# PI3K IN JUVENILE MYLEOMONOCYTIC LEUKEMIA

Charles B. Goodwin

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Medical and Molecular Genetics,
Indiana University

June 2013

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

_____

Rebecca J. Chan, MD, PhD, Chair

_____

Brittney-Shea Herbert, PhD

Doctoral Committee

_____

Kenneth E. White, PhD

March 20th, 2013

_____

Mervin C. Yoder, MD

ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Rebecca Chan for her endless support, patience, and encouragement over the past several years. With her help, I feel like I have truly grown and matured as a scientist, and that I could not have chosen a better lab to embark on my career as a physician-scientist.

I would also like to express my appreciation to my thesis committee, Dr. Brittney-Shea Herbert, Dr. Kenneth E. White, and Dr. Mervin C. Yoder, for their support, invaluable insight, enthusiasm, and tough questions, all of which inspired me in my development as a scientist.

I would also like to thank all the past and present members of the Chan lab, including Dr. Sarah Nabinger, Dr. Xingjun Li, Dr. Zhenyun Yang, and (before too long Dr.) Briana Richine for all their help with experiments and their support and friendship. Likewise, I would like to thank Linda Henson and Marilyn Wales for their behind-the-scenes administrative support.

I would like to thank my collaborators in the laboratory of Reuben Kapur at the Indiana University School of Medicine, Dr. Benjamin Neel at University Health Network in Toronto, and Dr. Brian Lannutti at Gilead Sciences, Inc.

I would like to thank everyone in the Indiana University School of Medicine's Medical Scientist Training Program, including Dr. Maureen Harrington, Dr. Wade Clapp, Dr. Raghu Mirmira, and Jan Receveur, for all their support and all the opportunities I have been given to grow as a future physician-scientist.

I would like to acknowledge my funding sources, the National Heart, Lung, and Blood Institute (National Research Service Award F30 HL104867), and the IU Simon

Cancer Center (Marilyn Hester Scholarship for Pediatric Hematology/Oncology Research).

Finally, I want to express my deepest appreciation and gratitude to my family, my parents Dr. Charles and Judy Goodwin, my brothers Tim and James, my sister, Emily, and my sister-in-law, Marie for all their love, patience, and support as I have continued my journey as a “professional student.”

ABSTRACT

Charles B. Goodwin

PI3K IN JUVENILE MYELOMONOCYTIC LEUKEMIA

Juvenile Myelomonocytic Leukemia (JMML) is rare, fatal myeloproliferative disease (MPD) affecting young children, and is characterized by expansion of monocyte lineage cells and hypersensitivity to Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) stimulation. JMML is frequently associated with gain-of-function mutations in the *PTPN11* gene, which encodes the protein tyrosine phosphatase, Shp2. Activating Shp2 mutations are known to promote hyperactivation of the Ras-Erk signaling pathway, but Akt is also observed to have enhanced phosphorylation, suggesting a potential role for Phosphatidylinositol-3-Kinase (PI3K)-Akt signaling in mutant Shp2-induced GM-CSF hypersensitivity and leukemogenesis.

Having demonstrated that Class IA PI3K is hyperactivated in the presence of mutant Shp2 and contributes to GM-CSF hypersensitivity, I hypothesized the hematopoietic-specific Class IA PI3K catalytic subunit p110δ is a crucial mediator of mutant Shp2-induced PI3K hyperactivation and GM-CSF hypersensitivity *in vitro* and MPD development *in vivo*. I crossed gain-of-function mutant Shp2 D61Y inducible knockin mice, which develop fatal MPD, with mice expressing kinase-dead mutant p110δ D910A to evaluate p110δ's role in mutant Shp2-induced GM-CSF hypersensitivity *in vitro* and MPD development *in vivo*. As a comparison, I also crossed Shp2 D61Y inducible knockin mice with mice bearing inducible knockout of the ubiquitously

expressed Class IA PI3K catalytic subunit, p110α. I found that genetic interruption of p110δ, but not p110α, significantly reduced GM-CSF-stimulated hyperactivation of both the Ras-Erk and PI3K-Akt signaling pathways, and as a consequence, resulted in reduced GM-CSF-stimulated hyper-proliferation *in vitro*. Furthermore, I found that mice bearing genetic disruption of p110δ, but not p110α, in the presence of gain-of-function mutant Shp2 D61Y, had on average, smaller spleen sizes, suggesting that loss of p110δ activity reduced MPD severity *in vivo*.

I also investigated the effects of three PI3K inhibitors with high specificity for p110δ, IC87114, GDC-0941, and GS-9820 (formerly known as CAL-120), on mutant Shp2-induced GM-CSF hypersensitivity. These inhibitors with high specificity for p110δ significantly reduced GM-CSF-stimulated hyperactivation of PI3K-Akt and Ras-Erk signaling and reduced GM-CSF-stimulated hyperproliferation in cells expressing gain-of-function Shp2 mutants.

Collectively, these findings show that p110δ-dependent PI3K hyperactivation contributes to mutant Shp2-induced GM-CSF hypersensitivity and MPD development, and that p110δ represents a potential novel therapeutic target for JMML.

Rebecca J. Chan, MD, PhD, Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# LIST OF ABBREVIATIONS

ALL ..... Acute Lymphoblastic Leukemia

AML ..... Acute Myeloid Leukemia

CML ..... Chronic Myelogenous Leukemia

eGFP ..... enhanced Green Fluorescent Protein

FACS ..... Fluorescence-Assisted Cell Sorting

FTI ..... Farnesyl Transferase Inhibitor

G-CSF ..... Granulocyte-Colony Stimulating Factor

GM-CSF ..... Granulocyte-Macrophage-Colony Stimulating Factor

HSCT ..... Hematopoietic Stem Cell Transplantation

JMML ..... Juvenile Myelomonocytic Leukemia

LDMNC ..... Low-Density Mononuclear Cell

MAPK ..... Mitogen-Activated Protein Kinase

M-CSF ..... Macrophage-Colony Stimulating Factor

MPD ..... Myeloproliferative Disease

MPN/MDS ..... Myeloproliferative Neoplasm/Myelodysplastic Syndrome

PI3K ..... Phosphatidylinositol 3-Kinase

RBD ..... Ras-Binding Domain

SCF ..... Stem Cell Factor

TPO ..... Thrombopoietin

WT ..... Wild type

# CHAPTER ONE

# INTRODUCTION

## Juvenile Myelomonocytic Leuekemia

Juvenile Myelomonocytic Leukemia (JMML) is a rare and often fatal myeloproliferative disease (MPD) of early childhood, typically affecting infants and toddlers. The World Health Organization (WHO) categorizes JMML as an overlap myeloproliferative neoplasm/myelodysplastic syndrome (MPN/MDS), accounting for 2-3% of childhood leukemias with an incidence of 1-2 cases per million per year and approximately 25-50 cases per year in the United States (Loh 2011). The typical initial presentation of a child with JMML is non-specific, with signs and symptoms that include fever, failure to thrive, lymphadenopathy, splenomegaly, and elevated white blood cell counts with monocytosis, which can make it difficult to distinguish from bacterial or viral infection, therefore potentially delaying proper diagnosis (Emanuel 2008; Loh 2011). WHO diagnostic criteria for JMML include absence of the BCR/ABL fusion gene, which characterizes Chronic Myelogenous Leukemia (CML), circulating monocyte count greater than $1 \times 10^9$/L, and less than 20% blasts observed in a bone marrow biopsy (Chan, Cooper et al. 2009). In addition, almost all patients develop splenomegaly at some point during the course of their disease, and for most patients it has already developed by the time of presentation (Loh 2011). Other common clinical features that may assist in the diagnosis of JMML include elevated Hemoglobin F, the presence of a cytogenetic abnormality (most commonly monosomy 7), and hypersensitivity of myeloid progenitor cells to Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (Emanuel, Bates

et al. 1991; Bergstraesser, Hasle et al. 2007; Niemeyer and Kratz 2008; Chan, Cooper et al. 2009).

At this time, the only curative therapy for JMML is allogeneic hematopoietic stem cell transplantation (HSCT), which under current protocols has around a 50% survival rate (Locatelli, Nollke et al. 2005; Bergstraesser, Hasle et al. 2007; Yoshimi, Mohamed et al. 2007). HSCT is plagued with many challenges including relatively high rates of relapse and complications such as infection, bleeding, and graft-versus-host disease (GVHD); therefore better treatment modalities for JMML are badly needed (Locatelli, Nollke et al. 2005). To that end, much work has been done over the past several years to understand the molecular mechanisms underlying JMML. Patients with JMML frequently exhibit mutations in genes encoding proteins involved in RAS signaling, suggesting that RAS hyperactivation is common etiologic feature in JMML. These mutations include gain-of-function mutations in *KRAS* and *NRAS* (together accounting for approximately 20% of cases) (Kalra, Paderanga et al. 1994; Miyauchi, Asada et al. 1994), as well as loss-of-function mutations in the RAS GAP, *NF1* (in 15% of cases) (Shannon, O'Connell et al. 1994), which negatively regulates RAS activity. Most recently, loss-of-function mutations in the E3 ubiquitin ligase, *CBL*, have been described (in 10-15% of cases) (Loh, Sakai et al. 2009). The most commonly mutated gene encountered in JMML patients, however, are gain-of-function mutations in *PTPN11*, which encodes the protein tyrosine phosphatase, SHP2, accounting for slightly more than one-third of JMML cases (Figure 1.1) (Tartaglia, Niemeyer et al. 2003). The mechanism by which SHP2 regulates RAS activity is not completely understood. Despite these

advances, causative mutations have not been identified in approximately 10-15% of JMML patients.

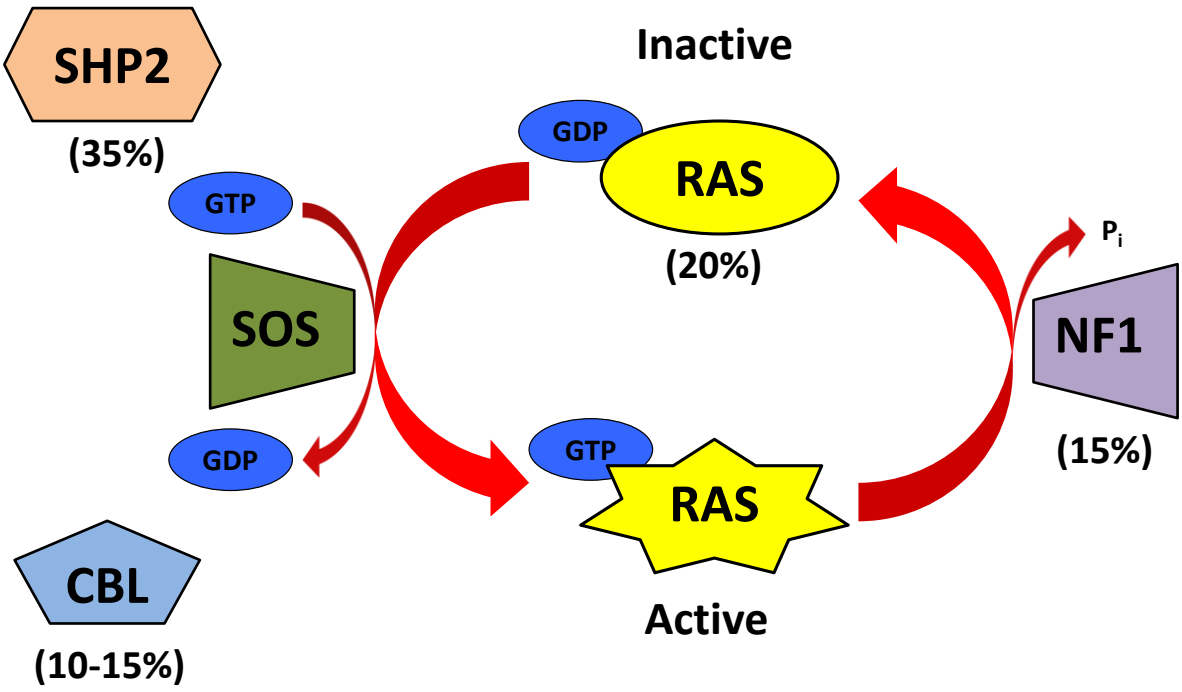



**Figure 1.1: Schematic diagram of RAS activation and its regulators highlighting mutations found in Juvenile Myelomonocytic Leukemia**

RAS is a GTPase molecular "switch," which is activated when it is in its GTP-bound state and inactive in its GDP-bound state. Activation of RAS is induced by the RAS Guanine Exchange Factor (GEF), SOS, which promotes that exchange of GDP for GTP. RAS is inactivated by its intrinsic, but extremely slow, GTPase activity, which promotes hydrolysis of the γ-phosphate group from GTP to yield GDP. This GTPase activity is stimulated by the RAS GTPase Activating Protein (GAP), NF1. The E3 ubiquitin ligase CBL negatively regulates RAS activity and the protein tyrosine phosphatase SHP2 positively regulates RAS activity both through mechanisms that remain incompletely defined. Loss-of-function mutations in *NF1* and *CBL* and gain-of-function mutations in *RAS* and *PTPN11* (SHP2) are found in JMML. Percentages in parentheses below proteins indicate the approximate frequency of JMML cases in which that protein is found to be mutated.

**The Protein Tyrosine Phosphatase, SHP2, and Juvenile Myelomonocytic Leukemia**

As stated above, somatic mutations in *PTPN11* (i.e., the gene encoding Shp2) occur in 35% of JMML cases, but are also found in childhood acute myeloid leukemia (AML, 4%), myelodysplastic syndrome (10%), acute lymphoid leukemia (7%) (Tartaglia, Niemeyer et al. 2003; Loh, Reynolds et al. 2004; Loh, Vattikuti et al. 2004; Tartaglia, Martinelli et al. 2004; Kratz, Niemeyer et al. 2005), and, rarely, in some solid tumors (Bentires-Alj, Paez et al. 2004; Loh, Martinelli et al. 2005; Martinelli, Carta et al. 2006). In addition, germline mutations in *PTPN11* are found in about half of patients with Noonan Syndrome, a relatively common autosomal dominant development disorder, in which patients often develop a self-limiting JMML-like MPD (Roberts, Allanson et al. 2013). More recently, germline mutations have also been found to be associated with a much rarer developmental disorder, called LEOPARD syndrome, which shares many clinical features with Noonan Syndrome (Kontaridis, Swanson et al. 2006; Marin, Keith et al. 2011). In addition to mutated forms of Shp2, recent work using murine models has demonstrated that wild type (WT) Shp2 participates in aberrant signaling induced by other oncogenes, such as Flt3-ITD-associated AML and mutant Kit in AML and mastocytosis (Mali, Ma et al. 2012; Nabinger and Chan 2012; Nabinger, Li et al. 2013).

Structurally, SHP2 is a non-receptor protein tyrosine phosphatase that contains two Src Homology 2 domains (N-SH2 and C-SH2) and an enzymatic phosphatase (PTPase) domain (Figure 1.2) (Chan and Feng 2007). In its inactive state, SHP2 is in a closed confirmation stabilized by intermolecular interactions between the N-SH2 and the PTPase domains, thereby blocking substrate access to enzymatic pocket of the PTPase domain. In the course of normal activation, the SHP2 SH2 domains are recruited to

phosphorylated tyrosine residues on activated receptors or adapter molecules, which results in an interruption of the auto-inhibitory interaction between the N-SH2 and PTPase domains, thereby allowing SHP2 to assume an open, active confirmation with unobstructed access to tyrosine-phosphorylated substrates. Leukemia-causing mutations typically affect amino acids in the N-SH2 or PTPase domains that participate in the stabilizing intermolecular interactions that contribute to the closed, inactive confirmation, such that the presence of such mutations results in a constitutively open and activated phosphatase (Chan and Feng 2007; Chan, Kalaitzidis et al. 2008). For this reason, *PTPN11* has been identified as a rare example of a phosphatase proto-oncogene, which promotes oncogenic signaling as a result of acquiring gain-of-function mutations.

Since the cloning of murine *Ptpn11* in 1992 (Freeman, Plutzky et al. 1992; Feng, Hui et al. 1993), numerous mechanistic studies have been conducted and published to clarify the role of Shp2 in Receptor Tyrosine Kinase (RTK)- and cytokine receptor-stimulated signaling pathways. As previously mentioned, it has been demonstrated that Shp2 is a positive regulator of Ras activity, though the mechanism of this regulation remains unclear. Studies have shown that the phosphatase activity of Shp2 is required to activate Ras, though the exact tyrosine-phosphorylated substrates that participate in this process remain to be fully elucidated (Neel, Gu et al. 2003; Mohi and Neel 2007). In addition to its enzymatic function, there is also evidence that Shp2 may promote signal transduction as a scaffolding protein via its two SH2 domains, and Shp2 is known to interact directly or in complexes with a number of proteins involved in signal transduction, including receptors such as the GM-CSF receptor, adapter molecules such as Gab2 and Grb2, and signaling molecules such as the Ras-GEF, Sos, and the p85

regulatory subunit of Phosphatidylinositide 3-Kinase (PI3K) (Neel, Gu et al. 2003; Mohi, Williams et al. 2005; Yu, Daino et al. 2006).

As Shp2 has been shown to be a positive regulator of signal transduction pathways that promotes proliferation, growth, and survival downstream of various growth factor and cytokine receptors and adhesion molecules such as integrins, it is hypothesized that leukemia-associated gain-of-function mutations in Shp2 lead to hyperactivation of these pro-growth and pro-survival signaling pathways and enhanced responsiveness to stimulation with specific cytokines and growth factors. Of particular importance to JMML is mitogenic signaling related to the GM-CSF receptor, since a common clinical diagnostic feature of JMML is hypersensitivity to GM-CSF, in which myeloid progenitors from JMML patients exhibit an enhanced proliferative response following GM-CSF stimulation (Emanuel, Bates et al. 1991). Our laboratory and others have shown that expression of Shp2 bearing leukemia-associated mutations such as E76K or D61Y in myeloid progenitor cells is sufficient to induce the GM-CSF hypersensitivity phenotype in these cells (Chan, Leedy et al. 2005; Mohi, Williams et al. 2005; Schubbert, Lieuw et al. 2005). Additionally, we have shown that cells expressing gain-of-function mutant Shp2 exhibit substantially increased signaling through both the Mitogen Activated Protein Kinase (MAPK) and PI3K pathways, as demonstrated by increased phosphorylation of Erk and Akt, respectively, compared to WT Shp2-expressing cells in response to GM-CSF stimulation (Yang, Li et al. 2008). This suggests a model in which activating Shp2 mutations result in enhanced MAPK and PI3K signaling that regulate down-stream effectors, which promote enhanced proliferation, growth, and survival following stimulation with GM-CSF and possibly other growth

factors and cytokines, leading to pathologic expansion of myeloid-lineage cells and the development of JMML. To date, much of the work on mutant Shp2-induced aberrant signaling and the pathogenesis of MPD has focused on the MAPK pathway. However, we have shown that Akt is hyper-phosphorylated in the presence of leukemia-associated Shp2 mutations, indicating a potential role for PI3K signaling, which is frequently hyperactivated in wide array of human malignancies, thereby contributing to their pathogenesis. Thus, there is significant rationale to explore the potential contribution of hyperactivated PI3K to mutant Shp2-induced GM-CSF hypersensitivity and leukemogenesis as a potential therapeutic target.

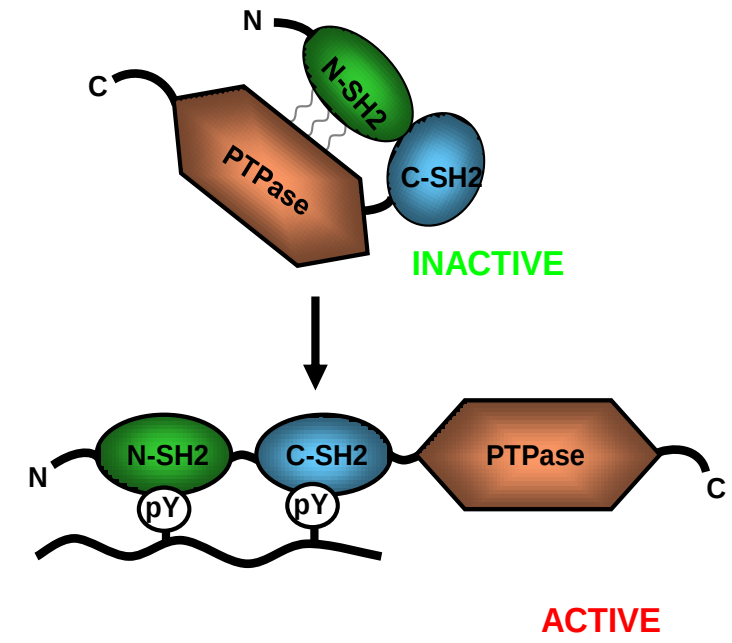



**Figure 1.2: Diagram showing typical mechanism of SHP2 activation and the consequences of common oncogenic mutations**

(A) In its inactive state, SHP2 forms a closed confirmation stabilized by intermolecular interactions between the N-SH2 and phosphatase (PTPase) domains. When the SH2 domains of SHP2 are engaged by phosphorylated tyrosine residues, SHP2 then assumes its open, active confirmation. (B) Oncogenic mutations in SHP2 often interrupt the intermolecular interactions between the N-SH2 and PTPase domains, so that Shp2 assumes an open, constitutively active confirmation (adapted from (Chan and Feng 2007)).

**Phosphatidylinositide 3-Kinase (PI3K) Structure and Function**

Class I Phosphatidylinositide 3-Kinases (PI3Ks) are lipid kinases, which phosphorylate the 3 position of plasma membrane-associated lipid, phosphatidylinositol (4,5) bisphosphate ($PIP_2$), to generate $PIP_3$, which serves to recruit Plekstrin Homology (PH)-domain-containing signaling kinases to the plasma membrane, especially PDK1, and its downstream effector, the serine-threonine kinase AKT (also known as Protein Kinase B, PKB), which it phosphorylates at Threonine 308 (Figure 1.3) (Alessi, James et al. 1997; Corvera and Czech 1998; Cantley 2002; Zhao and Vogt 2008). Activated AKT, by phosphorylating several downstream effectors, regulates multiple cellular processes, including protein synthesis (via mTOR), proliferation, survival, and motility (Zhao and Vogt 2008). Through a complex positive feedback loop, AKT is further activated by mTOR-mediated phosphorylation of Serine 473 (Figure 1.3) (Sarbassov, Guertin et al. 2005).

There are two subclasses of Class I PI3K. The Class IA PI3Ks are heterodimers, consisting of a p110 catalytic subunit and a regulatory subunit. The three catalytic subunits include p110α (encoded by *PIK3CA*), p110β (encoded by *PIK3CB*), and p110δ (encoded by *PIK3CD)* (Zhao and Vogt 2008). These three catalytic subunits can bind to any Class IA regulatory subunit, including p85α, and its splice variants p55α and p50α (encoded by *PIK3R1*), p85β (encoded by *PIK3R2*), and p55γ (encoded by *PIK3R3*) via an interaction between the adapter binding domain (ABD) of the catalytic subunit and the inter-SH2 (iSH2) domain of the regulatory subunit (Figure 1.4) (Walker, Perisic et al. 1999; Zhao and Vogt 2008). Class IB PI3K consists only of p110γ (encoded by *PIK3CG*), and its unique regulatory subunits, p84/87 and p101(Zhao and Vogt 2008).

The catalytic subunits p110α and p110β are expressed ubiquitously, while p110δ and p110γ are found predominantly in hematopoietic cells (Zhao and Vogt 2008).

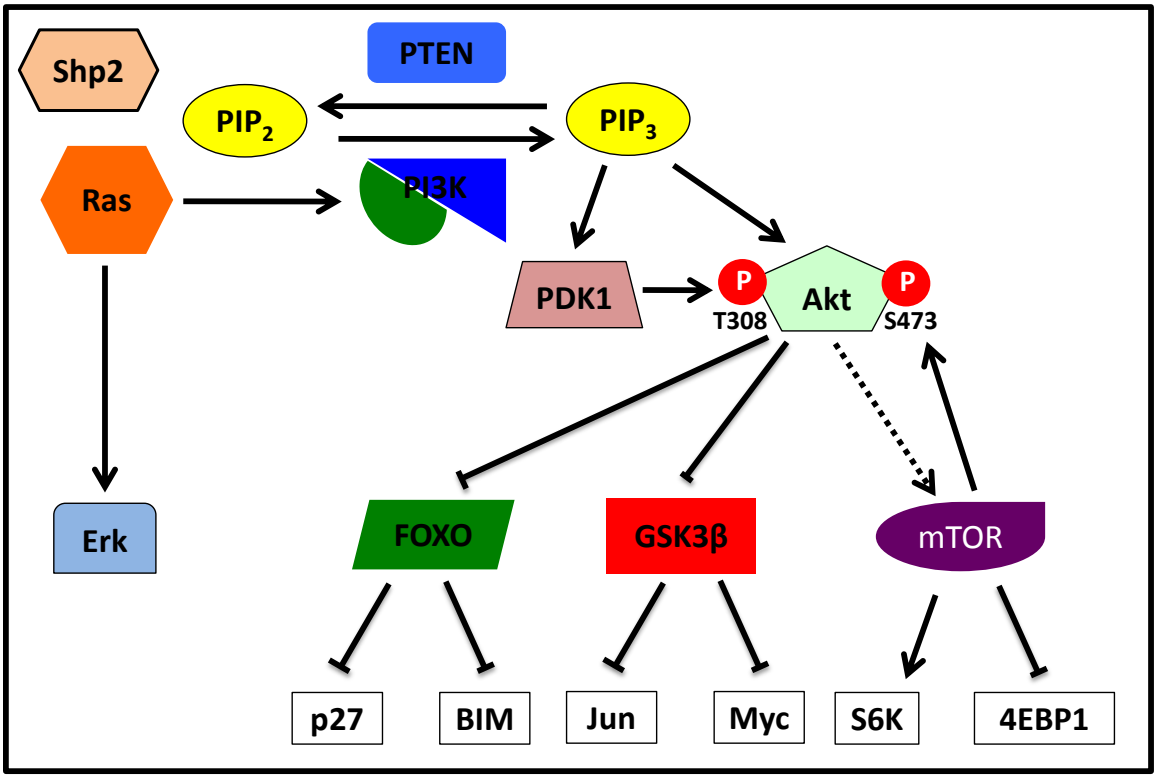



**Figure 1.3: Schematic diagram of PI3K-Akt signaling cascade**

Diagram showing the PI3K-Akt-mTOR signaling cascade, including Ras, a direct activator of PI3K activity, the major downstream effectors of PI3K, PDK1 and Akt, and the major downstream effectors of Akt, including FOXO, GSK3β, and mTOR. Notably, full activation of Akt requires phosphorylation at the Threonine 308 site, which is mediated by PDK1 and at the Serine 473 site, which is mediated by mTOR, while in the mTORC2 complex. mTOR is also positively regulated by Akt via several mediators not shown in this figure (represented by the dotted arrow from Akt to mTOR), creating a positive feedback loop that regulates Akt activity. Also shown is the tumor suppressor lipid phosphatase PTEN, which catalyzes the dephosphorylation of $PIP_3$ to generate $PIP_2$, thereby antagonizing the activity of PI3K.

The Class IA (p85) regulatory subunits help promote PI3K activity through two important mechanisms: (1) stabilizing the catalytic subunits, thereby preventing their rapid degradation, and (2) recruiting the catalytic subunits to phospho-tyrosine residues on activated receptors and adapter molecules via its two SH2 domains (Figure 1.4) (Zhao and Vogt 2008; Fruman 2010). It has also been demonstrated that the regulatory subunits also negatively regulate PI3K activity through an inhibitory interaction between its N-SH2 domain and the helical domain of the p110 catalytic subunit, which is relieved when the regulatory subunit N-SH2 domain interacts with a phospho-tyrosine residue (Yu, Zhang et al. 1998; Luo and Cantley 2005; Luo, Field et al. 2005; Zhao and Vogt 2008). In addition, GTP-bound RAS also promotes PI3K activation via an interaction with the RAS-binding domain (RBD) of the catalytic subunit (Figure 1.4) (Rodriguez-Viciana, Warne et al. 1994; Rodriguez-Viciana, Warne et al. 1996; Pacold, Suire et al. 2000; Chan, Rodeck et al. 2002; Zhao and Vogt 2008).

