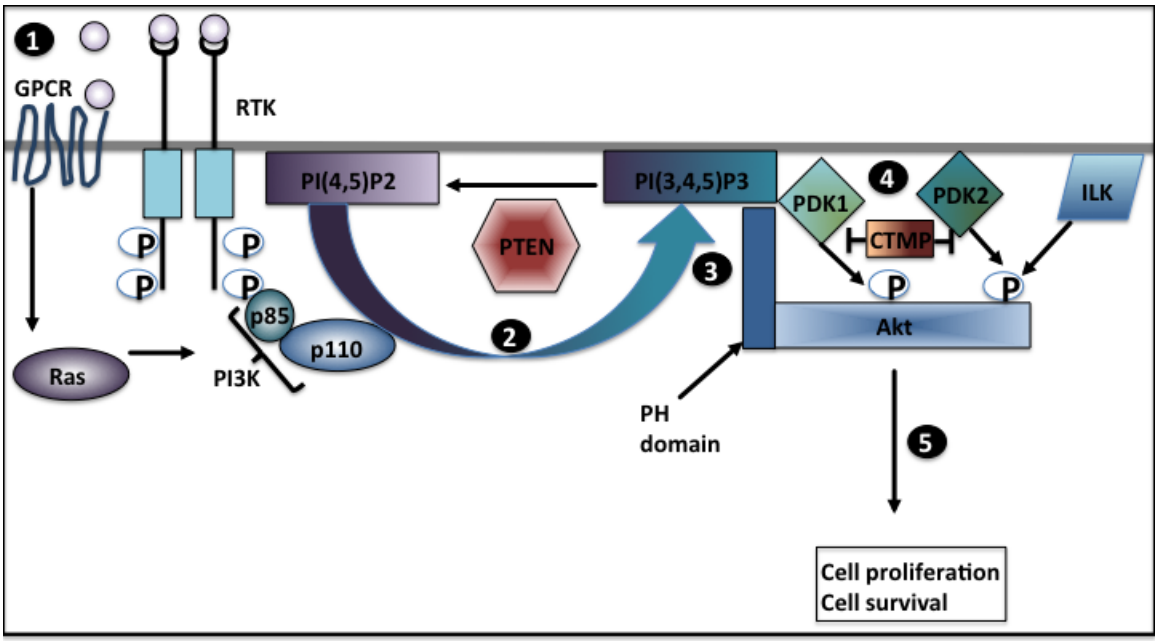


Figure 9: Regulation of the PI3K/Akt signal transduction pathway.

(1) Growth factor stimulation of receptor tyrosine kinases (RTK) and G protein-coupled receptor (GPCR) can lead to phosphorylation and activation of PI3K, which is composed of two subunits: p85 and p110. Alternatively, Ras can directly phosphorylate PI3K. (2) Activated PI3K can convert PI(4,5)P_2 to PI(3,4,5)P_3, which can be reversed by PTEN, a negative regulator of PI3K signaling. (3) PI(3,4,5)P_3 recruits PDK1 and Akt to the plasma membrane where they interact with PI(3,4,5)P_3 via its PH domain. (4) PDK1, PDK2, and ILK can phosphorylate residues important for Akt activation, which can be blocked by CTMP. (5) Activated Akt can promote expression of downstream targets that promote a variety of cellular functions, including cell proliferation and cell survival. Adapted from (Osaki, Oshimura et al. 2004).

mTOR complex, mTORC2, can then phosphorylate threonine and serine residues, respectively, thus activating the serine/threonine kinase Akt (also known as protein kinase B, PKB) (Vivanco and Sawyers 2002; Chalhoub and Baker 2009). The lipid phosphatase PTEN is an example of a negative regulator of PI3K signaling due to its ability to dephosphorylate PIP_3 to generate PIP_2 (Vivanco and Sawyers 2002; Chalhoub and Baker 2009).

Expression of dominant-negative forms of Akt in various cell lines results in apoptosis even in the presence of growth factors, suggesting that Akt is necessary for promoting cell survival (Datta, Brunet et al. 1999; Kandel and Hay 1999). Indeed, complementary cell line studies demonstrated that expression of a constitutively activated form of Akt is sufficient to prevent apoptosis in the absence of growth factor stimulation (Datta, Brunet et al. 1999; Kandel and Hay 1999). In addition to growth factor withdrawal, expression of constitutively active Akt also prevents against apoptosis induced by UV irradiation, c-Myc overexpression, matrix detachment, cell cycle discordance, DNA damage, anti-Fas antibody treatment, and treatment with TGFβ (Datta, Brunet et al. 1999; Kandel and Hay 1999).

Activated Akt can promote cell survival through a variety of different mechanisms. Akt can phosphorylate the pro-apoptotic protein BAD (BCL2-antagonist of death), which causes disassociation from Bcl-XL and other anti-apoptotic Bcl-2 family members

(Franke 2008). Phosphorylated BAD has increased affinity for 14-3-3 scaffolding proteins (Franke 2008). Upon binding the 14-3-3 proteins, BAD is sequestered in the cytoplasm, thus neutralizing its pro-apoptotic ability. On the other hand, dephosphorylated BAD can translocate to the mitochondria where it interacts and inactivates Bcl-2, causing cell death (Franke 2008). Additionally, Akt can promote cell survival by inhibiting the catalytic ability of caspase 9, an initiator caspase that can subsequently activate other downstream caspases, thus triggering the apoptotic program (Datta, Brunet et al. 1999; Rathmell and Thompson 1999).

Akt can also regulate Forkhead transcription factors, which are a family of proteins that can initiate genetic programs that promote apoptosis (Datta, Brunet et al. 1999; Vivanco and Sawyers 2002). In the presence of growth factors, Akt phosphorylation results in the sequestration of Forkhead family members in the cytoplasm (Datta, Brunet et al. 1999; Vivanco and Sawyers 2002). In the absence of growth factor signaling, Akt does not become activated, thus allowing Forkhead family members to translocate to the nucleus and initiate pro-apoptotic genetic programs (Datta, Brunet et al. 1999; Vivanco and Sawyers 2002). One such target is the FasL gene, a key component of the extrinsic pathway of apoptosis. Another target is Bim, an essential factor in the intrinsic apoptotic pathway (Strasser, O'Connor et al. 2000; Vivanco and Sawyers 2002).

In addition to inhibiting pro-apoptotic factors, Akt can also initiate genetic programs that promote the expression of anti-apoptotic genes (Vivanco and Sawyers 2002). Akt can phosphorylate and activate the IκB kinase, which can then induce the degradation of IκB, a negative regulatory of NFκB (Datta, Brunet et al. 1999; Vivanco and Sawyers 2002). Upon IκB degradation, NFκB can then translocate to the nucleus and initiate the expression of anti-apoptotic genes, such as Bfl-1/A1, and caspase inhibitors, such as c-IAP1 and c-IAP2 (Datta, Brunet et al. 1999; Vivanco and Sawyers 2002).

There are several genomic aberrations found in cancers that can upregulate PI3K/Akt signaling. These abnormalities include the following: activating mutations in *PIK3CA*, the gene encoding the p110α catalytic subunit of PI3K; *PIK3CA* amplification; deletion of PTEN; AKT mutations; and activation of receptor tyrosine kinases (Franke 2008; Chalhoub and Baker 2009). Among these, missense mutations in *PIK3CA* are the most common in human cancers (Chalhoub and Baker 2009). These mutations result in increased PI3K activity, which leads to growth-factor independent activation of Akt. On the other hand, silencing of the tumor suppressor, PTEN, is also frequently observed, especially in advanced cancers (Chalhoub and Baker 2009). PTEN is responsible for extinguishing downstream PI3K signaling by converting the secondary messenger PIP_3 to PIP_2, thus inhibiting Akt activation (Chalhoub and Baker 2009). While these data

provided researchers with correlative evidence that activation of the PI3K/Akt pathway may have been involved in tumorigenesis, direct proof was lacking until recently.

Of note, activation of the PI3K/Akt pathway has been observed in 50-70% of AML cases (Martelli, Nyakern et al. 2006). Mouse models were generated in order to determine whether activation of the PI3K/Akt pathway would be sufficient to cause disease in the hematopoietic system. Mutations in the helical and kinase domain of p110α, the catalytic subunit of PI3K, result in constitutive activation of the PI3K/Akt pathway and abrogate cytokine dependency in the Ba/F3 cell line (Horn, Bergholz et al. 2008). Inhibition of the PI3K/Akt pathway results in decreased proliferation and cell survival. In contrast, inhibition of the mTOR pathway results in a partial decrease in cellular proliferation and has no effect on cell survival (Horn, Bergholz et al. 2008). Furthermore, introduction of Ba/F3 cells stably expressing the p110α mutant in mice leads to the development of a hematopoietic neoplasia reminiscent of AML (Horn, Bergholz et al. 2008). Taken together, these data strongly suggest that activation of the PI3K/Akt pathway has strong transformation potential.

Myristoylation targets Akt to the cell membrane, which results in constitutive phosphorylation and activation of Akt (Datta, Brunet et al. 1999). Kharas and colleagues used a retroviral transduction system to introduce myristoylated AKT1 into whole bone marrow cells and found that only 10% of recipient mice develop AML, while a majority

of recipient mice develop MPN and T lymphoma (Kharas, Okabe et al. 2010). AML penetrance in this model is quite low, which could be due to the requirement for additional cooperating mutations.

Likewise, PTEN deletions have been observed in a number of cancers, including those of the hematopoietic system. Analysis of myeloid and lymphoid leukemic cell lines showed that 40% of cell lines studied contained mutations or hemizygous deletions (Dahia, Aguiar et al. 1999). Inducible PTEN knock-out mice were generated to determine if deletion of this phosphatase would have any impact on the hematopoietic system. Indeed, several groups have shown that PTEN-null mice develop MPN immediately after PTEN deletion, which progresses rapidly to T-ALL or AML (Yilmaz, Valdez et al. 2006; Guo, Lasky et al. 2008).

2. Materials and Methods

2.1 Mice

We used 6-12 weeks of age C57Bl/Ka-Thy1.1-Ly5.2, H2K-*BCL-2* transgenic, and RAG-2$^{-/-}$Ly5.2 mice in this study. All mice were maintained under specific pathogen-free conditions at the Duke University Animal Care Facility. All procedures were approved by IACUC at Duke University.

2.2 Flow Cytometry

FACS sorting and analysis were performed on a FACSVantage with a DiVa option equipped with a 488 nm argon, 599 nm dye, and 408 nm krypton lasers (BD Bioscience Flow Cytometry Systems). Cell cycle and apoptosis analysis was performed on a BD FACSCanto. Both machines are available at the FACS facility of Duke University Comprehensive Cancer Center. Data were analyzed with FlowJo software (Treestar). Cells were stained with propidium iodide (PI) to exclude dead cells from analyses and sorting.

2.2.1 Purification of HSCs

To purify HSCs (Lin^{-}Sca-1^{+}c-kit^{+}), we harvested bone marrow cells from tibia and femurs from C57Bl/Ka-Thy1.1-Ly5.2 or H2K-*BCL-2* transgenic mice at 6-12 weeks of age.

Bones were flushed with ice-cold staining medium (Hanks' balanced salt solution containing 2% FBS and 0.02% NaN_3) to create a single-cell suspension. Cells were kept on ice for the duration of the procedure, unless noted. Cells were pelleted by centrifugation at 1500 rpm for 5 min at 4^0C. Cells were then treated with ACK lysis buffer for 1 min at 25^0C to lyse red blood cells and washed with staining media.

Next, c-Kit$^+$ cells were enriched by staining whole bone marrow with anti-CD117/c-Kit microbeads (Miltenyi Biotec, Auburn, CA) and isolating positively labeled cells using the POSSEL_S mode with autoMACS cell separation. Cells were then incubated with rat IgG to block non-specific antibody binding. Cells were then stained with anti-Sca-1, anti-c-Kit, anti-Lineage (CD3, CD4, CD8, B220, Gr-1, Mac-1, Ter119) (KLS) antibodies for 20 min at 4^0C.

2.2.2 Purification of myeloid progenitors

To purify myeloid progenitors, we first harvested bones, generated single-cell suspensions, and enriched for c-Kit$^+$ cells as previously described. We then stained c-Kit$^+$ enriched bone marrow with KLS antibodies as previously described. We then sorted c-Kit$^+$ bulk myeloid progenitors that contain CMPs, GMPs, and MEPs.

2.2.3 Analysis of transplanted mice

Bone marrow and spleen were harvested for FACS analysis. Bone marrow cells were prepared as previously described and single-cell suspensions were made from mashing spleen. Bone marrow and spleen cells were washed with ice-cold staining medium, treated with ACK lysis buffer for 1 min at 25^0C to lyse red blood cells, washed again, and then incubated on ice with rat IgG to block non-specific antibody binding.

Peripheral blood was collected in a tube containing PBS+10mM EDTA via tail vein bleed. 2% dextran was added to the blood and incubated at 37^0C to allow for separation of red and white blood cells. The top layer was transferred to a new tube and treated with ACK lysis buffer for 5 min at 25^0C to lyse any remaining red blood cells. Cells were washed and incubated with rat IgG to block non-specific antibody binding as previously described.

Cells were then stained with a combination of the following antibodies as previously described: phycoerythrin (PE) anti-Thy1.2, PE-anti-CD8, PE-anti-Sca-1, PE/Cy5 anti-CD3, PE/Cy5 anti-CD4, PE/Cy5 anti-CD8, PE/Cy5 anti-B220, PE/Cy5 anti-CD19, PE/Cy5 anti-Gr-1, PE/Cy5 anti-Mac-1, PE/Cy5 anti-Ter119, PE/Cy5 anti-Thy1.1, PE/Cy5 anti-Thy1.2, PE/Cy7 anti-Gr-1, allophycocyanin (APC)-anti-Mac-1, APC anti-Thy1.2, APC anti-c-Kit, APC/Cy7 anti-B220, APC/Cy7 anti-Mac-1, APC/Cy7 anti-CD4, biotinylated CD3. All of the above antibodies were purchased from eBioscience (San Diego, CA), BioLegend (San Diego, CA), or R&D (Minneapolis, MN). Alexa Fluor

(Invitrogen, Carlsbad, CA) 594-anti-Sca-1 was prepared in our laboratory with a standard procedure. Biotin-conjugated antibodies were visualized with PE/Cy7-steptavidin (eBioscience).

2.3 Retroviral production and gene transfer

Active MEK cDNA was cloned into the MSCV-IRES-GFP vector. We produced virus using 293T cells, which were maintained in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% FBS. Briefly, 293T cells were transfected with viral constructs along with gag-pol and VSV-G constructs. Viral supernatants were collected for 4 days and concentrated by ultracentrifugation at 50,000*g* for 4 hrs at 4^0C. Virus was aliquoted and stored at -80^0C until use.

2.4 Transplantation

2.4.1 Retroviral bone marrow transplantation assay

HSCs were sorted as previously described and cultured at 37^0C overnight in X-Vivo 15 (BioWhittaker, Walkersville, MD) supplemented with 10% FBS, stem cell factor (SCF) (50 ng/mL, R&D Systems, Minneapolis, MN), thrombopoietin (10 ng/mL, R&D Systems, Minneapolis, MN), and IL-11 (10 ng/mL, R&D Systems, Minneapolis, MN). Active MEK or control retroviral supernatant was added to the cells the following day and cells were spun at 2000 revolutions per minute (rpm) at 25^0C for 2 hrs. Cells were

further cultured at 37^0C for 24 hrs to allow for gene expression. Cells were harvested and GFP^+ cells were sorted, washed with ice-cold staining medium, and resuspended in RPMI (Invitrogen, Carlsbad, CA)+10% FBS. Cells were injected into the tail vein of sublethally irradiated (400 rad) RAG-$2^{-/-}$ Ly5.2 mice. Following transplantation, recipient mice were maintained on antibiotic water (sulphamethoxazole and trimethorpim) and evaluated routinely for signs of morbidity, weight loss, hunchback appearance, and splenomegaly. Pre-morbid animals were sacrificed and tissues were harvested and analyzed by flow cytometry and histopathology.

2.4.2 Secondary transplantation of bone marrow and spleen cells

For secondary transplantations, unfractionated and fractionated bone marrow and spleen cells from individual primary transplanted mice were harvested as previously described, washed, and immediately injected into the tail vein of sublethally irradiated RAG-$2^{-/-}$ Ly5.2 mice. $1x10^5$-$1x10^6$ fractionated bone marrow, $1x10^5$-$2x10^6$ fractionated spleen, $5x10^5$-$5x10^6$ unfractionated bone marrow, or $1x10^6$-$10x10^6$ unfractionated splenocytes harvested from individual primary transplanted mice were injected into multiple recipient mice. Cells were fractionated based on GFP expression. Following transplantation, recipient mice were maintained on antibiotic water and evaluated routinely for signs of disease as described above.

2.5 Histopathology

2.5.1 Complete blood cell count

Tail vein bleeds were used to collect peripheral blood for weekly analysis of reconstituted animals. Blood was collected in a heparinized tube and differential cell counting was done with a HemaVet 950FS (Drew Scientific Inc.).

2.5.2 Bone marrow aspirates and cytology

Bone marrow was extracted from femurs using a 16 ½ gauge needle and smeared onto glass slides to generate bone marrow aspirates. Slides were dried and stained with Wright-Giemsa stain.

2.5.3 Histology

Organs of interest were collected immediately after mice were sacrificed and fixed in 4% paraformaldehyde at 4^0C. Samples were delivered to the pathology department at Duke University where they were further processed and embedded in paraffin blocks. 5 μm-thick tissue sections were cut from the paraffin blocks and mounted onto a glass slide. Slides were stained with hematoxylin and eosin.

2.6 In Vitro Culture Systems

2.6.1 Methylcellulose culture systems

HSCs were sorted and transduced with retrovirus as described above. After 48 hrs of infection, cells were harvested, stained with KLS antibodies as previously described, and GFP^+ HSCs cells were sorted. Sorted cells were washed with staining media, cells were resuspended to a concentration of 500 cells/10 uL of IMDM/well, and 500 uL of MethoCult 3534 (StemCell Technologies)/well was added directly to the cells. Tubes were capped and vortexed vigorously. Cells were incubated at 25^0C for 20-30 min and then plated in a 24-well plate using a syringe and 18 ½ gauge needle. Samples were plated in quadruplicate. Colonies were enumerated 5-8 days after plating.

Secondary platings were carried out after enumeration. Cells were harvested, washed twice with PBS, and counted. Cells were resuspended to a concentration of 50,000 cells/10 uL IMDM/well, and 500 uL of MethoCult 3534/well was added directly to the cells. Cells were processed as previously described. Samples were plated in quadruplicate. Colonies were enumerated 5-8 days after plating.

2.6.2 32D Cell line

32D cells were cultured in complete RPMI medium supplemented with 10% WEHI supernatant. 32D+active MEK transfectants were established using a retroviral

system followed by purification of GFP^+ cells by FACS sorting. Briefly, retroviral supernantant containing active MEK retrovirus was added to cells and spun at 2,000 revolutions per minute (rpm) at 25^0C for 2 hrs. Cells were further cultured at 37^0C for 48 hrs to allow for gene expression. Cells were harvested and GFP^+ cells (32D+active MEK) were sorted and cultured in complete RPMI at 37^0C. 32D+active MEK cells were maintained in culture for 72 hrs, GFP^+ cells were sorted again to ensure homogeneity, and cells were cultured in complete RPMI at 37^0C.

2.7 Quantitative PCR analysis

RNA purification and first-strand DNA synthesis were done as previously described (Lai and Kondo 2007). Briefly, cells were lysed using 1 mL of TRIzol reagent (Invitrogen). Total RNA was purified based on the manufacturer's instructions. First-strand cDNA was synthesized with Superscript III RT and random hexamers (Invitrogen). Quantitative PCR was performed in triplicate on MyIQ (Bio-rad). The expression level of the gene of interest was calculated and normalized to Rpl-13 or β-actin. PCR primers are listed below

β -actin-F: 5′-GGGAATGGGTCAGAAGGAT-3′

β -actin-R: 5′-GGGGTGTTGAAGGTCTCAAA-3′

Deltex1-F: 5′-CAGCCGCCTGGGAAGATGGAGTT-3′

Deltex-1-R: 5′-TGGATGCCTGTGGGGATGTCATAGAC-3′

Hes-1-F: 5′-AAAGCCTATCATGGAGAAGACGCG-3′

Hes-1-R: 5′-GGAATGCCGGGAGCTATCTTTCTT-3′

2.8 Cell cycle analysis

Cell populations were isolated and sorted based on specific markers described in the text as previously described. Cells were washed with staining media, pelleted, and the supernatant removed. Next, ice-cold 70% ethanol was added drop-wise to the sample while vigorously vortexing. Samples were stored in the dark at 4^0C for a minimum of 16 hrs and up to 48 hrs. Cells were pelleted and the ethanol was carefully aspirated from the top. Cells were resuspended in 200 uL of PI buffer (1X PBS+0.1% glucose containing PI and RNase A) and read on a flow cytometer.