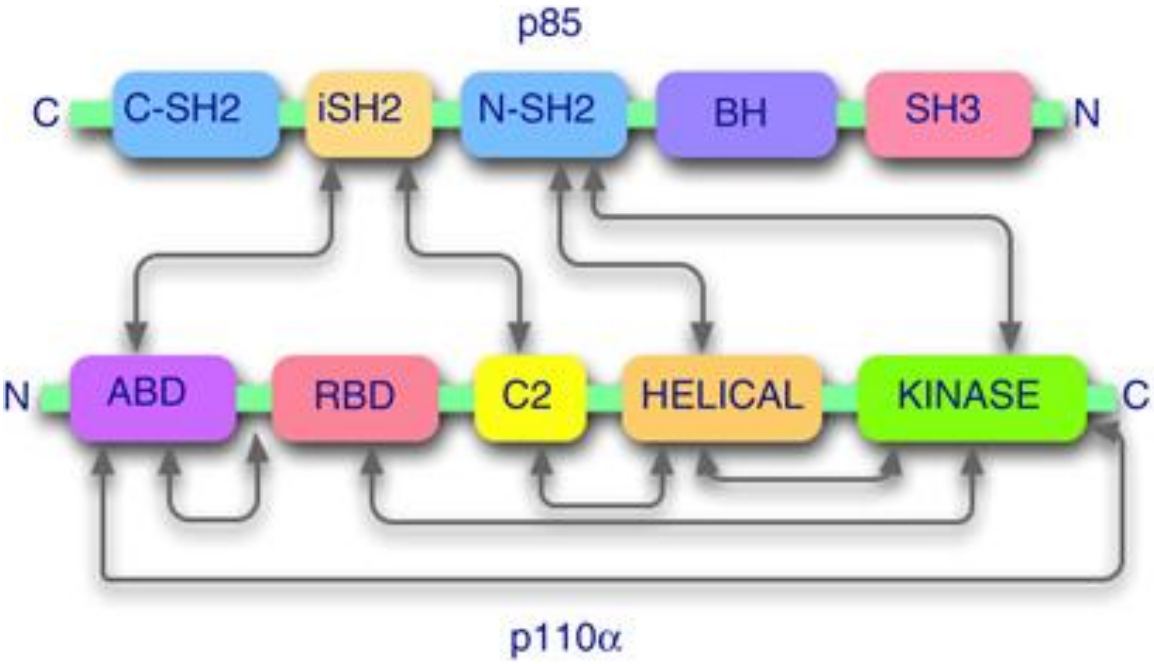



**Figure 1.4: Schematic diagram of the functional domains of and the intramolecular interactions between a PI3K catalytic subunit and a PI3K regulatory subunit**

Structurally, PI3K is a heterodimer, consisting of a regulatory subunit (p85) and a catalytic subunit (here, p110α). The regulatory subunit has two Src Homology-2 (SH2) domains, which interacts with phosphorylated tyrosine residues; a Src Homology-3 (SH3) domain, which interacts with proline-rich domains; a Breakpoint clustered Homology (BH) domain; and an inter-SH2 (iSH2), which is the principal interacting domain with the catalytic subunit. The catalytic subunit contains an Adapter-Binding Domain (ABD), which mediates the major interaction with the regulatory subunit; a Ras-Binding Domain (RBD), to which Ras binds, promoting activation of PI3K; a protein-kinase-C homology-2 (C2) domain, which mediates interactions with lipid membranes; and the Kinase domain, which mediates the lipid phosphorylation activity (Zhao and Vogt 2008).

**PI3K in Cancer and Leukemia**

Hyperactivation of PI3K signaling is found to be a common feature among a wide variety of human malignancies, which is not surprising given the role of PI3K in regulating proliferation, growth, and survival (Luo, Manning et al. 2003). Most often, this hyperactivation of PI3K is the result of oncogenic transformation of proteins such as receptors or kinases upstream of PI3K or through loss of expression or function of the

PI3K-antagonist lipid phosphatase PTEN, which removes the phosphate group at the 3 position, thereby converting the active lipid moiety $PIP_3$, which is the product of PI3K activity, to $PI(4,5)P_2$, the inactive lipid moiety that is also the original substrate of PI3K activity (Vivanco and Sawyers 2002; Luo, Manning et al. 2003; Salmena, Carracedo et al. 2008).

On the other hand, cancer-causing mutations in genes encoding the Class I catalytic or regulatory subunits are not as common. Of the four catalytic subunits, only p110α (*PIK3CA*) has been found to have cancer-causing mutations (Samuels, Wang et al. 2004), and more recently mutations in *PIK3R1*, which encodes the p85α regulatory subunit, have been identified in certain cancers. Interestingly, overexpression of p110δ and p110γ has also been observed in acute myeloid leukemia and chronic myelogenous leukemia, respectively (Skorski, Bellacosa et al. 1997; Sujobert, Bardet et al. 2005; Hickey and Cotter 2006).

In recent years, there has been significant progress in identifying the roles of individual PI3K catalytic subunits in particular physiologic processes and disease states, especially cancer. This research has been complemented by simultaneous efforts to develop newer classes of PI3K inhibitors that target particular catalytic subunits with several fold specificity, by taking advantage of subtle structural differences between the catalytic subunits (Marone, Cmiljanovic et al. 2008). The goal of this work is to inhibit only the activity of the particular catalytic subunit contributing to the pathogenesis of a disease, while sparing the activity of the other catalytic subunits, which may be mediating important normal functions, thus reducing the risk for off-target and potentially toxic side effects.

**The Hematopoietic-Specific Class IA PI3K Catalytic Subunit p110δ**

The Class IA PI3K catalytic subunit, p110δ, like the sole Class IB PI3K catalytic subunit, p110γ, is expressed predominantly in hematopoietic cells, in contrast to p110α and p110β, which are ubiquitously expressed, suggesting that p110δ and p110γ might be ideal pharmacologic targets in diseases involving the hematopoietic system such as autoimmune disorders and leukemias (Okkenhaug, Bilancio et al. 2002; Rommel 2010; Cushing, Metz et al. 2012). While p110γ is associated with signaling mediated by G-Protein-Coupled Receptors (GPCRs), p110δ is typically found promoting activation of PI3K signaling downstream of Receptor Tyrosine Kinases (RTKs) and cytokine receptors, indicating that there are unique functions for each catalytic subunit, though there has been much work recently demonstrating their potential cooperation in many diseases processes (Cushing, Metz et al. 2012). For our studies we focused on p110δ because we wanted to evaluate the role of PI3K in the context of the GM-CSF receptor, which is known to be hyper-responsive to its ligand in the presence of activating Shp2 mutations, and which is known to associate with p85, the major regulatory subunit of Class IA PI3K catalytic subunits, such as p110δ (Itoh, Liu et al. 1998; Chan, Leedy et al. 2005; Perugini, Brown et al. 2010).

A mouse model has been generated bearing a point mutation in the *Pik3cd* gene, which encodes p110δ, so that, as a result, endogenous levels of the kinase-dead mutant p110δ D910A are expressed (rather than a knockout model, in which there potentially may be compensatory upregulation and recruitment of the remaining Class IA catalytic subunits, p110α and p110β) (Okkenhaug, Bilancio et al. 2002). Although, this genetically inactivated *Pik3cd* gene is constitutively expressed, homozygous mutant mice

are born at normal Mendelian ratios and do not exhibit any overt phenotype except some impairment of B- and T-cell maturation and function and the development of a mild inflammatory bowel disease. The fact that p110δ$^{D910A/D910A}$ mice are completely viable with only modest health effects suggest that p110δ would be an ideal therapeutic target since pharmacologic inhibition of its activity would therefore be expected to have minimal side effects (Okkenhaug, Bilancio et al. 2002).

Because the effects of genetic inactivation of p110δ were mostly observed in B- and T-cells, much of the research on the role of p110δ in health and disease have focused on processes involving the lymphoid lineage, such a multiple myeloma, T-cell leukemia, and a variety of inflammatory conditions (Herman, Gordon et al. 2010; Ikeda, Hideshima et al. 2010; Rommel 2010; Lannutti, Meadows et al. 2011; Castillo, Furman et al. 2012). However, p110δ has been found to be overexpressed in Acute Myeloid Leukemia (AML) (Sujobert, Bardet et al. 2005), and it has been demonstrated to mediate proliferation and migration in macrophages (Papakonstanti, Zwaenepoel et al. 2008), which suggests it may also be relevant for JMML, an invasive MPD of monocyte-macrophage lineage cells.

IC87114 was the first PI3K inhibitor with high specificity for p110δ to be developed, but its low potency precluded it from entering clinical trials, though it still remains an important research tool (Berndt, Miller et al. 2010). More recently, GS-1101 (formerly known as CAL-101), an IC87114-derivative with greatly enhanced potency against p110δ has been developed, and has entered clinical trials for lymphoid malignancies (Castillo, Furman et al. 2012).

Another interesting aspect of p110δ is its apparent Ras-independence (Denley, Kang et al. 2008). The conserved structure of all Class I PI3K catalytic subunits contains a Ras-binding domain (RBD) (Figure 1.4) (Zhao and Vogt 2008), to which Ras can bind in order to promote full activation of PI3K signaling. It has been shown that the common oncogenic mutation H1047A in *PIK3CA* (encoding p110α)—along with other nearby mutations in the kinase domain—results in a conformational change similar to that induced by the binding of RAS, thereby promoting its constitutive activation (Zhao and Vogt 2008). These kinase domain mutations of p110α have been shown to promote RAS-independent PI3K hyperactivation as mutating a conserved lysine residue in the RBD (and, thus, abrogating RAS interactions) does not reduce AKT phosphorylation or cellular transforming ability (Zhao and Vogt 2008). Similarly, mutation of the WT p110δ RBD still retains its cellular transforming ability even in the absence of additional activating mutations, suggesting that p110δ, unlike the other Class I PI3K catalytic subunits, can mediate its signaling function independent of RAS interaction (Denley, Kang et al. 2008).

**Mechanisms of Shp2 Regulation of PI3K Activity**

Functional interactions between Shp2 and PI3K have been investigated previously in the context of various physiologic processes and disease states, and the interplay between Shp2 function and PI3K activity is not always consistent. In certain receptor-mediated signaling pathways such as Kit, PDGF receptor, and IGF receptor, it has been found that Shp2 positively regulates PI3K activity, while in the context of other receptors, such as EGF Receptor, Shp2 is apparently a negative regulator of PI3K activity

(Zhang, Tsiaras et al. 2002; Mattoon, Lamothe et al. 2004; Mali, Ma et al. 2012). For example, it has been reported that hyperactivated PI3K signaling, which results from loss-of-function mutations in *PTPN11* that significantly lower Shp2's phosphatase activity, contributes to the etiology of the cardiac defects found in LEOPARD syndrome, indicating that, at least, in regards to normal cardiac development, Shp2 function typically inhibits PI3K activity (Kontaridis, Yang et al. 2008; Edouard, Combier et al. 2010).

The GM-CSF receptor, a heterodimeric cytokine receptor, has been shown, however, to activate PI3K activity upon ligand binding (Perugini, Brown et al. 2010). Furthermore, our laboratory previously reported that murine bone marrow-derived macrophage progenitors transduced with Shp2 D61Y or Shp2 E76K have higher GM-CSF-stimulated activation of Akt compared to cells transduced with empty vector or WT Shp2 (Figure 1.5) (Yang, Li et al. 2008). Furthermore, Shp2 has been shown to positively regulate PI3K activity downstream of the IL-3 receptor, which shares its $\beta_c$ subunit with the GM-CSF receptor, and, therefore, is presumed to promote and regulate signal transduction pathways similarly to the GM-CSF receptor (Mohi, Williams et al. 2005; Yu, Daino et al. 2006). Based on these findings, we hypothesized that hyperactivation of class IA PI3K induced by activating *PTPN11* mutations contributes to the etiology of JMML, leading us to pursue studies examining various components of the PI3K signaling pathway in activating Shp2-induced leukemogenesis.

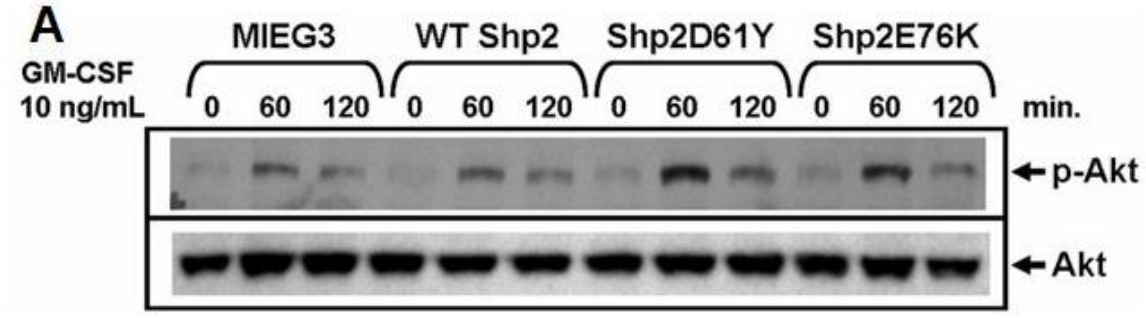



**Figure 1.5: Cells expressing gain-of-function mutant Shp2 D61Y and Shp2 E76K have higher levels of GM-CSF-stimulated Akt phosphorylation**

WT bone marrow-derived LDMNCs transduced with WT Shp2, gain-of-function mutant Shp2 D61Y, gain-of-function mutant Shp2 E76K, or empty vector (Mieg3) were cultured into adherent macrophages and stimulated with 10 ng/mL GM-CSF for 0, 60, and 120 minutes, at which point protein lysates were prepared for western blot analysis (adapted from Yang, Li et al. 2008).

The mechanisms by which Shp2 regulates PI3K remain to be fully elucidated. It is possible that Shp2 regulates PI3K indirectly through Ras, since it is well known that Shp2 promotes activation of Ras, and that PI3K is a direct downstream effector of Ras (Neel, Gu et al. 2003). However, it has been shown that gain-of-function Shp2's phosphatase activity is necessary for promoting full activation of Ras and GM-CSF hypersensitivity, but that a point mutation abrogating mutant Shp2's phosphatase activity has no effect on IL-3-stimulated Akt hyper-phosphorylation, though mutations targeting Shp2's SH2 domains do (Mohi, Williams et al. 2005; Yu, Daino et al. 2006). The authors of this study conclude that mutant Shp2 promotes IL-3-stimulated hyperactivation of PI3K signaling through its scaffolding function, rather than its enzymatic function (Yu, Daino et al. 2006). It has been shown that Shp2 forms a complex with the scaffolding molecules Grb2 and Gab2, and the p85 Class IA PI3K regulatory subunit, and it might be by participating in this complex that Shp2 is able to

promote PI3K activation (Neel, Gu et al. 2003; Yu, Daino et al. 2006; Chan, Kalaitzidis et al. 2008).

**Summary and Significance**

Juvenile Myelomonocytic Leukemia (JMML), although very rare, is still extremely challenging to treat. Unlike other more common forms of pediatric leukemia, such as Acute Lymphoblastic Leukemia (ALL), in which great progress in treatment has been made, JMML remains resistant to standard chemotherapy, and the only curative therapy is allogeneic hematopoietic stem cell transplantation (HSCT) (Loh 2011). Even with current HSCT protocols, survival is still around 50% (Locatelli, Nollke et al. 2005; Bergstraesser, Hasle et al. 2007; Yoshimi, Mohamed et al. 2007), and thus there is a great need for finding more effective targeted therapies. By better understanding the genetic aberrations found in JMML and their molecular consequences, we can identify potential therapeutic targets and improve outcomes in this disease.

Over the past few decades, the work of many researchers have identified a common signaling aberration underlying the pathogenesis of JMML: hyperactivated RAS signaling, either due to activating mutations in *RAS* itself, or in the RAS GAP, *NF1*, which is a direct negative regulator of RAS activity. Most recently, mutations have also been found in the E3 ubiquitin ligase, *CBL*, which promotes hyperactivation of RAS activity through mechanisms that remain incompletely defined. The most commonly mutated gene found in JMML, however, is the *PTPN11* gene, which encodes the protein tyrosine phosphatase, SHP2. SHP2 has also been shown to positively regulate RAS activity, and, like CBL, the mechanism through which this regulation occurs is still being

explored. It will be interesting to see if the remaining 10-15% of JMML cases, for which a causative gene mutation has not been identified, will also involve proteins that regulate RAS activity.

Although we have learned that RAS hyperactivation seems to be the common pathogenic feature of JMML, it still an open question as to how best to interrupt this RAS hyperactivation to achieve a desired therapeutic effect. Targeting RAS itself, although it would be the ideal solution, is complicated. RAS binds GTP with such high affinity, that GTP-competitive inhibitors—analogous to the ATP-competitive inhibitors used to target kinases—are unlikely to be successful (Gysin, Salt et al. 2011). Targeting activating post-translational modifications of RAS has also been explored as a potential therapeutic strategy. Specifically, investigators have explored farnesyl-transferase inhibitors (FTIs), such as tipifarnib, which block the enzymatic addition of hydrophobic farnesyl groups to mature RAS proteins, thereby targeting them to inner leaflet of the plasma membrane where they carry out their signal transduction function (Epling-Burnette and Loughran 2010; Liu, Sabnis et al. 2012). Although it showed promise as a therapy for JMML *in vitro* (Emanuel, Snyder et al. 2000), this strategy has had limited success in clinical trials for multiple cancers as a single agent, however, most likely because other membrane-targeting post-translational modifications, which are not affected by FTIs, such as geranylgeranylation, can circumvent the lack of farnesylation (Epling-Burnette and Loughran 2010).

Thus, if RAS itself cannot be inhibited, and if efforts to block its activation have also been unsuccessful, then another potential strategy to target oncogenic RAS activity and GM-CSF hypersensitivity in JMML is to inhibit one or more of its downstream

effector signaling pathways, such as the RAF-MEK-ERK MAPK pathway and the PI3K-AKT pathway. Much of the effort so far in investigating the aberrant signaling, which is induced by activating SHP2 mutations or other JMML-associated mutations, and which ultimately results in GM-CSF hypersensitivity and leukemogenesis, has focused on the MAPK pathway. Indeed, recent studies using mouse models of *Nf1* knockout- and gain-of-function mutant *KRas* knockin-induced MPD have shown that treatment with the clinical grade MEK inhibitor, PD0325901, can effectively reduce GM-CSF hypersensitivity and disease progression (Lyubynska, Gorman et al. 2011; Chang, Krisman et al. 2013). However, as MEK is ubiquitously expressed, pharmacologically targeting its activity runs the risk of significant toxic side effects. Therefore, it is appropriate to explore other potential targets, which either carry less such risk, or which can be simultaneously inhibited along with MEK, in such a way that lower doses of both inhibitors (with presumably reduced risk of side effects) can be used.

Our laboratory has shown previously, that Akt phosphorylation is also greatly enhanced in gain-of-function mutant Shp2-expressing cells following GM-CSF stimulation (Figure 1.5), suggesting that PI3K also might be an important player in GM-CSF hypersensitivity and the pathogenesis of JMML (Yang, Li et al. 2008). Certainly, hyperactivated PI3K is known to be involved in a wide array of human malignancies, and, as a result, there has been a significant effort to develop new classes of PI3K inhibitors with enhanced potency and specificity (Marone, Cmiljanovic et al. 2008). With this in mind, we wanted to investigate the potential role of PI3K in gain-of-function mutant Shp2-induced GM-CSF hypersensitivity and MPD development.

In the following studies we used genetic and pharmacologic approaches to investigate the role of Class IA PI3K in gain-of-function mutation Shp2-induced GM-CSF hypersensitivity and *in vivo* MPD development. We begin by demonstrating that the GM-CSF-stimulated Akt hyper-phosphorylation in mutant Shp2-expressing cells that our lab had previously reported was in fact due to enhanced PI3K activity (Chapter Three). We then show that Class IA PI3K is an important contributor to gain-of-function mutant Shp2-induced GM-CSF hypersensitivity, both pharmacologically—using the pan-PI3K inhibitor, LY294002—and genetically—by deletion of the *Pik3r1* gene, which encodes the major Class IA PI3K regulatory subunit, p85α, and its splice variants p55α, and p50α (Chapter Three) (Fruman, Snapper et al. 1999; Fruman, Mauvais-Jarvis et al. 2000). In both cases, there were significant reductions in GM-CSF-stimulated hyper-proliferation and Akt hyper-phosphorylation in mutant Shp2-expressing cells. Interestingly, we also found significant reductions in mutant Shp2-induced Erk hyper-phosphorylation, suggesting that Class IA PI3K signaling is an important positive regulator of mutant Shp2-induced hyperactivated MAPK signaling. To increase the potential translational application of our observations, we next wanted to explore the individual contributions of the three Class IA PI3K catalytic subunits, p110α, p110β, and p110δ, again using both genetic and pharmacologic approaches. Here we found that treatment with the p110δ-specific inhibitor, IC87114, or the clinical-grade PI3K inhibitor GDC-0941, which has high specificity for p110δ and p110α (Folkes, Ahmadi et al. 2008), both significantly reduced GM-CSF-stimulated hyper-proliferation and Akt and Erk hyper-phosphorylation, and increased apoptosis in gain-of-function mutant Shp2-expressing cells (Chapter Four). Interestingly, we also found that the highly potent p110δ-specific inhibitor, GS-9820

(formerly known as CAL-120), was able to reduce GM-CSF-stimulated hyper-phosphorylation of both Akt and Erk as well as GM-CSF-stimulated hyper-proliferation at clinically relevant nanomolar concentrations (Chapter Four). Finally, by crossing gain-of-function mutant Shp2 D61Y-inducible knockin mice, which provide an *in vivo* model of mutant Shp2-induced MPD (Chan, Kalaitzidis et al. 2009), with kinase-dead mutant p110δ D910A mice and p110α inducible knockout mice (Okkenhaug, Bilancio et al. 2002; Graupera, Guillermet-Guibert et al. 2008), we found that genetic interruption of p110δ, but not p110α, significantly reduced GM-CSF-stimulated hyper-proliferation and Akt and Erk hyper-phosphorylation *in vitro* in cells endogenously expressing gain-of-function mutant Shp2 D61Y (Chapter Five). We also found that genetic interruption of p110δ, but not p110α, significantly decreased mutant Shp2 D61Y-induced splenomegaly *in vivo* (Chapter Five).

Thus, based on the results of our studies, we propose a model in which activating Shp2 mutations induce hyperactivated PI3K signaling, as well as MAPK signaling, in response to GM-CSF (and possibly other cytokine and growth factor) stimulation, which together promote a pathologically enhanced proliferative and survival response that ultimately results in MPD development (Figure 1.6). In addition, we hypothesize that a crucial mediator of this gain-of-function mutant Shp2-induced PI3K hyperactivation is the hematopoietic-specific, Class IA PI3K catalytic subunit p110δ, and therefore, pharmacologic inhibition of p110δ activity, represents a potential novel therapeutic strategy for the treatment of JMML and other mutant Shp2-induced MPDs. Furthermore, because p110δ's expression is restricted to hematopoietic cells, it is an ideal target for

hematopoietic malignancies like JMML, because there is reduced risk of toxic systemic side effects.

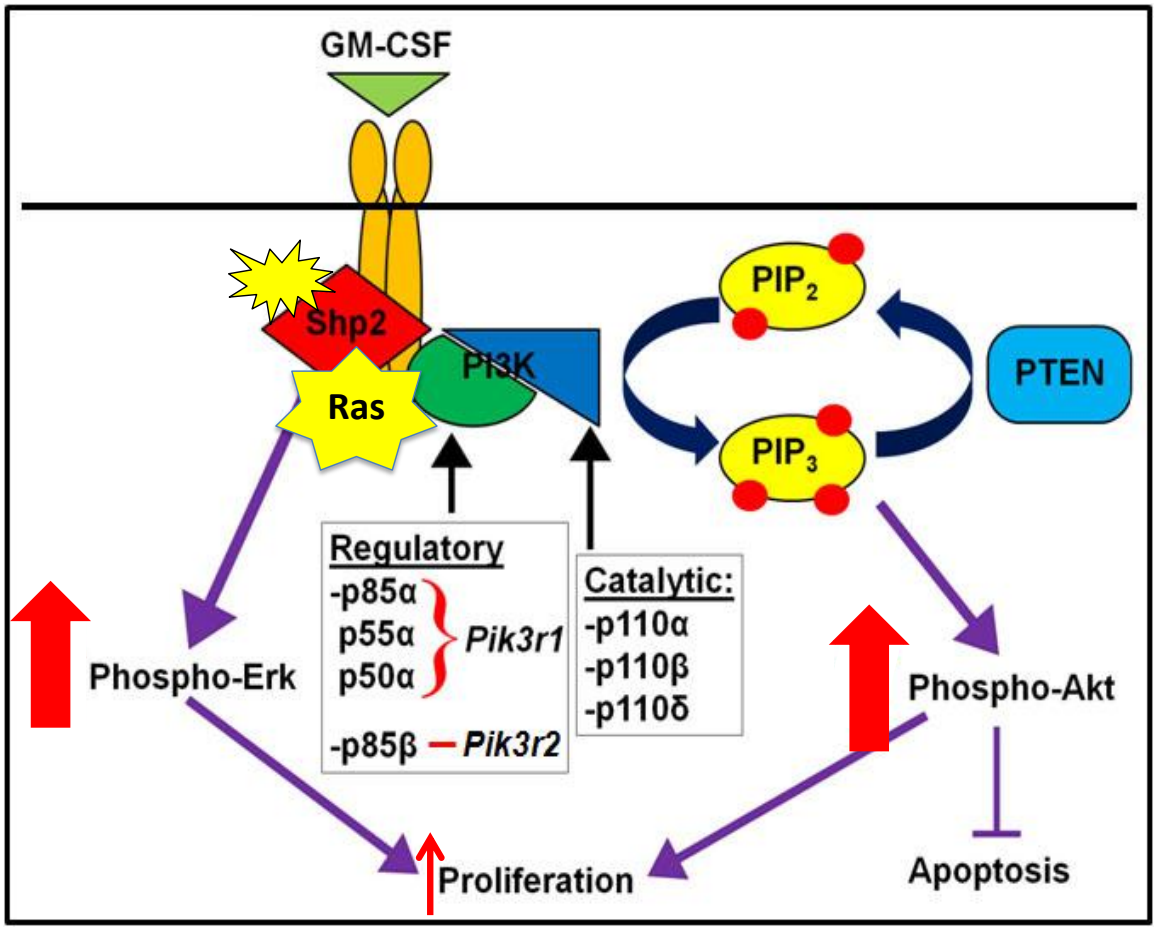



**Figure 1.6: Schematic diagram showing hypothesized mutant Shp2-induced dysregulated signaling that contributes to GM-CSF hypersensitivity**

We hypothesize that mutant Shp2 results in hyperactivation of both the Ras-Erk pathway and the PI3K-Akt pathway, which together promote hyper-proliferation in response to GM-CSF stimulation, ultimately leading to leukemogenesis.

Furthermore, because it has been shown that p110δ can induce oncogenic transformation of cells independently of Ras, we propose a model in which gain-of-function mutant Shp2 can promote hyperactivation of p110δ activity, in a Ras-independent manner. To explore this question, we combined FTI treatment (which prevents Ras activation) with genetic and pharmacologic interruption of PI3K activity. Specifically, we found that *Pik3r1*$^{-/-}$ cells expressing gain-of-function mutant Shp2 E76K were relatively insensitive to treatment with the FTI tipifarnib. We found that addition of the PI3K inhibitor, GDC-0941, resulted in further decreases in mutant Shp2-induced GM-CSF hypersensitivity beyond what was achieved with multiple doses of tipifarnib, suggesting the existence of non-Ras-dependent PI3K activity in the presence of gain-of-function mutant Shp2. Thus, these results suggest that even if successful Ras inhibition could be achieved in the context of activating Shp2 mutations, PI3K would still have to be targeted at least in part as an independent and parallel pathway. It also suggests an alternative model in which, mutant Shp2 can promote aberrant signaling and MPD development via Ras-independent mechanisms, instead of exclusively through promoting hyperactivation of Ras, as it is currently presumed (Figure 1.7).