2.9 Detecting apoptotic cells

HSCs were sorted and infected with retrovirus as previously described. Following retroviral transduction, cells were harvested and GFP^+ HSCs were sorted directly into IMDM+1% BSA. Cells were pelleted, resuspended in IMDM+1% BSA, and plated. Cells were harvested 6 or 12 hrs later and assayed for apoptosis. Cells were washed with Annexin V binding buffer, incubated with Annexin V and 7-AAD

antibodies for 15 min on ice, washed twice with Annexin V binding buffer, and analyzed using a flow cytometer.

2.10 Southern blot analysis

Bone marrow, spleen, and tumor cells were harvested from primary and secondary transplanted mice. Cells were lysed and genomic DNA was isolated. 10 ug of DNA was digested with PvuII restriction enzyme. 5 ug of DNA was subjected to electrophoresis through 0.7% agarose gel, blotted, and UV crosslinked to a Hybond-N membrane. Membranes were prehybridized at 42^0C overnight and hybridized at 60^0C in 50% formamide, 5X SSC, 50X Denhardt's, 10% SDS, 100 μg/mL boiled salmon sperm DNA, 1M Hepes (pH 7.0). Blots were washed twice with 1X SSC+0.5% SDS at 27^0C followed by an additional two washed with 0.1X SSC+0.1% SDS at 60^0C. Blots were then exposed to film.

GFP cDNA was amplified by PCR and cloned into Topo TA vector to generate a GFP-specific probe. Probes were gel purified and radiolabeled using random hexamer priming.

3. Constitutive MAP kinase activation in hematopoietic stem cells induces myelodysplastic disorder/myeloproliferative neoplasm

3.1 Introduction

Hematopoietic cells are divided into two groups: lymphoid and myeloid lineages (Kondo 2003). Leukemia is caused by neoplastic transformation of hematopoietic cells. It is estimated that annually, 5.6 and 5.8 out of 100,000 people are diagnosed with lymphoid or myeloid leukemias, respectively (Rodriguez-Abreu, Bordoni et al. 2007). Myeloid neoplasms are sub-divided by the World Health Organization (WHO) into four sub-groups based on morphology, immunophenotype, genetics, and clinical features, which include: acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), and a mixed MDS/MPN disease (Vardiman 2010). AMLs are marked by genetic abnormalities, such as chromosomal translocation, and increased blast counts in the peripheral blood or bone marrow. On the other hand, MDSs are generally viewed as pre-leukemic conditions that feature ineffective hematopoiesis that leads to cytopenia(s) (Vardiman, Thiele et al. 2009). MPNs are a heterogenous collection of diseases that includes leukemic and pre-leukemic conditions marked by increased proliferation of myeloid cells and effective maturation of neoplastic cells (Tefferi, Skoda et al. 2009). Lastly, the mixed MDS/MPN category, which was added in 2001, was specifically created to accommodate diseases such as juvenile

myelomonocytic leukemia (JMML) and chronic myelomonocytic leukemia (CMML) (Vardiman, Harris et al. 2002). This modification in the classification scheme was necessary since these diseases did not solely belong in the MPN or MDS category because many cases had both dysplastic and proliferative features (Vardiman, Harris et al. 2002; Vardiman, Thiele et al. 2009).

MDS/MPNs are a product of inappropriate activation or inactivation of molecules induced by genetic mutations (Emanuel 2008; Vardiman, Thiele et al. 2009). As a result, signaling pathways that are normally involved in growth factor receptor signaling are constitutively activated (Platanias 2003; Emanuel 2008). For example, activation of Ras, a membrane proximal signaling molecule and proto-oncogene, is frequently observed in various types of malignant cells generated by neoplastic transformation. In fact, mutations that result in activation of the Ras gene are found in 30% of human cancer cases including leukemia (Bos 1989; Downward 2003). Moreover, clinical findings also show that Ras mutations are observed in 20-60% of CMML cases and 20-25% of JMML cases (Emanuel 2008).

However, the role of Ras in transformation events is unclear because it is located upstream of several pathways, including MEK/ERK, PI3K/Akt, and Ral-GEF (Schubbert, Shannon et al. 2007). Various mouse models have been generated to further dissect each downstream pathway in order to determine its contribution to malignant transformation

of hematopoietic cells (Figure 10). For example, mouse models that feature expression of oncogenic K-Ras, either conferred by a point mutation or ablation of negative regulators such as NF1, result in MPNs that closely resembles human JMML (Braun, Tuveson et al. 2004; Chan, Kutok et al. 2004; Le, Kong et al. 2004; Sabnis, Cheung et al. 2009; Zhang, Wang et al. 2009).

Activation of the PI3K pathway can be achieved either through mutations that result in constitutive activation of Akt or ablation of PTEN, a negative regulator of Akt (Vivanco and Sawyers 2002; Chalhoub and Baker 2009). Regardless of the mode of activation, neoplasms that result from activation of the PI3K/Akt pathway begin as pre-leukemic myeloid diseases, but rapidly convert to a lymphoid disease (Figure 10) (Yilmaz, Valdez et al. 2006; Zhang, Grindley et al. 2006; Guo, Lasky et al. 2008; Kharas, Okabe et al. 2010). For example, only 10% of mice that expressed activated Akt in the bone marrow developed AML, while 65% developed an MDS that quickly progressed to T lymphoma (Kharas, Okabe et al. 2010). In mice where PTEN expression was ablated, 74% of mice developed T-acute lymphoblastic leukemia (T-ALL) while 26% developed a mixed AML/T-ALL phenotype (Yilmaz, Valdez et al. 2006; Zhang, Grindley et al. 2006; Guo, Lasky et al. 2008). On the other hand, activation of K-Ras primarily results in a myeloid disease. The heterogeneity in disease phenotype and latency strongly suggest

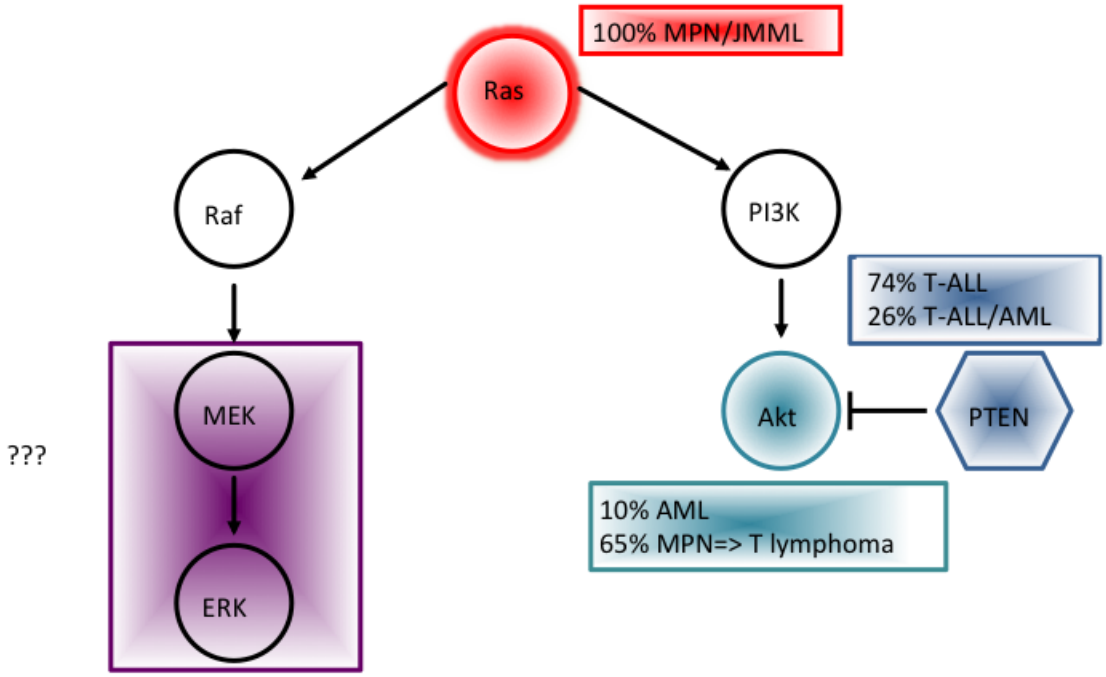


Figure 10: Disease phenotype and frequency in activated Ras and PI3K/Akt animal models.

Ras has many downstream targets, among them Raf/MEK/ERK and PI3K/Akt. Oncogenic Ras has been shown to give rise to MPN/JMMLs with complete penetrance. Activation of the PI3K/Akt pathway can be achieved by myristolyation of Akt, which yields 10% AML and 65% MPNs that quickly progress to T lymphoma in mouse models. Ablation of PTEN, a negative regulator of PI3K/Akt signaling, also results in activation of PI3K/Akt signaling. Almost three-quarters of PTEN-null mice develop T-ALL, while the remaining mice develop a mixed T-ALL/AML disease.

that PI3K/Akt signaling may not be the sole mediator of oncogenic K-Ras signaling and other downstream pathways such as the MEK/ERK pathway may be involved.

MEK/ERK are dual serine/threonine specific kinases that are part of the classical mitogen-activated protein kinase pathway (MAP kinase) (Raman, Chen et al. 2007). Of the five MAP kinase pathways, the MEK/ERK pathway is predominantly activated by a variety of extracellular stimuli including growth factors, serum, cytokines, and osmotic stress and is critically involved in regulating a variety of cellular processes, such as cell growth, migration, proliferation, differentiation, and survival (Platanias 2003; Raman, Chen et al. 2007).

Although activating MEK/ERK mutations have not been identified in any human samples or cancer cell lines, there is evidence suggesting a link between MEK/ERK signaling and malignant transformation (Chang, Steelman et al. 2000). This evidence comes from work that uses a constitutively active form of MEK1 (active MEK hereafter). Active MEK was generated in a two step process: first, a 20 amino acid stretch in the N-terminus that can regulate kinase activity was deleted; second, one of the Raf1-dependent regulatory serine phosphorylation sites, serine 222, was substituted with an aspartic acid residue (ΔN3-S222D) (Mansour, Matten et al. 1994). Expression of active MEK in NIH3T3 cells was sufficient to cause malignant transformation (Mansour,

Matten et al. 1994). Furthermore, expression of active MEK/ERK is sufficient to relieve growth factor dependency in hematopoietic cell lines (Blalock, Pearce et al. 2000). Therefore, activation of the MEK/ERK pathway may play a primary role in malignant transformation of hematopoietic cells, although this issue has not been directly investigated.

In this study, we found that expression of active MEK alone induced MDS/MPNs but not other types of hematopoietic malignancies from HSCs. Generation of MDS/MPNs from HSCs expressing active MEK was not affected by enhanced cell survival ability, which was conferred by ectopic expression of Bcl-2, an anti-apoptotic protein. MDS/MPNs induced by active MEK were not transplantable in the presence or absence of ectopic Bcl-2. Following a long latency period; however, T-ALL with fatal blast crisis was observed in naïve mice transplanted with cells harvested from a mouse with Bcl-2/active MEK-induced MDS/MPN. These results suggest that expression of active MEK is sufficient for development of myeloid malignancies from HSCs. Moreover, enhanced cell survival of MDS/MPNs could be necessary for the development of blast crisis, which mirrors a similar clinical course observed in human MDS/MPNs. Therefore, we concluded that the MEK/ERK pathway could play a significant role in neoplastic transformation of hematopoietic cells.

3.2 Results

3.2.1. Expression of active MEK relieves growth factor dependency in 32D cells.

MEK/ERK is downstream of many cytokine receptors that are important for myelopoiesis, such as GM-CSF, G-CSF, and IL-3 (de Groot, Coffer et al. 1998). Cytokine receptors have been shown to be involved in promoting cell survival, proliferation, and differentiation (Watowich, Wu et al. 1996). Our group has previously shown that MEK/ERK activity is necessary for myeloid lineage commitment during hematopoiesis from hematopoietic stem cells (Hsu, Kikuchi et al. 2007). To examine the significance of MEK/ERK in cytokine-mediated growth signaling, we introduced a constitutively activated form of MEK into the 32D cell line, a murine IL-3-dependent cell line that phenotypically resembles myeloid progenitors (Greenberger, Sakakeeny et al. 1983).

A constitutively active form of MEK (simply denoted active MEK hereafter) was introduced into 32D cells via retroviral infection and cultured in the presence or absence of IL-3 and cells were enumerated every day for 8 and 17 days, respectively. As shown in *Figure 11*A, expression of active MEK in 32D cells conferred a slight growth advantage. Moreover, 32D cells acquired cytokine-independent proliferation potential after introduction of an activated form of MEK (*Figure 11*B). 32D+MEK cells cultured in the absence of IL-3 were maintained in culture for at least one month in the absence of IL-3. These data suggest that active MEK can abrogate cytokine dependency of the 32D cell line, which is suggestive of transformation potential.

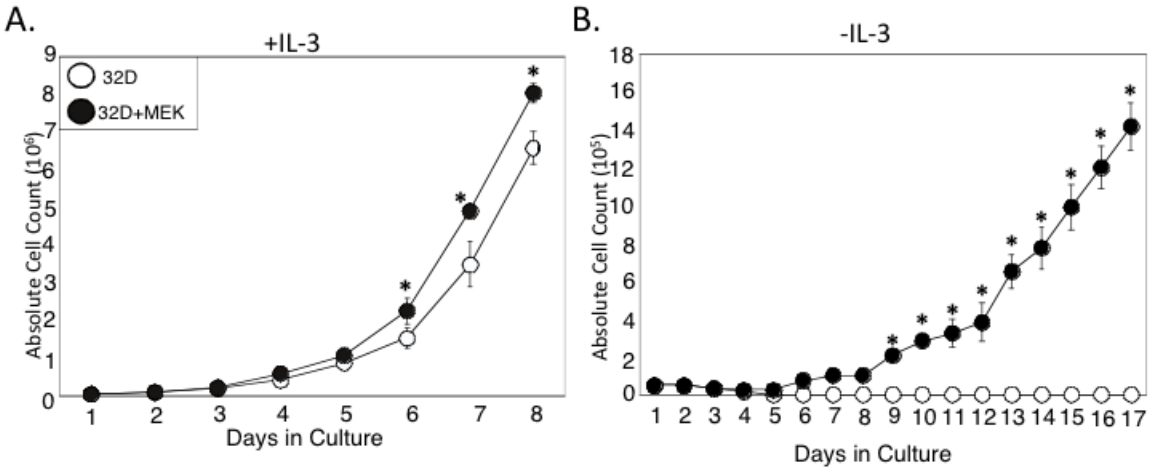


Figure 11: Effect of active MEK on growth in 32D cell line.

32D cells were transduced with retrovirus expressing active MEK (32D+MEK). (A) 32D and 32D+MEK were cultured in complete media containing IL-3 (10 ng/mL) and cells were enumerated daily. (B) 32D and 32D+MEK were cultured in complete media only and cells were enumerated daily. Trypan blue was used to exclude dead cells. Results shown are the mean value of triplicate wells. Error bars represent the standard deviation from three wells. *, $P<0.05$ (statistical significance) by Student *t* test.

3.2.2 HSCs expressing active MEK preferentially give rise to mature granulocyte/macrophage (G/M) cells *in vivo*, leading to MDS/MPN

To examine whether activation of the MEK/ERK pathway is sufficient to induce malignant transformation of primary blood cells, we purified c-Kit^{+}Lin^{-}Sca-1^{+} (KLS) cells, which are highly enriched for HSCs, from bone marrow of wild type (WT) C57Bl/Ka mice. Using a retroviral transduction system detailed in Figure 12, we introduced active MEK into HSCs and injected them into sublethally irradiated RAG-2$^{-/-}$ mice. The MSCV retroviral vector employed in our model contains an IRES-GFP cassette (Kondo, Scherer et al. 2000). Therefore, cells productively infected with retrovirus are positive for GFP expression. Approximately 6-8 weeks after injection, the mice transplanted with active MEK^{+} HSCs became lethargic, had a hunched back appearance, and experienced weight loss. Moreover, all mice died by 10 weeks post-injection compared to control mice, which remained healthy and free of disease for at least 100 days post-injection (**Figure 13**A).

Genesis of hematopoietic malignancies occurs in the bone marrow, a site used in clinical settings to diagnose disease. Therefore, we first examined the bone marrow for evidence of dysregulated hematopoiesis. While active MEK mice showed a modest increase in total number of bone marrow cells (**Figure 13**B), the majority of donor-derived cells expressed Mac-1 suggesting that myeloid cell numbers are increased in the

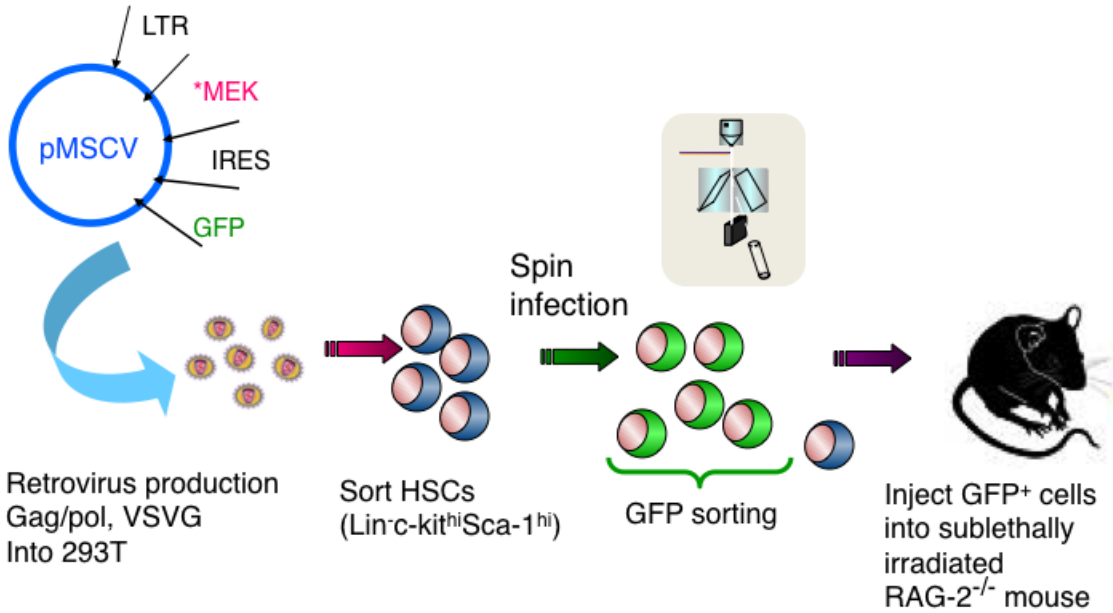


Figure 12: Retroviral transduction strategy used in our study.

A constitutively active form of MEK (*MEK) was cloned into the MSCV retroviral vector, which contains an IRES-GFP so infected cells are GFP^+. This retroviral vector construct was used to generate virus. Spin infection was used to infect sorted HSCs. After 24 hours post-infection, GFP^+ cells were sorted and injected into sublethally irradiated RAG-$2^{-/-}$ mice.

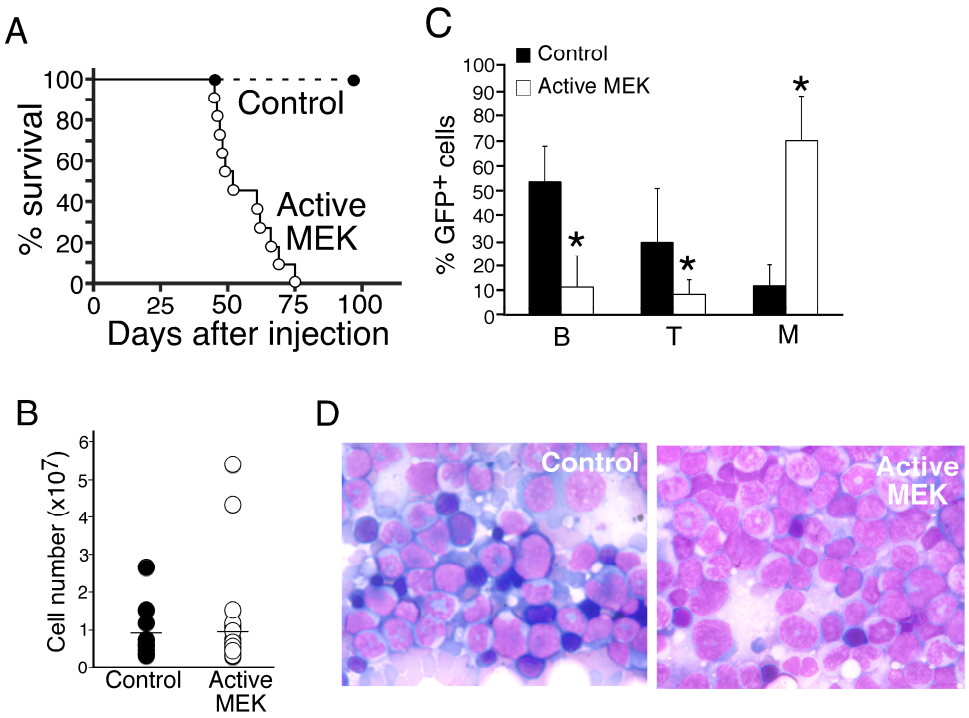


Figure 13: Expression of active MEK in HSCs led to the development of MDS/MPN.

(A) A Kaplan-Meier curve illustrating the survival time of mice reconstituted with hematopoietic stem cells (HSCs) infected with control (closed circles, n=7) or active MEK (open circles, n=13) retrovirus. Approximately 7,000 retrovirally infected (GFP^+) HSCs were injected into sublethally irradiated RAG-$2^{-/-}$ recipients. Survival of transplanted control mice was monitored for at least 100 days post-injection. (B) Absolute bone marrow cell counts were determined for mice injected with HSCs transduced with control (filled circles) or active MEK (empty circles) retrovirus at the terminal stage. Horizontal bar denotes the mean value for each group. (C) Contribution of active MEK^+ HSCs to different hematopoietic lineages. Bone marrow cells were harvested from transplanted mice at the terminal stage for FACS analysis of the various lineage-specific cell surface markers (B: $B220^+$ B cells, T: Thy^+ T cells, M: Mac-1^+ myeloid cells). The bar graph summarizes the mean

percentage of GFP^+ donor-derived cells for each cell type for control mice (black bar) and active MEK mice (white bar). Error bars represent the standard deviation from each group. *, $P<0.05$ (statistical significance) by Student *t* test. (D) Bone marrow aspirates from transplanted mice. Bone marrow was extracted from control (left) and active MEK mice (right), smeared onto a slide, and stained with Wright-Giemsa stain. Representative pictures are shown for each group. Zoom=400X.

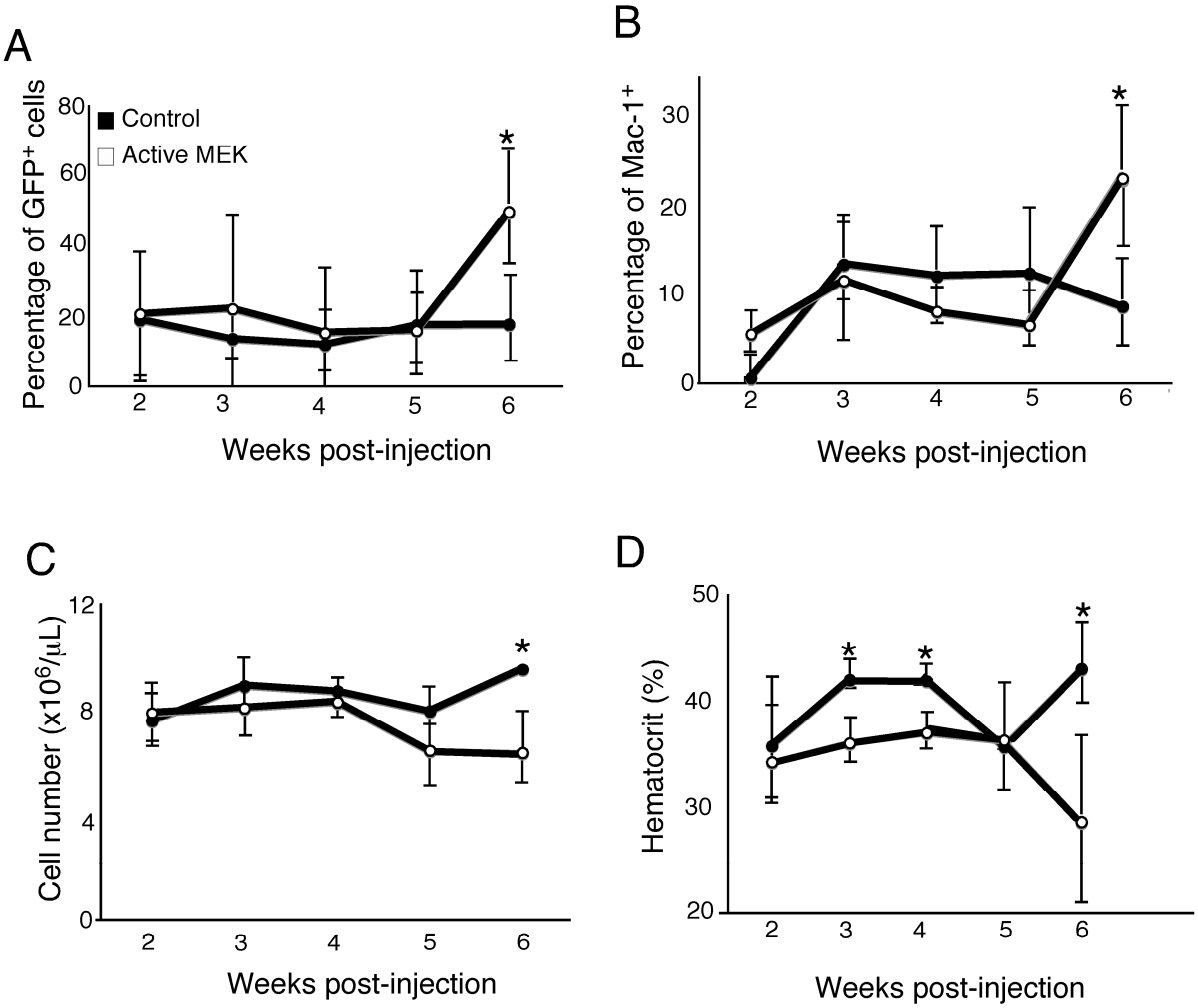


Figure 14: In vivo time course analysis of peripheral blood.

(A, B) Peripheral blood was collected from vector control (filled circle, n= 3) or active MEK mice (empty circle, n=5) two weeks post-injection and then weekly, thereafter. Cells were stained with antibodies and subjected to FACS analysis. Cells were pre-gated on GFP^+ cells and dead cells were excluded from the analysis by PI staining. (C) Differential cell counting was performed on peripheral blood harvested from control mice (filled circle) and active MEK mice (empty circle) using a HemaVet machine to assess red blood cell number and (D) hematocrit. Data shown are the mean values from all mice in each group. Error bars represent the standard deviation from each group of mice. *, $P<0.05$ (statistical significance) by Student *t* test.

presence of active MEK. (Figure 13C). This is in agreement with previous findings from our group, showing that expression of active MEK in HSCs predominantly gives rise to Mac-1$^+$ myeloid cells at the expense of both B and T cell development (Figure 13C) (Hsu, Kikuchi et al. 2007). Compared to control mice, bone marrow aspirates from active MEK mice contain more mature G/M cells, which are characterized by their donut-shaped nucleus (Figure 13D). Also, cells in the erythroid and lymphoid series are significantly decreased in the aspirates from active MEK mice when compared to control mice (Figure 13D).

Because hematopoietic cells generated in the bone marrow eventually enter the peripheral blood, we reasoned we would also find evidence of dysfunctional hematopoiesis in the peripheral blood. Peripheral blood collection from vector control and active MEK mice was initiated two weeks post-injection and then performed weekly. Complete blood cell counts and FACS were used to assess blood composition. We found that the percentage of GFP$^+$ cells in active MEK mice was comparable to vector control mice until week five, when GFP$^+$ cells in active MEK mice underwent a large expansion, which also mirrored the expansion of Mac-1$^+$ cells (Figure 14A,B). This data is in agreement with results obtained from the bone marrow, which also exhibited a large Mac-1$^+$ cell population (Figure 13C).

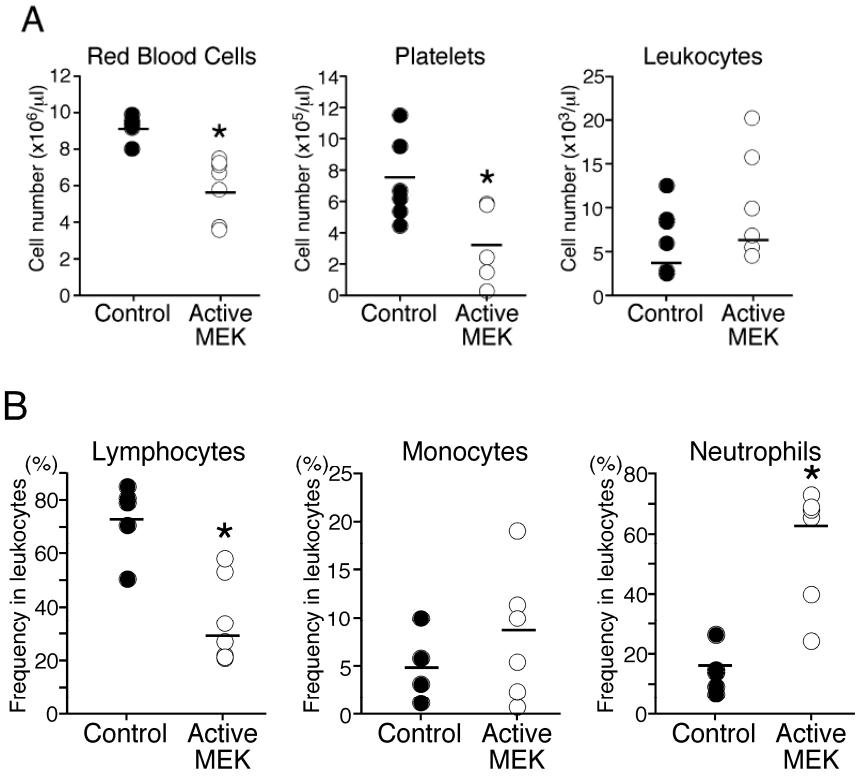


Figure 15: Dysfunctional hematopoiesis in terminally ill active MEK mice.

(A,B) Differential cell counting of blood was performed on peripheral blood harvested from control mice (filled circle, n=6) and active MEK mice (empty circle, n=6). Absolute cell counts were determined using a HemaVet machine. The horizontal bar denotes the mean value of each group. *, *P<0.05* (statistical significance) by Student *t* test.

Interestingly, we found that red blood cell numbers were low in active MEK mice, but that a significant decrease in production was not observed until the end point (Figure 14C). Additionally, hematocrit, which measures the percent volume occupied by red blood cells, was also decreased throughout the time course, with the biggest difference observed after six weeks post-injection (Figure 14D). Furthermore, these data also suggest that active MEK expression in HSCs does not directly suppress red blood cell production since mice do not develop anemia until later time points. Suppression of red blood cell production appears to be an indirect effect of active MEK expression in HSCs due to the developmental skew along the myeloid lineage and increase in Mac-1^{+} cells.

Examination of the peripheral blood at the terminal stage for control and active MEK mice also indicated a significant decrease in the number of red blood cells and platelets with a slight increase in the number of leukocytes (Figure 15A). Similar to results obtained from analysis of the bone marrow, we found a significant decrease in the percentage of lymphocytes in active MEK mice compared to control (Figure 15B). Also, we observed a significant increase in the percentage of neutrophils (Figure 15B). While monocytes were elevated in active MEK mice, the difference was not significant (Figure 15B). These results indicate that hematopoiesis is severely disrupted in active

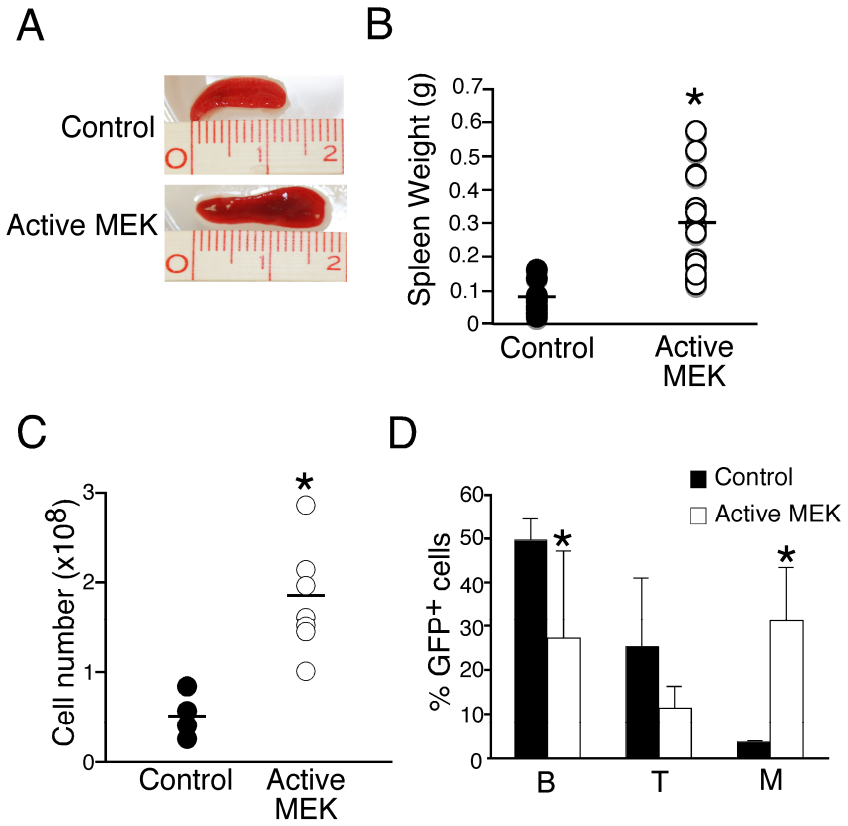


Figure 16: Active MEK mice have splenomegly.

(A) Enlarged spleens were observed in active MEK mice. (B) Whole spleens were harvested from moribund active MEK mice (empty circle n=13) and vector control mice (filled circle n=7) and weighed and (C) absolute splenocyte cell count was determined. The horizontal bar denotes the mean value of each group. (D) Contribution of active MEK$^+$ HSCs to different hematopoietic lineages. Spleens were harvested from transplanted mice at the terminal stage for FACS analysis of the various lineage-specific cell surface markers (B: B220$^+$ B cells, T: Thy$^+$ T cells, M: Mac-1$^+$ myeloid cells). The bar graph summarizes the mean percentage of GFP$^+$ donor-derived cells for each cell type for control mice (black bar) and active MEK mice (white bar). Error bars represent the standard deviation. *, $P<0.05$ (statistical significance) by Student *t* test.

MEK mice at the terminal stage. Moreover, mice were severely anemic and most likely died of bone marrow failure.

We found additional evidence of dysfunctional hematopoiesis in the spleen. Grossly, splenomegaly of active MEK$^+$ mice was obvious (Figure 16A). The mass (Figure 16B) and cellularity (Figure 16C) of the spleen was also significantly increased in active MEK$^+$ mice compared to control mice. Similar to the bone marrow, a marked increase in Mac-1$^+$ cells was observed along with a concomitant decrease in B and T cells (Figure 16D). Spleens from control mice showed clear demarcation between the red and white pulp (Figure 17, white arrow), while the splenic architecture of active MEK mice was completely disorganized (Figure 17, black arrow). However, myeloid cells were not limited to lymphoid organs. Infiltration into the peribronchial and perivascular areas of the lung was observed (Figure 17, black arrow). Mice also exhibited hepatomegly and evidence of myeloid infiltration into the periportal areas of the liver was also noted (Figure 17).

In summary, the disease we observed is marked by dysfunctional hematopoiesis that results in anemia, thrombocytopenia, and decreased lymphocytes. These types of cytopenias are characteristic of MDS. On the other hand, myeloid cells are able to undergo full and effective maturation. However, the hyperproliferation of mature

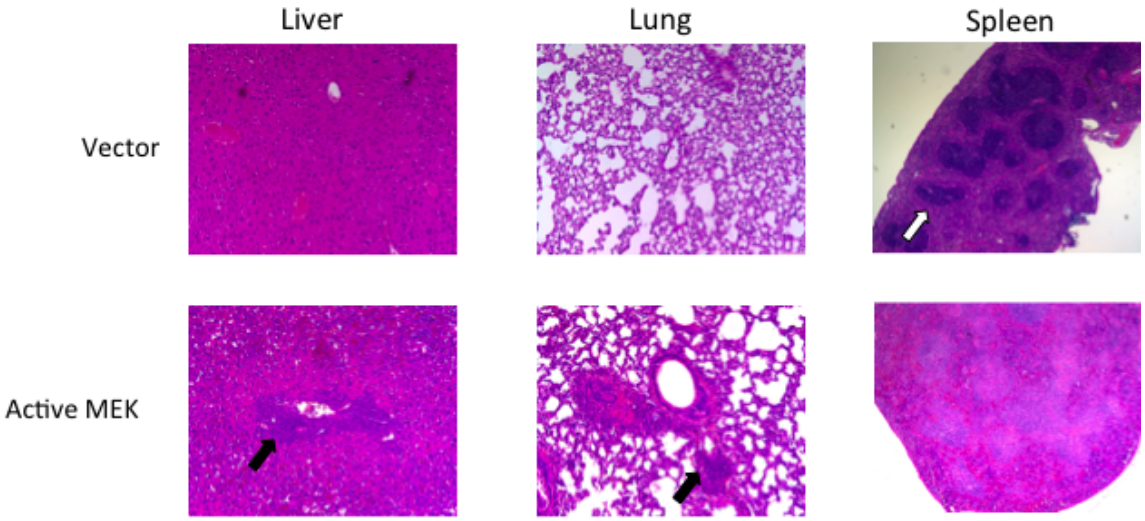


Figure 17: Impact of enhanced myeloid cell production on organs of active MEK mice.

Organs were harvested from active MEK mice (bottom row) at the terminal stage or control mice (top row), fixed in 4% paraformaldehyde, and embedded in paraffin blocks. 5 μM thick sections were cut, mounted on a glass slide, and stained with hematoxylin and eosin. Zoom=400X. One representative tissue section from vector control and active MEK mice are shown. Myeloid cell infiltration is observed exclusively in the liver and lung of active MEK mice and is denoted with a black arrow. An example of a region containing white pulp in the spleen of a control mouse is denoted with a white arrow.

myeloid cells is indicative of a MPN. MPNs are also marked by leukocytosis, which was observed to a moderate degree in our model. Splenomegly and hepatomegly, two other common signs of MPNs, were also observed in our mice. Taken together, these data suggest that expression of active MEK in HSCs resulted in a mixed MDS/MPN disease.

3.2.3 Active MEK expression has limited effect on survival and self-renewal properties of hematopoietic progenitor populations.

Data from the 32D cell line suggests that active MEK may have some transforming properties (Figure 11). Indeed, MEK signaling has been shown to be important in a variety of cellular functions including cell survival and proliferation, which are also important acquired capabilities for cancer cells (Hanahan and Weinberg 2000; Raman, Chen et al. 2007). Specifically, MEK signaling has been shown to enhance cell survival by upregulating anti-apoptotic genes (Scholl, Dumesic et al. 2007). The percentage of Annexin V positive cells was similar in control (Figure 18, left panel) and active MEK mice (Figure 18, right panel), suggesting that expression of active MEK did not prevent cells from entering early states of apoptosis. Thus, expression of active MEK does not appear to affect the survival of HSCs when cultured in the absence of growth factor (Figure 18).