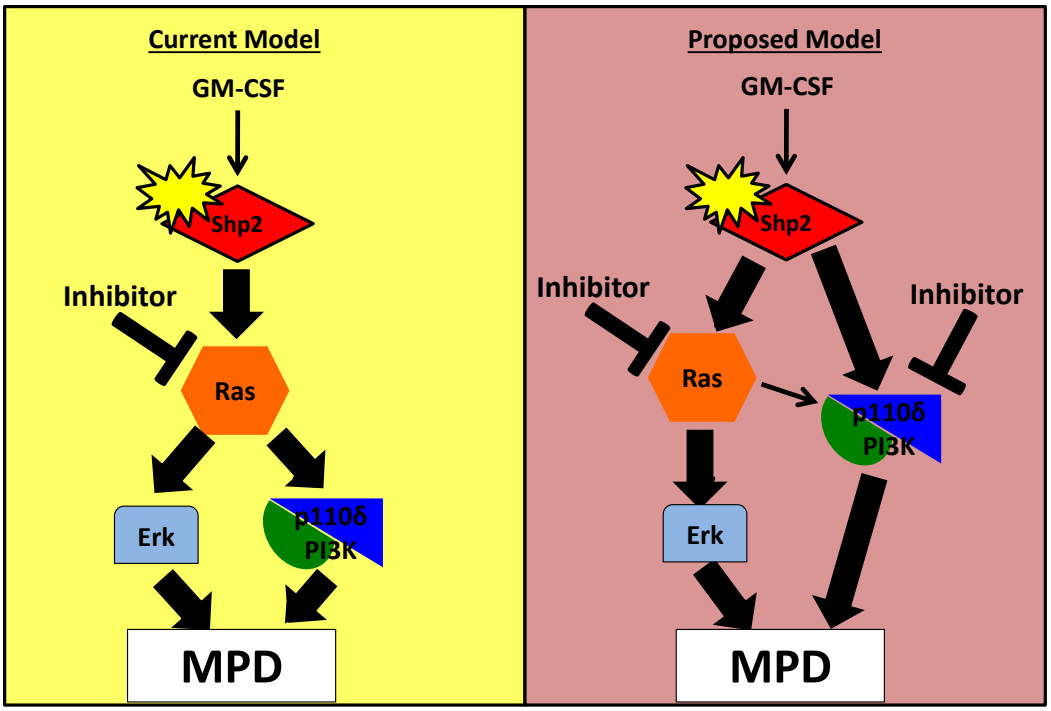



**Figure 1.7: Comparative models of gain-of-function mutant Shp2-induced hyperactivation of PI3K as a Ras-dependent versus Ras-independent process**

The current model of gain-of-function mutant Shp2-induced aberrant signaling leading to MPD development is presumed to be an entirely Ras-dependent process. In comparison, we propose a new model in which gain-of-function mutant Shp2 can promote hyperactivation of p110δ-mediated PI3K hyperactivation in at least a partially Ras-independent manner, suggesting that PI3K must be targeted separately in gain-of-function mutant Shp2-induced MPD.

**Significance**

Although JMML is a rare condition, it still portends a very dismal prognosis, especially compared to other childhood leukemias like ALL, which generally responds favorably to chemotherapy. JMML, on the other hand is resistant to standard chemotherapy, and, thus, the only curative therapy is allogeneic hematopoietic stem cell transplantation, which still only has a survival rate around 50%. Thus, JMML is a disorder desperately in need of more effective therapeutic options.

In these studies, we identify Class IA PI3K signaling, particularly that mediated by p110δ, as an important contributor to mutant Shp2-induced GM-CSF hypersensitivity and MPD development. We found p110δ to be an intriguing target for several reasons. First, both genetic interruption and pharmacologic inhibition of p110δ activity significantly reduced not only mutant Shp2-induced Akt hyper-phosphorylation, but Erk hyper-phosphorylation as well. Thus, rather than observing a compensatory increase in a parallel oncogenic signaling pathway upon pharmacological blockade of PI3K, we found, instead, that we able to reduce flux through two major oncogenic signaling pathways. In contrast, we found that the MEK inhibitor PD0325901, although very efficiently reducing MAPK signaling, had no effect on PI3K signaling.

Another significant aspect of p110δ is the fact that its expression is largely restricted to hematopoietic cells, making it an ideal target for hematologic malignancies. Thus, it can be expected that pharmacologically inhibiting p110δ would have minimal toxic side effects, since very few if any other cell types in the body would have significant p110δ-dependent activities. To illustrate this potential, it should be noted that mice constitutively expressing germline kinase-dead p110δ D910A are viable and fertile

with no significant deficits or gross phenotypes, except for some defects in lymphoid function (Okkenhaug, Bilancio et al. 2002).

Finally, p110δ is also an interesting target to investigate in the context of oncogenic Shp2 signaling, because it has been reported that p110δ, unlike the other Class I PI3K catalytic subunits, can induce oncogenic transformation in a Ras-independent manner (Denley, Kang et al. 2008). Mechanistically, this observation is intriguing because it is traditionally thought that gain-of-function mutant Shp2 promotes aberrant signaling, GM-CSF hypersensitivity, and, ultimately, leukemogenesis by promoting hyperactivation of Ras, which then mediates all downstream dysregulated signaling processes. Thus, this work is significant because it provides evidence that gain-of-function mutant Shp2 can promote aberrant mitogenic signaling at least in part in a Ras-independent manner. As a result, this work has important implications for strategies to treat mutant Shp2-induced MPD by directly targeting Ras hyperactivation, because it suggests that p110δ-mediated PI3K signaling would also have to be targeted as an independent and parallel pathway.

Together, the results of our research demonstrate Class IA PI3K signaling, and particularly PI3K activity mediated by p110δ**,** as an important component of gain-of-function mutant Shp2-induced GM-CSF hypersensitivity and a key mediator of the aberrant oncogenic signal transduction processes that promote the features of enhanced proliferation and survival, which contribute to the pathogenesis of JMML and other forms of mutant Shp2-associated MPD. Thus, we believe this work implicates p110δ as potential novel therapeutic target for the treatment of JMML.

CHAPTER TWO

MATERIALS AND METHODS

## A. Materials

### 1. Plasmids

#### a. pMieg3

pMieg3: WT Shp2, Shp2 D61Y, Shp2 E76K, Shp2 E76K/R32K, Shp2 E76K/R138K, and Shp2 E76K/C463A constructs were expressed using the pMieg3 plasmid. cDNA from murine WT Shp2 and mutated forms of Shp2 generated by site-directed mutagenesis in the bicistronic pMieg3 vector, co-expressing enhanced green fluorescent protein (eGFP) as a marker. Shp2 D61Y and Shp2 E76K are gain-of-function mutant forms of Shp2 commonly found in patients with Juvenile Myelomonocytic Leukemia (Yang, Li et al. 2008). Shp2 E76K/R32K, Shp2 E76K/R138K, and Shp2 E76K/C463A are loss-of-function mutant forms of Shp2 E76K that specifically inactivate the N-SH2, the C-SH2, and the phosphatase functional domains of Shp2 E76K, respectively.

### 2. Primers

Standard PCR reactions were performed using oligonucleotides synthesized by Invitrogen.

**Table 2.1: Genotyping primers**

| Gene | Forward (5'-3') | Reverse (3'-5') |
|---|---|---|
| *Pik3r1*-WT | 5'-TCCAAATGAAAAGAACGGCATC-3' | 5'-TGTACCAAAGTCACTGAAAAATCTGTT-3' |
| *Pik3r1*-Neo | 5'-AAGATAATATTGAAGCTGTAGGGAAAAA-3' | 5'-TGTTAAGAAGGGTGAGAACAGAGTACC-3' |
| Shp2 Exon 3 (Shp2 D6Y) | 5'-CAAGGTGAGTGGGCGTTTCATTTTAAC-3' | 5'-ACCTTTCAGAGGTAGGGTCTGCAC-3' |

**Table 2.1: Genotyping primers (continued)**

| Gene | Forward (5'-3') | Reverse (3'-5') |
|---|---|---|
| p110δ D910A | 5'-GCGTAACAGAGAGCAAAG TCCC-3' | 5'-AGGGAACCGCCGTATGAC-3' |
| p110α flox | 5'-GGATGCGGTCTTTATTGTC-3' | 5'-TGGCATGCTGCCGAATTG-3' |
| Mx1-Cre | 5'-GCCTGCATTACCGGTCGAT GCAACGAGTG-3' | 5'-CTGGCAATTTCGGCTATAC GTAACAGGGTG-3' |

**Table 2.2: Site-directed mutagenesis primers**

| Gene | Forward (5'-3') | Reverse (3'-5') |
|---|---|---|
| Shp2 R32K | 5'-AAGTTTGCCACTTTGGCTA AACTGGTTCAGTATTACA-3' | 5'-TGTAATACTGAACCAGTTT AGCCAAAGTGGCAAACTT-3' |
| Shp2 R138K | 5'-CATGGCAGCTTCCTCGTTA AAGAGAGCCAGAGCCACC-3' | 5'-GGTGGCTCTGGCTCTCTTT AACGAGGAAGCTGCCATG-3' |
| Shp2 C463A | 5'-CGGCCAATCCCAGCGCTGG CGTGAACCACGACAGGGC-3' | 5'-GCCCTGTCGTGGTTCACGC CAGCGCTGGGATTGGCCG-3' |

## 3. Mice

### a. C57Bl/6

C57Bl/6 mice were received from Harlan and were used to isolate wild type bone marrow low-density mononuclear cells (LDMNCs).

### b. *Pik3r1*$^{-/-}$

*Pik3r1*$^{-/-}$ mice were a generous gift from Dr. Lewis C. Cantley (Fruman, Mauvais-Jarvis et al. 2000).

### c. LSL-Shp2$^{D61Y/+}$

LSL-Shp2$^{D61Y/+}$ mice were a generous gift from Dr. Benjamin G. Neel. They were crossed with Mx1-Cre mice, p110δ$^{D910A/D910A}$ mice, and p110α$^{flox/flox}$ mice (Chan, Kalaitzidis et al. 2009).

### d. Mx1-Cre

Mx1-Cre mice were a generous gift from Dr. Reuben Kapur.

### e. p110δ$^{D910A/D910A}$

p110δ$^{D910A/D910A}$ mice were a generous gift from Dr. Reuben Kapur (Okkenhaug, Bilancio et al. 2002).

### f. p110α$^{flox/flox}$

p110α$^{flox/flox}$ mice were a generous gift from Dr. Reuben Kapur (Graupera, Guillermet-Guibert et al. 2008).

## 4. Antibodies

**Table 2.3: Primary antibodies**

| Primary Antibodies | Company | Clone |
|---|---|---|
| Total Akt | Cell Signaling | N/A |
| Phospho-Akt (S473) | Cell Signaling | 587F11 |
| Phospho-Akt (T308) | Cell Signaling | 244F9 |
| Total Erk | Cell Signaling | N/A |
| Phospho-Erk | Cell Signaling | N/A |
| Total Shp2 | Santa Cruz | C-18 |
| Phospho-Shp2 (Y580) | Cell Signaling | N/A |
| p85α | AbD Serotec | U5 |
| p110α | Cell Signaling | C73F8 |
| p110β | Santa Cruz | S-19 |
| p110δ | BD Biosciences | 29 |
| GAPDH | Biodesign International | 6C5 |
| Annexin V-APC | BD Pharmigen | N/A |

**Table 2.4: HRP Secondary antibodies**

| Secondary Antibody | Vendor |
|---|---|
| Goat Anti-Rabbit IgG HRP | Santa Cruz |
| Goat Anti-Mouse IgG HRP | Santa Cruz |

## 5. Inhibitors

**Table 2.5: Inhibitors**

| Inhibitor | Vendor/Source | Target |
|---|---|---|
| LY294002 | Selleck | Pan-PI3K |
| IC87114 | Selleck | PI3K p110δ |
| GDC-0941 | Selleck | Pan-PI3K (p110δ and p110α) |
| TGX-221 | Cayman | p110β |
| GS-9820 | Gift from Gilead Sciences | p110δ |
| Tipifarnib | Selleck | Ras (via Farnesyl-Transferase) |
| PD0325901 | Selleck | MEK |

## 6. Kits

**Table 2.6: Kits**

| Kit | Vendor | Catalog Number |
|---|---|---|
| Plasmid Maxi-Prep Kit | Invitrogen | K210007 |
| SuperSignal West Dura Extended Duration Substrate | Thermo Scientific | 34076 |
| Mini-prep kit | Qiagen | 27106 |
| Profection Mammalian Transfection System | Promega | E1200 |
| Annexin V Apoptosis Kit | BD Pharmigen | 556547 |
| QuikChange Site-Directed Mutagenesis Kit | Agilent | 200518-5 |
| QIAquick Gel Extraction Kit | Qiagen | 28704 |
| QIAquick PCR Purification Kit | Qiagen | 28104 |

## B. Methods

### 1. Cell Culture

#### a. Primary Murine Bone Marrow-Derived Low-Density Mononculear Cells (LDMNCs)

Primary murine bone marrow-derived LDMNCs were isolated from total bone marrow cells by Histopaque ficoll gradient (Sigma-Aldrich), and cultured in IMDM (Invitrogen), 20% FBS (Hyclone), 2% Penicillin/Streptomycin (Invitrogen), Granulocyte-Colony-Stimulating Factor (G-CSF), Stem Cell Factor (SCF), and Thrombopoietin (TPO) at 100 ng/mL concentration each. LDMNCs were maintained at 37˚C and 5% $CO_2$. LDMNCs are cultured for a maximum of 9-10 days, changing their media every 2-4 days.

#### b. Primary Murine Fetal Liver Cell-Derived Hematopoietic Progenitor Cells

Primary murine fetal liver cell-derived hematopoietic progenitor cells were isolated from day 14.5 embryos and cultured in IMDM, 20% FBS, 2% Penicillin/Streptomycin, G-CSF, SCF, and TPO at 100 ng/mL concentration each. They were maintained at 37˚C and 5% $CO_2$ and were cultured for a maximum of 9-10 days, changing their media every 2-4 days.

#### c. Adherent Macrophage Progenitor Cells

Primary murine LDMNCs or fetal liver cell-derived hematopoietic progenitors were cultured in IMDM, 20% FBS, 2% Penicillin/Streptomycin, and 50 ng/mL Macrophage-Colony-Stimulating Factor (M-CSF) for up to 7-10 days 37˚C and 5% $CO_2$ to generate adherent macrophage progenitors. A full media change was required after they become adherent at day 5-6, and every 2-3 days thereafter.

**d. Primary Murine Splenocytes**

Primary murine splenocytes were isolated by Histopaque ficoll gradient from total spleen cells.

**e. Eco-Phoenix Packaging Cells**

Eco-Phoenix packaging cells were cultured in DMEM (Invitrogen), 10% FBS, 1% Penicillin/Streptomycin, 1% L-glutamine (Invitrogen), and 1% Sodium pyruvate (Invitrogen) at 37˚C and 5% $CO_2$. Cells were split every 2-3 days.

**2. Retroviral Supernatant Production**

Retroviral supernatants were produced by transfecting the Eco-Phoenix cells with retroviral vector plasmids using calcium phosphate transfection (ProFection® Mammalian Transfection System, Promega, Madison, WI). Supernatants, collected at 48, 72, and 96 hours post-transfection were filtered through 0.45 μm filters and concentrated 3-6X by ultra-centrifugation.

**3. Retroviral Transduction of Primary Murine Cells**

Primary murine bone marrow-derived LDMNCs or fetal liver cell-derived hematopoietic progenitors were transduced with 2 mL pMieg3-Shp2, pMieg3-Shp2 D61Y, pMieg3 E76K, pMieg3-Shp2 E76K/R32K, pMieg3-Shp2 E76K/R138K, pMieg3-Shp2 E76K/C463A retroviral supernatant, supplemented with 100 ng/mL each G-CSF, SCF, and TPO in 6-cm non-tissue culture plates coated with Retronectin ® (Takara) for 24 hours at 37˚C. After 24 hours, cells were collected, transduced again with fresh retroviral supernatant and growth factors, and incubated overnight at 37˚C. The following

morning, cells were collected and plated back in IMDM, 20% FBS, 2% Penicillin/Streptomycin and 100 ng/mL each G-CSF, SCF, and TPO for 24 hours before performing Fluorescence-Activated Cell Sorting (FACS).

**4. Cell Sorting**

Retrovirally transduced cells were sorted using FACS to collect enhanced green fluorescent protein-positive (eGFP+) cells.

**5. Isolation of Total Cellular Protein Lysates**

For isolation of total cellular protein lysates, cells were washed twice with cold PBS and then incubated on ice for 5-10 minutes on ice in a lysis buffer containing 50 mM Hepes, 150 mM NaCl, 10% Glycerol, 1% Triton X, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, and 10 mM $NaPP_i$ with sodium orthovanadate, $ZnCl_2$, PMSF, and protease inhibitor cocktail (Sigma). Protein lysates were than centrifuged at 13000 rpm for 15 minutes at 4˚C and quantified using Bradford reagent.

**6. Immunoblot Analysis**

Protein lysates were separated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane, which was then incubated overnight in primary antibody (see Table 2.3) at 4˚C with shaking. The following day, the membrane was washed and incubated with HRP-conjugated secondary antibody (see Table 2.4), developed with Supersignal and exposed to X-Ray film or by using a Biorad Imager

## 7. PI3K Activity Assay

Protein lysates were added to lipid micelles formed by sonication containing Phosphatidylinositol (PI; Avanti Polar Lipids), Phosphatidylinositol-(4,5)-bisphosphate ($PIP_2$; Avanti Polar Lipids), and Phosphatidyl-serine (PS; Avanti Polar Lipids). To this mixture, was added [$^{32}$P]-labeled ATP, which was then allowed to incubate at 37˚C for 5 minutes, so that the [$^{32}$P]-labeled γ-phosphate of ATP could be incorporated into $PIP_2$ by PI3K activity to form $PIP_3$. The lipids from this reaction were then spotted on a plate an allowed to separate by thin layer chromatography (TLC) overnight. The following morning, the TLC plate was exposed to X-ray film to assess the amount of [$^{32}$P]-labeled $PIP_3$ generated by the PI3K activity of the protein lysates.

## 8. [$^3$H]-Thymidine Incorporation Assay

Cells were starved for 4-6 hours in IMDM containing 0.2% Bovine Serum Albumin (BSA) (Roche) and then plated in 96-well plate (200 μL IMDM with 10% FBS and 2% Penicillin/Streptomycin/well) for [$^3$H]-Thymidine incorporation assays (1-5 x $10^4$ cells/well) either at baseline (no growth factor) or in the presence of 0.01 ng/mL, 0.1 ng/mL, and 1 ng/mL Granulocyte Macrophage-Colony-Stimulating Factor (GM-CSF), with or without indicated inhibitors and incubated overnight at 37˚C. The following morning, cells were pulsed with 1 μCi [$^3$H]-Thymidine and incubated for 5-6 hours at 37˚C. [$^3$H]-Thymidine incorporation was measured using an automated 96-well cell harvester and scintillation counter (Brandel, Gaithersburg, MD).

## 9. Apoptosis Assay

Cells were then collected and counted. $5 \times 10^4$ cells were plated into 12-well non-tissue culture plates in 0.5 mL IMDM with 10% FBS and 2% Penicillin/Streptomycin and 0 ng/mL GM-CSF or 10 ng/mL GM-CSF with 1 μM IC87114 or 0.5 μM GDC-0941 or no inhibitor and incubated at 37˚C for 48 hours. The cells were then collected into individual 5-mL polystyrene tubes, washed with PBS and resuspended in 100 μL 1X buffer (BD Pharmingen FITC-Apoptosis Detection Kit I #556547 with APC-Annexin V #51-65874X (556420)) supplemented with 5 μL propidium iodide (BD#51-66211E(556463)) and 5 μL Annexin V-APC. Cells were incubated in the dark at room temperature for 30 minutes. Finally, 200-400 μL of 10X Annexin V Binding Buffer (BD# 51-66121E) was added and FACS analysis performed using an Accuri C6 Flow Cytometer.

## 10. Methylcellulose Colony-Forming Assays

Bone Marrow Low-Density Mononuclear Cells ($1.0 \times 10^5$) were plated in duplicate in 1 mL progenitor assays containing 1% methylcellulose (ES-Cult® M3120, Stem Cell Technologies), 30% FBS, 2% penicillin/streptomycin, 1% glutamine, and 80 μM β-mercaptoethanol in the absence or presence of increasing concentrations of GM-CSF (0.1-10 μM). Methylcellulose assays were incubated at 37˚C for 7-10 days before total number of colonies were counted.

**11. Statistical Analysis**

Statistics for Figure 3.4A were performed using Prentice's rank sum test for replicated block data (Prentice 1979) and reported as mean +/- standard error of the mean (SEM). Statistics for all other experiments were performed using unpaired, two-tailed student's test, and figures show mean +/- standard deviation (s.d.) or mean +/- standard error of the mean (SEM), as indicated in the figure.

# CHAPTER THREE

# GENETIC DISRUPTION OF p85α, p55α, AND p50α REDUCES GAIN-OF-FUNCTION MUTANT SHP2-INDUCED GM-CSF HYPERSENSITIVITY

## Introduction

Our laboratory has previously shown that Akt is hyper-phosphorylated following GM-CSF stimulation in murine bone marrow low-density mononuclear cell (LDMNC)-derived macrophage progenitors expressing gain-of-function mutant Shp2 (Figure 1.5) (Yang, Li et al. 2008). However, the role of mutant SHP2 in promoting hyperactivation of PI3K remains to be demonstrated, as does the potential contribution of PI3K signaling to gain-of-function mutant SHP2-induced GM-CSF hypersensitivity—a hallmark of Juvenile Myelomonocytic Leukemia (JMML)—and, as a result, to the pathogenesis of mutant SHP2-associated myeloproliferative disease (MPD). Hyperactivation of PI3K signaling is frequently observed in most cancers, and consequently, much work in recent years has been dedicated to developing new classes of PI3K inhibitors with higher potency and specificity and assessing their suitability as a potential targeted therapy for a variety of human neoplasms and other diseases (Luo, Manning et al. 2003; Marone, Cmiljanovic et al. 2008).

Gain-of-function mutations in SHP2 are known to cause hyperactivation of RAS, which is thought to promote hyperactivation of growth and survival-promoting signals that contribute to the initiation and progression of MPDs such as JMML. The causative role of RAS hyperactivation in leukemogenesis is further evidenced by the fact that commonly encountered mutations in JMML, other than activating *PTPN11* mutations, include gain-of-function mutations in *RAS* itself, and loss of function mutations in the

negative regulator of *RAS*, *NF1*. Although RAS is known to regulate several signaling pathways that promote proliferation, growth, and survival, much of the attention has focused on SHP2's effects on the RAF-MEK-ERK pathway. However, it has been demonstrated that RAS can directly bind and activate PI3K (Rodriguez-Viciana, Warne et al. 1994; Rodriguez-Viciana, Warne et al. 1996; Chan, Rodeck et al. 2002), suggesting that PI3K may also play a role mutant SHP2-induced hyper-proliferation in response to GM-CSF stimulation, as well as mutant SHP2-induced MPD development.

Structurally, Class IA PI3K is a lipid kinase heterodimer, composed of one regulatory subunit, and one of three catalytic subunits, p110α, p110β, or p110δ. The regulatory subunit serves to stabilize the expression of the catalytic subunits by preventing their rapid degradation, and to recruit the catalytic subunits to specific phospho-tyrosine residues on activated receptors and scaffolding molecules, thus facilitating activation of PI3K signaling (Zhao and Vogt 2008). Genetically ablating Class IA PI3K signaling by targeting the catalytic subunits would be technically challenging, as it would require genetic interruption of all three genes encoding the Class IA catalytic subunits. Instead, we chose the approach of using an available mouse model, in which the *Pik3r1* gene, encoding the main Class IA PI3K regulatory subunit, p85α and its splice variants p55α and p50α, has been genetically disrupted (Fruman, Mauvais-Jarvis et al. 2000), resulting in dramatically reduced expression of the Class IA catalytic subunits and, thus, reduced Class IA PI3K activity.

By introducing WT Shp2 or gain-of-function mutant Shp2 E76K, a mutation commonly found in JMML patients, into *Pik3r1*$^{-/-}$ hematopoietic progenitor cells, we were able to assess the effects of the loss of p85α, p55α, and p50α on gain-of-function

mutant Shp2-induced hyper-proliferation and hyperactivation of PI3K signaling in response to GM-CSF stimulation.

## Results

### *Murine Bone Marrow Low-Density Mononuclear Cell-Derived Macrophages expressing gain-of-function mutant Shp2 E76K have increased PI3K activity*

Previously, our lab has shown the bone marrow LDMNC-derived macrophage progenitors expressing activating Shp2 mutants have higher levels of Akt phosphorylation following GM-CSF stimulation than WT Shp2-expressing cells (Yang, Li et al. 2008). We hypothesized that this increase in Akt activation was a result of gain-of-function mutant Shp2-induced hyperactivation of PI3K, which we measured using an *in vitro* kinase assay.

Bone marrow-derived LDMNCs from WT mice were isolated by Histopaque ficoll gradient, and plasmids containing WT Shp2 or Shp2 E76K were introduced by fibronectin-assisted retroviral transduction in the presence of 100 ng/mL each, Granulocyte-Colony-Stimulating Factor (G-CSF), Stem Cell Factor (SCF), and Thrombopoietin (TPO). The retroviral vectors expressed each Shp2 cDNA in tandem with enhanced Green Fluorescent Protein (eGFP), allowing successfully transduced cells to be selected by Fluorescence-Activated Cell Sorting (FACS). The transduced progenitor cells were then cultured into adherent macrophages, and following 7-10 days in culture, the adherent macrophages were starved overnight in IMDM, and the following morning were stimulated for 60 minutes with either 0 ng/mL GM-CSF or 10 ng/mL GM-CSF, followed by lysis to collect total cellular protein. The protein lysates were then

used for an *in vitro* PI3K activity assay. We found that both at baseline, and following a 60-minute stimulation with 10 ng/mL GM-CSF, Shp2 E76K-expressing cells had higher levels of PI3K activity, indicated by the darker spots on the X-ray film, which correlates with the amount [$^{32}$P]-labeled phosphate incorporated into the phosphatidyl-(4,5)-bisphosphate to produce $PIP_3$, the active second messenger product of PI3K (Figure 3.1) (Goodwin, Yang et al. 2012). Indeed, WT Shp2-expressing cells had an increase in $PIP_3$ production, following GM-CSF stimulation, but it was markedly lower than that seen in the Shp2 E76K-expressing cells, indicating that GM-CSF stimulation promotes activation of PI3K, which is significantly enhanced in the presence of an activating Shp2 mutation.

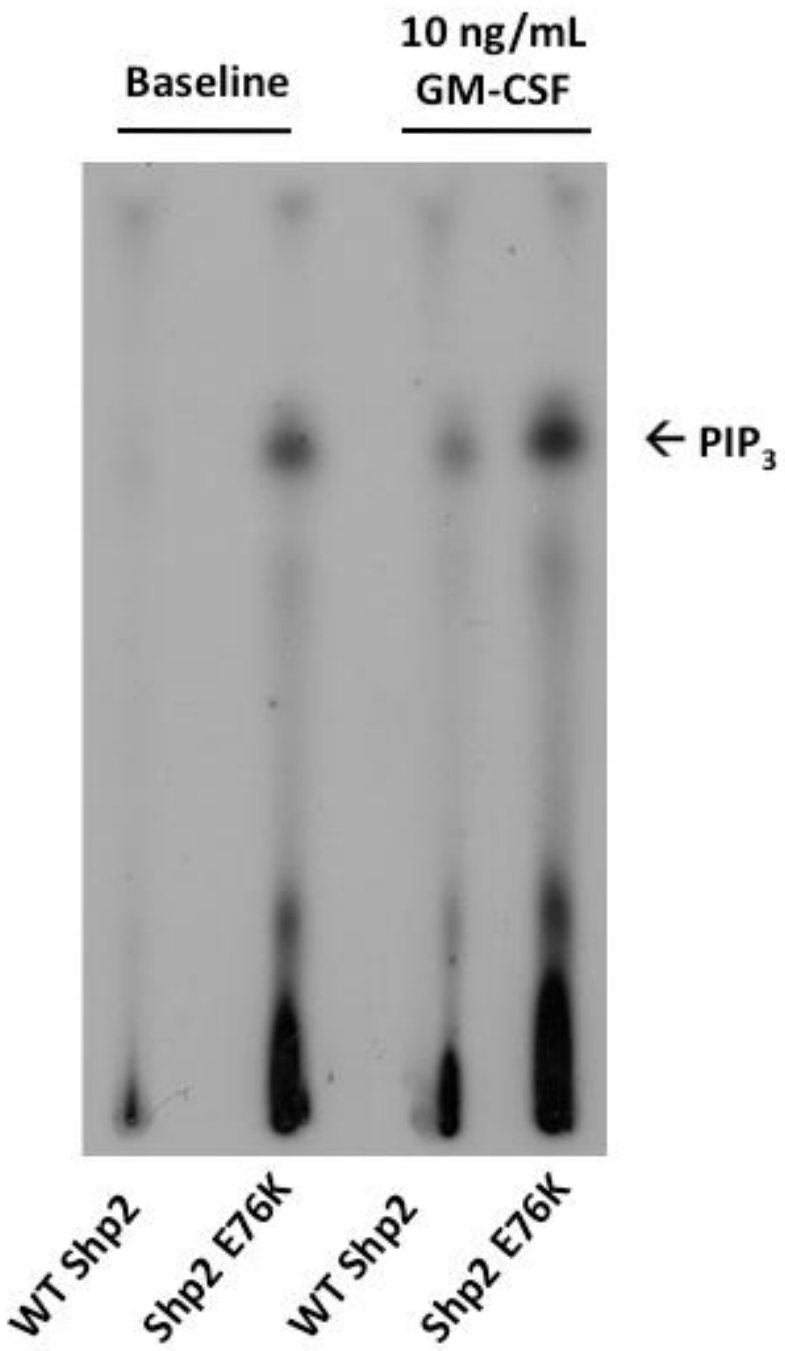



**Figure 3.1: Gain-of-function mutant Shp2 E76K-expressing cells demonstrate increased PI3K activity**

PI3K activity assay showing increased Phosphatidylinositide-(3,4,5)-triphosphate ($PIP_3$) production both at baseline and following a 60-minute stimulation with 10 ng/mL GM-CSF in adherent macrophages expressing Shp2 E76K compared to those expressing WT Shp2, indicating increased basal and GM-CSF-stimulated PI3K activity in the presence of activating mutant Shp2 E76K (Goodwin, Yang et al. 2012).

***Pharmacologic inhibition of PI3K using the pan-PI3K inhibitor, LY294002, reduces gain-of-function mutant Shp2-induced hyper-proliferation in response to GM-CSF stimulation***

Having demonstrated that PI3K is hyperactivated in the presence of gain-of-function Shp2 mutations, we next wanted to determine whether the substantially increased PI3K activity in response to GM-CSF stimulation contributed to the classic phenotype of GM-CSF hypersensitivity—or enhanced proliferation response following GM-CSF stimulation—that is observed in hematopoietic progenitors expressing gain-of-function mutant Shp2. To do this, we performed to [$^{3}$H]-Thymidine incorporation assays to measure GM-CSF-stimulated proliferation of Shp2 E76K-expressing cells in the presence of LY294002, a commonly used pan-PI3K inhibitor. Proliferation of WT Shp2 and Shp2 E76K-transduced cells were assessed in the presence of 0 ng/mL GM-CSF or 1 ng/mL GM-CSF with 0 μM, 5 μM, or 15 μM LY294002 (Figure 3.2A). Alternatively, cells were plated in the presence or absence of 5 μM LY294002 with increases concentrations of GM-CSF (Figures 3.2B). As previously described, hematopoietic progenitors expressing gain-of-function mutant Shp2 E76K demonstrate enhanced proliferation both in the absence of growth factors, and in the presence of increasing concentrations of GM-CSF. We found that treatment with LY294002 caused a concentration-dependent decrease in the proliferation of Shp2 E76K-expressing cells stimulated with 1 ng/mL GM-CSF (Figure 3.2A) (Goodwin, Yang et al. 2012). Similarly, we found that treatment with 5 μM LY294002 significantly reduced the hyper-proliferative response in Shp2 E76K-expressing cells induced by the increasing concentrations of GM-CSF (Figure 3.2B) (Goodwin, Yang et al. 2012). These data

suggest that cells expressing activating Shp2 mutations not only promote hyperactivation of PI3K in response to GM-CSF stimulation, but that this hyperactivated PI3K signaling contributes to the hyper-proliferative phenotype.

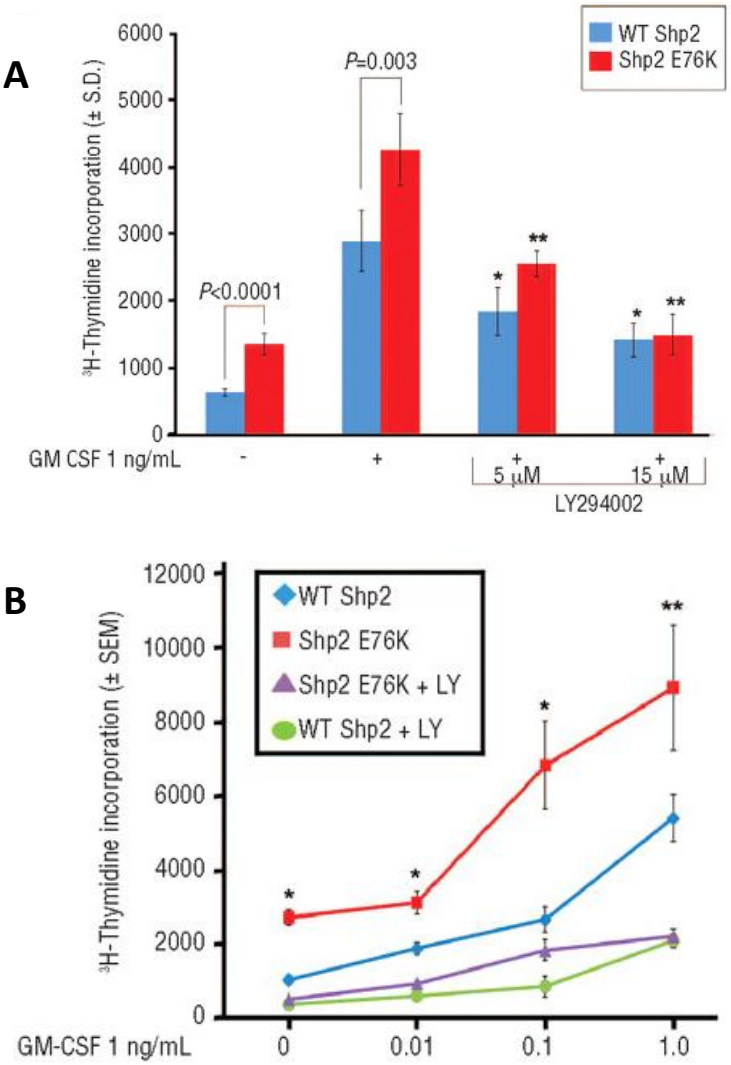



**Figure 3.2: Pharmacologic inhibition of PI3K with LY294002 reduces gain-of-function mutant Shp2 E76K-induced GM-CSF hypersensitivity**

(A) [$^3$H]-Thymidine incorporation assay measuring 1 ng/mL GM-CSF-stimulated proliferation in WT Shp2- and gain-of-function mutant Shp2 E76K-transduced cells in the presence of increasing concentrations of the PI3K inhibitor, LY204002 (0, 5, and 15 μM). Data are representative of two independent experiments; n= 4-6 samples; *p < 0.005 comparing WT Shp2-expressing cells in the absence to the presence of LY294002 at 5 μM and 15 μM; **p < 0.001 comparing Shp2 E76K-expresing cells in the absence to the presence of LY294002 at 5 μM and 15 μM; statistics performed using unpaired, two-tailed student's t-test. (B) [$^3$H]-Thymidine incorporation assay measuring proliferation in response to increasing concentrations of GM-CSF (0-1 ng/mL GM-CSF) in the presence or absence of 5 μM LY294002; n = 3 samples; *p < 0.05 and **p = 0.06 comparing Shp2 E76K-expressing cells in the presence (purple line) or absence (red line) of LY294002; statistics performed using unpaired, two-tailed student's t-test (Goodwin, Yang et al. 2012).

### *Genetic disruption of the* **Pik3r1***-encoded Class IA PI3K regulatory subunits, p85α, p55α, and p50α, results in reduced gain-of-function mutant Shp2-induced GM-CSF hypersensitivity*

While LY294002 inhibition of proliferation provides useful insight toward the relevance of PI3K in gain-of-function Shp2-mediated GM-CSF hypersensitivity, interpretation of these data is limited as LY294002 is able to inhibit all classes of PI3K (IA, IB, II, and III) as well as to inhibit the function of PI3K-related protein kinases (Knight 2010). To further explore the hypothesis that gain-of-function mutant Shp2-induced GM-CSF hypersensitivity is at least in part mediated by hyperactivated Class IA PI3K signaling, we next used a genetic approach. We used an existing mouse model in which the *Pik3r1* gene, which encodes the major Class IA regulatory subunit, p85α, along with its splice variants, p55α and p50α, has been constitutively disrupted. Based on our previous results (Figure 3.2), we hypothesized the genetic disruption of the *Pik3r1*-encoded regulatory subunits would result in significantly reduced PI3K signaling and Akt hyperactivation. Because of the cross-talk between the PI3K and MAPK signaling pathways, we also predicted a reduction in GM-CSF-stimulated Erk hyperactivation. By diminishing these two pro-growth signaling pathways, we hypothesized that genetic disruption of p85α and its splice variants would result in reduced gain-of-function mutant Shp2-induced hyper-proliferation in response to GM-CSF stimulation (Figure 3.3).

Because mice with homozygous deletion of the *Pik3r1* gene typically die around embryonic day 15, timed matings between heterozygous males and females were performed in which hematopoietic progenitor cells were isolated from fetal livers of

homozygous *Pik3r1*$^{-/-}$ embryos and from WT littermates. Plasmids containing WT Shp2 and gain-of-function mutant Shp2 E76K were introduced into these hematopoietic progenitors by Fibronectin-assisted retroviral transduction. Positively transduced cells were then used for [$^{3}$H]-Thymidine incorporation to measure proliferation in response to GM-CSF stimulation or cultured into adherent macrophages for biochemical analyses.

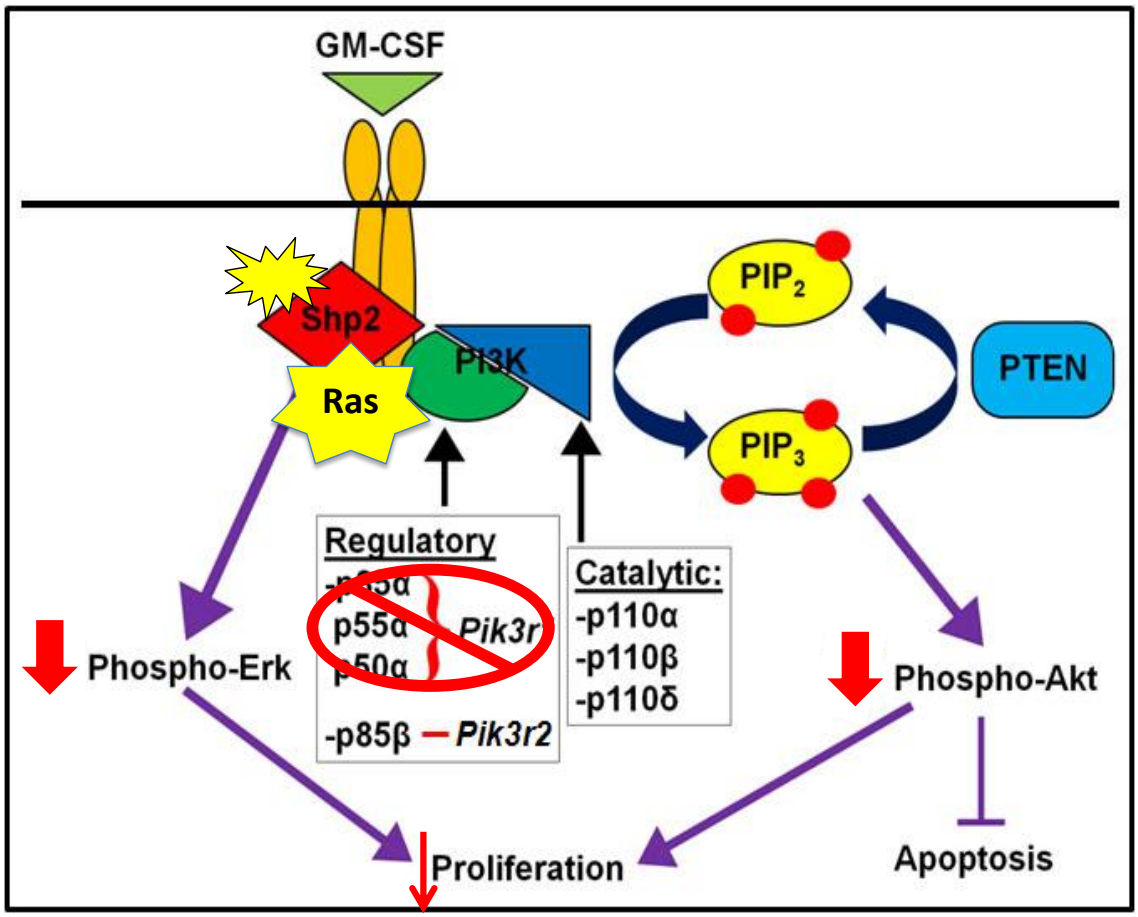



**Figure 3.3: Schematic representation of hypothesized role of Class IA PI3K in gain-of-function mutant Shp2-induced GM-CSF hypersensitivity**

We hypothesize that Class IA PI3K signaling is a major contributor to gain-of-function mutant Shp2-induced GM-CSF hypersensitivity, such that genetic disruption of the *Pik3r1* gene, which encodes the major Class IA PI3K regulatory subunits p85α, p55α, and p50α, will result in reduced GM-CSF-stimulated PI3K-Akt signaling, Erk activation, and resulting GM-CSF hyper-proliferation.

We found that genetic disruption of the *Pik3r1*-encoded regulatory subunits resulted in a significant, but partial, reduction in mutant Shp2 E76K-induced hyper-proliferation in response to increasing concentrations of GM-CSF stimulation. A complete correction in proliferation similar to that of WT Shp2-expressing cells was not achieved, indicating that the hyperactivated PI3K and/or MAPK signaling pathways were only partially decreased by the loss of p85α and its splice variants (Figure 3.4A) (Goodwin, Yang et al. 2012).

Biochemical analyses of the WT and *Pik3r1*$^{-/-}$ cells expressing either WT Shp2 or Shp2 E76K revealed that WT cells expressing Shp2 E76K had higher levels of phospho-Akt (both Serine 473 and Threonine 308) and phospho-Erk than those cells expressing WT Shp2, both at baseline and following a 60-minute stimulation with 10 ng/mL GM-CSF, consistent with previous findings (Figure 3.4B, compare lanes 1 and 2 and lanes 5 and 6) (Goodwin, Yang et al. 2012). Interestingly, we found that genetic disruption of *Pik3r1* substantially reduced mutant Shp2-induced Akt hyper-phosphorylation at both Serine 473 and Threonine 308, both at baseline and following GM-CSF stimulation (Figure 3.4B, compare lanes 2 and 4 and lanes 6 and 8). Furthermore, Erk hyper-phosphorylation was also substantially reduced in *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K (Figure 3.4B, compare lanes 2 and 4 and lanes 6 and 8). This suggests that PI3K plays a positive regulatory role in Erk activation, and that the gain-of-function Shp2-mediated hyperactivation of Erk is in part due to or reinforced by hyperactivated PI3K signaling. When targeting one signaling pathway, it is important to consider the effects on other signaling pathways, which may become disinhibited or hyperactivated when another pathway is interrupted. In this case, it is an interesting observation that genetic disruption

of the Class IA signaling pathway results in similar decrease in Erk activation, rather than a compensatory hyperactivation.

These biochemical findings correlate with the proliferation data above (Figure 3.4A), and together they suggest that the *Pik3r1*-encoded regulatory subunits are necessary for full hyperactivation of PI3K in cells expressing mutant Shp2, and that intact Class IA PI3K signaling is required for mutant Shp2-induced GM-CSF hypersensitivity. We observed, however, that in the absence of p85α and its splice variants, that Akt hyper-phosphorylation, although substantially reduced, was not completely abrogated, indicating that these mutant Shp2 E76K-expressing cells retained some residual PI3K activity inducible by GM-CSF stimulation that was higher in *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K compared to those expressing WT Shp2 (Figure 3.4B, compare lanes 7 and 8). We therefore wished to confirm the presence of this residual PI3K activity and to investigate the mechanisms by which it might be maintained.

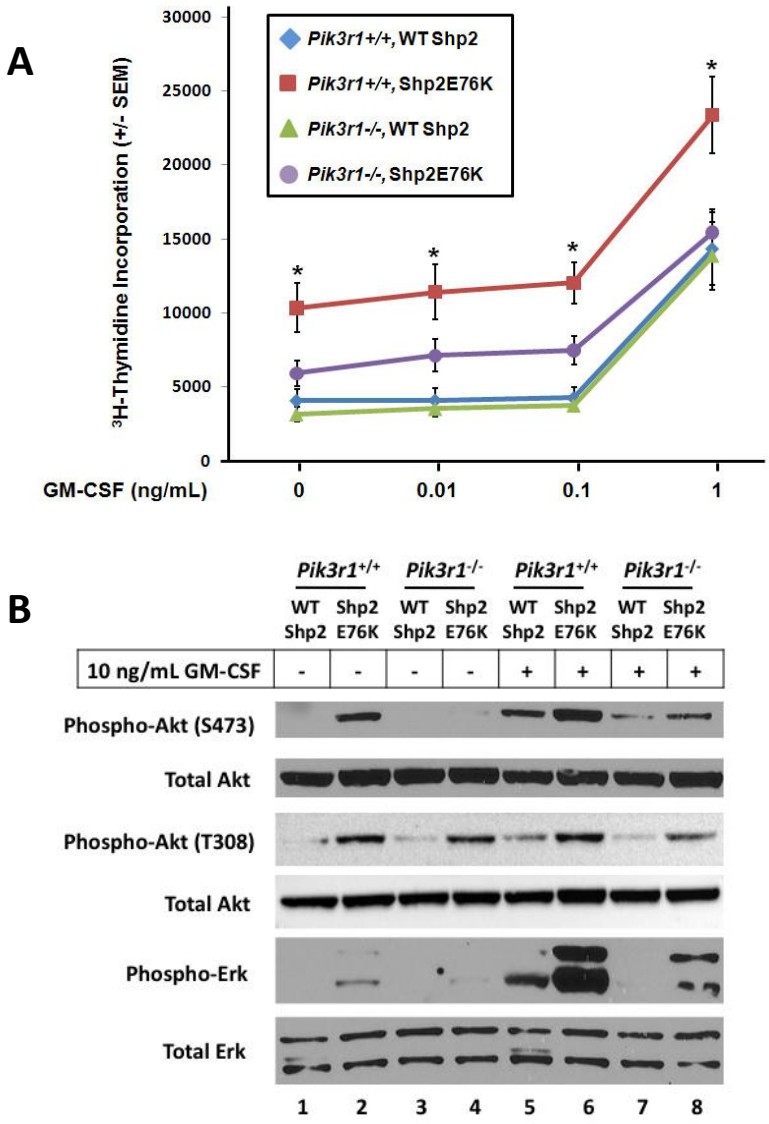



**Figure 3.4: Genetic disruption of the PI3K regulatory subunits, p85α, p55α, and p50α results in significant reduction of mutant Shp2 E76K-induced GM-CSF hypersensitivity**

(A) [$^3$H]-Thymidine incorporation assay measuring proliferation in response to increasing concentrations of GM-CSF (0-1 ng/mL) in WT and *Pik3r1*$^{-/-}$ fetal liver cell-derived hematopoietic progenitors transduced with either WT Shp2 or gain-of-function mutant Shp2 E76K; 3 independent experiments combined with n=3 replicates per each experiment, *$p < 0.01$ for *Pik3r1*$^{+/+}$; Shp2 E76K (red) vs. *Pik3r1*$^{-/-}$; Shp2 E76K (purple) at each concentration of GM-CSF; statistics performed using Prentice's rank sum test for replicated block data (Prentice 1979) . (B) Western blot analyses demonstrating reduced Akt (Serine 473 and Threonine 308) hyper-phosphorylation and Erk hyper-phosphorylation in *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K compared to WT cells expressing Shp2 E76K (compare lanes 4 and 2 and lanes 8 and 6). Representative of three independent experiments (Goodwin, Yang et al. 2012).

### **Pik3r1$^{-/-}$** ***cells expressing gain-of-function mutant Shp2 E76K retain residual GM-CSF-stimulated PI3K activity***

In order to demonstrate that hematopoietic cells expressing gain-of-function mutant Shp2 E76K retained some PI3K activity that was inducible by GM-CSF stimulation in the absence of the p85α and its splice variants, p55α and p50α, we cultured WT and *Pik3r1*$^{-/-}$ adherent macrophages transduced with either WT Shp2 and Shp2 E76K, which were then starved overnight in IMDM without serum or growth factors, and then stimulated 16-18 hours later for 60 minutes with 10 ng/mL GM-CSF in the presence of either 5 μM of the pan-PI3K inhibitor, LY294002, or DMSO as a control. Again, we found that following stimulation with GM-CSF, Shp2 E76K-induced hyper-phosphorylation of Akt and Erk were substantially reduced in the absence of the *Pik3r1* regulatory subunits (Figure 3.5, compare lanes 3 and 7). Additionally, treatment of *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K with LY294002 further reduced Akt phosphorylation (Figure 3.5, compare lanes 7 and 8), indicating that in the absence of p85α and its splice variants, there was residual PI3K activity that was inducible by GM-CSF stimulation, and which could be inhibited pharmacologically. We also observed that, while treatment with LY294002 resulted in a reduction of Shp2 E76K-induced Erk hyper-phosphorylation in those cells with the *Pik3r1* gene intact (Figure 3.5, compare lanes 3 and 4), this same inhibitor caused an increase in Erk phosphorylation in *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K (Figure 3.5, compare lanes 7 and 8), indicating the complicated nature of the cross-talk between these two pathways.

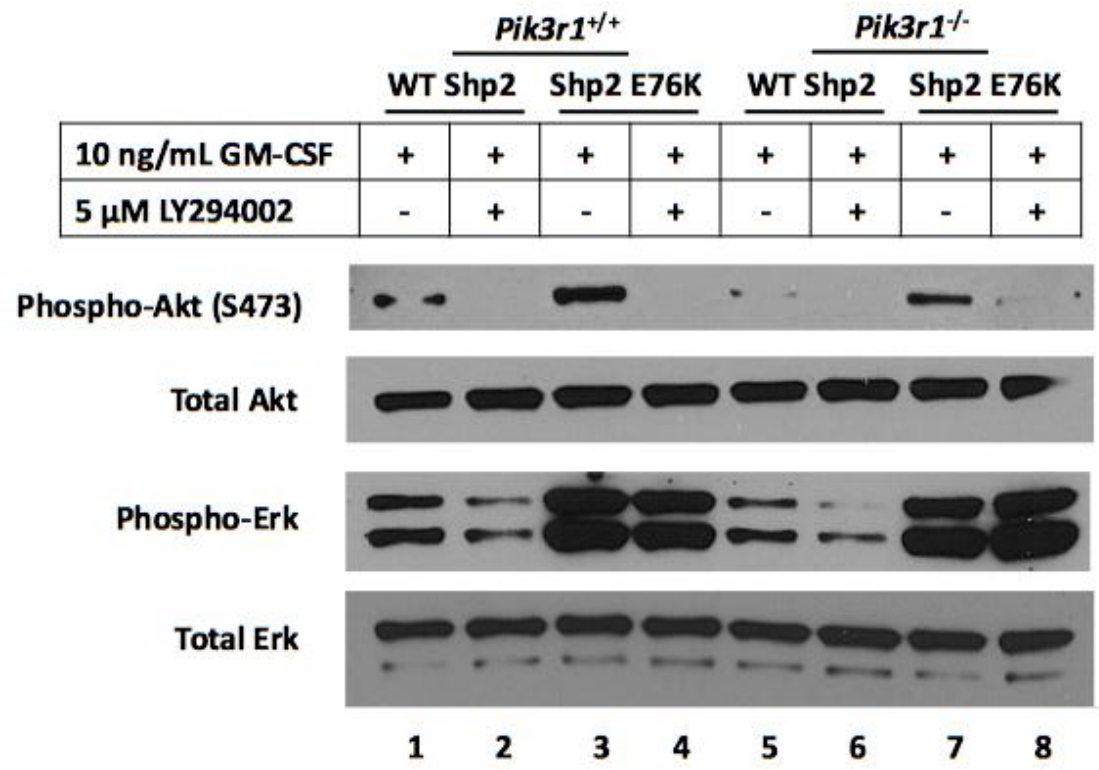



**Figure 3.5: Treatment of *Pik3r1*$^{-/-}$ cells expressing gain-of-function mutant Shp2 E76K with the pan-PI3K inhibitor, LY294002, inhibits residual PI3K activity**

Western blot analysis of adherent macrophages cultured from WT and *Pik3r1*$^{-/-}$ fetal liver cell-derived hematopoietic progenitors, demonstrating that there is residual PI3K activity in the cells in the absence of the *Pik3r1*-encoded regulatory subunits, which can be inhibited pharmacologically with the pan-PI3K inhibitor, LY294002, as evidenced by the near complete abrogation of Akt phosphorylation in *Pik3r1*$^{-/-}$ cells expressing mutant Shp2 E76K in the presence of LY294002 (compare lanes 7 and 8). Note, however, that there is an increase in Erk phosphorylation in the *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K upon treatment with LY294002 (compare lanes 7 and 8) Representative of two independent experiments.

***Genetic disruption of* Pik3r1*-encoded Class IA PI3K regulatory subunits, p85α, p55α, and p50α, results in significantly reduced expression of Class IA catalytic subunits, with preferential stabilization of p110δ in the presence of gain-of-function mutant Shp2 E76K***

To begin exploring possible mechanisms for the residual PI3K activity observed in *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K, we assessed the protein expression levels of the three Class IA catalytic subunits, p110α, p110β, and p110δ, because it has been reported that the stability of these catalytic subunits is substantially decreased in the absence of p85α and its splice variants, and their expression levels are reduced as result of rapid degradation.

WT and *Pik3r1*$^{-/-}$ cells transduced with either WT Shp2 or Shp2 E76K were cultured into adherent macrophages, starved and stimulated before total cellular protein lysates were collected as described above. Western blots analyses revealed that in the presence of the *Pik3r1*-encoded regulatory subunits, there was no difference in the expression of p110α, p110β, or p110δ between the WT Shp2- or Shp2 E76K-expressing cells, either at baseline or following stimulation (Figure 3.6A, compare lanes 1and 2, and lanes 5 and 6) (Goodwin, Yang et al. 2012), indicating that the increased PI3K activity caused by Shp2 E76K expression is not due to increased protein expression of one or more Class IA catalytic subunits. In the absence of p85α and its splice variants, expression of p110α and p110δ is markedly reduced, while expression of p110β is almost completely abrogated (Figure 3.6A). The residual p110α and p110δ expression might result from stabilizing interactions with the remaining regulatory subunits p85β and p55γ. We noticed, however, that *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K had slightly higher levels

of p110δ compared to *Pik3r1*$^{-/-}$ cells expressing WT Shp2 (Figure 3.6A, compare lanes 3 and 4 and lanes 7 and 8). We confirmed this observation with four additional experiments, and, using densitometric analysis, we found that the expression levels of p110δ were approximately two-fold higher in the Shp2 E76K-expressing cells compared to the WT Shp2-expressing cells (Figure 3.6B) (Goodwin, Yang et al. 2012). This observation suggests that the catalytic subunit p110δ is being preferentially stabilized in Shp2 E76K cells lacking p85α and its splice variants, and we hypothesize that this may result from enhanced or compensatory stabilizing interactions with the regulatory subunit p85β. Attempts to assess the interaction between p110δ and p85β by co-immunoprecipitation were unsuccessful due to a lack of commercially available p85β antibodies of sufficient quality. These results suggest that the residual GM-CSF-stimulated PI3K activity and incomplete reduction in Shp2 E76K-induced hyper-proliferation following GM-CSF simulation may be due to retained expression of some of catalytic subunits, especially p110δ. These findings also suggest that p110δ may be the principal mediator of mutant Shp2-induced PI3K hyperactivation in response to GM-CSF stimulation, and therefore p110δ may be a potential therapeutic target for the treatment of activating mutant Shp2-associated MPD.