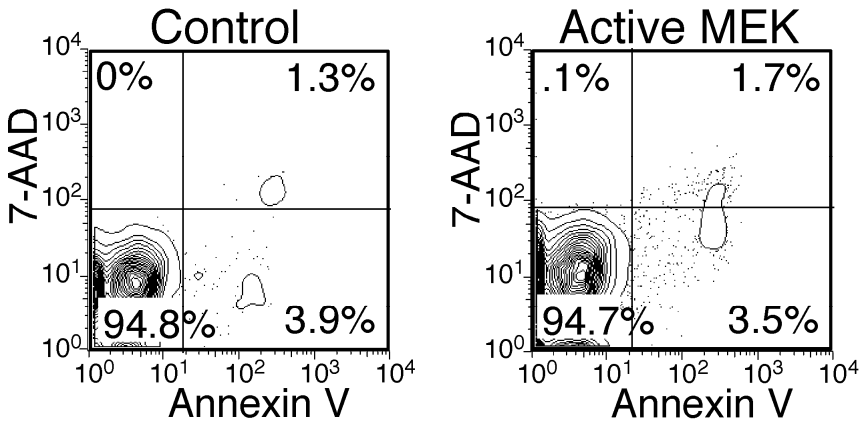


Figure 18: Active MEK does not enhance cell survival in HSCs.

HSCs were sorted and infected with retrovirus expressing active MEK (right panel) or vector control (left panel). GFP^+ HSCs were sorted 48 hrs post-infection and plated in IMDM+1%BSA. Cells were incubated at 37^0C for 12 hrs, harvested, washed, stained with AnnexinV and 7-AAD, and read on a flow cytometer. Results shown are representative of three independent experiments.

Unlimited self-renewal capacity is a unique feature of HSCs (Kondo, Wagers et al. 2003). To determine if active MEK expression impacted self-renewal capacity, we plated HSCs transduced with retrovirus expressing vector control or active MEK on methylcellulose media. Serial replating of cells on methylcellulose media can only be accomplished with HSCs because other progenitor populations have limited self-renewal capacity (Kondo, Wagers et al. 2003). The primary plating may result in numerous colonies because certain progenitor populations have high proliferative capacity. However, subsequent platings will yield fewer colonies because these progenitor populations have low self-renewal capacity compared to HSCs. Thus, progenitors quickly become exhausted and are unable to form new colonies upon subsequent platings (Kondo, Wagers et al. 2003).

We found that HSCs expressing a vector control and active MEK gave rise to the same number of colonies during the primary plating (Figure 19A). Moreover, the colony sizes were similar (Figure 19B) and similar numbers of cells were harvested from the primary plating (Figure 19C), suggesting that, unlike the case in 32D cells, expression of active MEK does not confer a growth advantage in HSCs. Furthermore, secondary plating of vector control and active MEK cells resulted in similar numbers of colonies (Figure 19D), suggesting that active MEK expression in HSCs did not affect self-renewal.

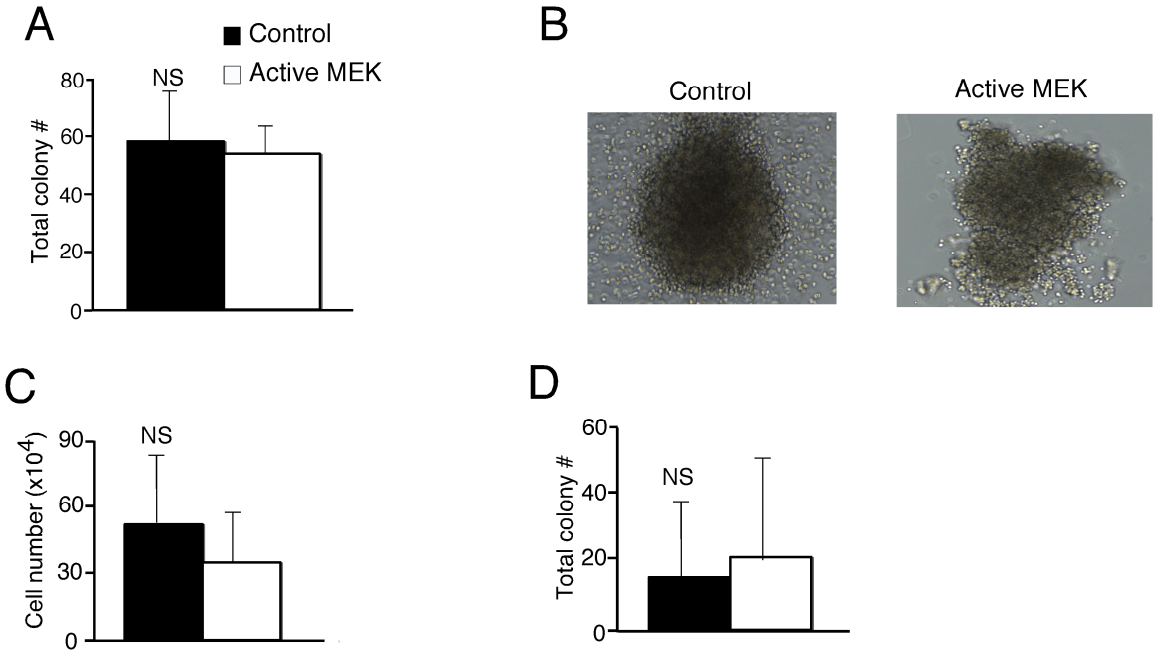


Figure 19: Expression of active MEK in HSCs does not enhance self-renewal.

(A) HSCs were transduced with retrovirus expressing vector control (black bars) or active MEK (white bars). Following transduction, GFP^+ cells were sorted, 500 cells/well were plated on methylcellulose media, and colonies were enumerated 5-8 days later. (B) Representative colonies from HSCs transduced with control (left panel) or active MEK (right panel) retrovirus. (C) Absolute cell numbers following primary plating were determined for each group. (D) Wells were harvested and 50,000 cells/well were plated on methylcellulose media for secondary plating. Colonies were enumerated 5-8 days later. All samples were plated in quadruplicate. Error bars represent the standard deviation from 4 wells. NS, $P>0.05$ (statistical significance) by Student *t* test. Results shown are representative of three independent experiments.

3.3.4 Active MEK expression enhances proliferation in mature myeloid cells.

Average bone marrow chimerism judged by GFP expression derived from vector control and active MEK mice at the terminal stage was 6.1% and 56%, respectively, suggesting that cells expressing active MEK have more proliferative potential *in vivo* (Figure 20A, left panels). The population of cells that was most prevalent in mice with active MEK-induced MDS/MPN was Gr-1$^+$Mac-1$^+$ cells (Figure 20A, left and center panels), which is most likely due to the increased percentage of cells in cycle. While expression of active MEK did not appear to enhance proliferative capacity of the HSC population (Figure 19A-C), its effects on more downstream populations was unclear. The expansion of Mac-1$^+$ cells could be mediated by two possible populations: immature progenitors or mature cells.

To distinguish which population was responsible for the expansion of myeloid cells and subsequent MDS/MPN, we sorted both populations from moribund active MEK or vector control and subjected them to cell cycle analysis. HSCs are the most primitive and quiescent progenitor population (Kondo, Wagers et al. 2003; Passegué, Wagers et al. 2005). However, proliferative capacity increases as cells commit to a lineage as evidenced by an increase in the percentage of cycling cells among committed progenitors when compared to HSCs (Passegué, Wagers et al. 2005). In agreement with previously published data, we found that a quarter of myeloid

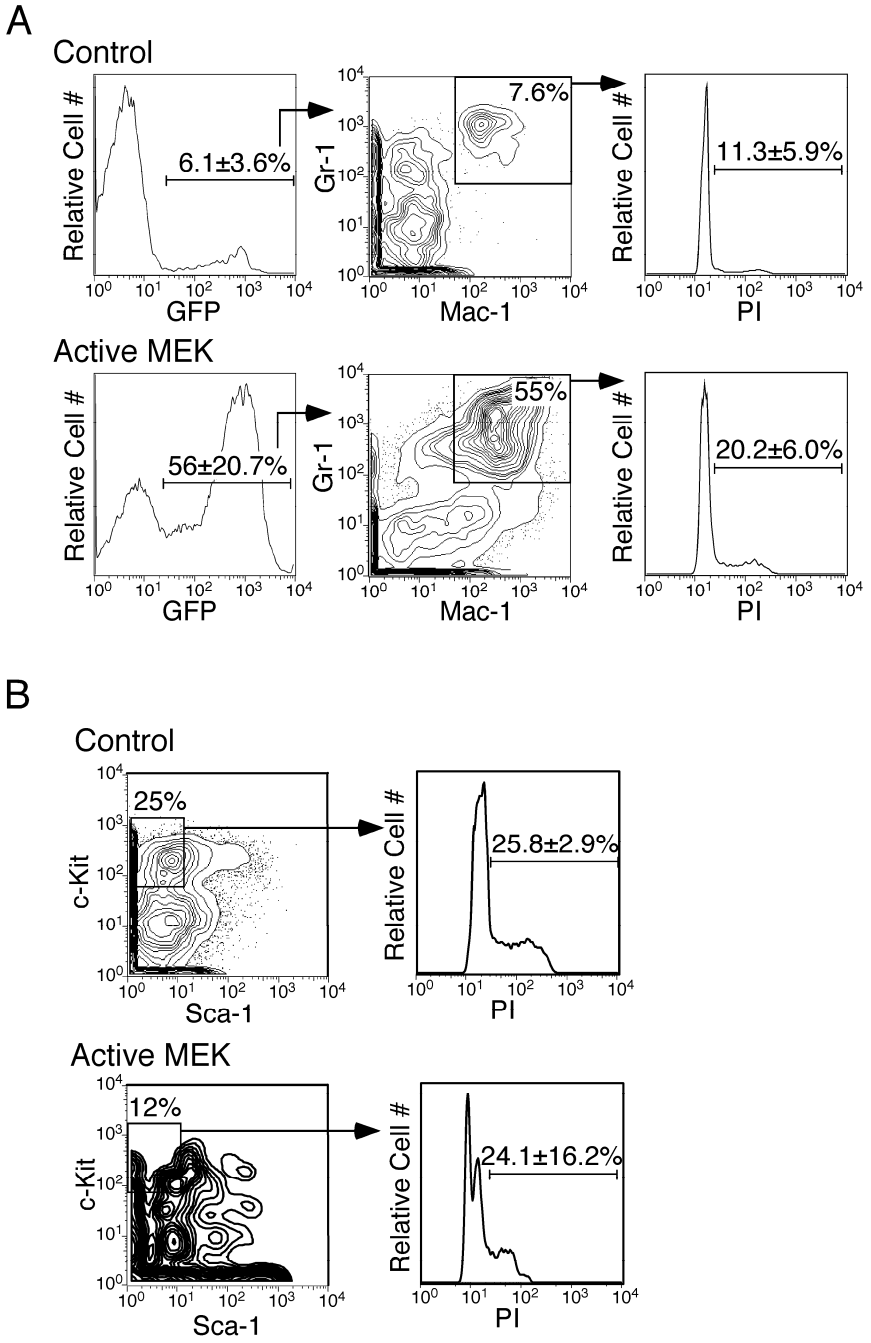


Figure 20: Cell cycle status of myeloid progenitors and mature myeloid cells in transplanted mice.

(A) GFP^+ cells in the bone marrow of control (top row, n=4) or active MEK mice at the terminal stage (bottom row, n=5) were gated and the percentage of mature myeloid cells ($Gr\text{-}1^+$ $Mac\text{-}1^+$) was determined. $Gr\text{-}1^+$ $Mac\text{-}1^+$ cells were sorted and cell cycle status was examined using intracellular PI staining. The mean percentage of cells in G_2/M ± std dev is indicated. (B) GFP^+ myeloid progenitors (Lin^- $c\text{-}kit^+$) were sorted from bone marrow of active MEK mice at the terminal stage (bottom row, n=3) or control mice (top row, n=3). DNA content was assessed by intracellular PI staining. The mean percentage of cells in G_2/M ± std dev is indicated.

progenitors from vector control mice were in cycle (Figure 20B, right panels) (Passegué, Wagers et al. 2005). However, the proliferation rates of myeloid progenitors isolated from the bone marrow of vector control and active MEK mice did not differ (Figure 20B). Once cells have fully differentiated, they can exit the cell cycle and return to a quiescient state until stimulated (Passegué, Wagers et al. 2005). In agreement with this observation, we found that the percentage of Mac-1$^+$Gr-1$^+$ cells from vector control mice in cycle was far less than that observed in the myeloid progenitor population (11.3% vs 25.8%) (Figure 20A,B). However, the percentage of mature myeloid cells from active MEK mice in cycle was far greater than its vector control counterpart (20.2% vs 11.3%) and more similar to that observed in the myeloid progenitor population (20.2% vs 24.1%) (Figure 20A,B). These data suggest that MEK signaling may specifically affect cell cycle status of mature G/M cells in the bone marrow.

3.2.5 c-Myc expression is enhanced in the presence of active MEK.

In the presence of active MEK, ERK is constitutively phosphorylated resulting in permanent activation of the MEK/ERK pathway (Zheng and Guan 1993; Pearson, Robinson et al. 2001). Active ERK can subsequently phosphorylate its target molecules and initiate signaling cascades that eventually lead to activation of gene transcription.

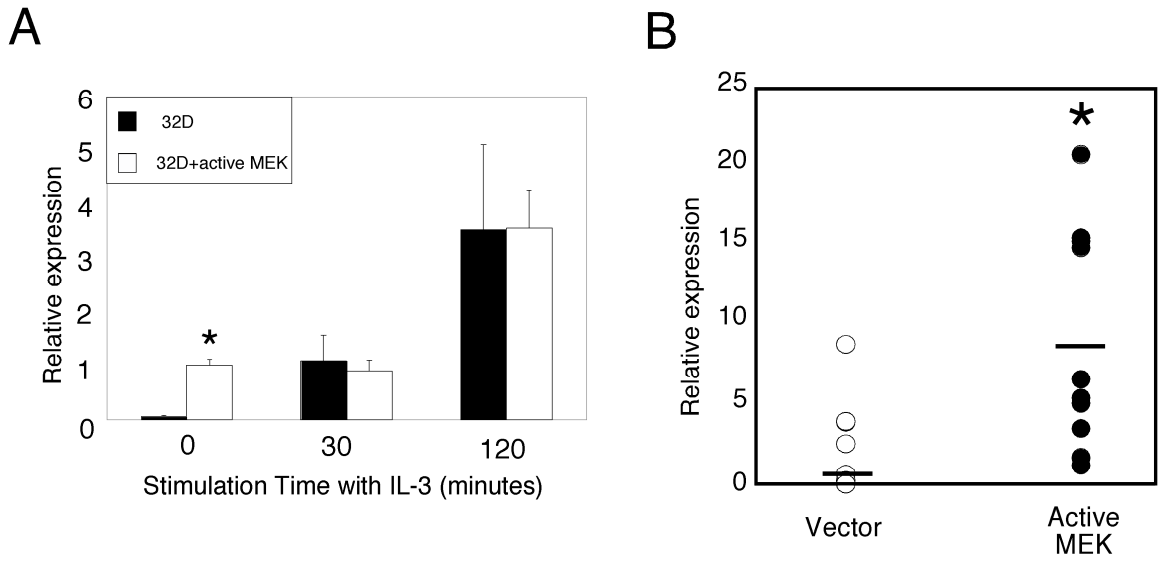


Figure 21: Impact of active MEK on c-Myc expression in 32D cell line and primary cells.

(A) 32D and 32D+MEK were starved of IL-3 for 6 hrs and stimulated with IL-3 (10 ng/mL) for the indicated times. Cells were harvested and total RNA was purified and subjected to quantitative RT-PCR. Relative expression level is normalized to Rpl13 expression and plotted on a bar graph. Data shown are the mean value of triplicate reactions. Results shown are representative of three independent experiments. (B) GFP^+ bone marrow cells were harvested from terminal active MEK mice (filled circles, n=13) or control mice (open circles, n=7) and prepared as described in (A). Relative expression level is normalized to Rpl13 expression and plotted. Data shown are the mean value of triplicate reactions. The horizontal bar denotes the mean value of each group. *, $P<0.05$ (statistical significance) by Student *t* test.

One such target of the MEK/ERK pathway, c-Myc, is an oncogene that plays a critical role in cytokine-mediated proliferation signaling (Grandori, Cowley et al. 2000). To determine how active MEK affects c-Myc levels, we starved 32D and 32D+active MEK of IL-3 for 12 hours. We found that c-Myc levels in 32D+MEK cells 12 hours after IL-3 depletion were higher than parental 32D cells, but returned to comparable levels after 30 minutes of IL-3 stimulation (Figure 21A). This suggests that activation of the MEK/ERK pathway alone is sufficient for transcriptional initiation of the c-Myc gene in 32D cells.

Active MEK signaling can increase c-Myc transcription in 32D cells (Figure 21A) and enhance proliferation of mature myeloid cells (Figure 21B); however, the connection between the two events is unclear. We next asked if c-Myc expression was upregulated in bone marrow cells ofactive MEK-induced MDS/MPN mice. We harvested GFP^+ bone marrow cells from moribund active MEK and vector control mice and subjected them to quantitative RT-PCR. We found that c-Myc expression was significantly higher in cells expressing active MEK (Figure 21B). This suggests that enhanced c-Myc expression mediated by active MEK signaling may drive mature myeloid cell proliferation in MPD/MPN.

3.2.6 Active MEK-induced MDS/MPNs are not frank leukemias.

The MDS/MPN designation includes various classes of myeloid malignancies, some of which are classified as pre-leukemic (Van Etten and Shannon 2004; Vardiman, Thiele et al. 2009). A hallmark of leukemia is the ability to recapitulate disease in a naïve animal following transplantation (Reya, Morrison et al. 2001). To determine the transplantability of the disease, we harvested unfractionated bone marrow or spleen cells from individual moribund active MEK^{+} mice and injected them into multiple naïve, sublethally irradiated RAG-2$^{-/-}$ mice (Figure 22). We routinely monitored the mice for signs of disease development, but did not observe the development of any myeloid diseases up to 200 days post-injection. This suggests that the MDS/MPN we observed was a pre-leukemic condition and not a frank leukemia.

However, we did observe one case of an aggressive lymphoid disease. In contrast to the primary active MEK mice that had very few donor-derived T cells, donor-derived cells from the secondary transplanted mouse were predominantly T cells (Figure 23A). Specifically, GFP^{+} cells from the bone marrow, spleen, and peripheral blood were CD4^{+}CD8^{+} (Figure 23A). This population appears similar to double positive (DP) cells that comprise a majority of the thymus, but are rarely seen in the periphery (Ciofani and Zúñiga-Pflücker 2007). Also, bone marrow sections indicate that the bone marrow is hypercellular and packed with lymphoid cells (Figure 23B). Furthermore,

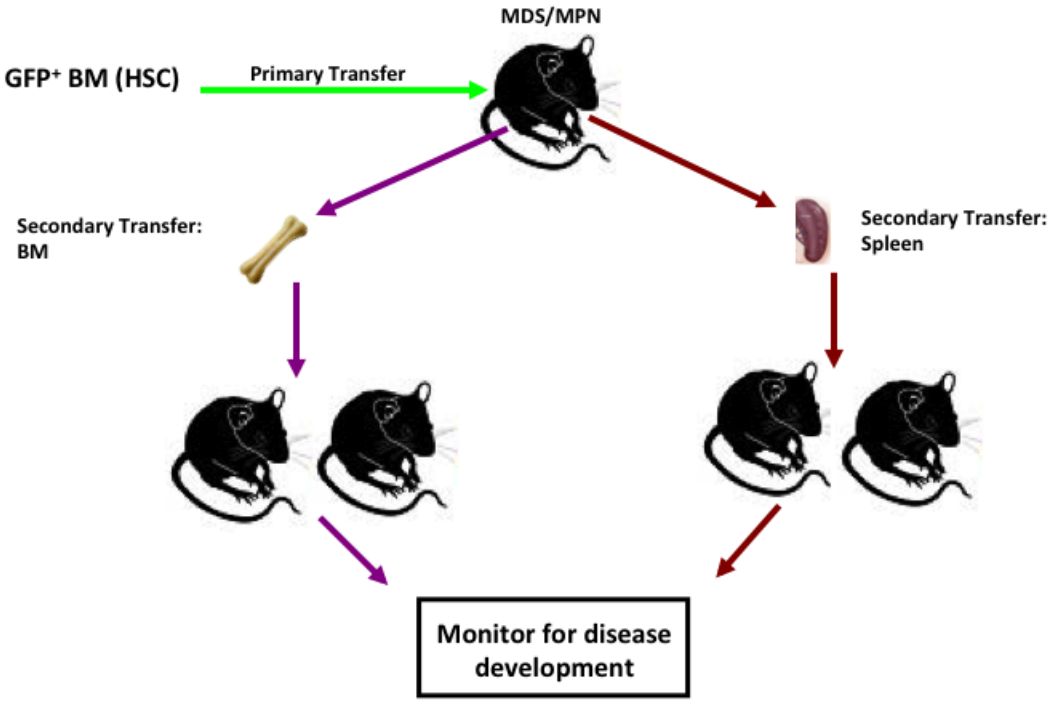


Figure 22: Secondary transplantation of MDS/MPN.