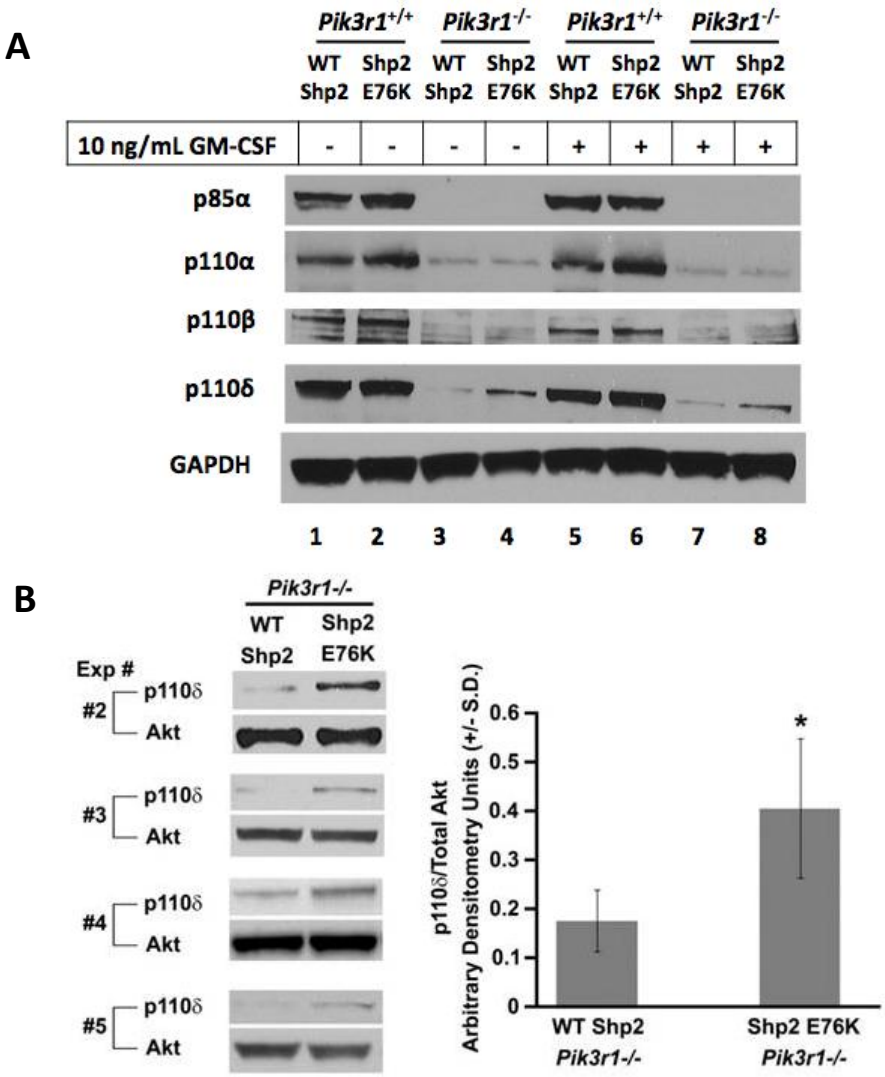



**Figure 3.6: Expression of Class IA PI3K catalytic subunits is substantially decreased in the absence of *Pik3r1*-encoded regulatory subunits, but the catalytic subunit p110δ is preferentially stabilized in *Pik3r1*$^{-/-}$ cells expressing gain-of-function mutant Shp2 E76K**

(A) Western blot analysis of adherent macrophages cultured from WT and *Pik3r1*$^{-/-}$ fetal liver cell derived-hematopoietic progenitors transduced with WT Shp2 or gain-of-function mutant Shp2 E76K, showing dramatically reduced expression of the Class IA PI3K catalytic subunits, p110α, p110β, and p110δ in the absence of the *Pik3r1*-encoded regulatory subunits. Unlike the other catalytic subunits, p110δ is expressed at slightly higher levels in *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K compared to *Pik3r1*$^{-/-}$ cells expressing WT Shp2 (compares lanes 3 and 4 and lanes 7 and 8). (B) Four additional immunoblots showing relatively increased p110δ protein levels in *Pik3r1*$^{-/-}$ expressing Shp2 E76K compared to those expressing WT Shp2 and quantitation using densitometry normalized to total Akt expression; n = 5; *p = 0.02; statistics performed using unpaired, two-tailed student's t-test (Goodwin, Yang et al. 2012).

***Cells expressing Shp2 E76K in the absence of p85α, p55α, and p50α are relatively resistant to treatment with the Ras inhibitor, tipifarnib***

Because one of consequences of gain-of-function Shp2 mutations is Ras hyperactivation, we wished to explore whether the mutant Shp2-induced hyperactivation of PI3K was entirely Ras-dependent or at least partially Ras-independent. This question is of clinical relevance, since a demonstration that the mutant Shp2-induced PI3K hyperactivation was entirely dependent on Ras would suggest that a therapeutic approach of solely targeting Ras hyperactivation would theoretically be sufficient for the treatment of mutant Shp2-associated MPD, while a demonstration that mutant Shp2 can hyperactivate PI3K independently of Ras would suggest that PI3K would need to be targeted in addition to the Ras-MAPK pathway. To address this question, we used the farnesyl-transferase inhibitor (FTI), tipifarnib, which prevents the farnesylation of Ras and, as a consequence, prevents its targeting to the inner leaflet of the plasma membrane where it conducts its signal transducing activities.

WT and *Pik3r1*$^{-/-}$ macrophages transduced with Shp2 E76K were pretreated for 48 hours with 10 μM of tipifarnib or vehicle control, before overnight starvation in IMDM and a 60-minute stimulation with 10 ng/mL GM-CSF in the continued presence or absence of tipifarnib. Western blot analyses of these cells revealed that treatment of WT cells expressing Shp2 E76K with tipifarnib caused a modest decrease in GM-CSF-stimulated hyperactivation of Akt and Erk (Figure 3.7A, compare lanes 1 and 2) (Goodwin, Yang et al. 2012). As already observed, *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K have significantly lower levels of phosphorylated Akt and Erk following GM-CSF stimulation (Figure 3.7A, compare lanes 1 and 3); however treatment with tipifarnib does

not bring a further reduction in Akt and Erk activation (Figure 3.7A, compare lanes 3 and 4), suggesting that the residual PI3K activity that remains in the absence of the *Pik3r1*-encoded regulatory subunits is largely Ras-independent. These biochemical results correlate with the proliferation data, in which, as previously observed, genetic disruption of *Pik3r1* results in partial correction of the Shp2 E76K-induced hyper-proliferative response following GM-CSF stimulation, but treatment with 1 μM tipifarnib caused a 50% decrease in GM-CSF-stimulated proliferation in Shp2 E76K-transduced WT cells, while only causing a 30% decrease in proliferation in Shp2 E76K-transduced *Pik3r1*$^{-/-}$ cells (Figure 3.7B) (Goodwin, Yang et al. 2012). This suggests that *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K are less sensitive to Ras inhibition, and that therefore, the residual GM-CSF-stimulated PI3K activity in the absence of p85α and its splice variants is, at least in part, induced by gain-of-function mutant Shp2 in a Ras-independent manner. This finding is particularly significant given the relatively increased expression of p110δ observed in *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K, since it has been reported that p110δ, in contrast to the other PI3K catalytic subunits, can induce oncogenic transformation in a Ras-independent manner (Denley, Kang et al. 2008).

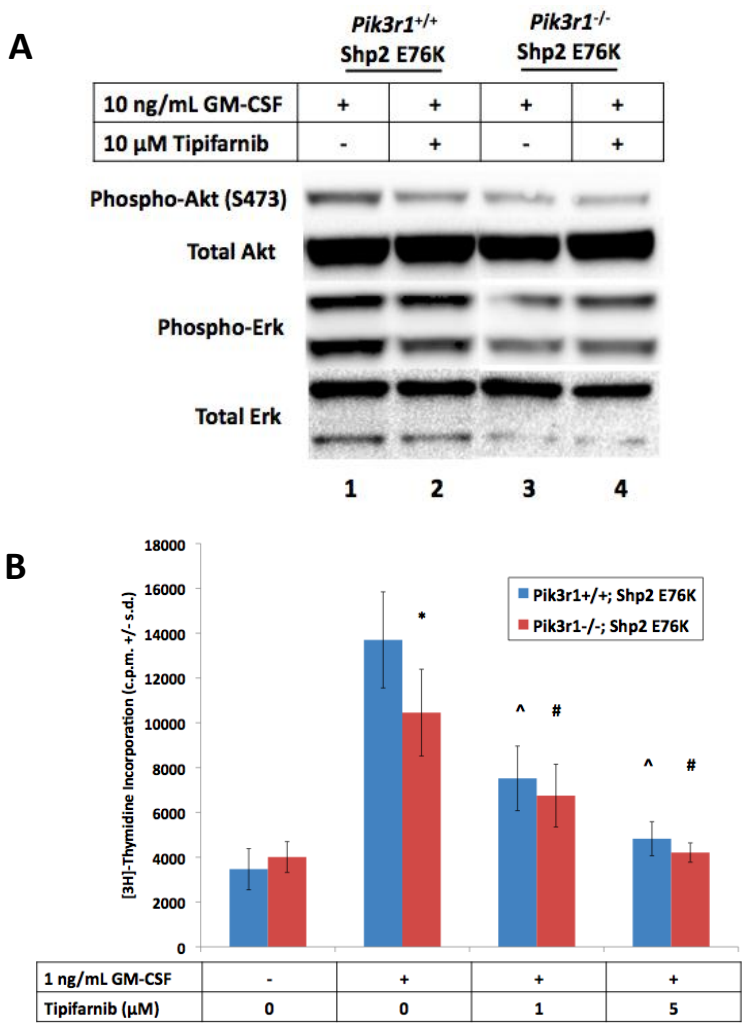



**Figure 3.7: *Pik3r1*$^{-/-}$ cells expressing gain-of-function mutant Shp2 E76K are relatively resistant to Ras inhibition mediated by the farnesyl-transferase inhibitor tipifarnib**

(A) Western blot analyses of adherent macrophages cultured from WT and *Pik3r1*$^{-/-}$ fetal liver cell-derived hematopoietic progenitors transduced with gain-of-function mutant Shp2 E76K stimulated for 60 minutes with 10 ng/mL GM-CSF in the presence or absence of 10 µM tipifarnib, a farnesyl-transferase inhibitor which blocks activation of Ras. (B) [$^3$H]-Thymidine incorporation assay measuring 1 ng/mL GM-CSF-stimulated proliferation of WT and *Pik3r1*$^{-/-}$ fetal liver cell-derived hematopoietic progenitors transduced with gain-of-function mutant Shp2 E76K in the presence of increasing doses of tipifarnib (0, 1, or 5 µM); representative of 2 independent experiments; n = 8; *$p < 0.02$ comparing *Pik3r1*$^{+/+}$ Shp2 E76K vs. *Pik3r1*$^{-/-}$ Shp2 E76K cells in response to1 ng/mL GM-CSF in the absence of tipifarnib; ^$p < 0.001$ comparing *Pik3r1*$^{+/+}$ Shp2 E76K cells in absence to the presence of tipifanib at 1 µM and 5 µM; #$p < 0.005$ comparing *Pik3r1*$^{-/-}$ Shp2 E76K cells in the absence to the presence of tipifarnib at 1 µM and 5 µM; statistics performed using unpaired, two-tailed student's t-test (Goodwin, Yang et al. 2012).

**Conclusions**

Gain-of-function mutations in *PTPN11* are the most common genetic aberrations in JMML patients. It is already well known that these gain-of-function mutations, as well as the other identified mutated genes in JMML, promote hyperactivation of the RAS-MAPK pathway, which helps promote the observed hypersensitivity to GM-CSF stimulation that is thought to be an important underlying mechanism of the pathogenesis of JMML (Chan and Feng 2007; Mohi and Neel 2007). Until now, the contribution of PI3K—a known effector of RAS—to GM-CSF hypersensitivity and leukemogenesis has not been thoroughly examined. In these studies, we confirm that PI3K is hyperactivated in the presence of activating Shp2 mutations, and, using the pan-PI3K inhibitor LY294002, we show that hyperactivated PI3K contributes to mutant Shp2-induced GM-CSF hypersensitivity.

In addition, we initiated genetic studies to investigate the role of Class IA PI3K in mutant Shp2-induced GM-CSF hypersensitivity, using a genetic mouse model in which the main Class IA PI3K regulatory subunit, p85α, and its splice variants, p55α and p50α, encoded by the *Pik3r1* gene, is constitutively knocked out (Fruman, Mauvais-Jarvis et al. 2000). Using this mouse model, we found that genetic interruption of the *Pik3r1*gene reduces mutant Shp2 E76K-induced hyper-proliferation in response to GM-CSF stimulation as well as hyper-phosphorylation of Akt, a major downstream effector of PI3K activity. In addition we found that *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K also had a significant reduction in the hyper-phosphorylation of Erk, indicating that PI3K is a positive regulator of Erk activity. Thus, mutant Shp2-induced hyperactivation of PI3K may be promoting GM-CSF hypersensitivity not only through Akt or other common

effectors of PI3K, but it may also be doing so through the augmentation of the Raf-MEK-Erk pathway that is already directly hyperactivated by enhanced Ras signaling.

Although mutant Shp2-induced PI3K hyperactivation and resulting GM-CSF hypersensitivity were reduced by genetic disruption of *Pik3r1*, we found there to be residual PI3K activity that could be pharmacologically inhibited. This residual GM-CSF-stimulated PI3K activity is likely due to the retained expression—though at greatly reduced levels—of two of the Class IA PI3K catalytic subunits, p110α and p110δ, the latter of which we observed to be expressed at higher levels in *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K compared to those cells expressing WT Shp2. This unexpected finding suggests that p110δ may serve as the principal mediator of mutant Shp2-induced PI3K hyperactivation, and it may also explain why the residual PI3K activity in the absence of p85α and its splice variants is relatively resistant to Ras inhibition, since it has been previously reported that the transforming capabilities of p110δ is Ras-indpendent, unlike the other Class IA catalytic subunits (Denley, Kang et al. 2008). Importantly, these data suggest that PI3K hyperactivity, particularly that mediated by p110δ, may represent a potential therapeutic target for gain-of-function mutant Shp2-induced MPD, and that this PI3K hyperactivation must be targeted independently of Ras.

# CHAPTER FOUR

# PHARMACOLOGIC INHIBITION OF PI3K WITH CATALYTIC SUBUNIT-SPECIFIC INHIBITORS REDUCES GAIN-OF-FUNCTION MUTANT SHP2-INDUCED GM-CSF HYPERSENSITIVITY

## Introduction

Having demonstrated the role of Class IA PI3K signaling in gain-of-function mutant SHP2-induced GM-CSF hypersensitivity, a hallmark of Juvenile Myelomonocytic Leukemia (JMML) which is thought to contribute to the pathogenesis of the disease, we next wanted to explore newer classes of catalytic subunit-specific inhibitors, many of which have already entered clinical trials for a variety of malignant and non-malignant human diseases. In recent years, there has been increasing focus on investigating the role of individual PI3K catalytic subunits in particular physiologic processes and, especially, specific disease states with the hope of targeting the activity of the one or two catalytic subunits involved in the pathogenesis of that disease rather than indiscriminately inhibiting all PI3K activity (Marone, Cmiljanovic et al. 2008). This focus would reduce the risk of unintended side effects which may occur, given the relatively universal importance of PI3K signaling in normal human physiology. Likewise, we wanted to determine whether a particular Class IA catalytic subunit was the principal mediator of mutant Shp2-induced PI3K hyperactivation in response to GM-CSF stimulation, so that we might be able to take advantage of newly developed catalytic subunit-specific inhibitors for more efficient targeting of this pathway.

We were particularly interested in focusing on the role of p110δ for a number of reasons. Based on our previous studies using murine cells bearing genetic disruption of

the *Pik3r1* gene, which encodes the major Class IA PI3K regulatory subunit, p85α and its splice variants, p55α and p50α, we found that p110δ expression, though greatly reduced like the other catalytic subunits, was relatively higher in Shp2 E76K-expressing *Pik3r1*$^{-/-}$ cells compared to the WT Shp2-expressing cells (Figure 3.6). We postulated that this preferential stabilization of p110δ in the presence of the activating Shp2 mutation might account for the residual PI3K activity that remained in the absence of the *Pik3r1*-encoded regulatory subunits and it might also explain the relative insensitivity to Ras inhibition in the *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K, given previous studies that suggest that p110δ can induce oncogenic transformation independent of Ras (Denley, Kang et al. 2008). Therefore, based on these observations, we hypothesized that p110δ might be the principal mediator of GM-CSF-stimulated PI3K hyperactivation in the presence of activating Shp2 mutations. Finally, we were also interested in p110δ because its expression is mostly confined to hematopoietic cells, unlike the other two Class IA catalytic subunits, p110α and p110β, which are ubiquitously expressed (Okkenhaug, Bilancio et al. 2002). This would make p110δ an ideal target for hematologic malignancies, thereby reducing the risk of systemic side effects that may occur from inhibiting PI3K activity more globally with a less specific inhibitor.

At the same, we also wanted to explore the role of p110α, because it is frequently associated with human malignancies, being the only Class IA PI3K catalytic subunit found to have somatic mutations in human cancer (Zhao and Vogt 2008). In our studies using the *Pik3r1*$^{-/-}$, we noted that p110α expression, though substantially reduced, was also not completely abolished by the loss of p85α and its splice variants. Unlike p110δ,

however, there was no difference in p110α expression between *Pik3r1*$^{-/-}$ cells expressing mutant Shp2 E76K and those expressing WT Shp2.

For these *in vitro* studies, we initially used two commercially available inhibitors, IC87114, a p110δ-specific inhibitor, and GDC-0941, a clinical-grade pan-PI3K inhibitor with highest specificity for p110δ and p110α (Table 4.1). Additionally, we also made use of the p110β-specific inhibitor, TGX-221 (Table 4.1). Finally, in collaboration with Gilead Sciences, we initiated studies with the highly potent and specific p110δ inhibitor, GS-9820 (formerly known as CAL-120). GS-9820 is structurally similar to the inhibitor, GS-1101 (formerly known as CAL-101), which is in clinical trials for lymphoid malignancies, but the former has a much better pharmacodynamic profile in mice, and therefore is often used in mouse studies (BJ Lannutti, Gilead Sciences, Inc, Seattle, WA, personal communication).

In addition to performing these studies using WT murine bone marrow-derived low-density mononuclear cells (LDMNCs) transduced with WT Shp2 and gain-of-function mutant Shp2 E76K, we also used bone marrow LDMNCs from LSL-Shp2$^{D61Y/+}$; Mx1-Cre+ mice. These mice express endogenous levels of gain-of-function mutant Shp2 D61Y, a commonly found mutation in JMML patients (Chan, Kalaitzidis et al. 2009). This expression is inducible primarily in hematopoietic cells upon activation of the Cre recombinase under the Mx1 promoter following a polyI:polyC-stimulated γ-interferon response. Using these cells has the advantage of providing a more physiologically relevant model, since the gain-of-function mutation Shp2 D61Y is expressed at endogenous levels, unlike in the transduction model where the mutated Shp2 is significantly overexpressed.

We hypothesized that treatment of cells expressing gain-of-function Shp2 mutations with the IC87114 (p110δ-specific), GDC-041 (p110α- and p110δ-specific), or the clinical-grade p110δ-specific inhibitor, GS-9820, would reduce mutant Shp2-induced hyper-proliferation as well as Akt and Erk hyperactivation following GM-CSF stimulation. We also hypothesized that these inhibitors would also increase apoptosis in mutant Shp2-expressing cells. Together, we believe these studies will demonstrate the potentially utility of PI3K catalytic subunit-specific inhibitors, particularly those with high specificity for p110δ, in the treatment of mutant Shp2-induced myeloproliferative disease.

$IC_{50}$

| Inhibitor | p110α | p110β | p110δ | p110γ |
|---|---|---|---|---|
| GDC-0941 | **3 nM** | 33 nM | **3 nM** | 75 nM |
| TGX-221 | 5000 nM | **5 nM** | 100 nM | 3500 nM |
| IC87114 | > 1-200 uM | 1-75 uM | **0.06-0.5 uM** | 17-29 uM |
| GS-1101* | 820 nM | 565 nM | **2.5 nM** | 89 nM |

*Gilead Sciences

**Table 4.1: PI3K catalytic subunit-specific inhibitors**

Table showing four PI3K inhibitors, along with their $IC_{50}$ concentrations for each of the four Class I PI3K catalytic subunits. Lowest $IC_{50}$ concentrations for particular catalytic subunits are shown in bold for each inhibitor to emphasize the principal specificity for each inhibitor.

## Results

### *Inhibition of PI3K with catalytic subunit-specific inhibitors, IC87114, GDC-0941 and TGX-221, reduces GM-CSF-stimulated hyper-proliferation in cells with exogenously overexpressed gain-of-function mutant Shp2 E76K*

WT bone marrow LDMNCs were transduced with WT Shp2 and gain-of-function mutant Shp2 E76K, and their GM-CSF-stimulated proliferation was measured in the presence of increasing concentrations of the PI3Kcatalytic subunit-specific inhibitors, IC87114, GDC-0941, and TGX-221. IC87114 is specific for p110δ, while TGX-221 is specific for p110β, and GDC-0941 is a clinical-grade pan-PI3K inhibitor with highest specificity for p110δ and p110α. We hypothesized that p110δ is the principal mediator of gain-of-function mutant Shp2-induced PI3K hyperactivation, which contributes to the enhanced proliferative response following GM-CSF stimulation, and that therefore the inhibitors with highest specificity for p110δ would be most effective in reducing GM-CSF stimulated proliferation in these cells.

We found that all three inhibitors, IC87114 (Figure 4.1A), GDC-0941 (Figure 4.1B), and TGX-221 (Figure 4.1C) caused a concentration-dependent decrease in GM-CSF-stimulated hyper-proliferation in cells transduced with mutant Shp2 E76K. Treatment with IC87114 efficiently inhibited GM-CSF-stimulated proliferation in the 5-25 μM range (Figure 4.1A) (Goodwin, Yang et al. 2012). This relatively high concentration is most likely due to the fact that IC87114, though highly specific for p110δ is not very potent (Table 4.1). TGX-221 demonstrated efficient inhibition of GM-CSF-stimulated proliferation in the 1-5 μM range (Figure 4.1C), but at these concentrations, it is possibly losing specificity for p110β and beginning to also have an

inhibitory effect on p110δ, for which it also has a relatively low $IC_{50}$ (Table 4.1). By far, the inhibitor that decreased GM-CSF-stimulated proliferation the most efficiently was GDC-0941, which achieved significant reductions in GM-CSF-stimulated proliferation in the 0.1-0.5 μM range (Figure 4.1B) (Goodwin, Yang et al. 2012). The efficacy of GDC-0941 could be the result of its extremely low $IC_{50}$ for p110δ, 3 nM (Table 4.1), or because it is also a very potent inhibitor of p110α, which would suggest that both p110δ and p110α are responsible for mediating gain-of-function mutant Shp2-induced GM-CSF hypersensitivity, and that therefore the activity of both p110δ and p110α must be interrupted in order to effectively inhibit mutant Shp2-induced PI3K hyperactivation and resulting GM-CSF hypersensitivity.

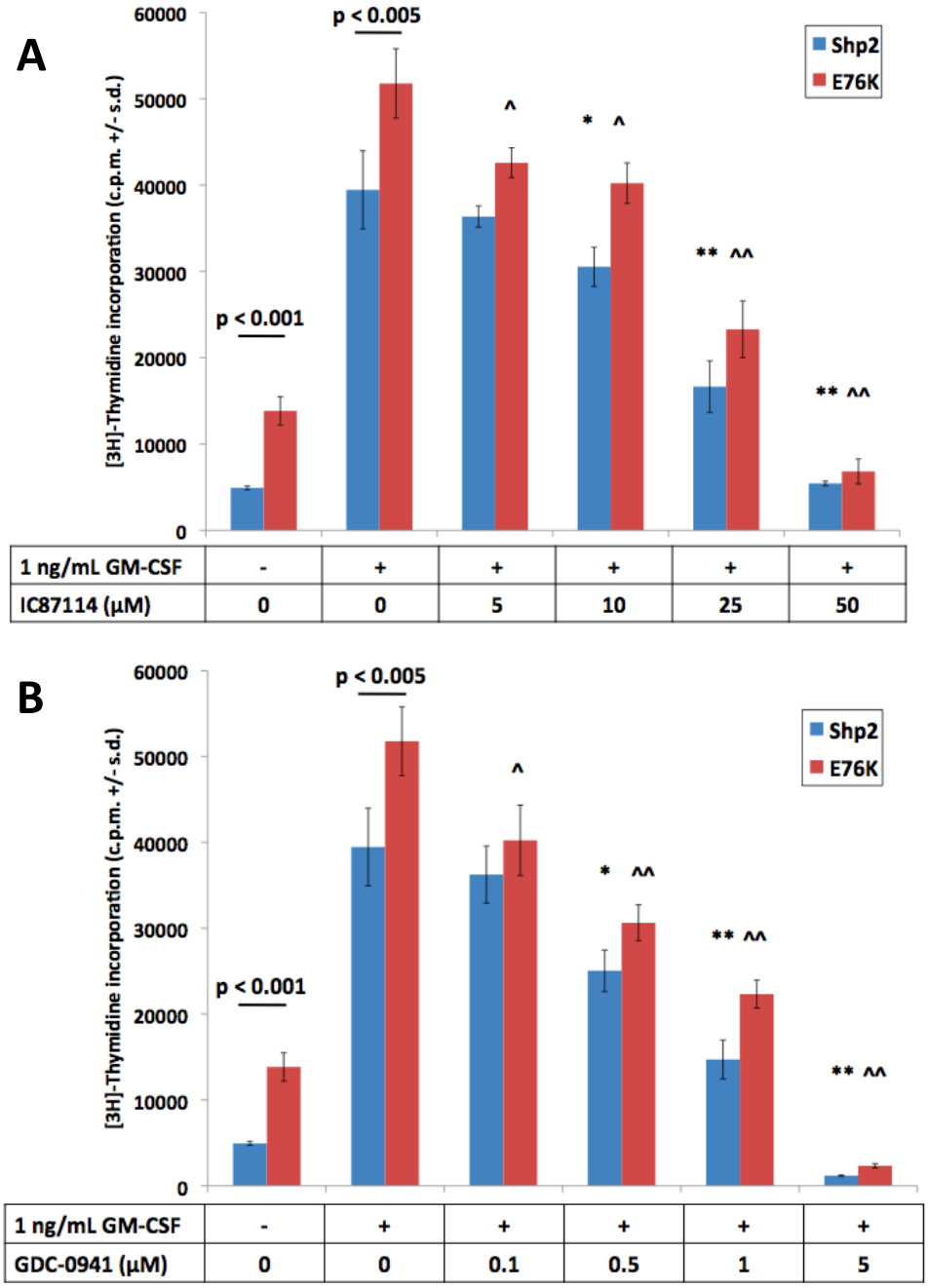



**Figure 4.1: Catalytic subunit-specific PI3K inhibitors, especially those with high specificity for p110δ reduce gain-of-function mutant Shp2 E76K-induced hyper-proliferation in response to GM-CSF stimulation**

[$^3$H]-Thymidine incorporation assays measuring GM-CSF-stimulated proliferation in bone marrow-derived LDMNCs transduced with WT Shp2 or Shp2 E76K in the presence of three different catalytic subunit-specific PI3K inhibitors: (A) IC87114, a specific inhibitor for p110δ, (B) GDC-0941, a highly potent inhibitor of p110δ and p110α, and (C) TGX-221, a specific inhibitor for p110β (continued on next page).

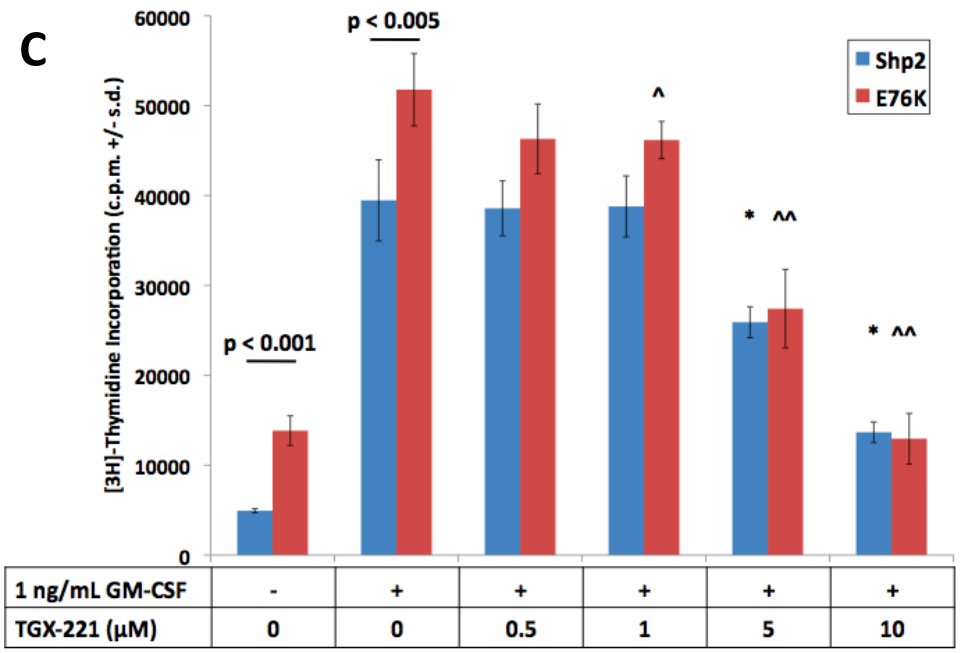



**Figure 4.1: Catalytic subunit-specific PI3K inhibitors, especially those with high specificity for p110δ reduce gain-of-function mutant Shp2 E76K-induced hyper-proliferation in response to GM-CSF stimulation (continued)**

(A) IC87114 inhibits GM-CSF-stimulated hyper-proliferation in mutant Shp2 E76K-expressing cells in concentration-dependent manner (0-50 μM); representative of 2 independent experiments; n = 4-5; *$p < 0.05$ in WT Shp2-expressing cells comparing the absence and the presence of IC87114 at 10 μM; **$p < 0.001$ in WT Shp2-expressing cells comparing the absence and the presence of IC87114 at 25 μM and 50 μM; ^$p < 0.005$ in Shp2 E76K-expressing cells comparing the absence and the presence of IC87114 at 5 μM and 10 μM; ^^$p < 0.001$ in Shp2 E76K-expressing cells in the absence and the presence of IC87114 at 25 μM and 50 μM. (B) GDC-0941 inhibits GM-CSF in mutant Shp2 E76K-expressing cells in a concentration-dependent manner (0-5 μM); representative of 2 independent experiments; n = 4-5; *$p < 0.005$ in WT Shp2-expressing cells comparing the absence and the presence of GDC-0941 at 0.5 μM; **$p < 0.001$ in WT Shp2-expressing cells comparing the absence and the presence of GDC-0941 at 1 μM and 5 μM; ^$p < 0.005$ in Shp2 E76K-expressing cells comparing the absence and the presence of GDC-0941 at 0.1 μM; ^^$p < 0.001$ in Shp2 E76K-expressing cells in the absence and the presence of GDC-0941 at 0.5 μM, 1 μM, and 5 μM. (C) TGX-221 inhibits GM-CSF in mutant Shp2 E76K-expressing cells in a concentration-dependent manner (0-10 μM); representative of 2 independent experiments; n = 4-5; *$p < 0.01$ in WT Shp2-expressing cells comparing the absence and the presence of TGX-221 at 5 μM; **$p < 0.001$ in WT Shp2-expressing cells comparing the absence and the presence of TGX-221 at 10 μM; ^$p < 0.05$ in Shp2 E76K-expressing cells comparing the absence and the presence of TGX-221 at 1 μM; ^^$p < 0.001$ in Shp2 E76K-expressing cells in the absence and the presence of TGX-221 at 5 μM and 10 μM; statistics performed using unpaired, two-tailed student's t-test (Goodwin, Yang et al. 2012).

***PI3K inhibitors with high specificity for p110δ reduce GM-CSF-stimulated Akt and Erk hyper-phosphorylation in cells transduced with gain-of-function mutant Shp2 E76K***

To confirm that these inhibitors were causing a decrease in GM-CSF-stimulated hyper-proliferation by specifically blocking hyperactivated PI3K signaling, we performed biochemical analysis of adherent macrophages expressing exogenous WT Shp2 or Shp2 E76K in the presence of these inhibitors. We found that at these doses, the inhibitors caused a substantial decrease in GM-CSF-stimulated Akt hyper-phosphorylation both at Serine 473 and Threonine 308 in mutant Shp2 E76K-expressing cells, which we believed to be the result of the inhibitors blocking the activity of PI3K, particularly that mediated by p110δ (Figure 4.2, compare lanes 7 and 8 with lane 6) (Goodwin, Yang et al. 2012). We also found that these inhibitors reduced GM-CSF-stimulated hyperactivation of Erk, which is consistent with our observations in our earlier studies with *Pik3r1*$^{-/-}$ cells that PI3K has a positive regulatory effect on MAPK signaling (Figure 4.2, compare lanes 7 and 8 with lane 6). Mirroring our proliferation data above, GDC-0941 appeared to be a more potent inhibitor of GM-CSF-stimulated PI3K hyperactivation, as it was able to achieve greater reductions in Akt and Erk hyper-phosphorylation at lower doses than IC87114 (Figure 4.2 compares lanes 7 and 8), correlating with its ability to reduce GM-CSF-stimulated proliferation at lower doses. Based on these results, it appears that the inhibitors are causing a reduction in GM-CSF-stimulated hyper-proliferation in cells expressing gain-of-function mutant Shp2 E76K by specifically reducing GM-CSF-stimulated hyperactivation of PI3K, and that GDC-0941 is a more potent inhibitor of this

PI3K hyperactivation than IC87114 either because of its lower $IC_{50}$ for p110δ, or because it is also able to inhibit the activity of p110α, or both.

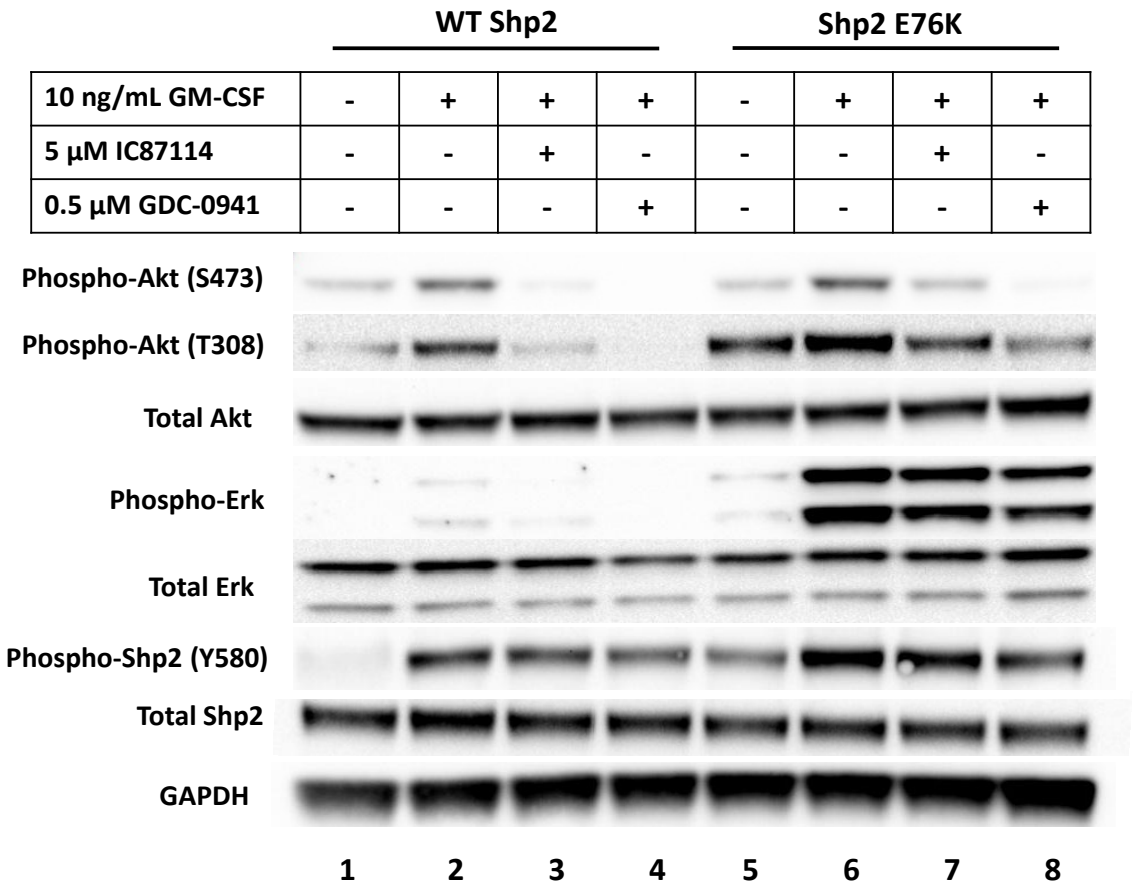



**Figure 4.2: PI3K inhibitors with high specificity for p110δ reduce GM-CSF-stimulated hyperactivation of PI3K and MAPK signaling in mutant Shp2 E76K-expressing cells**

Western blot analyses of adherent macrophages cultured from bone marrow-derived LDMNCs transduced with WT Shp2 or gain-of-function mutant Shp2 E76K stimulated for 60 minutes with the 10 ng/mL GM-CSF in the presence of 5 µM IC87114 or 0.5 µM GDC-0941. Treatment with either inhibitor reduced GM-CSF-stimulated hyper-phosphorylation of Akt (at both serine 473 and threonine 308), Erk, and Shp2 (at tyrosine 580) in cells exogenously expressing gain-of-function mutant Shp2 E76K. Data representative of 2 independent experiments (Goodwin, Yang et al. 2012).

***Pharmacologic inhibition of both Ras and PI3K cooperate to reduce gain-of-function mutant Shp2 E76K-induced GM-CSF hypersensitivity***

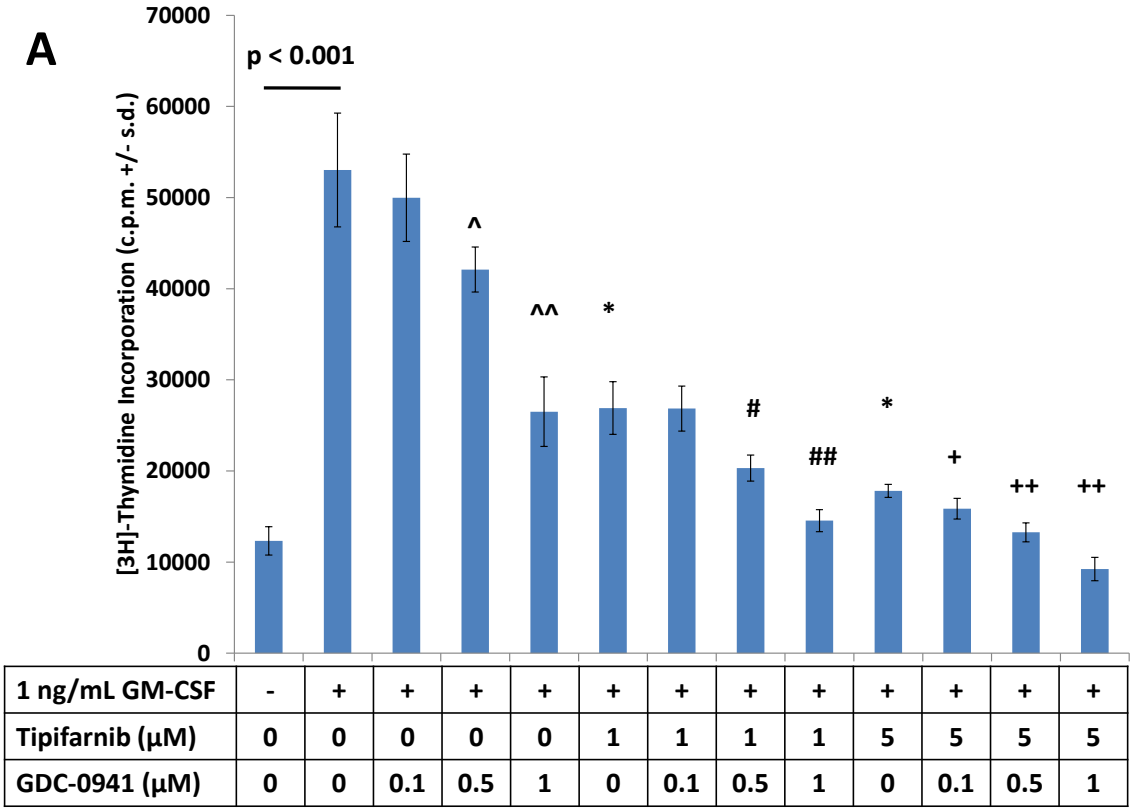
To further explore the question of Ras-dependent and Ras-independent hyperactivation of PI3K in the presence of gain-of-function mutant Shp2, we measured proliferation of Shp2 E76K-transduced cells with a combination of different doses of the PI3K inhibitor, GDC-0941 and the farnesyl-transferase inhibitor, tipifarnib, to block Ras activation. Specifically, we treated cells stimulated with 1 ng/mL GM-CSF with either 0 μM, 1 μM, or 5 μM tipifarnib in the presence of increasing concentrations of GDC-0941 (0 μM, 0.1 μM, 0.5 μM, and 1 μM). We found that treating these cells with tipifarnib (either 1 μM or 5 μM) caused a significant reduction in GM-CSF-stimulated proliferation, and that at each given concentration of tipifarnib, a further decrease in proliferation could be achieved with GDC-0941 in the concentration range of 0.5-1 μM (Figure 4.3A) (Goodwin, Yang et al. 2012).

Inhibition of Erk and Akt phosphorylation as examined by western blot in cells treated with these inhibitors correlated with the reduced level of observed proliferation. Starved adherent macrophages were stimulated for 60 minutes in the presence of either 0 μM, 1 μM, 5 μM, or 10 μM tipifarnib, and for each concentration of tipifarnib, cells were also treated with increasing concentrations of GDC-0941 (0 μM, 0.1 μM, and 0.5 μM). Similar to the proliferation data, for each concentration of tipifarnib, addition of GDC-0941 resulted in a further reduction in GM-CSF-stimulated hyper-phosphrylation of Akt and Erk in a concentration-dependent manner (Figure 4.3B, compare lanes 6 and 7 with lane 5, lanes 9 and 10 with lane 8, and lanes 12 and 13 with lane 11) (Goodwin, Yang et al. 2012). We observed, however, that the magnitude of the reduction of GM-CSF-

stimulated hyper-proliferation achieved with a given dose of tipifarnib did not appear to be commensurate with the magnitude of reduction of GM-CSF-stimulated hyper-phosphorylation of Akt or Erk, so it is difficult to conclude whether the proliferation inhibitory effect of tipifarnib is solely the result of blocking Ras activity or a non-specific toxicity effect. For instance, treatment with 1 μM tipifarnib results in a nearly two-fold reduction in GM-CSF-stimulated hyper-proliferation in Shp2 E76K-expressing cells, while the same concentration resulted in a modest reduction at best in GM-CSF-stimulated Akt and Erk hyper-phosphorylation (Figure 4.3). However, it should be noted that we frequently observed differences in the magnitude of biochemical and proliferative effects of a variety of inhibitors in our studies. This may be due to the fact that different cells are being used for the different assays—myeloid progenitors for [$^3$H]-Thymidine incorporation assays and adherent macrophages for biochemistry analyses—or due to the fact that the cells are usually treated for different time periods with the inhibitor under investigation—approximately 24 hours for [$^3$H]-Thymidine incorporation assays and typically one hour for biochemistry analyses. The latter factor does not, however, apply to the differences in the biochemical and functional effects observed with tipifarnib treatment, because the cells are treated for approximately 24 hours for both proliferation and biochemical assays, since this inhibitor is targeting an activating post-translational modification of Ras, rather than directly blocking its activity. Unfortunately, at this time, FTIs represent the only option for pharmacologically targeting Ras activity. Despite the complexities introduced by using an inhibitor like tipifarnib, we believe the general conclusions of these results still hold; i.e., tipifarnib is able to produce some reduction in

Ras-mediated PI3K hyperactivation, which contributes to a reduction in GM-CSF-stimulated hyper-proliferation.

Thus, with this in mind, we believe that inhibition of mutant Shp2 E76K-induced hyperactivation of Ras with tipifarnib is able to cause a reduction in GM-CSF-stimulated PI3K hyperactivation and resulting proliferation, but there remains residual PI3K activity that can be blocked pharmacologically, suggesting that this PI3K activity is Ras-independent. This correlates well with our previous genetic data in which *Pik3r1*$^{-/-}$ cells expressing mutant Shp2 E76K were relatively insensitive to Ras inhibition with tipifarnib (Figure 3.7), and together these data suggest that gain-of-function mutant Shp2 E76K promotes hyperactivation of Ras-MAPK and PI3K signaling, which work in concert to promote GM-CSF hypersensitivity, suggesting that both pathways most likely must be targeted to have an efficient therapeutic effect in the treatment of gain-of-function mutant Shp2-induced MPD.




| 1 ng/mL GM-CSF | - | + | + | + | + | + | + | + | + | + | + | + | + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tipifarnib (μM) | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 5 | 5 | 5 | 5 |
| GDC-0941 (μM) | 0 | 0 | 0.1 | 0.5 | 1 | 0 | 0.1 | 0.5 | 1 | 0 | 0.1 | 0.5 | 1 |

**Figure 4.3: Simultaneous inhibition of Ras and PI3K cooperatively reduce gain-of-function mutant Shp2 E76K-induced GM-CSF hypersensitivity**

(A) [$^3$H]-Thymidine incorporation assay measuring 1 ng/mL GM-CSF-stimulated proliferation in the presence of combined increasing concentrations of the farnesyl-transferase inhibitor tipifarnib (0-5 μM) and the PI3K inhibitor GDC-0941 (0-1 μM); representative of 3 independent experiments; n = 5-7; *$p < 0.001$ comparing Shp2 E76K-expressing cells in the absence vs. the presence of tipifarnib at 1 μM and 5 μM in the absence of GDC-0941; ^$p < 0.01$ comparing Shp2 E76K-expressing cells not treated with tipifarnib in the absence vs. the presence of GDC-0941 at 0.5 μM; ^^$p < 0.001$ comparing Shp2 E76K-expressing cells not treated with tipifarnib in the absence vs. the presence of GDC-0941 at 1 μM; #$p < 0.005$ comparing Shp2 E76K-expressing cells treated with 1 μM tipifarnib in the absence vs. the presence of GDC-0941 at 0.5 μM; ##$p < 0.001$ comparing Shp2 E76K-expressing cells treated with 1 μM tipifarnib in the absence vs. the presence of GDC-0941 at 1 μM; +$p < 0.01$ comparing Shp2 E76K-expressing cells treated with 5 μM tipifarnib in the absence vs. the presence of GDC-0941 at 0.1 μM; ++$p < 0.001$ comparing Shp2 E76K-expressing cells treated with 5 μM tipifarnib in the absence vs. the presence of GDC-0941 at 0.5 μM and 1 μM; statistics performed using unpaired, two-tailed student's t-test (Goodwin, Yang et al. 2012) (Figure continued on next page).

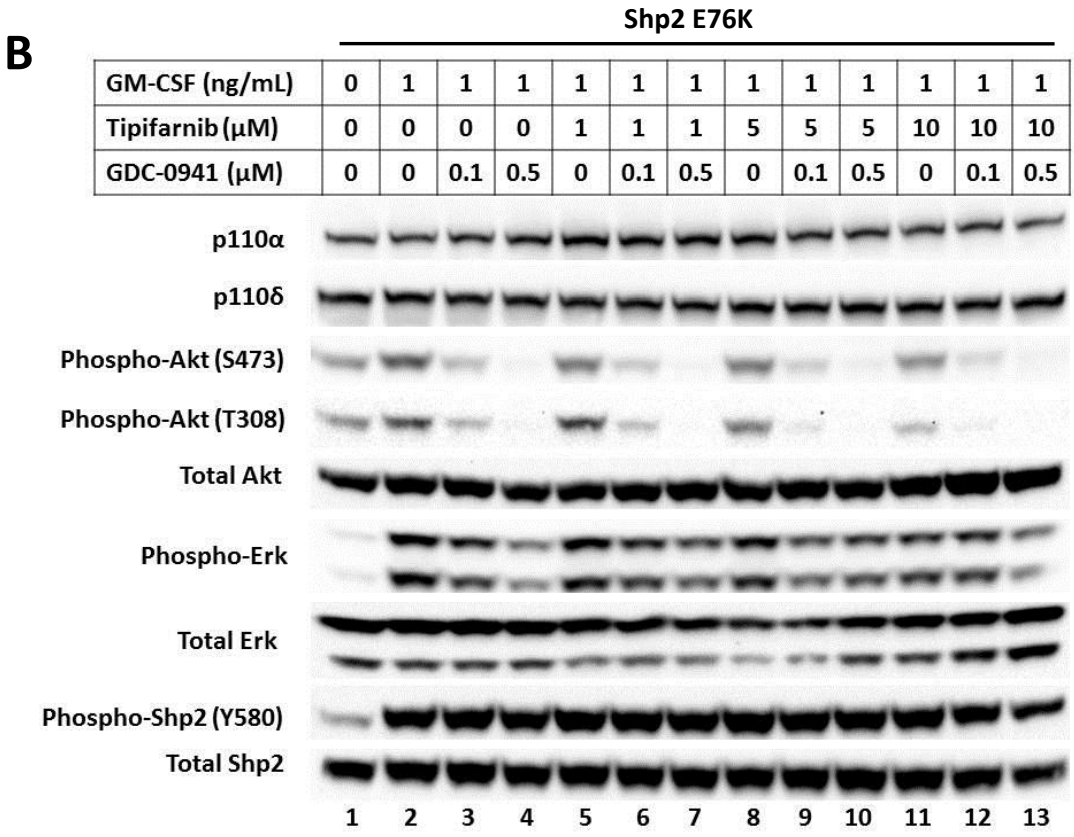



**Figure 4.3: Simultaneous inhibition of Ras and PI3K cooperatively reduce gain-of-function mutant Shp2 E76K-induced GM-CSF hypersensitivity (continued)**

(B) Western blot analyses of macrophages cultured from WT bone marrow-derived LDMNCs exogenously expressing gain-of-function mutant Shp2 E76K stimulated for 60 minutes with 10 ng/mL GM-CSF in the presence of increasing combined concentrations of tipifarnib (0-10 μM) and GDC-0941 (0-0.5 μM). Representative data from 2 independent experiments (Goodwin, Yang et al. 2012).

***PI3K inhibitors with high specificity for p110δ increase apoptosis in cells transduced with gain-of-function mutant Shp2 E76K***

In addition to imparting an enhanced GM-CSF-stimulated proliferative response, activating Shp2 mutations also reduce apoptosis, thereby providing the cells that bear them a survival advantage as well as a proliferation advantage. PI3K is well known to negatively regulate apoptosis in many cellular contexts, and so, not surprisingly, a frequent consequence of the hyperactivated PI3K signaling found in many neoplasms is enhanced survival. We next wished to investigate whether gain-of-function mutant Shp2-induced hyperactivated PI3K activity likewise contributes to reduced apoptosis, which can be reversed pharmacologically with inhibitors with high specificity for p110δ, as was found to be the case with GM-CSF-stimulated hyper-proliferation.

Sorted WT bone marrow-derived LDMNCs transduced with WT Shp2 or mutant Shp2 E76K were plated for 48 hours in the absence or presence of 10 ng/mL GM-CSF with 1 μM IC87114, 0.5 μM GDC-0941, or no inhibitor at all, before measuring the percentage of Annexin V-positive cells by FACS to assess apoptosis levels. In the absence of growth factors, Shp2 E76K-expressing cells had significantly lower Annexin V-positive cells (Figure 4.4). In the presence of GM-CSF, apoptosis was reduced in both WT Shp2-expressing and mutant Shp2 E76K-expressing cells. However, upon treatment with PI3K inhibitors with high specificity for p110δ—either 0.5 μM IC87114 or 1 μM GDC-0941—there was a significant induction of apoptosis in Shp2 E76K-expressing cells, but not in WT Shp2-expressing cells (Figure 4.4), thus implying that cells bearing activating Shp2 mutations are more sensitive to PI3K inhibition than WT cells in terms of regulating survival. Therefore, hyperactivated PI3K signaling, particularly that mediated

by p110δ, contributes to both the hyper-proliferative and enhanced survival phenotypes, which characterize cells expressing gain-of-function Shp2 mutations and likely contribute to the pathogenesis of JMML. Because pharmacologic inhibition of PI3K, especially p110δ, is able to reduce gain-of-function mutant Shp2-induced hyper-proliferation and enhanced survival, this suggests that p110δ would be an effective therapeutic target for the treatment of mutant Shp2-associated MPD.

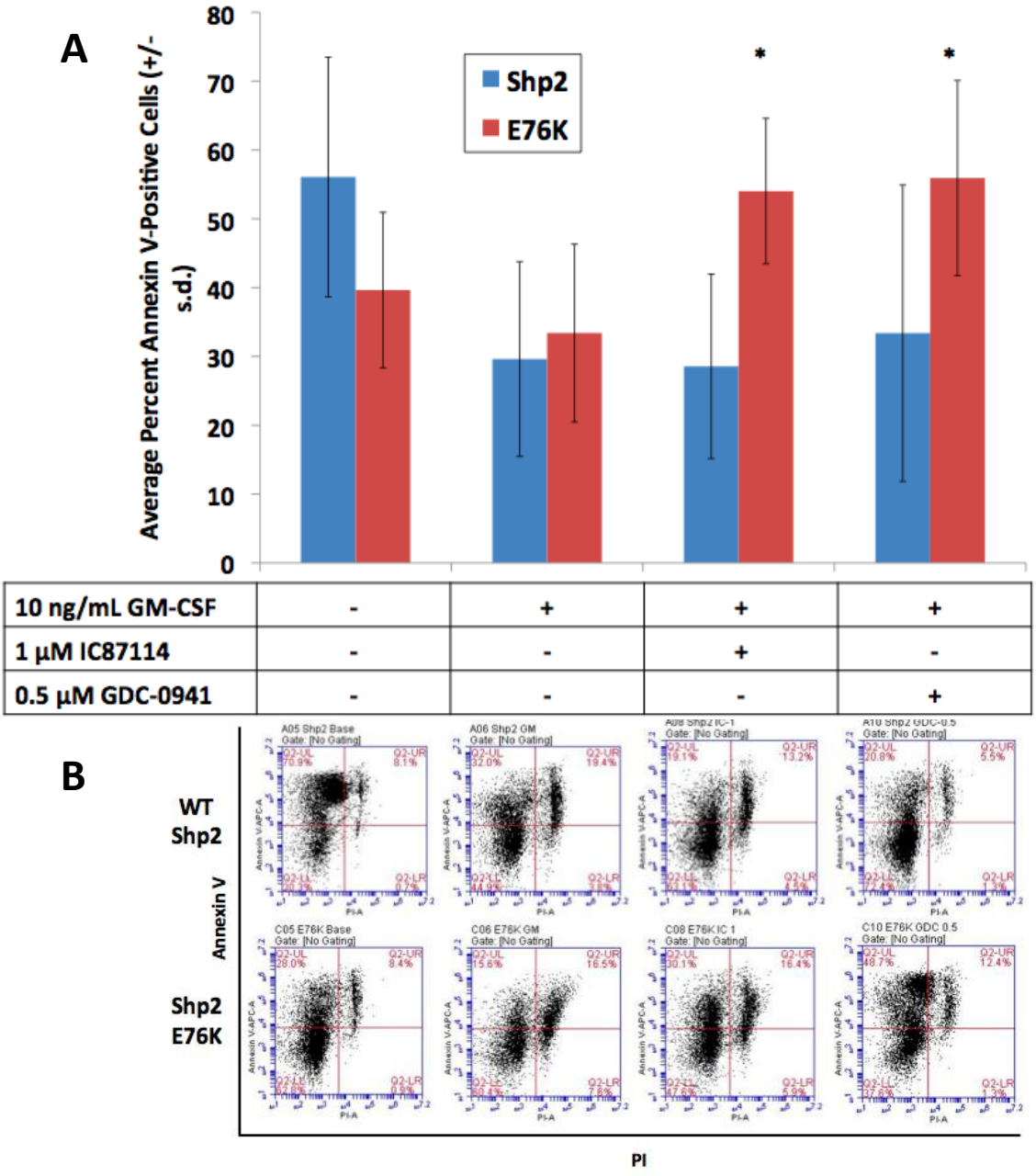



**Figure 4.4: PI3K inhibitors with high specificity for p110δ induce apoptosis in Shp2 E76K-expressing cells**

(A) Average percent of Annexin V-positive cells in WT bone marrow LDMNCs transduced with either WT Shp2 or gain-of-function mutant Shp2 E76K in the presence or absence of 10 ng/mL GM-CSF and treated with 1 µM IC87114 or 0.5 µM GDC-0941 or no inhibitor. Combined data from 5 independent experiments; *$p < 0.05$ comparing Shp2 E76K-expressing cells in the absence of inhibitors with Shp2 E76K-expressing cells in the presence of either 1 µM IC87114 or 0.5 µM GDC-0941; statistics performed using unpaired, two-tailed student's t-test. (B) Representative flow analysis data of one experiment (Annexin V-positive cells in upper left and right quadrants).