Primary transplants were performed with bone marrow cells that were harvested and transduced with retrovirus expressing active MEK (Figure 12). Next, GFP+ cells were sorted and injected into a naïve recipient mouse. Secondary transplanted mice were generated by harvesting bone marrow and spleen cells from moribund primary transplanted mice that had developed MDS/MPN and immediately injected into multiple naïve recipient mice. Mice were monitored routinely for disease development.

blast cells were observed in the peripheral blood (Figure 23C). Taken together, these results suggest that the sole case of disease from a secondary transplanted active MEK-induced MDS/MPN mouse resulted in T-ALL.

3.2.7 Enhanced cell survival in HSCs expressing active MEK also result in MDS/MPN

Cancer is often the result of multiple oncogenic "hits" (Hanahan and Weinberg 2000). We have shown that expression of active MEK primarily drives cellular proliferation, which is an important acquired capability for a cancer cell to have. Another important acquired capability is the ability to evade apoptosis. Cells that are refractory to apoptosis induced by extracellular stresses such as growth factor withdrawal or DNA damage are more susceptible to malignant transformation in part due to the increased likelihood of acquiring genetic mutations (Hanahan and Weinberg 2000).

However, enhanced cell survival is not sufficient to cause disease, thus additional, cooperating oncogenes are necessary for disease development (Strasser, Harris et al. 1990; McDonnell and Korsmeyer 1991). We chose Bcl-2 as a stereotypical anti-apoptotic protein to determine whether coupling active MEK expression with a factor capable of enhancing cell survival would alter disease phenotype or disease progression. Bcl-2 is an anti-apoptotic protein that promotes cell survival by inhibiting

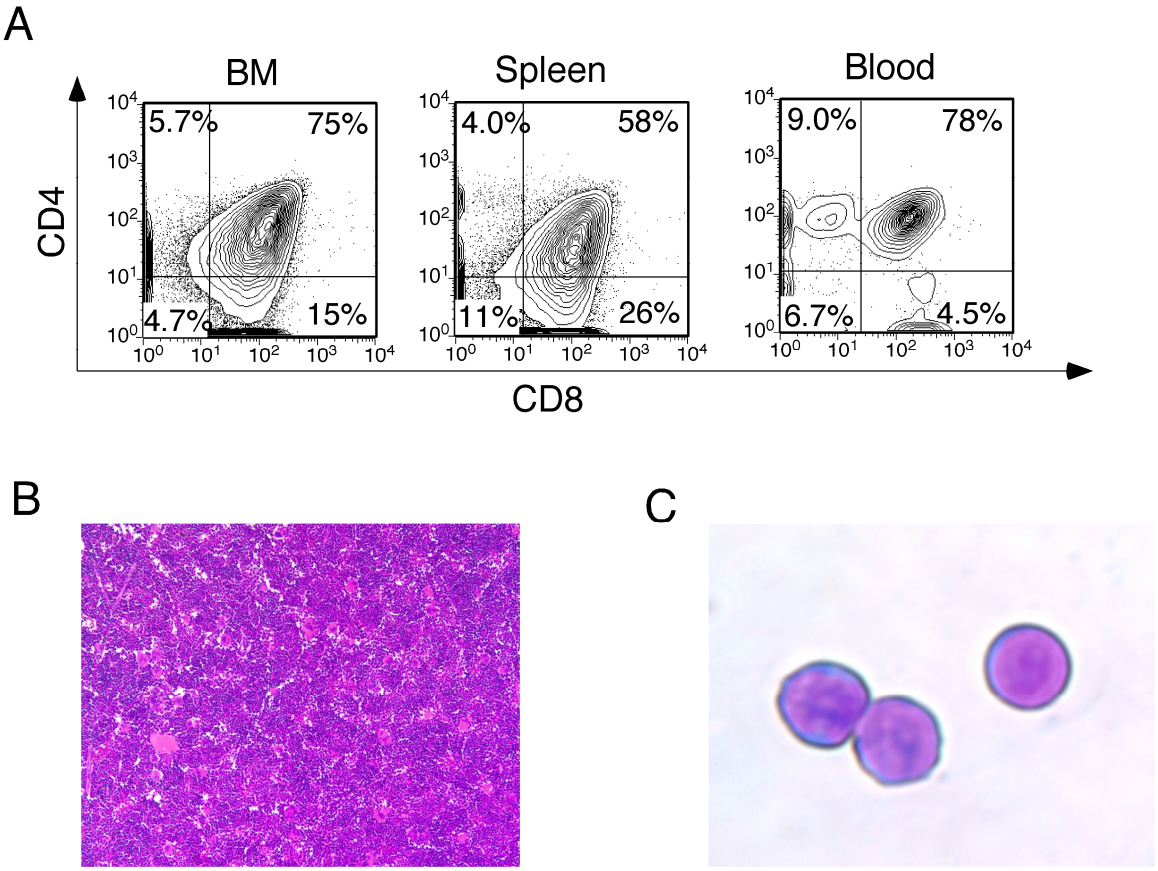


Figure 23: Sole case of T-ALL development in secondary transplanted active MEK mice.

(A) Cells from the bone marrow (left), spleen (middle), and peripheral blood (right) of a terminal secondary transplanted active MEK mouse was harvested. Cells are pre-gated on PI$^-$GFP$^+$ cells and CD4 and CD8 expression was assessed by FACS. (B) A femur was harvested, fixed in 4% paraformaldehyde, and embedded in a paraffin block. 5 μM thick sections were cut, mounted on a glass slide, and stained with hematoxylin and eosin. Zoom=100X. (C) Peripheral blood was collected, smeared on a slide, and stained with Wright-Giemsa stain. Zoom=400X

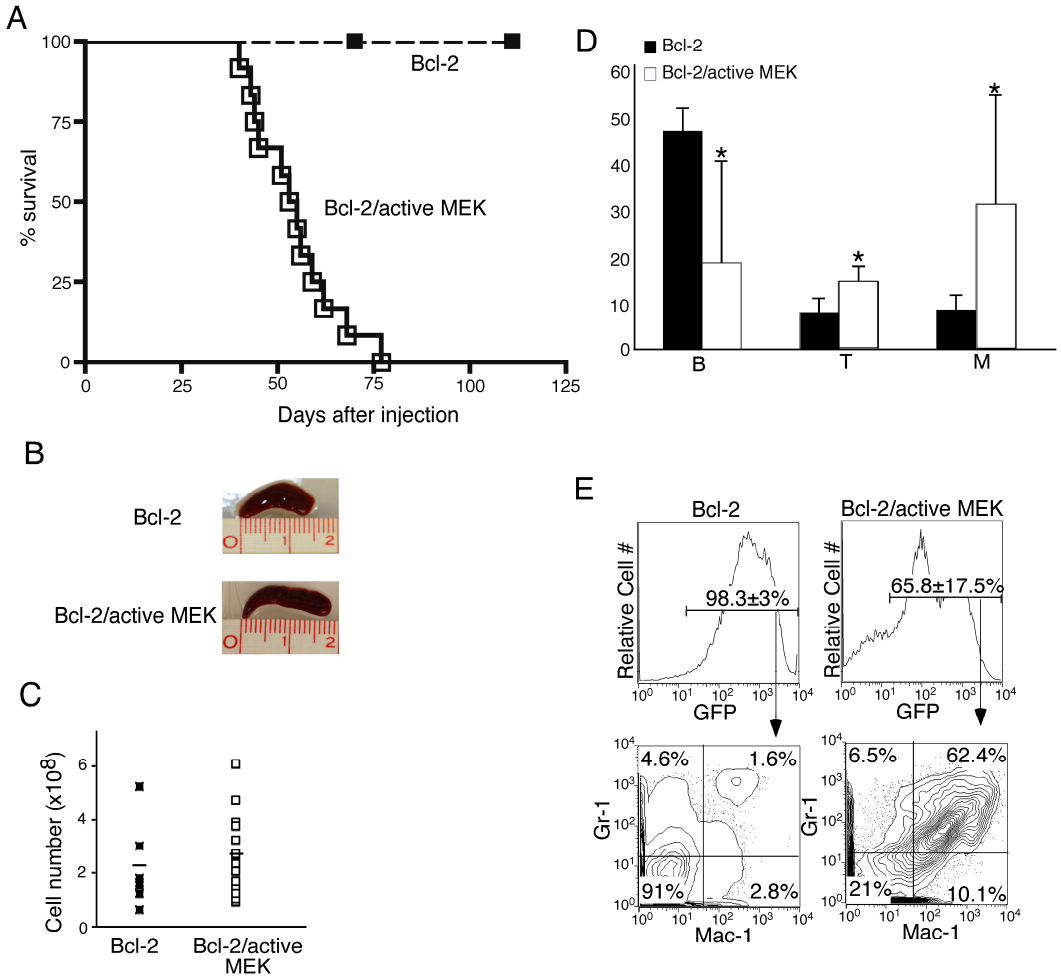


Figure 24: Coupling Bcl-2 and active MEK expression results in MDS/MPN.

(A) A Kaplan-Meier curve illustrating the survival time of mice reconstituted with Bcl-2^{+} HSCs infected with control (closed squares, n=8) or active MEK (open squares, n=17) retrovirus. Approximately 10,000 retrovirally infected GFP^{+} Bcl-2^{+} HSCs were injected into sublethally irradiated RAG-2$^{-/-}$ recipients. Survival of transplanted mice was monitored for at least 120 days post-injection. (B) Mild spleen enlargement in

Bcl- 2/active MEK mice. (C) Absolute number of spleen cells. (D) Contribution of Bcl-2/active MEK HSCs to different hematopoietic lineages. Spleen cells were harvested from transplanted mice at the terminal stage for FACS analysis of the various lineage-specific cell surface markers (B: $B220^+$ B cells, T: Thy^+ T cells, M: $Mac\text{-}1^+$ myeloid cells). The bar graph summarizes the mean percentage of GFP^+ donor-derived cells for each cell type from $Bcl\text{-}2^+$ control mice (black bars) and Bcl-2/active MEK mice (white bars). Error bars represent the standard deviation. *, $P<0.05$ (statistical significance) by Student *t* test. (E) FACS analysis of bone marrow cells from transplanted mice at the terminal stage. FACS plots were pre-gated on GFP^+ donor-derived cells (top row). Data shown is the mean percentage of GFP^+ cells ± std dev from $Bcl\text{-}2^+$ control (n=8) and Bcl-2/active MEK mice (n=17). GFP^+ cells were further analyzed by examining Gr-1 and Mac-1 expression.

pro-apoptotic proteins from disrupting the integrity of the mitochondrial membrane (Chonghaile and Letai 2009; Giam, Huang et al. 2009). Bcl-2 has also been shown to cooperate with other oncogenes that drive cellular proliferation, such as c-Myc, to promote leukemia and lymphoma development (Strasser, Harris et al. 1990; McDonnell and Korsmeyer 1991).

To drive enforced Bcl-2 expression, we used a mouse line that expresses the human Bcl-2 transgene under the control of the MHC class I promoter (Domen, Gandy et al. 1998). This system ensures that Bcl-2 expression is achieved in all hematopoietic cells, including HSCs. Using the retroviral transduction system that was previously described (Figure 12), we transduced purified Bcl-2^{+} HSCs with retrovirus expressing active MEK and injected them into sublethally irradiated RAG-2$^{-/-}$ mice. All recipientmice injected with Bcl-2/active MEK HSCs became sick and reached the terminal stage at the same time as mice injected with HSCs expressing active MEK (Figure 13A and Figure 24A). A much milder case of splenomegly was observed in Bcl-2/active MEK mice compared to active MEK mice (Figure 24B,C). Similar to active MEK mice, Bcl-2/active MEK mice displayed a very prominent skew towards mature myeloid cell development at the expense of lymphoid cell development (Figure 13, 14 Figure 15Figure 16, Figure 24D,E).

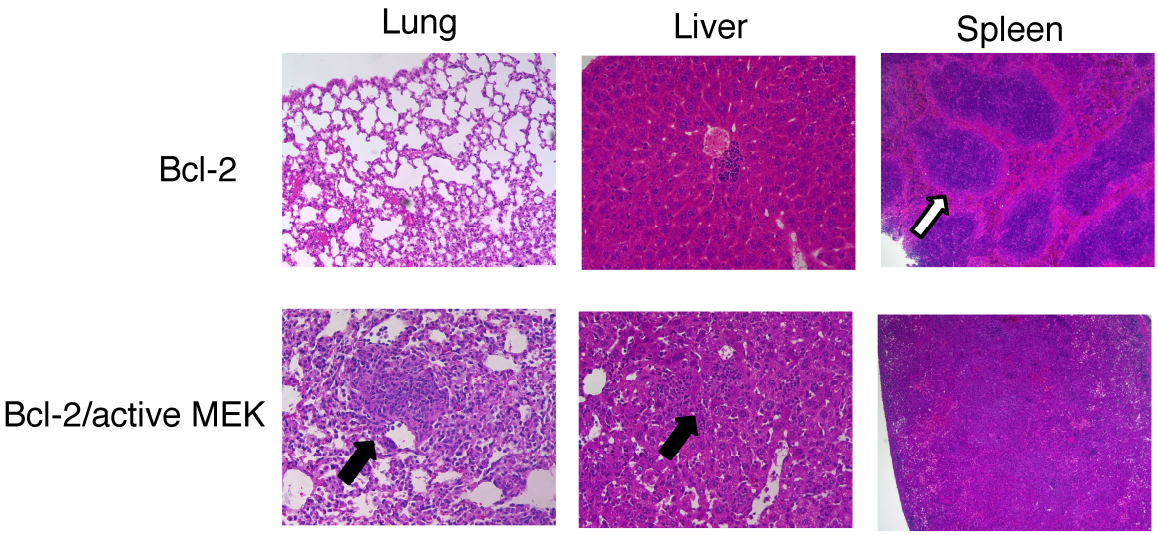


Figure 25: Impact of increased myeloid cell production on certain organs of Bcl-2/active MEK mice.

Organs were harvested from Bcl-2/active MEK mice (bottom row) at the terminal stage or control Bcl-2 mice (top row) and fixed in 4% paraformaldehyde and embedded in paraffin blocks. 5 μM thick sections were cut, mounted on a glass slide, and stained with hematoxylin and eosin. Zoom=400X. One representative tissue section from vector control and Bcl-2/active MEK mouse are shown. Myeloid cell infiltration is observed exclusively in the liver and lung of Bcl-2/active MEK mice and is denoted with a black arrow. An example of a region containing white pulp in the spleen of a control Bcl-2 mouse is denoted with a white arrow.

The expansion of myeloid cells also spills into the periphery and into non-lymphoid organs. Myeloid cell infiltration is once again observed in the perivascular and peribronchial regions of the lung and the periportal region of the liver (Figure 25, black arrows). Disorganization of the spleen, which results in the absence of clear red and white pulp regions, is also observed in Bcl-2/active MEK mice (Figure 25, white arrows).

Taken together, our analysis shows that Bcl-2/active MEK mice developed an MDS/MPN very similar to those observed in active MEK only mice (Figure 13 and Figure 24). We did not observe any difference in the phenotype of recipient mice that received WT HSCs expressing active MEK and Bcl-2 HSCs expressing active MEK, which suggests that enforced Bcl-2 expression does not influence the neoplastic transformation potential of active MEK in HSCs.

3.2.8 Enhanced cell survival alters the course of hematological malignancies induced by active MEK.

Coupling Bcl-2 expression with active MEK did not impact disease phenotype, however; its effect on disease progression was unclear. To address this question, we performed secondary transplants with bone marrow and spleen cells harvested from primary mice that had Bcl-2/active MEK-induced MDS/MPN, as previously described (Figure 22).

Table 1: Disease frequency and phenotype induced by expression of active MEK and Bcl-2/active MEK.

	MEK	Bcl-2/active MEK
Primary Transplant	13/13	17/17
%MDS/MPN	100%	100%
Secondary Transplant	1/17	5/18
%T-ALL	5.9%	27.8%

We noticed that some secondary transplanted showed signs of illness more than 120 days post-injection, almost twice as long as primary Bcl-2/active MEK mice (Figure 24A and Figure 26A). Only one mouse transplanted with active MEK-induced MDS/MPN developed disease (5.9%). In contrast, significantly more mice transplanted with Bcl-2/active MEK-induced MDS/MPN became fatally ill (27.8%, $p<0.05$) (**Table 1**).

We further examined the disease phenotype in mice transplanted with Bcl-2/active MEK-induced MDS/MPN at the terminal stage. Different from primary MDS/MPN mice, we did not observe massive expansion of myeloid cells. Rather, the mice had enlarged thymi and Thy-1$^+$ T cells became dominant in the bone marrow, spleen, and peripheral blood of the mice (Figure 26B,C). A majority of these T cells expressed both CD4 and CD8, suggesting that expanded T cells in the mouse had an immature T cell phenotype similar to CD4$^+$CD8$^+$ DP cells, which are normally abundant in the thymus but not in the periphery (Figure 26B,C). In accordance with their immature phenotype, T cells from bone marrow in the secondary transplanted mice were CD3$^-$ (Figure 26C, bottom right panel). Moreover, peripheral blood smears obtained from these mice show the presence of blast cells in the peripheral blood (Figure 26B).

Similar to primary mice with MDS/MPNs, secondary transplanted mice also had cellular infiltration in non-lymphoid organs such as the lung and the liver (Figure 27).

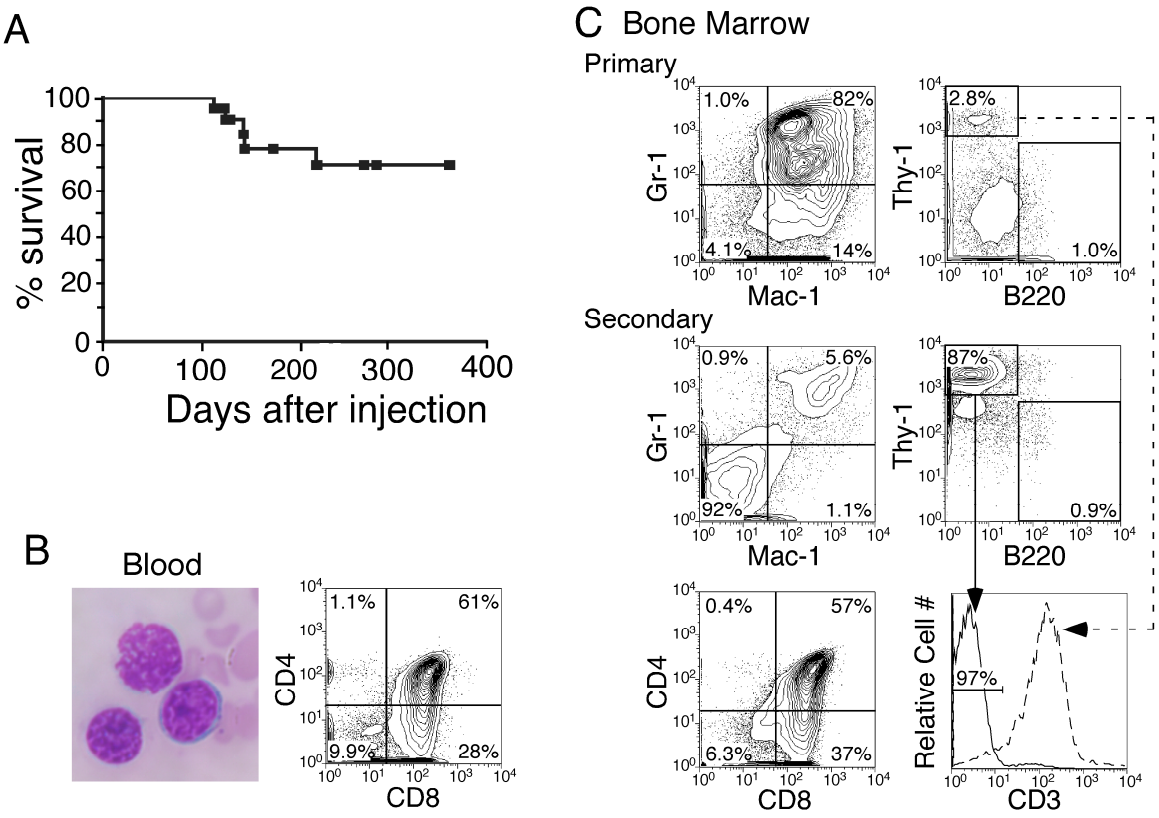


Figure 26: Transplantation of Bcl-2/active MEK-induced MDS/MPN results in increased cases of T-ALL.