***PI3K inhibitors with high specificity for p110δ significantly reduce GM-CSF hypersensitivity in cells expressing endogenous gain-of-function mutant Shp2 D61Y***

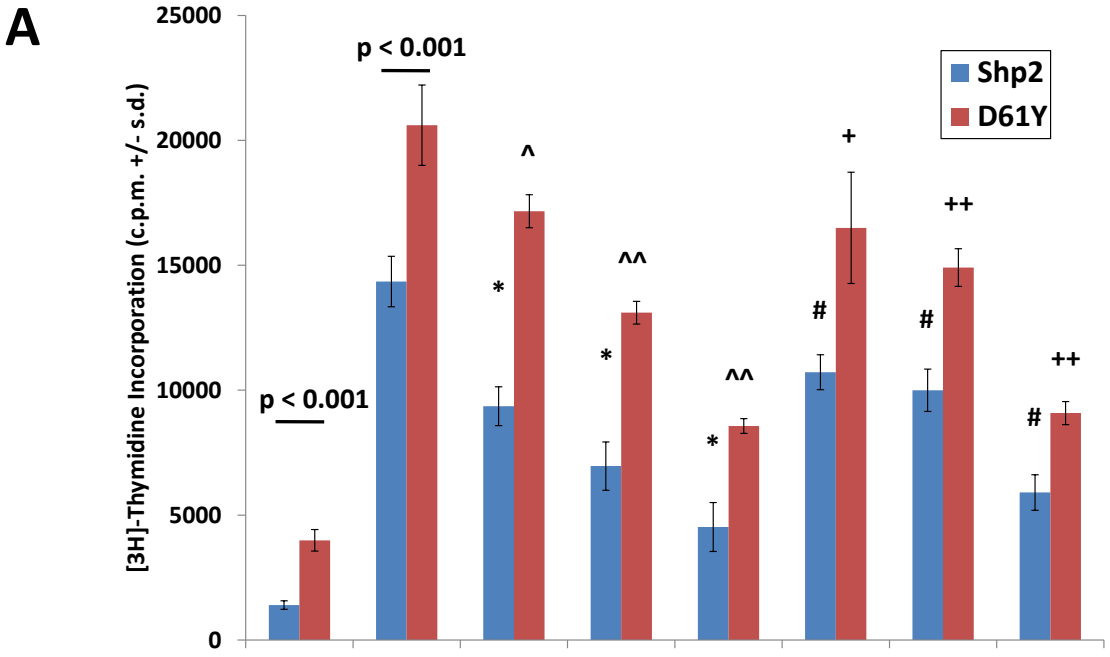
We next wanted to confirm the pharmacologic findings in cells expressing endogenous levels of activating mutant Shp2 to ensure that the observed PI3K hyperactivation was not the artifact of exogenous overexpression of an oncogene. Accordingly, we used cells from LSL-Shp2$^{D61Y/+}$; Mx1-Cre+ mice, which expresses gain-of-function mutant Shp2 D61Y under its endogenous promoter in hematopoietic cells, when induced by Cre recombinase activity following a polyI:polyC-induced γ-interferon response. These mice provide a more physiologically relevant system for investigating oncogene-induced aberrant signal transduction *in vitro* as well as an *in vivo* model for gain-of-function mutant Shp2-induced MPD.

For these studies, bone marrow-derived LDMNCs were isolated 8-12 weeks following polyI:polyC treatment to allow sufficient time for disease onset, and these cells were used for [$^{3}$H]-Thymidine incorporation assays to measure proliferation or cultured into adherent macrophages for biochemical analyses. As with Shp2 E76K-transduced cells, we found that gain-of-function mutant Shp2 D61Y-expressing cells demonstrated a greater proliferative response following GM-CSF stimulation than WT Shp2-expressing cells (LSL-Shp2$^{D61Y/+}$; Mx1-Cre-; Figure 4.5A). Similarly, treatment of these cells with PI3K inhibitors that have high specificity for p110δ, IC87114 and GDC-0941, caused a dose-dependent decrease in GM-CSF-stimulated hyper-proliferation (Figure 4.5A). As with the Shp2 E76K-transduced cells, GDC-0941 was able to inhibit proliferation at much lower concentrations—in the 0.1-0.5 μM range—compared to IC87114 , which achieved significant inhibition in the 10-25 μM range (Figure 4.5A). Again, it is not

clear if this is the consequence of GDC-0941 having a much lower $IC_{50}$ for p110δ than IC87114, or because GDC-0941 is also able to efficiently inhibit p110α (Table 4.1).

Western blot analyses of the Shp2 D61Y cells treated with IC87114 and GDC-0941 supported the observations of the proliferation assays, and recapitulated the results from the pharmacologic studies using cells transduced with gain-of-function mutant Shp2 E76K. Serum and growth factor-starved adherent macrophages were stimulated for 60 minutes with 0 ng/mL GM-CSF or 10 ng/mL GM-CSF in the presence of 5 μM IC87114, 0.5 μM GDC-0941, or no inhibitor at all. As previously reported, GM-CSF stimulation produced substantial Erk and Akt hyperactivation in cells endogenously expressing mutant Shp2 D61Y (Figure 4.5B, compare lanes 2 and 6) (Chan, Kalaitzidis et al. 2009). Additionally, at these concentrations, both inhibitors were able to achieve a substantial reduction in GM-CSF-stimulated hyper-phosphorylation of Akt at both Serine 473 and Threonine 308, indicating that endogenous mutant Shp2 D61Y is promoting hyperactivation of PI3K in response to GM-CSF stimulation (Figure 4.5B, compare lanes 7 and 8 with lane 6). Furthermore, both inhibitors reduce Erk hyperactivation, confirming that our previous observation of PI3K positively regulating Erk activity in the presence of activating Shp2 mutants, is not an artifact of oncogene overexpression (Figure 4.5B, compare lanes 7 and 8 with lane 6). These results demonstrate that endogenously expressed mutant Shp2 D61Y promotes hyperactivation of PI3K similarly to exogenously overexpressed mutant Shp2 E76K, which in both cases contributes to GM-CSF hypersensitivity that can be substantially reduced with PI3K inhibitors with high specificity for p110δ.

Additionally, we wanted to investigate the relationship between Ras and mutant Shp2-induced PI3K hyperactivation in the more physiologically relevant context of endogenously expressed mutant Shp2 D61Y. Again, we combined Ras inhibition, using the farnesyl-transferase inhibitor, tipifarnib, with PI3K inhibition, using GDC-0941. Similar to the exogenous Shp2 E76K-expressing cells, we found that treatment of endogenous Shp2 D61Y-expressing cells with 0.5 μM or 1 μM tipifarnib resulted in a significant decrease in GM-CSF-stimulated hyper-proliferation (Figure 4.5C). Furthermore, we found that addition of GDC-0941 again caused a concentration-dependent decrease in GM-CSF-stimulated hyper-proliferation beyond what tipifarnib was able to achieve alone (Figure 4.5C). These results confirm our previous observations (Figures 3.7 and 4.4) that Ras inhibition alone is insufficient to fully block mutant Shp2-induced PI3K hyperactivation, suggesting that this PI3K hyperactivation is at least in part Ras-independent.




| 1 ng/mL GM-CSF | - | + | + | + | + | + | + | + |
|---|---|---|---|---|---|---|---|---|
| GDC-0941 (μM) | 0 | 0 | 0.1 | 0.5 | 1 | 0 | 0 | 0 |
| IC87114 (μM) | 0 | 0 | 0 | 0 | 0 | 5 | 10 | 25 |

**Figure 4.5: PI3K inhibitors with high specificity for p110δ significantly reduce GM-CSF hypersensitivity in cells endogenously expressing gain-of-function mutant Shp2 D61Y**

(A) [$^3$H]-Thymidine incorporation assay measuring 1 ng/mL GM-CSF-stimulated proliferation of bone marrow-derived LDMNCs endogenously expressing WT Shp2 or mutant Shp2 D61Y from Shp2 D61Y inducible knockin mice in the presence of increasing concentrations of the PI3K inhibitors GDC-0941 (0 – 1 μM) or IC87114 (0 – 25 μM). Data representative of 2 independent experiments; n = 4-6; *$p < 0.001$ comparing WT Shp2-expressing cells in the absence of inhibitors or the presence of GDC-0941 at 0.1 μM, 0.5 μM, or 1 μM; #$p < 0.001$ comparing WT Shp2-expressing cells in the absence of inhibitors or the presence of IC87114 at 5 μM, 10 μM, or 25 μM; ^$p < 0.005$ comparing Shp2 D61Y-expressing cells in the absence of inhibitors or the presence of GDC-0941 at 0.1 μM; ^^$p < 0.001$ comparing Shp2 D61Y-expressing cells in the absence of inhibitors or the presence of GDC-0941 at 0.5 μM or 1 μM; +$p < 0.05$ comparing Shp2 D61Y-expressing cells in the absence of inhibitors or the presence of IC87114 at 5 μM; ++$p < 0.001$ comparing Shp2 D61Y-expressing cells in the absence of inhibitors or the presence of IC87114 at 10 μM or 25 μM; statistics performed using unpaired, two-tailed student's t-test (Figure continued on next page).

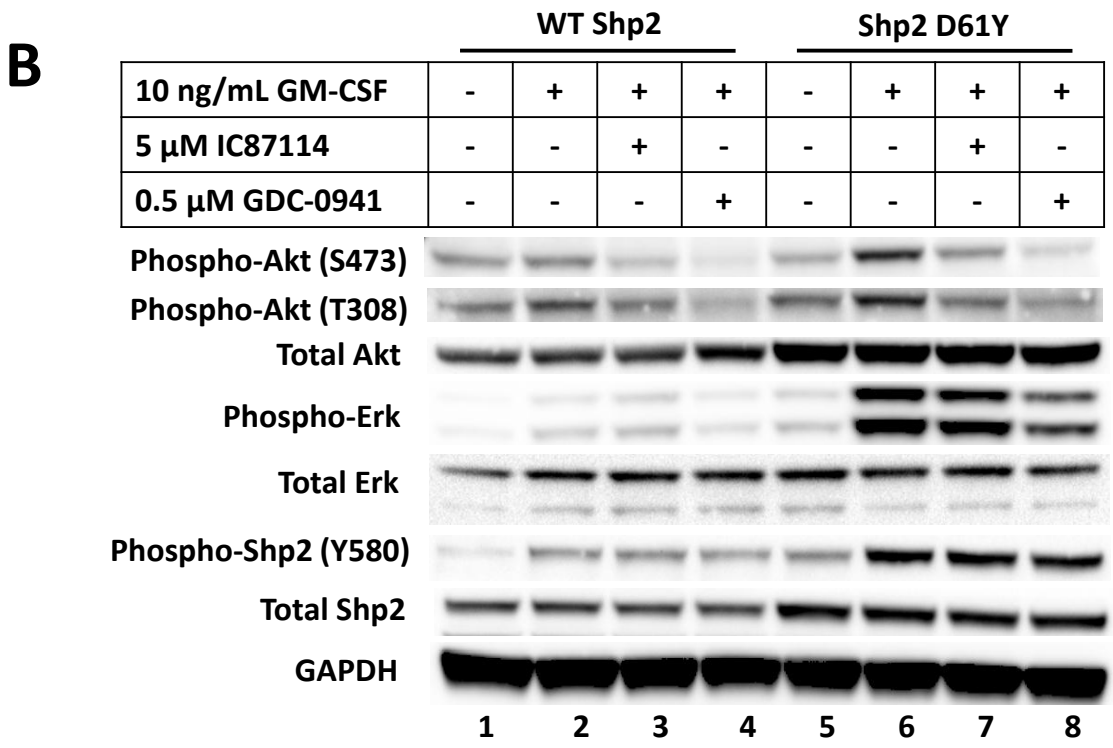
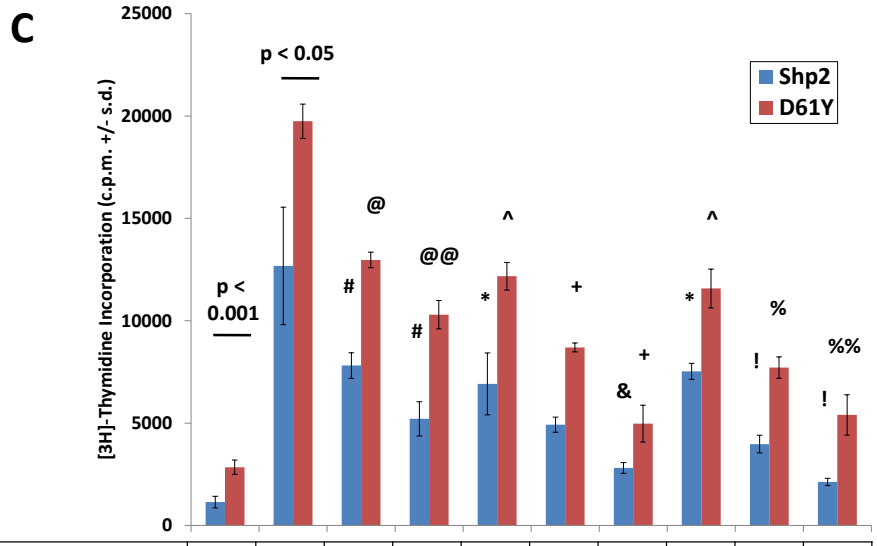



**Figure 4.5: PI3K inhibitors with high specificity for p110δ significantly reduce GM-CSF hypersensitivity in cells endogenously expressing gain-of-function mutant Shp2 D61Y (continued)**

(B) Western blot analyses of adherent macrophages cultured from bone marrow-derived LDMNCs endogenously expressing WT Shp2 or Shp2 D61Y from Shp2 D61Y inducible knockin mice stimulated for 60 minutes with 10 ng/mL GM-CSF in the presence of 5 µM IC87114 or 0.5 µM GDC-0941. Data representative of 3 independent experiments (Figure continued on next page).




| 1 ng/mL GM-CSF | - | + | + | + | + | + | + | + | + | + |
|---|---|---|---|---|---|---|---|---|---|---|
| Tipifarnib (μM) | 0 | 0 | 0 | 0 | 0.5 | 0.5 | 0.5 | 1 | 1 | 1 |
| GDC-0941 (μM) | 0 | 0 | 0.1 | 0.5 | 0 | 0.1 | 0.5 | 0 | 0.1 | 0.5 |

**Figure 4.5: PI3K inhibitors with high specificity for p110δ significantly reduce GM-CSF hypersensitivity in cells endogenously expressing gain-of-function mutant Shp2 D61Y (continued)**

(C) [$^3$H]-Thymidine incorporation assay measuring 1 ng/mL GM-CSF-stimulated proliferation of bone marrow-derived LDMNCs endogenously expressing WT Shp2 or mutant Shp2 D61Y from Shp2 D61Y inducible knockin mice in the presence of increasing combined concentrations of farnesyl-transferase inhibitor, tipifarnib (0 – 1 μM), and the PI3K inhibitor, GDC-0941 (0 – 0.5 μM); data representative of 2 independent experiments; n = 3-4; *$p < 0.05$ comparing WT Shp2-expressing cells not treated with GDC-0941 in the absence versus the presence of tipifarnib at 0.5 μM or 1 μM; ^ $p < 0.001$ comparing Shp2 D61Y-expressing cells not treated with GDC-0941 in the absence versus the presence of tipifarnib at 0.5 μM or 1 μM; #$p < 0.05$ comparing WT Shp2-expressing cells not treated with tipifarnib in the absence versus the presence of GDC-0941 at 0.1 μM or 0.5 μM; &$p < 0.05$ comparing WT Shp2-expressing cells treated with 0.5 μM tipifarnib in the absence versus the presence of GDC-0941 at 0.5 μM; !$p < 0.001$ comparing WT Shp2-expressing cells treated with 1 μM tipifarnib in the absence versus the presence of GDC-0941 at 0.1 μM or 0.5 μM; @$p < 0.005$ comparing Shp2 D61Y-expressing cells not treated with tipifarnib in the absence versus the presence of GDC-0941 at 0.1 μM; @@$p < 0.001$ comparing Shp2 D61Y-expressing cells not treated with tipifarnib in the absence versus the presence of GDC-0941 at 0.5 μM; +$p < 0.001$ comparing Shp2 D61Y-expressing cells treated with 0.5 μM tipifarnib in the absence versus the presence of GDC-0941 at 0.1 μM or 0.5 μM; %$p < 0.005$ comparing Shp2 D61Y-expressing cells treated with 1 μM tipifarnib in the absence versus the presence of GDC-0941 at 0.1 μM; %%$p < 0.001$ comparing Shp2 D61Y-expressing cells treated with 1 μM tipifarnib in the absence versus the presence of GDC-0941 at 0.5 μM. Statistics performed using unpaired, two-tailed student's t-test.

***MEK inhibition reduces gain-of-function mutant Shp2-induced GM-CSF hypersensitivity***

Thus far, we have demonstrated that PI3K inhibitors, particularly those with high specificity for p110δ, are very effective at reducing gain-of-function mutant Shp2-induced hyper-proliferation, most likely by correcting hyperactivated mitogenic signaling pathways, including both PI3K and MAPK. We next wanted to assess the effect of an inhibitor that specifically targets the MAPK pathway alone on mutant Shp2-induced GM-CSF hypersensitivity, using PD0325901, an extremely potent MEK inhibitor. This inhibitor has been shown to be highly effective in reducing GM-CSF hypersensitivity induced by gain-of-function mutations in *KRas* and loss of *Nf1* activity, two other molecular aberrations encountered in JMML (Lyubynska, Gorman et al. 2011; Chang, Krisman et al. 2013).

To assess the inhibitory effect of targeting in MEK in bone marrow LDMNCs from Shp2 D61Y inducible knockin mice, we measured GM-CSF-stimulated proliferation by [$^3$H]-Thymidine incorporation assay in the presence of a range of concnetrations of PD0325901 (0-10 μM). We found that PD0325901 was a very potent inhibitor of mutant Shp2-induced, GM-CSF-stimulated hyper-proliferation, achieving a significant reduction at concentrations as low as 0.1 μM (Figure 4.6A).

Western blot analyses of adherent macrophages expressing Shp2 D61Y confirmed that PD0325901 inhibits MEK activity very efficiently, as GM-CSF-stimulated Erk hyper-phosphorylation was completely abrogated in the presence of just 0.1μM of the inhibitor (Figure 4.6B, compare lanes 7 and 8 with lane 6). However, treatment with PD0325901 had no apparent effect on mutant Shp2-induced hyperactivated PI3K

signaling, as GM-CSF-stimulated Akt hyper-phosphorylation was not altered by the presence of the MEK inhibitor (Figure 4.6B, compare lanes 7 and 8 with lane 6). These results show that a MEK inhibitor like PD0325901 can also significantly reduce mutant Shp2-induced hyper-proliferation in response to GM-CSF stimulation, but it does so exclusively by targeting the MAPK pathway, leaving the hyperactivated PI3K pathway apparently unaffected.

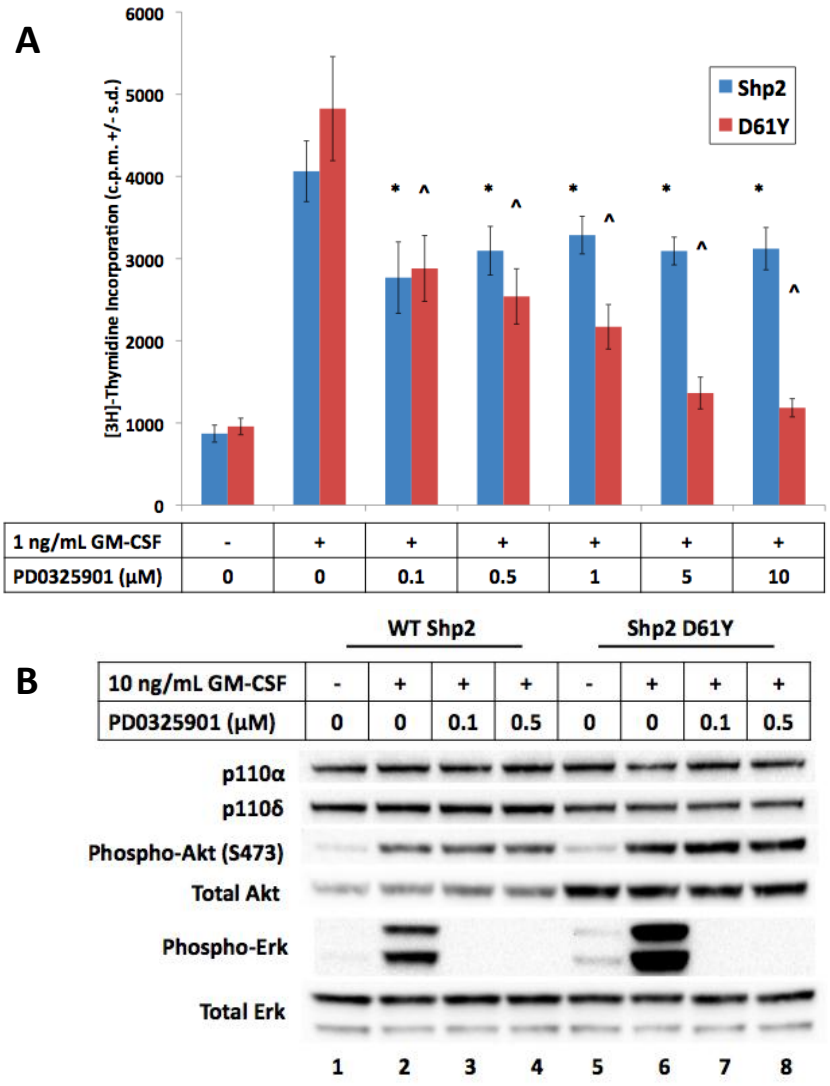



**Figure 4.6: Pharmacologic inhibition of MEK reduces GM-CSF hypersensitivity in gain-of-function mutant Shp2 D61Y-expressing cells**

(A) [$^3$H]-Thymidine incorporation assay measuring 1 ng/mL GM-CSF-stimulated proliferation of bone marrow-derived LDMNCs endogenously expressing WT Shp2 or mutant Shp2 D61Y from Shp2 D61Y inducible knockin mice in the presence of increasing concentrations of the MEK inhibitor PD0325901 (0 – 10 μM). Data representative of 2 independent experiments; n = 7; *$p < 0.001$ comparing WT Shp2-expressing cells in the absence versus the presence of PD0325901 at 0.1 μM, 0.5 μM, 1 μM, 5 μM, or 10 μM; ^$p < 0.001$ comparing Shp2 D61Y-expressing cells in the absence versus the presence of PD0325901 at 0.1 μM, 0.5 μM, 1 μM, 5 μM, or 10 μM; statistics performed using unpaired, two-tailed student's t-test. (B) Western blot analyses of adherent macrophages cultured from bone marrow-derived LDMNCs endogenously expressing WT Shp2 or Shp2 D61Y from Shp2 D61Y inducible knockin mice stimulated for 60 minutes with 10 ng/mL GM-CSF in the presence of 0.1 μM or 0.5 μM PD0325901.

***The highly potent p110δ-specific inhibitor, GS-9820, reduces both GM-CSF-stimulated hyperactivation of Akt and Erk, as well as GM-CSF-stimulated hyper-proliferation of Shp2 D61Y-expressing cells***

Thus far, our data has suggested that gain-of-function mutant Shp2 induces hyperactivation of PI3K signaling and that this increased PI3K activity plays an important contributory role to the GM-CSF hypersensitivity phenotype, which is thought to be an important underlying mechanism for mutant Shp2-induced leukemogenesis. Furthermore, our results suggest that the hematopoietic-specific PI3K catalytic subunit p110δ is a crucial mediator of this hyperactivated PI3K activity, and therefore it represents a potential therapeutic target for the treatment of mutant Shp2-associated MPD. The p110δ-specific inhibitor that we used for our initial studies, IC87114, is adequate for proof-of-concept experiments, but is not sufficiently potent for clinical use. Recently, however, a clinical-grade p110δ-specific inhibitor, GS-1101 (formerly known as CAL-101), has been developed. This inhibitor has been investigated for lymphoid malignancies, and has entered clinical trials for them as a result, but its potential use for myeloid malignancies of any kind has not been studied as far as we know. We initiated a collaboration with Gilead Sciences, which produces the inhibitor, and acquired GS-9820, which is structurally similar to GS-1101, but has a better pharmacodynamic profile in mice (BJ Lannutti, Gilead Sciences, Inc., Seattle, WA, personal communication).

We measured GM-CSF-stimulated proliferation by [$^3$H]-Thymidine incorporation assay of cells from Shp2 D61Y-inducible knockin mice, and found that pre-treating the cells for two hours with GS-9820 over a range of concentrations from 0.1 to 10 μM cause a significant concentration-dependent decrease in 1 ng/mL GM-CSF-stimulated hyper-

proliferation with concentrations as low as 0.1 μM (Figure 4.7A). Furthermore, the GS-9820 showed a significant therapeutic index as GM-CSF-stimulated proliferation of WT Shp2-expressing cells were not significantly affected excepted at higher concentrations, beginning around 5 μM or higher.

To determine whether the inhibitor was having the expected biochemical effect, we performed western blot analyses of WT Shp2 and Shp2 D61Y-expressing macrophages, which had been starved and then stimulated with either 0 ng/mL GM-CSF or 10 ng/mL GM-CCSF in the presence of either 0.1 μM GS-9820 or 0.5 μM GS-9820 or no inhibitor at all. We found that in the presence of GS-9820, there was a concentration-dependent decrease in GM-CSF-stimulated hyper-phosphorylation of Akt at both Serine 473 and Threonine 308 (Figure 4.7B, compare lanes 7 and 8 with lane 6), indicating that it was effectively blocking mutant Shp2-induced PI3K hyperactivity, as the other PI3K inhibitors had. Furthermore, GS-9820, like the other PI3K inhibitors we investigated, caused substantial reductions in GM-CSF-stimulated Erk hyperactivation (Figure 4.8B, compare lanes 7 and 8 with lane 6). Thus, GS-9820 had a similar biochemical profile as the other PI3K inhibitors, and it achieved reductions in aberrant signaling pathways at clinically relevant concentrations (0.1-0.5 μM). As with previous drug studies the magnitude of biochemical effect was not completely commensurate with the functional consequences of the inhibitor. We found that 0.5 μM GS-9820 almost completely abrogated Akt phosphorylation at Serine 473, though the same concentration resulted in only a 30% decrease in GM-CSF-stimulated proliferation. Furthermore, noticeable reductions in Akt and Erk hyper-phosphorylation were observed in WT Shp2-expressing cells at concentrations of 0.1 and 0.5 μM, though no reduction in proliferation were

observed in [$^3$H]-Thymidine Incorporation assays. Again, this lack of congruence may stem from the fact that slightly different cells are being used for the different assay: myeloid progenitor cells for the [$^3$H]-Thymidine Incorporation assays and adherent macrophages for biochemical analyses. It may also be due to the fact that the cells are treated for different time periods: the cells in the [$^3$H]-Thymidine Incorporation assay were pre-treated for two hours with the GS-9820, followed by an approximate 24-hour incubation with 1 ng/mL GM-CSF and the GS-9820, while the adherent macrophages used for biochemical analyses were stimulated for only one hour with 10 ng/mL GM-CSF and the GS-9820. Nevertheless, because GS-9820 was able to produce reductions in both GM-CSF-stimulated hyperactivated mitogenic signaling and hyper-proliferation at low, clinically relevant concentrations, we believe these data both support our hypothesis that p110δ is an important mediator of mutant Shp2-induced hyperactivated PI3K signaling and resulting GM-CSF-stimulated hyper-proliferation, and that, therefore, highly potent inhibitors targeting p110δ, such as GS-9820 (or GS-1101) represent potential therapies for mutant Shp2-associated MPD.