(A) A Kaplan-Mier curve illustrating the survival time of Bcl-2/active MEK secondary transplanted mice. (B) Histology of peripheral blood from secondary transplanted mice. Peripheral blood smears were stained with Wright-Giemsa stain (left panel). Zoom=400X. Peripheral blood was also subjected to FACS analysis to examine CD4 and CD8 expression. Cells were pre-gated on PI^{-}GFP^{+} cells. One representative smear and FACS plot is shown for five analyzed mice (right panel). (C) At the terminal stage, bone marrow was harvested from moribund primary transplanted (top panels) and moribund secondary transplanted (middle panel) mice. Secondary transplanted mice were

generated as described previously (Figure 22). Cells were stained with various lineage antibodies and subjected to FACS analysis. CD3 expression on Thy-1^{+} T cells was further analyzed in primary (dotted arrow) and secondary (solid arrow) mice (bottom right panel). CD4 and CD8 expression in bone marrow was also examined in secondary transplanted mice. All plots are pre-gated on PI^{-}GFP^{+} cells (bottom left panel). Data is from one representative mouse out of five mice.

However, compared to the myeloid cell infiltration observed in primary transplanted mice, the lymphoid infiltration in the organs of secondary transplanted animals was much greater (Figure 27). Lymphoid cells encompassed a greater area of both the lung and liver and moved further away from the blood supply to extend further into the tissue (Figure 27). Additionally, more organ involvement was observed in secondary transplanted mice. For example, both the kidney and small intestine of primary transplanted animals were clear of any cellular infiltration. However, secondary transplanted animals exhibited prominent lymphoid cell infiltration in both the kidney and small intestine (Figure 27). Disorganized splenic architecture was also observed in secondary mice; however, the spleen became packed with lymphoid cells (Figure 27).

Taken together, these data suggest that transplantation of Bcl-2/active MEK-induced MDS/MPN can also result in T-ALL. Moreover, there is a greater proportion of T-ALL cases among Bcl-2/active MEK secondary transplanted mice when compared to active MEK secondary transplanted mice (**Table 1**). The T-ALL only occurs following a long latency period, however; the disease is extremely aggressive. The bone marrow, spleen, and peripheral blood are filled with lymphoblasts (Figure 26). Additionally, lymphoid cells begin to invade multiple non-lymphoid organs including the liver and

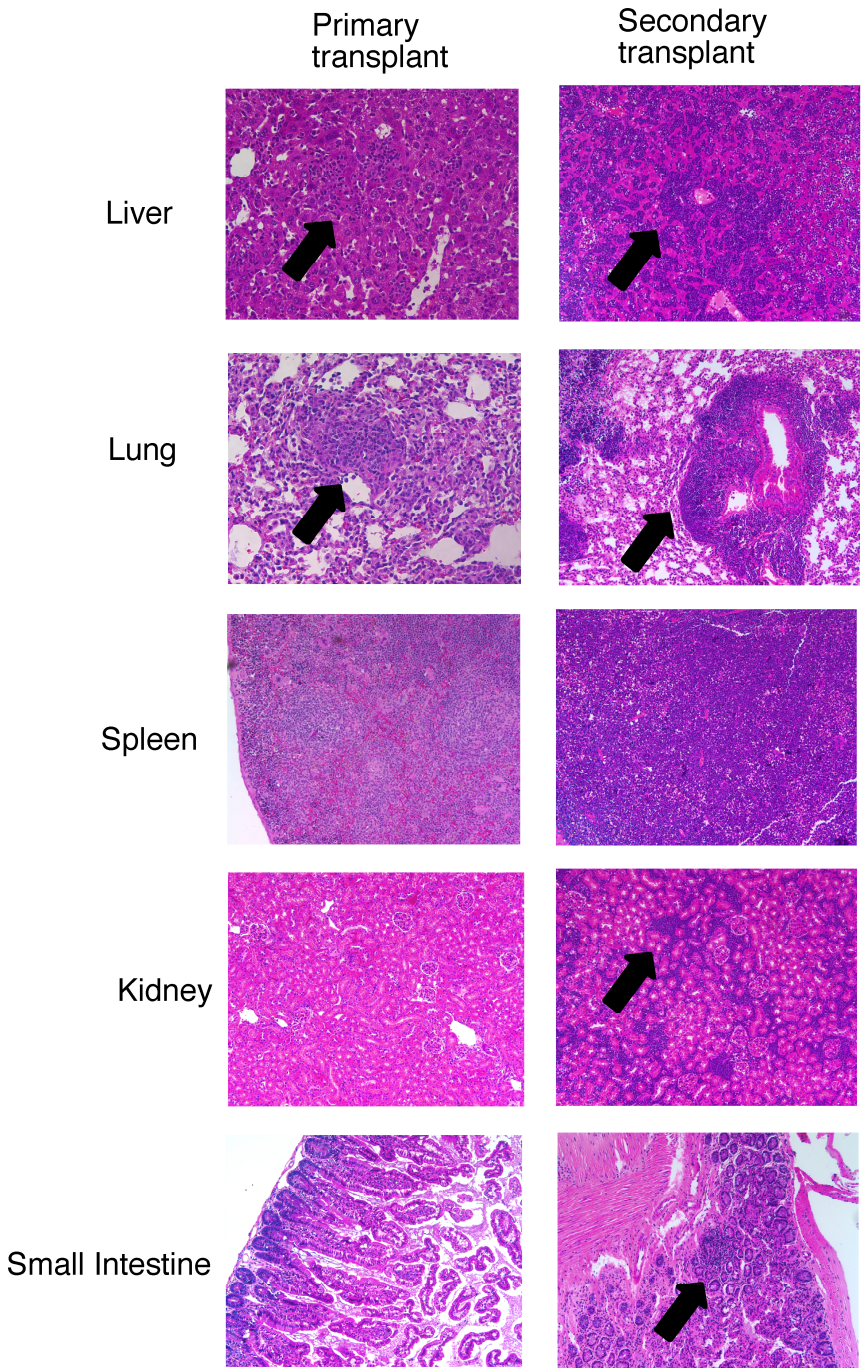


Figure 27: Severe lymphocyte infiltration in mice with T-ALL.

Organs were harvested from primary Bcl-2/active MEK mice (left column) or secondary transplanted mice with T-ALL (right column) at the terminal stage and fixed in 4% paraformaldehyde and embedded in paraffin blocks. 5 μM thick sections were cut, mounted on a glass slide, and stained with hematoxylin and eosin. Zoom=400X. One representative tissue section from primary Bcl-2/active MEK mice and secondary transplanted mice with T-ALL are shown. Myeloid cell infiltration is observed exclusively in the liver and lung of Bcl-2/ active MEK mice and is denoted with a black arrow (left column). Massive lymphoid cell infiltration is observed in several organs of secondary transplanted mice with T-ALL and is denoted with a black arrow (right column).

lung, which are also observed during primary disease development, but also the kidney and small intestine (Figure 27). We should note that secondary transplanted Bcl-2/active MEK mice that did not develop T-ALL were monitored for at least 365 days post-injection and did not develop other hematopoietic malignancies (Figure 26A).

3.2.9 Notch pathway is exclusively activated in mice with T-ALL

Greater than 50% of human T-ALL cases have been found to harbor mutations that result in aberrant activation of the Notch pathway, which may be necessary for the generation and/or maintenance of disease (Weng, Ferrando et al. 2004). Moreover, certain Notch mutations have been shown to enhance the malignancy of tumors generated from introduction of active K-Ras (Chiang, Xu et al. 2008).

To determine whether activation of the Notch pathway is involved in T-ALL generation in the secondary transplanted mice with bone marrow and spleen cells derived from MDS/MPN mice co-expressing active MEK and Bcl-2, we examined expression of Notch target genes as performed previously (Lai and Kondo 2007). As shown in Figure 28, T-ALL cells expressed significantly higher levels of Hes1, Deltex1, and c-Myc when compared to phenotypically similar WT double positive thymocytes (DP), which only express basal levels of these notch target genes. Among hematopoietic

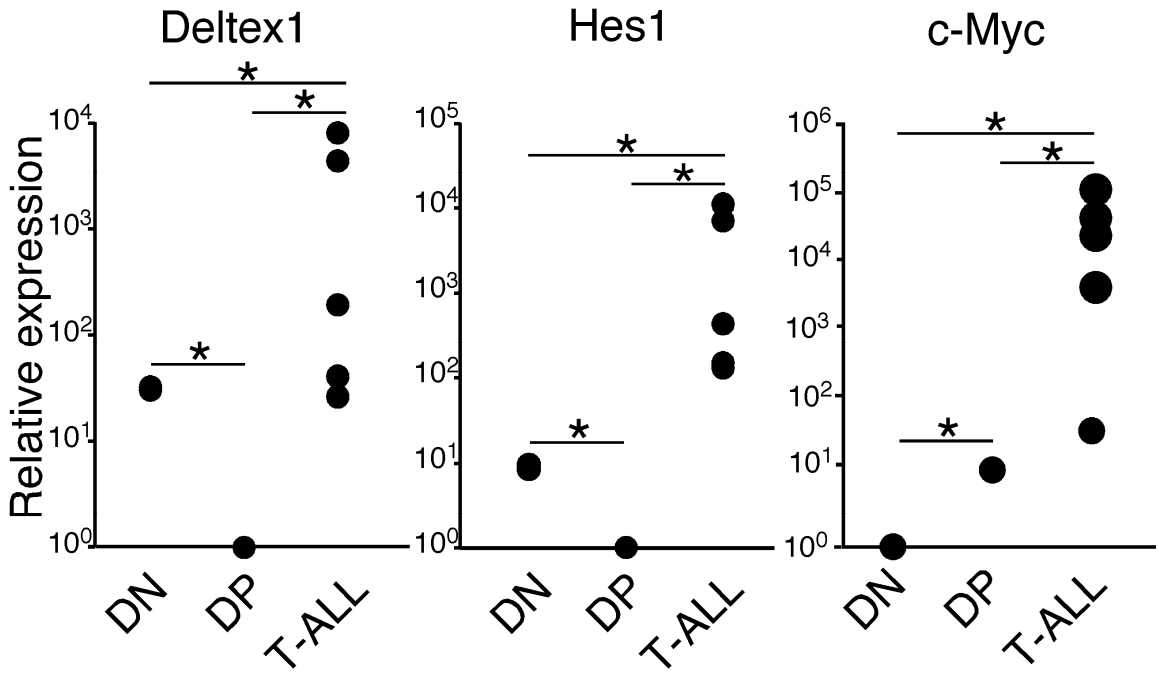


Figure 28: Notch pathway is activated in T-ALLs.

Quantitative RT-PCR analysis of Deltex-1 (left panel), Hes-1 (middle panel), and c-Myc (right panel) in WT $CD4^-CD8^-$ DN thymocytes (n=3), WT $CD4^+CD8^+$ DP thymocytes (n=3), and GFP^+ bone marrow cells from mice with T-ALL (n=5). Relative expression level for each gene is normalized to β-actin expression level and plotted. The mean normalized expression level for each group was calculated and used to determine fold induction above DP cells. Expression level of the gene was arbitrarily set at 1 for DP cells. Fold induction over DP is expressed for each corresponding group. Data shown represent the mean value from triplicate reactions. The horizontal bar denotes the mean value of each group. Standard deviation was calculated for each sample assayed in triplicate. *, $P<0.05$ (statistical significance) by Student *t* test.

cells, $CD4^{-}CD8^{-}$ double negative (DN) thymoctyes exhibit the highest levels of Notch signaling (Deftos, Huang et al. 2000). However, the level of Notch signaling observed in our diseased animals exceeds even those found in DN thymoctes, which is in agreement with previous studies showing that the levels of Notch signaling observed in T-ALLs far exceeds those necessary to mediate physiological functions (Chiang, Xu et al. 2008). Therefore, activation of the Notch pathway may be necessary to complete malignant transformation of cells initiated by active MEK.

3.2.10 MDS/MPN are oligoclonal diseases while T-ALLs are clonal diseases.

Recipient mice that received either WT HSCs expressing active MEK or Bcl-2 HSCs expressing active MEK died relatively quickly when compared to secondary transplanted mice (6-10 weeks vs 17-30 weeks) (Figure 13,Figure 24, and Figure 26). The quickness in which disease onset occurred suggested that multiple clones could have contributed to disease development. To address this issue, we harvested bone marrow and spleen cells from moribund active MEK and Bcl-2/active MEK mice that had developed MDS/MPN and examined them using blot hybridization with a GFP-specific probe. We reasoned that the retrovirus would randomly insert itself into the host genome, so that no two infected cells would share the same exact integration sites. Restriction enzyme digest with PvuII cuts at known sites within the MSCV vector and

many sites within the host genome, thus creating a unique signature for every clone. A heterogeneous population of cells that have random viral integrations into the genome should yield a ladder of different band sizes. Indeed, we find that most active MEK and all Bcl-2/active MEKmice analyzed have unique banding patterns (Figure 29A). Banding intensity is varied within each sample, suggesting that some clones may be more dominant. There are two active MEK samples that appear to have very similar banding patterns and intensity (Figure 29A, left panel, far right two lanes). Both of these mice were injected with the same pool of transduced HSCs at the same time so it is possible that both mice received the same daughter cells. It is also possible that other unique clones are not observed in these samples because of the dominance of one particular clone. For example, there appears to be one band (Figure 29A, left panel, denoted with *) that has much higher intensity than the other bands in both samples, suggesting that this may be a dominant clone. Taken together, these results suggest that the MDS/MPN we observe in our model is an oligoclonal disease.

The long latency period we observed in T-ALL development suggests that it may be a clonal disease. We observed that Notch signaling is activated in all T-ALLs (Figure 28), suggesting that a mutation that could activate the Notch pathway had to be acquired (Figure 29B). While it is possible that a large population of cells all acquired

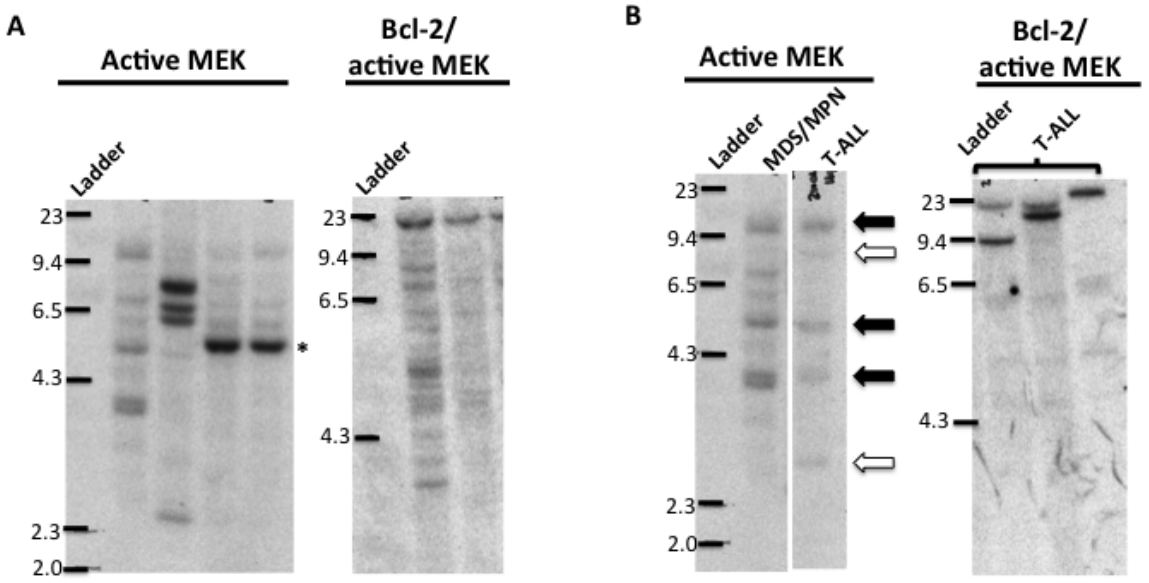


Figure 29: Southern blot analysis of the disease clonality of leukemic cells.

(A) Bone marrow or spleen cells were harvested from mice with active MEK-induced MDS/MPN and Bcl-2/active MEK-induced MDS/MPN. Genomic DNA was extracted and digested with PvuII. 5 ug of DNA were subjected to electrophoresis and transferred to Hybond-N membrane. A GFP-specific probe was labeled with ^{32}P and used to probe membranes. Representative samples from mice with active MEK-induced MDS/MPN are on the left (n=4) and Bcl-2/active MEK-induced MDS/MPN are on the right (n=2). (B) Tumor cells from secondary transplanted mice with T-ALL were harvested and processed as descried above. Spleen cells from a primary active MEK mouse (left panel, left lane) were transplanted into a naïve recipient and gave rise to T-ALL (left panel, right lane). Black arrows denote bands that are similar in size between the primary and secondary mouse and white arrows denote bands unique to the T-ALL sample. Note: both samples were run on the same gel, but not in neighboring lanes. Representative samples from Bcl-2/active MEK secondary transplanted mice that developed T-ALL (n=3). Molecular weight marker is on the left-hand side of every blot.

activating Notch mutations, it is much more likely that a single cell acquired an oncogenic "hit" and expanded, giving rise to a clonal disease.

To determine whether observed T-ALLs were of clonal origin, we harvested bone marrow and tumor cells from moribund secondary transplanted mice and subjected them to the same analysis as primary transplanted mice. As previously mentioned, we had one active MEK mouse that gave rise to T-ALL upon secondary transplantation of spleen cells (Figure 23). We compared the banding pattern of bone marrow from the primary transplanted mouse to the tumor from the secondary transplanted mouse. We observed a few bands of similar size (Figure 29B, left panel, black arrows) in the primary and secondary mouse samples, suggesting that these clones were selected for following secondary transplantation. Interestingly, we also observed two bands in the secondary transplanted sample (Figure 29B, left panel, white arrows) that do not appear to be present in the primary transplanted sample. These bands are lower in intensity, suggesting that they may be minor clones.

We do not have cells from the primary transplanted Bcl-2/active MEK mouse to compare with the secondary transplanted mice. Regardless, only a few bands are present in the secondary transplanted Bcl-2/active MEK-induced T-ALLs (Figure 29B, right panel), suggesting that the observed T-ALLs are most likely of clonal origin.

3.3 Discussion

MEK/ERK transformation potential has been demonstrated in various cell line systems; however, direct proof in primary hematopoietic cells was lacking (Mansour, Matten et al. 1994; Blalock, Pearce et al. 2000; Blalock, Pearce et al. 2001; Chang, Steelman et al. 2003). In this report, we provide direct evidence that introduction of active MEK into HSCs is sufficient to give rise to a mixed MDS/MPN disease. The MDS/MPN we observed has features of both CMML and JMML such as lack of the *BCR/ABL* fusion gene, peripheral blood monocytosis, and non-elevated myeloblast or monoblast counts in the bone marrow and peripheral blood (Figure 13, 14,Figure 15) (Emanuel 2008; Vardiman, Thiele et al. 2009). However, our mice also show signs of anemia and thrombocytopenia, which are also characteristic of myeloid dysplasias observed in CMMLs (Figure 14 Figure 15) (Emanuel 2008; Vardiman, Thiele et al. 2009).

Interestingly, our mice developed myeloid dysplasias at later time points, which also coincide with an increase in the percentage of donor-derived cells in peripheral blood (Figure 14A). These GFP^+ cells were mainly Mac-1^+, suggesting that active MEK signaling does not directly effect erythropoiesis and platelet production (Figure 14A-D). However, the Gr-1^+Mac-1^+ population in the bone marrow of active MEK mice was found to be cycling more than vector control mice (Figure 20A). These data suggest that active MEK signaling may indirectly contribute to cytopenias. The anemia and

thrombocytopenia observed at the terminal stage of active MEK mice (**Figure 15**) could be a consequence of enhanced mature myeloid cell proliferation, which could out-compete normal cells for space in the bone marrow resulting in dysfunctional hematopoiesis. It is also interesting to note that enhanced proliferation observed among MDS/MPN cells was correlated with higher c-Myc expression, suggesting that downstream active MEK proliferative signals may be mediated by c-Myc in mature myeloid cells (**Figure 21**).

Additionally, our mice display organomegaly and infiltration of mature myeloid cells into non-hematopoietic organs, which is more often associated with JMML (Figure 16 andFigure 17)(Emanuel 2008; Vardiman, Thiele et al. 2009). While a majority of JMML cases have been linked to activated Ras, the etiology of CMML has been found to be more heterogeneous, suggesting that dysregulation of multiple pathways can lead to similar disease phenotypes (Emanuel 2008).

The phenotype of active MEK-mediated myeloid disorders observed in our study is similar to oncogenic K-Ras- but not PI3K/Akt-mediated myeloid neoplasms (Braun, Tuveson et al. 2004; Chan, Kutok et al. 2004; Sabnis, Cheung et al. 2009; Zhang, Wang et al. 2009). Additionally, inhibition of MEK signaling in oncogenic K-Ras animals enhances survival and improves myeloid progenitor function (Lyubynska, Gorman et al. 2011). These data suggest that MEK may be the primary mediator of

oncogenic K-Ras signaling. Activation of the PI3K/Akt pathway appears to favor development of mixed lineage diseases that begin as MPNs, but rapidly progress to more aggressive diseases, namely T cell lymphomas and T-ALLs, while only a small percentage develop AML (Figure 10) (Yilmaz, Valdez et al. 2006; Zhang, Grindley et al. 2006; Guo, Lasky et al. 2008; Kharas, Okabe et al. 2010). Therefore, MEK/ERK and PI3K share some overlapping functions, but also have distinct roles in neoplastic transformation of hematopoietic cells.

The PI3K/Akt pathway is primarily associated with cell survival; however, it is well-known that sole enhancement of cell survival is not sufficient to cause malignant transformation (Datta, Brunet et al. 1999). For example, translocation events that placed Bcl-2 under the control of the immunoglobulin heavy chain enhancer coupled with dysregulated c-Myc expression resulted in lymphoid disease. However, ectopic expression of Bcl-2 alone is insufficient to promote oncogenesis (Vaux, Cory et al. 1988; Strasser, Harris et al. 1990). These data suggest that dysregulation of the cell cycle and uncontrolled proliferation may be primary transformation events while prolonged cell survival may play a supportive role, which allows susceptible pre-leukemic cells to acquire additional mutations that can lead to blast crisis.

Active MEK-induced MDS/MPN was not transplantable into naïve mice, highlighting the pre-leukemic nature of the disease (**Table 1**). One potential explanation

for this could be that expression of active MEK does not affect self-renewal capacity of HSCs (Figure 19). Thus, the numerous progenies that resulted from enhanced cell cycle status of mature myeloid cells (Figure 20A) cannot be sustained and do not give rise to myeloid disease. On the other hand, even if transformed progenitors capable of initiating lymphoid disease were present, we would not be able to detect them in the primary mice transplanted with active MEK^+ HSCs because the MDS/MPN was lethal and had a relatively short latency period (Figure 13).

While MEK signaling has been shown to be involved in promoting cell survival, active MEK signaling did not enhance cell survival of HSCs following growth factor withdrawal (Figure 18). Pairing active MEK expression with Bcl-2 did not alter the MDS/MPN disease phenotype observed in primary transplanted mice (Figure 24). However, ectopic expression of Bcl-2 could extend the short latency period associated with MDS/MPN; thus, allowing T-ALLs to develop at a greater frequency in Bcl-2/active MEK secondary transplanted mice compared to secondary transplanted active MEK only mice (Figure 30A-C). All T-ALLs, regardless of the source of primary MDS/MPN tumors, were similar. The bone marrow and spleen were packed with lymphoid cells (Figure 23CFigure 27, and Figure 23B). Moreover, lymphoblasts were observed in the peripheral blood and the cellular infiltration of multiple organs (Figure 23C, Figure 26, and Figure 27). These lymphoblasts expressed both CD4 and CD8 on their cell surface,

but not CD3 (Figure 26B-C). The observed immunophenotype is in good agreement with their immature morphology.

Further analysis showed that T-ALLs had activated Notch signaling, whereas MDS/MPNs did not (Figure 28). Our data is in agreement with models of oncogenic K-Ras where T-ALLs develop following a long latency period and harbor additional genetic aberrations such as activating mutations and chromosomal alterations (Aifantis, Raetz et al. 2008; Sabnis, Cheung et al. 2009; Zhang, Wang et al. 2009). This suggests that enhanced cell survival may have contributed to the acquisition of additional mutations that promote leukemogenesis (Figure 30C).

Similarly, active inhibition of apoptosis has been suggested to be necessary for lymphoid disease development (Luo, Li et al. 2005). Overexpression of the oncogene c-Myc in hematopoietic cells resulted in increased apoptosis and AML development in primary and secondary transplanted mice. Coupling c-Myc and Bcl-2, an anti-apoptotic gene that is able to counteract c-Myc-induced apoptosis, resulted in lymphoid and myeloid diseases in the primary transplant, but only lymphoid disease in the secondary transplant (Luo, Li et al. 2005). These data suggest that aberrant expression of oncogenes that promote cell survival may allow lymphoid leukemia initiators to acquire additional oncogenic "hits" and these newly acquired "hits" can cooperate with existing oncogenes, which can significantly affect disease progression and phenotype (Figure 30).

MEK/ERK signaling has been implicated in a variety of cellular functions including proliferation, survival, and differentiation. The results shown in this paper clearly demonstrate that active MEK can function as an oncogene in hematopoietic cells, suggesting that MEK/ERK signaling must be tightly regulated in this cell type. However, we should note that only myeloid disorders were observed in recipient mice injected with HSCs expressing active MEK despite the fact that HSCs have multipotent hematopoietic differentiation potential. We previously demonstrated that activation of the MEK/ERK pathway promotes granulocyte/macrophage differentiation from HSCs both *in vivo* and *in vitro*, most likely through its ability to upregulate C/EBPα, a myeloid-specific transcription factor, which has also been shown to regulate HSC self-renewal (Zhang, Iwasaki-Arai et al. 2004; Hsu, King-Fleischman et al. 2006; Hsu and Kondo 2007). In addition, ERK can phosphorylate c-Myc, a well-known cell-cycle regulator, protecting the protein from proteasomal degradation (Sears, Leone et al. 1999). Reciprocal expression of C/EBPα and c-Myc has been observed in monocyte cell lines and they have also been shown to negatively regulate the expression of each other (Li, Nerlov et al. 1994; Antonson, Pray et al. 1995; Johansen, Iwama et al. 2001). This underscores the important of differentiation and proliferation as mutually exclusive cellular events that occur at distinct times. MEK signaling fluctuates as HSCs commit to

the myeloid lineage and differentiate into mature cells; thus, turning off MEK signaling is important not only to control cellular proliferation, but also to balance HSC output.

Taken together, our findings provide new insight into the novel role of the MEK/ERK pathway in leukemogenesis. Our data suggests that activated MEK can serve as an oncogene in hematopoietic cells because its activation is sufficient to drive development of MDS/MPN (Figure 13). Moreover, enforced expression of Bcl-2 allows transformed cells to acquire additional oncogenic hits, such as activated Notch signaling (Figure 28). It will be interesting to examine the interplay between the MEK/ERK and Notch pathways because both pathways have been shown to be important for differentiation. Our experimental systems are unique and may be helpful for dissecting T-ALL development from MDS/MPNs.

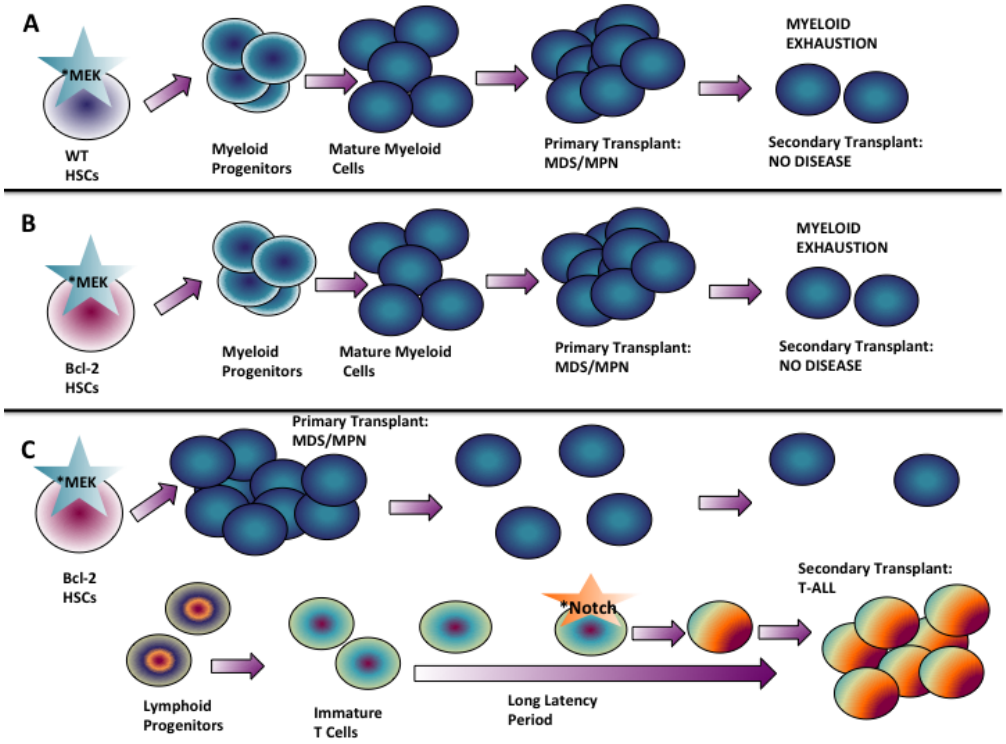


Figure 30: Model of leukemia development induced by active MEK, Bcl-2, and active Notch signaling.

(A) Active MEK expression in HSCs leads to differentiation along the myeloid lineage. Mature myeloid cells have enhanced proliferative capacity and expand, giving rise to a MDS/MPN. Transplantation of MDS/MPN does not result in secondary disease. This observation suggests that myeloid cells have acquired enhanced proliferative capacity, but not enhanced self-renewal capacity, and eventually become "exhausted". (B) Similarly, coupling Bcl-2 expression with active MEK also results in a MDS/MPN, presumably through the same mechanisms observed in active MEK mice. (C) T cell development is observed in both active MEK and Bcl-2/active MEK mice, albeit to a limited extent when compared to control mice. Multipotent progenitor populations capable of giving rise to T cells are transferred along with the MDS/MPN tumors into naïve recipient mice. Active

suppression of apoptosis allows lymphoid-skewed progenitors to persist longer in mice transplanted with Bcl-2/active MEK-induced MDS/MPN. Enhanced cell survival provides lymphoid progenitors with more opportunities to acquire additional oncogenic "hits", such as mutations that can activate the Notch signaling pathway. Activation of the Notch pathway results in full transformation of malignant cells, giving rise to T-ALL, a frank leukemia.

Chapter 4: General discussion and future directions

4.1 Role of MEK/ERK signaling in MDS/MPN development

Our study showed that constitutive activation of the MEK/ERK pathway in HSCs is sufficient to give rise to a lethal MDS/MPN that resembles human JMML and CMML (Figure 13, 14Figure 15Figure 16 Figure 17). This MDS/MPN is marked by the expansion of myeloid cells in the bone marrow and spleen at the expense of lymphoid cell development (Figure 13Figure 16). Myeloid cell infiltration is also observed in non-lymphoid organs. Mice transplanted with either bone marrow or spleen cells from active MEK-induced MDS/MPN mice did not develop any type of disease, with the exception of one mouse that developed T-ALL (**Table 1** and Figure 23). Coupling active MEK with enforced Bcl-2 expression, an anti-apoptotic molecule, did not affect MDS/MPN development (Figure 24). However, mice transplanted with Bcl-2/active MEK-induced MDS/MPNs developed T-ALLs at a higher frequency, following a long latency period, compared to primary transplanted active MEK mice (Figure 13Figure 26). Interestingly, every T-ALL case examined exhibited activation of the Notch pathway (Figure 28). Taken together, our data showed that specific activation of MEK/ERK has transformation potential in hematopoietic cells. Furthermore, coupling active MEK expression with Bcl-2 expression resulted in cooperation among these oncogenes that can lead to additional oncogenic "hits".

While our data clearly shows that activation of MEK signaling can cause disease, the mechanisms employed are still unclear. It is interesting to note that we only observed myeloid disease development in primary transplanted mice, even though active MEK was expressed in HSCs, a pluripotent population capable of giving rise to cells of the lymphoid and myeloid lineage. This suggests that MEK signaling can affect disease phenotype; however the exact mechanism is unclear. Previous work from our laboratory has shown that inhibition of MEK signaling in HSCs results in decreased plating efficiency in methylcellulose culture, indicating that MEK signaling is important for the transition from HSCs to CMPs (Hsu, Kikuchi et al. 2007). Additionally, the expression of C/EBPα, a transcription factor required for granulopoiesis, is decreased in the presence of MEK inhibitors (Figure 32) (Zhang, Zhang et al. 1997; Hsu, King-Fleischman et al. 2006). While low levels of C/EBPα expression have been detected in HSCs and CMPs, C/EBPα primarily functions in the transition from CMPs to GMPs, which can occur in the absence of MEK signaling (Figure 32) (Zhang, Iwasaki-Arai et al. 2004; Hsu, Kikuchi et al. 2007). Moreover, there is some evidence that suggests C/EBPα expression during this early phase does not directly contribute to granulocyte differentiation; rather, it serves to suppress erythroid development (Suh, Gooya et al. 2006). These data suggest that MEK signaling during the early stages of myelopoeisis

does not explicitly instruct multipotent progenitors to proceed along the myeloid lineage pathway.

Alternatively, MEK signaling appears to contribute to disease development by promoting cellular differentiation via downstream targets such as c-Myc and C/EBPα (Figure 31). c-Myc is an oncogene that is best known for its ability to drive cellular proliferation (Grandori, Cowley et al. 2000; Pelengaris, Khan et al. 2002). However, c-Myc has also been shown to play a role in apoptosis and differentiation (Grandori, Cowley et al. 2000; Pelengaris, Khan et al. 2002). Specifically, c-Myc can downregulate the expression of adhesion molecules responsible for retaining HSCs in the stem cell niche, thus allowing HSCs to migrate out of this protective microenvironment and begin the process of differentiation (Wilson, Murphy et al. 2004; Adams and Scadden 2006). While this does not skew development of HSCs toward the myeloid lineage, it does allow HSCs to differentiate.

Similarly, C/EBPα has been found to regulate stem cell activity. In competitive reconstitution experiments, C/EBPα-deficient HSCs were found to have enhanced repopulating ability (Zhang, Iwasaki-Arai et al. 2004). Moreover, C/EBPα-deficient HSCs exhibited higher Bmi-1 expression, a gene that controls self-renewal in HSCs, than WT cells (Iwama, Oguro et al. 2004; Zhang, Iwasaki-Arai et al. 2004). Taken together, these data suggest that one potential role for MEK signaling may be to prime HSCs to

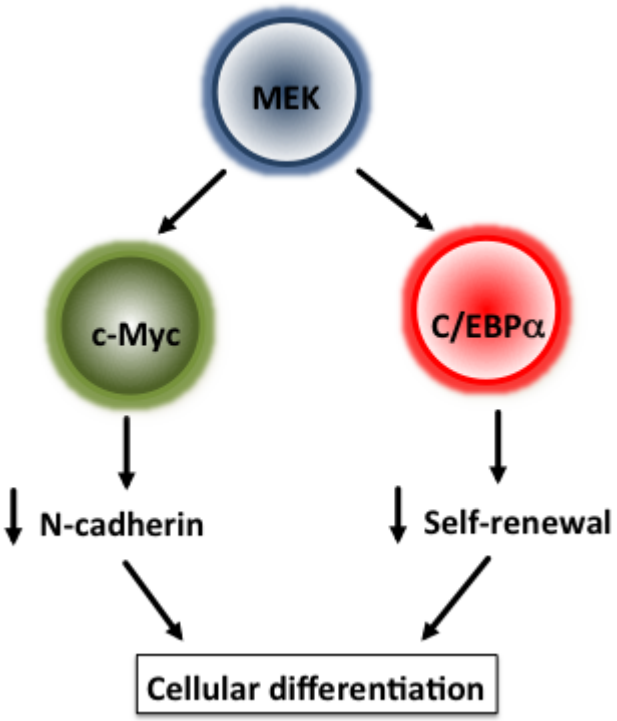


Figure 31: Downstream MEK targets that affect HSC differentiation.

MEK signaling can upregulate c-Myc and C/EBPα expression. c-Myc contributes to HSC differentiation by downregulating N-cadherin, allowing HSCs to leave the HSC niche. C/EBPα drives HSC differentiation by down-regulating self-renewal capacity of HSCs.

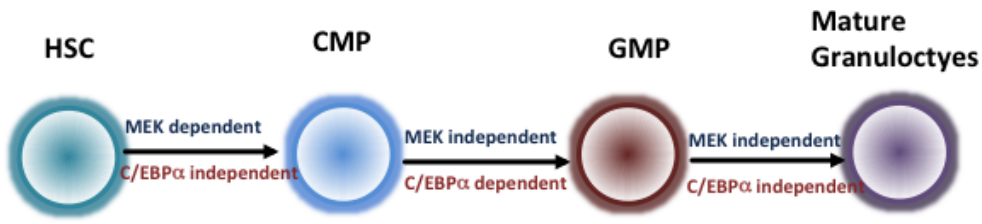
differentiate by augmenting the expression of genes such as c-Myc and C/EBPα, which serve to release HSCs from the protective stem cell niche and suppress self-renewal capacity of HSCs, respectively (Figure 31). Closer examination of C/EBPα and c-Myc expression along with their downstream targets would help to clarify their role in differentiation of HSCs expressing active MEK.

A distinct feature of our model is the massive expansion of myeloid cells in the spleen and bone marrow (Figure 13 and Figure 16). Mature myeloid cells are generally quiescent in the absence of stimulation; however, mature myeloid cells in the bone marrow of mice with active MEK-induced MDS/MPN were found to be cycling (Figure 20A) (Passegué, Wagers et al. 2005). Moreover, these mice have increased c-Myc transcription levels in bone marrow (Figure 21B). This is to be expected given that c-Myc is a positive regulator of cell cycle entry and its expression is found to be higher in cycling cells than quiescent cells (Grandori, Cowley et al. 2000; Pelengaris, Khan et al. 2002).

Interestingly, we did not observe an increase in cell cycle among c-Kit$^+$ myeloid progenitors because committed progenitor populations, such as CLP, CMP, and GMP, are the most actively cycling populations in the bone marrow of WT mice (Figure 20B) (Passegué, Wagers et al. 2005). Additionally, oncogenic K-Ras models of MDS/MPN feature increased proliferation among myeloid progenitors in both the bone marrow and

spleen (Van Meter, Diaz-Flores et al. 2007). Although cell cycling is increased in the myeloid progenitor populations, the bone marrow is hypocellular which could trigger homeostatic proliferation in these progenitor populations (Van Meter, Diaz-Flores et al. 2007). Moreover, oncogenic K-Ras mice had splenomegaly and HSCs and myeloid progenitor populations were increased in excess of 100-fold, suggesting that the spleen had become a site of extramedullary hematopoiesis (Van Meter, Diaz-Flores et al. 2007). While MDS/MPNs are generally thought to originate from HSCs, data from our lab and other groups suggest that different cell types can perpetuate MDS/MPNs (Van Meter, Diaz-Flores et al. 2007). Studies on oncogenic K-Ras-induced MDS/MPN have focused mainly on progenitor populations, thus it would be interesting to examine and compare the cell cycle status of mature myeloid cells and expression of cell cycle regulators in this model to our model.

It is interesting to note that C/EBPα and c-Myc can negatively regulate the expression of each other (Li, Nerlov et al. 1994; Antonson, Pray et al. 1995; Johansen, Iwama et al. 2001). While c-Myc is viewed as an oncogene due to its ability to drive cellular proliferation, C/EBPα has been characterized as a tumor suppressor due to its ability to suppress cellular proliferation and self-renewal (Grandori, Cowley et al. 2000; Pabst, Mueller et al. 2001; Pelengaris, Khan et al. 2002; Tenen 2003). c-Myc and C/EBPα



	HSC	CMP	GMP	Mature Granuloctyes
C/EBPα expression	+	+	+++	+
c-Myc expression	++	+++	+++	+
% cycling	10%	21%	38%	10%

Figure 32: Requirement for MEK signaling and expression of its downstream targets in myeloid-skewed hematopoietic progenitors.

An abbreviated diagram of hematopoiesis is presented here. The requirement for MEK signaling and C/EBPα expression to transition to each stage of development is noted. C/EBPα and c-Myc expression are summarized in the table below the diagram. Also, the percentage of cells in each population that are actively cycling is also given.

display reciprocal gene expression patterns in certain monocyte cell lines following differentiation signals (Johansen, Iwama et al. 2001). The interaction between the two transcription factors is particularly important with regards to cellular differentiation. Specifically, downregulation of c-Myc expression inhibits cellular proliferation, which is necessary for myeloblasts to undergo terminal differentiation into neutrophils, but is not sufficient for granulocyte development, which requires C/EBPα expression (Johansen, Iwama et al. 2001; Wang, Cleaves et al. 2003).

The crucial balance between these two factors is evident as disrupting that balance can lead to cancer. For example, increased myeloid progenitor proliferation and a block in granulocyte differentiation in AMLs are found to be related to mutated forms of C/EBPα, thus underscoring the importance of two distinct regions of the C/EBPα protein that control proliferation and differentiation (D'Alo', Johansen et al. 2003; Keeshan, Santilli et al. 2003; Wang, Cleaves et al. 2003; Porse, Bryder et al. 2005). Introduction of c-Myc into C/EBPα-expressing AML cell lines completely rescues the block in proliferation, as evidenced by increased cell numbers in culture, but only partially halts differentiation (Matsushita, Nakajima et al. 2008). These data further reiterate the role of c-Myc in driving cellular proliferation while highlighting the requirement of other factors and overall complexity of differentiation.

Understanding the relationship between these transcription factors and how MEK signaling upregulates their expression is important in disease initiation as well as disease maintenance. C/EBPα and c-Myc have opposing functions but both are upregulated by MEK signaling, thus understanding when expression of one is more favorable than the other and how that is controlled is important for understanding disease genesis (Antonson, Pray et al. 1995; Zhang, Zhang et al. 1997; Johansen, Iwama et al. 2001; Hsu, King-Fleischman et al. 2006). Once disease is established, certain mechanisms must be in place to maintain disease. Our work clearly shows that active MEK signaling is sufficient to cause disease; however, it is unclear what role, if any, it has in disease maintenance. Determining the role of MEK signaling in disease maintenance is particularly important for developing effective therapies.

4.2 The role of Notch signaling in T-ALL development against a backdrop featuring active MEK signaling and Bcl-2 expression

We observed T-ALL development in one-third of mice that received secondary transplants from Bcl-2/active MEK-induced MDS/MPN and one lone case in mice transplanted with active MEK-induced MDS/MPN (**Table 1**). The remaining mice did not develop T-ALL, nor any other disease, and were disease-free for at least 52 weeks post-injection (Figure 26). All T-ALL cases exhibited increased Notch signaling while

MDS/MPN samples and samples from T-ALL-free secondary transplanted animals did not (Figure 28).

It is also interesting to note that T-ALL development only occurred after a long latency period in our model and those of other groups, suggesting that activating Notch mutations were acquired late in the transformation process (Figure 28) (Kindler, Cornejo et al. 2008; Sabnis, Cheung et al. 2009). Indeed, mathematical models have been developed to better our understanding of the transformation process and provide us with a conceptual framework in which to examine our findings. These models predict that the ability to be self-sufficient in growth signals is one of the early hits because it provides multiple opportunities for mutations to occur (Hanahan and Weinberg 2000; Spencer, Gerety et al. 2006). Additionally, evasion of apoptosis is a necessary ability to acquire early during the transformation process because it allows mutations that would normally trigger cell death pathways to persist (Spencer, Gerety et al. 2006). In our model, active MEK signaling confers enhanced proliferation and Bcl-2 enhances cell survival. Overall, this results in higher numbers of mutations at the population level, which increases the likelihood of those mutations activating additional oncogenes or inactivating tumor suppressors (Spencer, Gerety et al. 2006). While the contribution of activated Notch signaling in our model is unclear, it would also be interesting to

examine if the acquisition order of oncogenic capabilities had any effect on disease phenotype or progression.

Notch signaling has very defined roles in physiological T cell development, but aberrant Notch signaling can also result in leukemogenesis (Rothenberg and Taghon 2005; Aster, Pear et al. 2008). While translocation events are rare and only found in less than 1% of T-ALL patients, gain-of-function Notch mutations are found in 55% of T-ALL cases (Ellisen, Bird et al. 1991; Weng, Ferrando et al. 2004). Curiously, many gain-of-function Notch mutations do not result in disease (Weng, Millholland et al. 2006; Chiang, Xu et al. 2008). This is because the mutations exhibit weak signal strength that can affect hematopoiesis, but are insufficient to induce T-ALL without additional oncogenic factors (Aster, Pear et al. 2008; Chiang, Xu et al. 2008; Kindler, Cornejo et al. 2008). These findings suggest that levels of Notch signaling required for T-ALL development far exceeds that necessary to mediate physiological functions.

More than 20 different genetic mouse backgrounds, including models that feature overexpression of oncogenes or ablation of tumor suppressors that are involved in cell cycle regulation, differentiation/cell fate, and DNA repair have been shown to be predisposed to T-ALL (Aster, Pear et al. 2008). Also, a majority of these mouse models feature mutations that activate Notch signaling. These observations suggest that aberrant Notch signaling is extremely important in T-ALL development and other

pathways cannot substitute for activated Notch signaling, especially in the context of leukemogenesis (Aster, Pear et al. 2008). Furthermore, data from T-ALL cell line studies show that withdrawal of Notch signaling results in cell cycle arrest and eventual apoptosis, suggesting that T-ALL cells are "addicted" to Notch signaling (Aster, Pear et al. 2008).

One salient feature of Notch signaling is its ability to upregulate c-Myc expression. Specifically, Notch signaling in T-ALLs has been shown to increase c-Myc expression that is correlated with increased proliferation (Sharma, Calvo et al. 2006; Weng, Millholland et al. 2006). Also, c-Myc expression is rapidly downregulated following Notch withdrawal (Weng, Millholland et al. 2006). Moreover, T-ALL cell lines that depend on transgenic c-Myc expression for growth can be rescued by introducing a constitutively active form of Notch1 capable of upregulating endogenous c-Myc (Weng, Millholland et al. 2006). Furthermore, genome-wide analysis shows that Notch and c-Myc regulate interconnected transcriptional programs (Palomero, Lim et al. 2006). However, only a subset of T-ALL cell lines dependent on Notch signaling could be rescued by c-Myc expression following treatment with γ-secretase inhibitors (Aster, Pear et al. 2008). Thus, c-Myc expression does not mediate all Notch functions and the impact of Notch signaling extends beyond its ability to upregulate c-Myc.

In our model, we also observed increased c-Myc expression in T-ALLs as well as in primary MDS/MPNs (Figure 21B andFigure 28). Levels of c-Myc expression in T-ALL samples were significantly higher than those in MDS/MPN, which were higher than vector control. Whether activation of both MEK and Notch signaling had a synergistic effect in terms of c-Myc expression is unclear. It should be noted that ERK phosphorylation of c-Myc has been shown to stabilize c-Myc protein while ICN has been found to directly bind to the c-Myc promoter, thus increasing gene expression (Sears, Leone et al. 1999; Sharma, Calvo et al. 2006). Together Notch and MEK signaling can regulate c-Myc expression at two levels: transcription and post-translation.

While the mechanism is not well understood, overexpression of c-Myc has also been shown to drive apoptosis concomitantly with proliferation (Luo, Li et al. 2005). Although we did not directly examine apoptosis in terminal MDS/MPN mice, we did find that expression of active MEK did not affect apoptosis of HSCs during short-term culture (Figure 18). Moreover, Bcl-2 has been suggested to counteract c-Myc function by promoting cell survival and inhibiting cell cycle progression, thus halting cellular proliferation. Similarly, c-Myc can counteract Bcl-2 function by upregulating Puma and Noxa, two pro-apoptotic proteins that can neutralize the pro-survival function of Bcl-2. Thus, Bcl-2 expression in Bcl-2/active MEK mice may raise the tolerance threshold for c-Myc expression (Figure 33) (Askew, Ashmun et al. 1991; Luo, Li et al. 2005). It would be

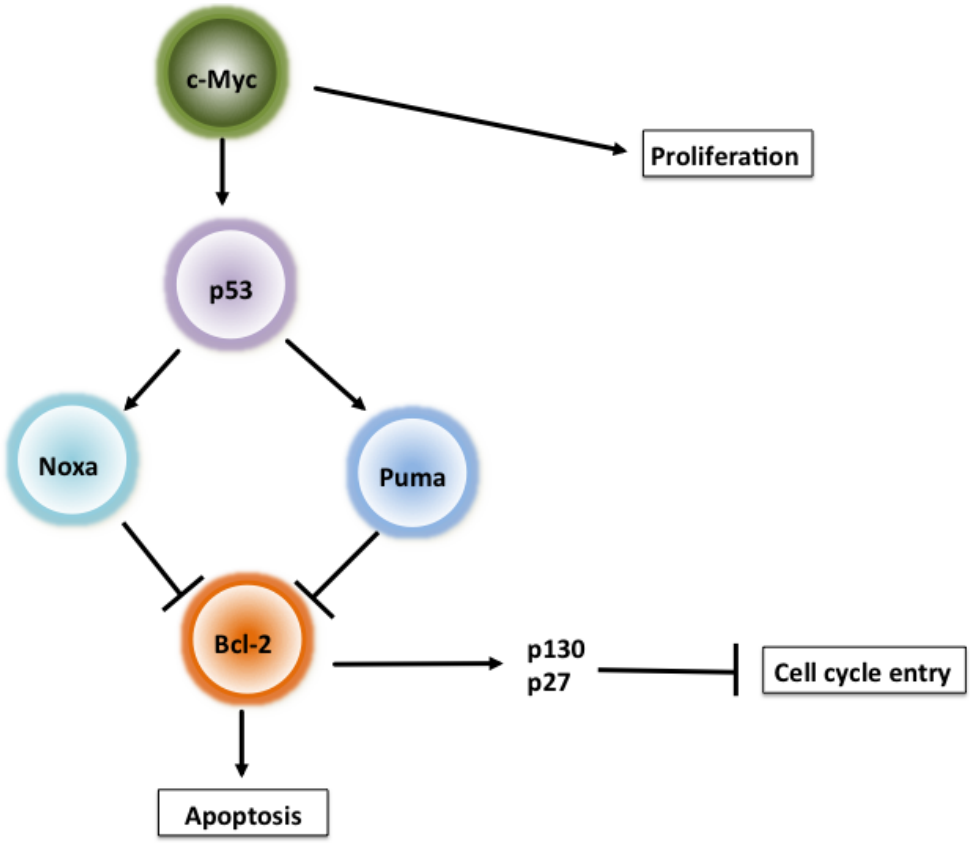


Figure 33: Antagonistic relationship between c-Myc and Bcl-2.

c-Myc is a pleiotropic molecule that can promote proliferation as well as apoptosis by inhibiting Bcl-2 function. c-Myc can increase expression of the tumor suppressor, p53. Increased expression of p53 leads to increased transcription of pro-apoptotic proteins, Puma and Noxa, which can inhibit Bcl-2 function, resulting in apoptosis. Bcl-2 can also retard entry into the cell cycle, thus decreasing cell proliferation. Bcl-2 can increase the expression of p130 and p27, members of the retinoblastoma family of proteins that control cell cycle progression. Increased p130 and p27 expression results in the formation of repressive complexes with E2F transcription factors, thus inhibiting the expression of genes that promote cell cycle progression. Adapted from (Cory, Huang et al. 2003).

interesting to investigate how multiple layers of regulation affect overall c-Myc expression. Also, examining how c-Myc expression correlates with function would be beneficial in developing a better understand of the malignant transformation process.

In addition to regulating cell proliferation and cell growth, Notch signaling has also been implicated in the regulation of other cancer hallmarks, although its role is somewhat controversial. Gain-of-function experiments in HSCs and hematopoietic progenitor cell lines showed that increased Notch signaling enhances "stemness" by increasing self-renewal capacity and decreasing differentiation (Carlesso, Aster et al. 1999; Varnum-Finney, Xu et al. 2000; Stier, Cheng et al. 2002; Kunisato, Chiba et al. 2003; Burns, Traver et al. 2005). Enhanced Notch signaling was achieved through a variety of different methods including: exposure to Notch ligands *in vitro*; overexpression of constitutively active Notch alleles; overexpression of downstream Notch targets, such as Hes1; and activation of Notch ligands (Varnum-Finney, Purton et al. 1998; Carlesso, Aster et al. 1999; Varnum-Finney, Xu et al. 2000; Karanu, Murdoch et al. 2001; Stier, Cheng et al. 2002; Kunisato, Chiba et al. 2003; Varnum-Finney, Brashem-Stein et al. 2003; Burns, Traver et al. 2005; Suzuki, Yokoyama et al. 2006; Yu, Alder et al. 2006).

However, the genetic loss-of-function models do not appear to support the conclusions garnered by the gain-of-function studies (Radtke, Wilson et al. 1999; Mancini, Mantei et al. 2005; Maillard, Koch et al. 2008). To assess Notch signaling in

HSC function and maintenance, multiple loss-of-function models were generated, including: inactivation of Notch1; inactivation of Notch1 ligands, such as Jagged1; and inactivation of CSL/RBP-Jκ, a DNA-binding factor that is required for signaling through all four mammalian Notch receptors (Radtke, Wilson et al. 1999; Mancini, Mantei et al. 2005; Maillard, Koch et al. 2008). These models found that HSC engraftment, pluripotency, and overall fitness are not altered in the absence of Notch signaling. While studies on the role of Notch signaling in self-renewal and differentiation of HSCs are inconclusive, studies examining other progenitor populations may shed more light on Notch function in the context of leukemogenesis. High levels of Notch signaling in DN3 thymocytes is necessary for their survival and expansion, but Notch signaling must be downregulated promptly following β-selection to allow thymocytes to progress to later stages of development (Figure 34)(Sambandam, Maillard et al. 2005; Maillard, Tu et al. 2006). The mechanism responsible for downregulating Notch signaling at the β-selection juncture is unknown; however, one intriguing idea is that dysregulation of this mechanism may contribute to leukemogenesis.

One piece of evidence to support this idea is that many mouse models of T-ALL featuring aberrant Notch signaling have tumors that are pre-TCR$^+$, suggesting that the thymocytes had arrived at the β-selection checkpoint but failed to mature to the SP stage (Asnafi, Beldjord et al. 2003; Weng, Ferrando et al. 2004). Recall that Notch signaling is

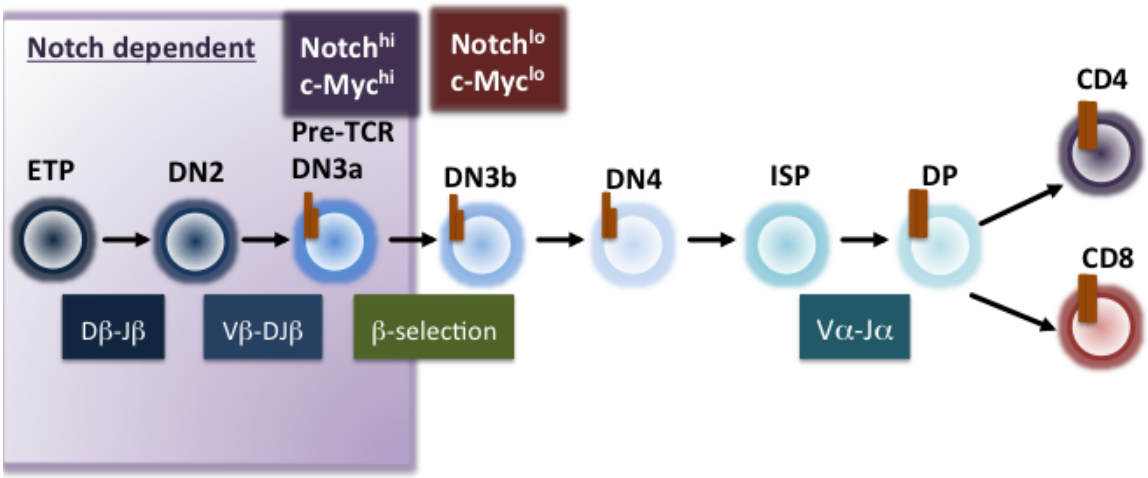


Figure 34: Notch 1 and T cell development.

T cell development from the early thymic progenitor (ETP) to mature single positive CD4 and CD8. Key T cell development events, such as gene rearrangement and selection events are shown here. Notch1 and c-Myc expression are noted along with stages of T cell development that are dependent on Notch signaling. Adapted from (Aster, Pear et al. 2008).

necessary to promote proliferation and survival in DN3 cells; thus, any pre-existing mutation in a DN3 cell could easily be propagated and expanded at the β-selection stage (Maillard, Tu et al. 2006). Also, the ability of Notch signaling to promote cell survival allows damaged cells that would otherwise undergo apoptosis to persist. Finally, persistent Notch signaling blocks differentiation. Damaged DN3 cells that are allowed to mature to the SP stage exit the cell cycle and become quiescent, thus limiting the pool of malignant cells. However, persistent Notch signaling at the β-selection checkpoint blocks the ability of DN3 cells to fully mature so they are continually maintained in a state of enhanced proliferation and cell survival.

4.3 Closing remarks

Cancer is the second leading cause of death in the United States. While every cancer is different due to the tissue type they affect, they all share similar acquired capabilities, or cancer hallmarks (Hanahan and Weinberg 2000). Leukemia is a particularly good model to use in studying cancer for many reasons: hematopoietic populations are well-defined and easy to isolate; human cells lines are available; animal models that recapitulate a variety of leukemias have been generated; and the tumors are not solid so they do not need to undergo angiogenesis or metastasize, which allows for

the focus to be placed on hallmarks acquired early during disease development (Kennedy and Barabe 2008).

Activation of Ras has been implicated in a variety of different cancers, including leukemias (Bos 1989; Downward 2003). Due to its ability to activate multiple signal transduction pathways, the link between the downstream effector pathway and disease were not always clear (Schubbert, Shannon et al. 2007). We have shown in our study that activation of MEK, a downstream target of Ras signaling, in HSCs is sufficient to cause a MDS/MPN. A myeloid disease phenotype is somewhat expected given that MEK signaling has been shown to be involved in physiological myeloid cell development (Hsu, Kikuchi et al. 2007). However, the role of MEK signaling may not be to drive myeloid development, but to affect HSC differentiation ability (Wilson, Murphy et al. 2004; Zhang, Iwasaki-Arai et al. 2004).

We also showed that active MEK signaling specifically drives proliferation of mature myeloid cells, perhaps through its ability to upregulate c-Myc, an oncogene and cell cycle regulator. However, C/EBPα, a tumor suppressor and transcription factor important for granulopoiesis, is also upregulated by MEK signaling (Hsu, King-Fleischman et al. 2006; (Grandori, Cowley et al. 2000; Pabst, Mueller et al. 2001; Pelengaris, Khan et al. 2002; Tenen 2003). The two transcription factors have opposing functions and have been shown to regulate the expression of the other (Li, Nerlov et al.

1994; Antonson, Pray et al. 1995; Johansen, Iwama et al. 2001). However, it is not clear at which stage of hematopoiesis MEK signaling would play a role, if any, in regulating their expression.

Moreover, pairing the ability of active MEK signaling to drive proliferation with the ability of Bcl-2 to promote survival resulted in increased incidence of T-ALL. Disease onset occurred only after a long latency period and all T-ALLs harbored mutations that resulted in activated Notch signaling, a phenotype observed in 50% of human T-ALL cases (Weng, Ferrando et al. 2004). We speculate that enhanced cell survival enabled lymphoid progenitors, which have been found to be more sensitive to pro-apoptotic signals elicited by other oncogenes, to persist and acquire Notch mutations (Luo, Li et al. 2005). Additionally, activation of Notch signaling was necessary to transition the pre-leukemic MDS/MPN into full-blown leukemia. Identification of the cancer stem cell population will help better our understanding of how oncogene cooperation manifests in disease and the population targeted for transformation.

Our model follows a similar disease course observed in humans and may be helpful in investigating how pre-leukemic diseases transition to blast crisis. Given that some cancers become "addicted" to MEK signaling, our model may be also useful in screening potential cancer therapies.

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