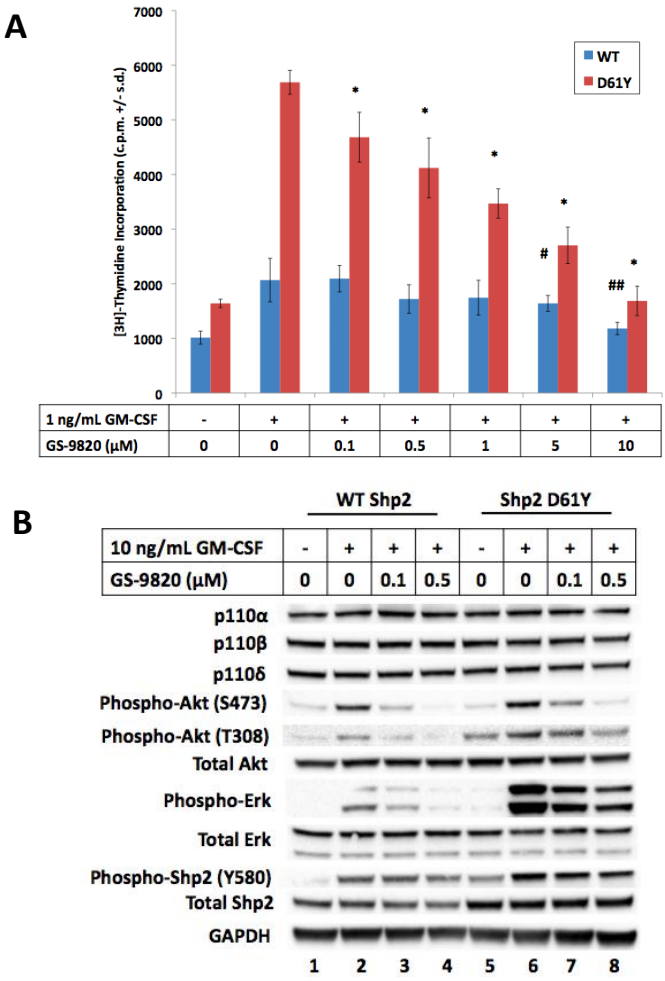



**Figure 4.7: The highly potent p110δ-specific inhibitor GS-9820 reduces gain-of-function mutant Shp2 D61Y-induced Akt and Erk hyperactivation and resulting hyper-proliferation**

(A) $^{3}$H]-Thymidine incorporation assay measuring 1 ng/mL GM-CSF-stimulated proliferation of bone marrow-derived LDMNCs endogenously expressing WT Shp2 or mutant Shp2 D61Y from Shp2 D61Y inducible knockin mice in the presence of increasing concentrations of the highly potent p110δ-specific inhibitor GS-9820 (0-10 μM). Representative data of 2 independent experiments; n = 6-7; *$p<0.001$ comparing Shp2 D61Y-expressing cells in the absence versus the presence of GS-9820 at 0.1 μM, 0.5 μM, 1 μM, 5 μM or 10 μM; #$p<0.05$ comparing WT Shp2-expressing cells in the absence versus the presence of GS-9820 at 5 μM; ##$p<0.05$ comparing WT Shp2-expressing cells in the absence versus the presence of GS-9820 at 10 μM; no significant difference was found comparing WT Shp2-expressing cells in the absence versus the presence of GS-9820 at 0.1 μM, 0.5 μM, or 1 μM; statistics performed using unpaired, two-tailed student's t-test. (B) Western blot analyses of adherent macrophages cultured from bone marrow-derived adherent macrophages endogenously expressing WT Shp2 or Shp2 D61Y from Shp2 D61Y inducible knockin mice stimulated for 60 minutes with 10 ng/mL GM-CSF in the absence or presence of 0.1 μM or 0.5 μM GS-9820.

**Conclusions**

Our earlier genetic studies with *Pik3r1*$^{-/-}$ mice suggested that mutant Shp2-induced hyperactivated Class IA PI3K signaling is an important contributor to the GM-CSF hypersensitivity that characterizes JMML. These initial studies also implicated p110δ as a crucial mediator of mutant Shp2-induced PI3K hyperactivation, which, coupled with its hematopoietic-specific expression profile, makes it a particularly attractive therapeutic target to investigate for mutant Shp2-associated MPD.

To this end, we first focused on evaluating the effectiveness of two commercially available inhibitors with high specificity for the p110δ catalytic subunit: IC87114, which is highly specific for p110δ, but not very potent, and GDC-0941, a clinical-grade pan-PI3K inhibitor with very high specificity for p110α as well as p110δ (Table 4.1). We found that both inhibitors significantly reduced GM-CSF-stimulated hyper-proliferation as well as hyperactivation of both the PI3K and MAPK signaling pathways in cells transduced with gain-of-function mutant Shp2 E76K. These results recapitulated the observations of our genetics studies, thereby supporting our hypothesis that hyperactivated PI3K signaling plays an important role in mutant Shp2-induced GM-CSF hypersensitivity and providing more direct evidence that p110δ is a crucial mediator of this enhanced PI3K activity. In addition, we found that both of these inhibitors increased apoptosis in Shp2 E76K-expressing cells, indicating that PI3K hyperactivation contributes not only to mutant Shp2-induced hyper-proliferation, but also to enhanced survival, suggesting that targeting PI3K, especially p110δ, would be therapeutically beneficial on both accounts. Finally, by combining the PI3K inhibitor with the farnesyl-transferase inhibitor, tipifarnib, which provides a pharmacologic means of blocking

mutant Shp2-induced Ras hyperactivation, we found that the two inhibitors work cooperatively to reduce GM-CSF hypersensitivity, which suggests that the mutant Shp2-induced PI3K hyperactivation is at least in part independent of Ras. Because it has been reported that p110δ alone of the Class I PI3K catalytic subunits can induce oncogenic transformation in a Ras-independent manner, these results further implicate an important role for p110δ.

Because both the previous genetic studies and these initial pharmacologic studies involved retroviral transduction of an exogenous mutant Shp2 allele, we wanted to confirm that the observed PI3K hyperactivity and its contribution to GM-CSF hypersensitivity were not merely an artifact of oncogene overexpression. Therefore, we repeated many of the pharmacologic studies using bone marrow-derived LDMNCs from Shp2 D61Y inducible knockin mice (LSL-Shp2$^{D61Y/+}$; Mx1-Cre+), which express endogenous levels of mutant Shp2 under its native promoter, and found that the inhibitors produced similar reductions in GM-CSF-stimulated hyper-proliferation and hyper-phosphorylation of Akt and Erk. Thus, the Shp2 D61Y knockin mice provide a more physiologically relevant *in vitro* model for investigating the effects of mutant Shp2 on aberrant signal transduction, as well as a potential *in vivo* model of mutant Shp2-induced MPD with which we can explore the role of PI3K in MPD development and progression.

In addition to PI3K inhibitors, we also assessed the MEK inhibitor, PD0325901, which has shown great promise as a therapy in other mouse models of JMML including gain-of-function *Kras* mutations and loss of *Nf1* activity. We found that this inhibitor very efficiently reduced mutant Shp2-induced hyper-proliferation in response to GM-

CSF stimulation and almost completely abrogated hyperactivated MAPK signaling, but without having any apparent effect on PI3K hyperactivation.

Finally, we next wanted to assess the therapeutic potential of a clinical-grade p110δ-specific inhibitor with much higher potency than IC87114. We collaborated with Gilead Sciences to initiate studies using GS-9820, an inhibitor that is structurally similar to GS-1101, but which has better pharmacodynamics in mice. GS-1101 is in clinical trials for lymphoid malignancies, but has never been investigated for myeloid malignancies to the best of our knowledge. Interestingly, we found that pretreating cells for two hours with GS-9820 significantly reduced GM-CSF-stimulated hyper-proliferation in gain-of-function mutant Shp2 D61Y-expressing cells at clinically relevant, nanomolar concentrations. Furthermore, it was able to decrease substantially both Akt and Erk hyper-phosphorylation, recapitulating the biochemical effects observed in prior studies targeting PI3K genetically and pharmacologically. The results of these studies are particularly noteworthy, since GS-1101 (which is chemically related to GS-9820) is already in clinical trials for lymphoid malignancies, suggesting that a highly potent p110δ-specific inhibitor, such as GS-1101, may have therapeutic benefit for the treatment of gain-of-function mutant Shp2-associated MPD.

In sum, our studies suggest that p110δ-mediated PI3K hyperactivation plays an important role in mutant Shp2-induced GM-CSF hypersensitivity, and because p110δ expression is largely restricted to hematopoietic cells, pharmacologically targeting it carries a lower risk of systemic side effects. PI3K inhibitors with high potency for p110δ might also be used in combination with inhibitors that specifically target the mutant Shp2-induced hyperactivated MAPK pathway, such as the MEK inhibitor, PD0325901.

Because MEK is ubiquitously expressed, it can be expected that pharmacologically targeting it in JMML patients might produce unwanted toxic side effects. However, if PD0325901 is used in conjunction with a p110δ-specific inhibitor, which by itself can reduce Erk hyperactivation, it can be expected that lower doses of PD0325901 might be used with reduced risk of side effects. Furthermore, combining inhibitors also has the added advantage of lowering the risk for the development of drug resistance, which is a common concern with any monotherapy approach.

# CHAPTER FIVE

# GENETIC DISRUPTION OF THE PI3K CATALYTIC SUBUNIT p110δ, BUT NOT p110α, REDUCES GAIN-OF-FUNCTION MUTANT SHP2-INDUCED GM-CSF HYPERSENSITIVITY

## Introduction

The results of our previous studies have demonstrated the important role that PI3K plays in gain-of-function mutant Shp2-induced GM-CSF hypersensitivity. Using mice bearing genetic disruption of the *Pik3r1* gene, which encodes the main Class IA PI3K regulatory subunits p85α, p55α, and p50α, we were able to achieve a significant correction in GM-CSF-stimulated hyper-proliferation. This correction, though significant, was not complete, which we hypothesized to be the consequence of some residual PI3K activity, potentially mediated by the p110α and p110δ catalytic subunits, whose expression levels were significantly reduced in the absence of the *Pik3r1*-encoded regulatory subunits, though not completely absent. Their stabilization is possibly mediated through interactions with the remaining Class IA regulatory subunits, p85β or p55γ. We noticed that p110δ levels were significantly higher in the *Pik3r1*$^{-/-}$ cells expressing gain-of-function mutant Shp2 E76K compared to those expressing WT Shp2, suggesting that p110δ may play an important role in mediating gain-of-function mutant Shp2-induced PI3K hyperactivation. This hypothesis was further supported by the observation that *Pik3r1*$^{-/-}$ cells expressing Shp2 E76K were relatively insensitive to treatment with the farnesyl-transferase inhibitor, tipifarnib (Figure 3.7), which blocks Ras activity. This observation is significant since it has been reported that p110δ, unlike the other Class I PI3K catalytic subunits, can transform cells in a Ras-independent manner.

To further investigate the role of p110δ, we used three inhibitors with high specificity for p110δ, IC87114, GDC-0941, and GS-9820, and we found that all three reduced gain-of-function mutant Shp2-induced GM-CSF hypersensitivity. These studies were motivated by a desire to find the most efficient way of inhibiting hyperactivated PI3K signaling in the context of mutant Shp2-associated MPD. In recent years, much work has been dedicated to identifying the specific activities of the different Class I PI3K catalytic subunits in normal physiologic processes and, in particular, in a variety of malignant and non-malignant disease processes. This progress has been coupled with continued development of newer classes of PI3K inhibitors with increased potency and higher specificity for particular catalytic subunits. The goal of this line of research is to target only the PI3K catalytic subunit involved in a particular disease process, while sparing the others, and, in so doing, reducing the risk of unwanted side effects, since PI3K is important in many normal physiologic processes. The catalytic subunit p110δ is particularly well suited for this line of inquiry since it is expressed predominantly in a hematopoietic-specific manner, unlike the other Class IA PI3K catalytic subunits, p110α and p110β, which are ubiquitously expressed. Therefore, p110δ can be targeted in hematopoietic disease with reduced risk of potentially toxic systemic side effects.

In our pharmacologic studies, we first investigated two commercially available inhibitors, IC87114 and GDC-0941. IC87114 is very specific for p110δ, but its $IC_{50}$ is still much too high to be clinically relevant (Table 4.1), though it is appropriate for proof-of-concept studies. GDC-0941, on the other hand, is a clinical-grade inhibitor, and it has a very low $IC_{50}$ for p110δ, but its $IC_{50}$ is equally low for p110α, which makes it impossible to determine if the inhibitory effects that we observed in our studies are due to

its highly potent anti-p110δ activity or if it is due to the fact that it is also blocking p110α. To investigate this issue further, we collaborated with Gilead Sciences to assess the efficacy of GS-9820, which is structurally similar to the clinical-grade p110δ-specific inhibitor, GS-1101, and found that it very efficiently reduced Akt and Erk hyperactivation, and significantly decreased GM-CSF-stimulated hyper-proliferation in mutant Shp2 D61Y-expressing cells at clinically relevant, nanomolar concentrations. All together, these pharmacologic data suggest that p110δ is the principal mediator of gain-of-function mutant Shp2-induced PI3K hyperactivation, which is an important contributor to the phenomenon of GM-CSF hypersensitivity, and therefore, p110δ represents a potential therapeutic target for mutant Shp2-associated MPD.

Because it is difficult to demonstrate that small molecule inhibitors are only interfering with the activity of their intended target, we also wanted to explore the relative importance of Class IA PI3K catalytic subunits in mediating mutant Shp2-induced PI3K activation using a genetic approach. We believed this line of inquiry was especially important given the relatively similar structures of the Class IA PI3K catalytic subunits, despite the fact these inhibitors have been rationally designed to take advantage of subtle structural differences to achieve substantial specificity.

Therefore, to avoid some of the potential ambiguities raised by the p110δ-specific inhibitors investigated in the previous studies, we used a genetic approach to assess the role of p110δ in gain-of-function mutant Shp2-induced GM-CSF hypersensitivity *in vitro* and MPD development *in vivo*. We took advantage of a mouse model in which the *Pik3cd*, the gene encoding p110δ, has been genetically inactivated, such that endogenous levels of kinase-dead p110δ D910A are expressed (Okkenhaug, Bilancio et al. 2002).

This model provides a genetic mimic of very specific p110δ pharmacologic inhibition and prevents compensatory recruitment of the other Class IA PI3K catalytic subunits, which might occur in a model in which p110δ expression is completely knocked out. These mice have the *Pik3cd* gene constitutively inactivated throughout the entire germline, and yet these mice breed normally and have no other overt defects. This is significant, because it suggests that efforts to target p110δ pharmacologically should be well tolerated in humans, since genetic loss p110δ function is compatible with life and relatively normal function.

In a similar way, we also wished to examine the function of p110α, to evaluate its potential role in gain-of-function mutant Shp2-induced PI3K hyperactivation, since its expression was also retained in *Pik3r1*$^{-/-}$ cells transduced with Shp2 E76K, though, in contrast to p110δ, at the same levels as those cells transduced with WT Shp2. Furthermore, aberrant p110α activity is frequently encountered in a variety of cancers, making it a candidate worthy of further investigation. For these studies, we used an inducible knockout mouse model of p110α$^{flox/flox}$; Mx1-Cre+ (Graupera, Guillermet-Guibert et al. 2008), in which the *Pik3ca* gene can be inducibly disrupted by Cre recombinase activity in hematopoietic cells following a polyI:polyC-stimulated γ-interferon response.

For both catalytic subunits—p110δ and p110α—interactions with mutant Shp2 were investigated in two ways: first, by adding the mutant Shp2 E76K allele by retroviral transduction, and second, by crossing the previously described mice—p110δ$^{D910A/D910A}$ and p110α$^{flox/flox}$; Mx1-Cre+—with the Shp2 D61Y inducible knockin mice (Shp2$^{D61Y/+}$; Mx1-Cre+) (Chan, Kalaitzidis et al. 2009). The latter approach has the advantage of

allowing us to explore the function of p110δ and p110α in the more physiologically relevant context of endogenously expressed gain-of-function mutant Shp2 *in vitro* as well as providing a model for evaluating the role of these two catalytic subunits in MPD development *in vivo*.

## Results

### *Genetic inactivation of p110δ reduces GM-CSF-stimulated hyper-phosphorylation of Akt and Erk, but does not reduce GM-CSF-stimulated hyper-proliferation of cells transduced with gain-of-function mutant Shp2 E76K*

To begin investigating the role of the Class IA PI3K catalytic subunit p110δ in gain-of-function mutant Shp2-induced PI3K hyperactivation and GM-CSF hypersensitivity using a genetic approach, we transduced WT and $p110\delta^{D910A/D910A}$ bone marrow low-density mononuclear cells (LDMNCs) with WT Shp2 and gain-of-function mutant Shp2 E76K. Based on our previous observations suggesting an important role for p110δ, we hypothesized that genetic interruption of p110δ activity would significantly decrease GM-CSF-stimulated hyper-proliferation and Akt hyperactivation, in a manner similar to genetic disruption of the *Pik3r1* gene or pharmacologic inhibition of p110δ activity. Because of the consistently observed positive regulatory role that PI3K has on MAPK signaling, we also predicted a significant decrease in Erk hyperactivation (Figure 5.1).

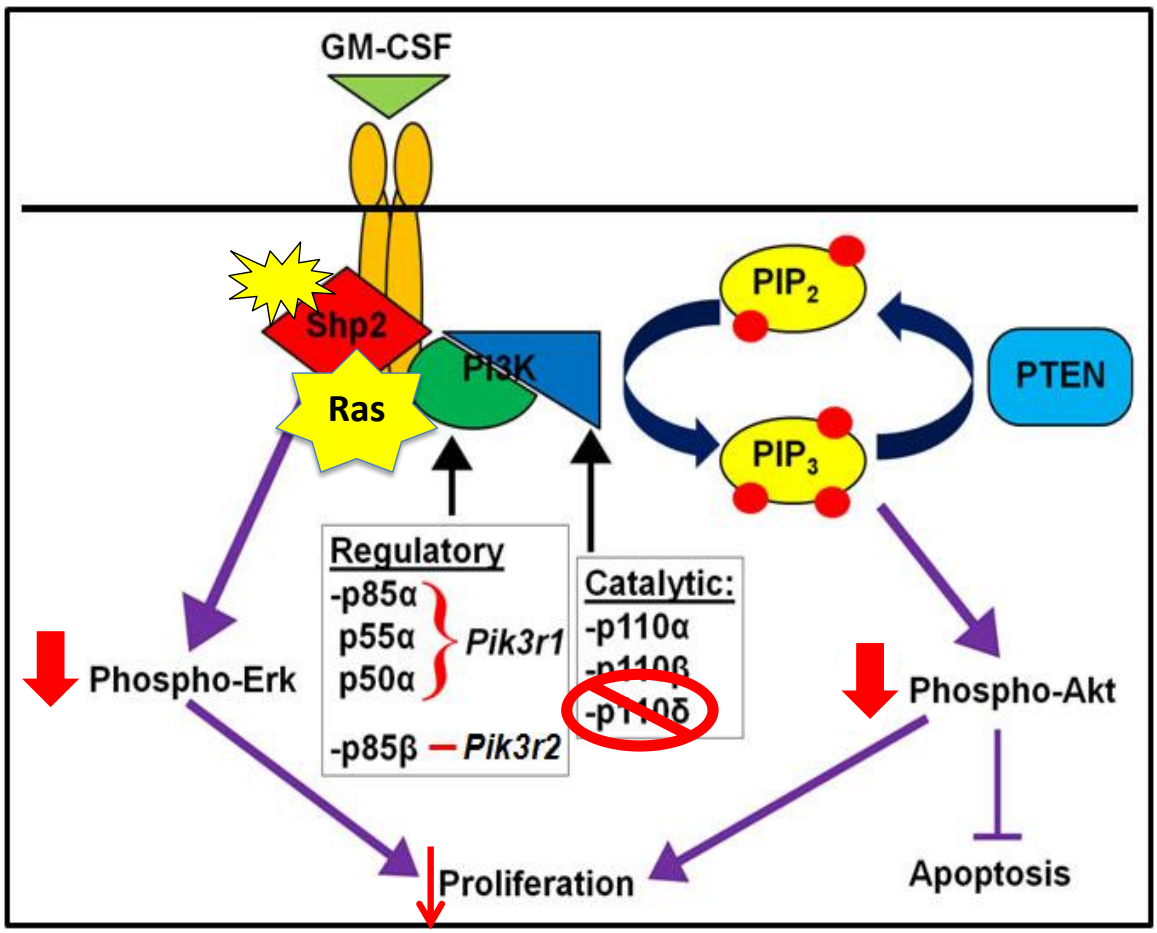



**Figure 5.1: Schematic representation of hypothesized role of the Class IA PI3K catalytic subunit p110δ in gain-of-function mutant Shp2-induced GM-CSF hypersensitivity**

We hypothesize that the hematopoietic-specific Class IA PI3K catalytic subunit p110δ is the major mediator of gain-of-function mutant Shp2-induced PI3K hyperactivation, which contributes to the phenotype of GM-CSF hypersensitivity. We hypothesize that genetic disruption of the *Pik3cd* gene, which encodes p110δ, will result in reduced GM-CSF-stimulated PI3K-Akt signaling, Erk activation, and resulting GM-CSF hypersensitivity.

When we measured GM-CSF-stimulated proliferation by [$^3$H]-Thymidine incorporation, we found, as usual, that WT cells expressing mutant Shp2 E76K had a more robust proliferative response to each given dose of GM-CSF stimulation than those

cells expressing WT Shp2 (Figure 5.2A). We were intrigued, however, to find that p110δ$^{D910A/D910A}$ (kinase-dead p110δ knockin) cells expressing gain-of-function mutant Shp2 E76K had a statistically identical proliferative response to GM-CSF stimulation as WT cells expressing Shp2 E76K (Figure 5.2A), indicating that genetic disruption of p110δ activity had no apparent effect on GM-CSF-stimulated hyper-proliferation of cells transduced with gain-of-function mutant Shp2 E76K.

Western blot analyses of WT and p110δ$^{D910A/D910A}$ adherent macrophages transduced with WT Shp2 or mutant Shp2 E76K revealed that genetic disruption of p110δ activity did, however, affect gain-of-function mutant Shp2 E76K-induced hyperactivated PI3K and MAPK signaling. Again, as previously observed, GM-CSF-stimulated phosphorylation of Erk and Akt (both Serine 473 and Threonine 308) was higher in WT cells expressing mutant Shp2 E76K than those expressing WT Shp2 (Figure 5.2B, compare lanes 5 and 6). Interestingly, p110δ$^{D910A/D910A}$ cells expressing Shp2 E76K had modestly, though consistently, lower levels of both Akt and Erk phosphorylation than cells expressing Shp2 E76K in the presence of intact p110δ (Figure 5.2B, compare lanes 6 and 8). These modest decreases in hyperactivated PI3K and MAPK signaling, however, were apparently not sufficient to reduce proliferation, as we had observed (Figure 5.2A). We had predicted more significant reductions in GM-CSF-stimulated Akt and Erk hyperactivation and, as a result, a measurable decrease in hyper-proliferation. The absence of such a result could not be accounted for by a compensatory upregulation of p110α, which could sustain mutant Shp2-induced PI3K hyperactivation in the absence of functional p110δ, as there was no difference in the expression levels of p110α between WT and p110δ$^{D910A/D910A}$ cells expressing Shp2 E76K (Figure 5.2B,

compare lanes 2 and 4 and lanes 6 and 8). We also noted that expression levels p110δ D910A were similar to WT p110δ. Because we were able to observe biochemical consequences of genetic interruption of p110δ activity that did not translate into a functional effect, we suspected that exogenous overexpression of an oncogene, specifically, gain-of-function mutant Shp2 E76K, resulted in an overly exacerbated hyperactivation of PI3K and MAPK signaling, thereby preventing proper assessment of the role of p110δ in gain-of-function mutant Shp2-induced PI3K hyperactivation. This suspicion led us to believe that this question could be more effectively explored in the more physiologically relevant context of endogenously expressed mutant Shp2.

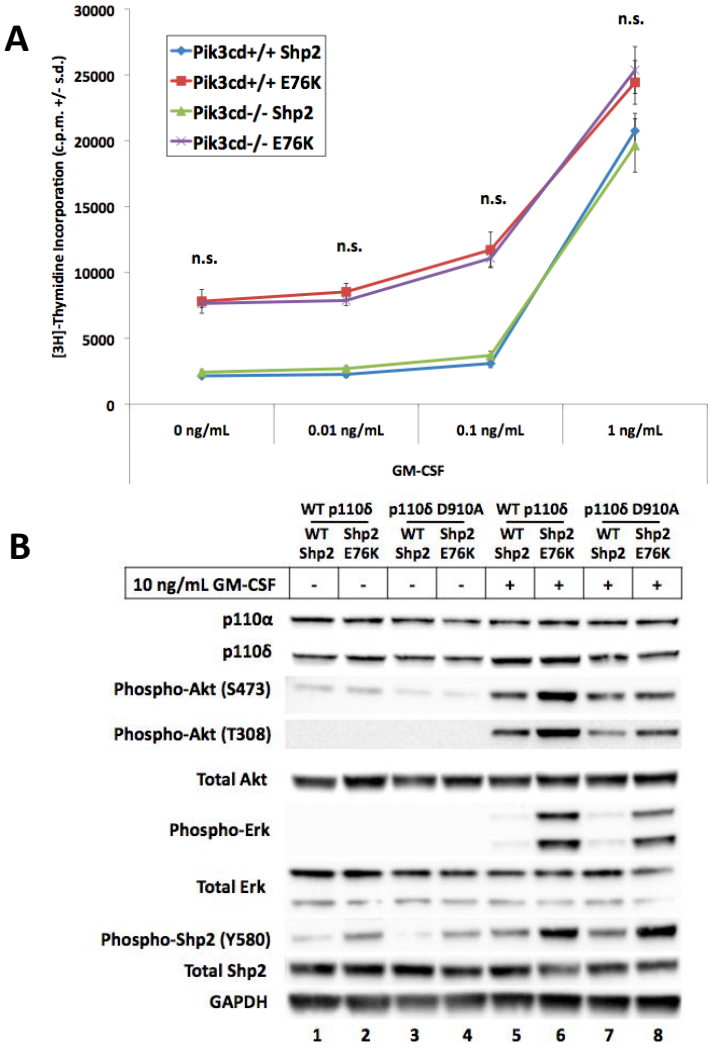



**Figure 5.2: Genetic inactivation of p110δ reduces Akt and Erk hyper-phosphorylation, but does not affect GM-CSF-stimulated hyper-proliferation in cells exogenously overexpressing gain-of-function mutant Shp2 E76K**

(A) [$^3$H]-Thymidine incorporation assay measuring proliferation of bone marrow-derived LDMNCs from WT and kinase-dead mutant p110δ D910A knockin mice transduced with either WT Shp2 or gain-of-function mutant Shp2 E76K in response to increasing concentrations of GM-CSF (0-1 ng/mL). Data representative of 2 independent experiments; n = 6; no significant (n.s.) differences were found comparing WT cells versus p110δ D910A cells transduced with WT Shp2 at any GM-CSF concentration; no significant (n.s.) differences were found comparing WT cells versus p110δ D910A cells transduced with Shp2 E76K at any GM-CSF concentration; statistics performed using unpaired, two-tailed student's t-test. (B) Western blot analyses of macrophages cultured from bone marrow-derived LDMNCs from WT and kinase-dead mutant p110δ D910A knockin mice transduced with WT Shp2 or gain-of-function mutant Shp2 E76K and stimulated for 60 minutes with 0 or 10 ng/mL GM-CSF. Data representative of 2 independent experiments.

***Genetic deletion of p110α has no effect on GM-CSF-stimulated hyper-proliferation or Akt and Erk hyperactivation in cells transduced with gain-of-function mutant Shp2 E76K***

Using a similar genetic approach to assess the role of the Class IA PI3K catalytic subunit p110α in gain-of-function mutant Shp2-induced PI3K hyperactivation and resulting GM-CSF hypersensitivity, we introduced, by retroviral transduction, WT Shp2 and gain-of-function mutant Shp2 E76K into bone marrow LDMNCs lacking p110α expression ($p110\alpha^{flox/flox}$; Mx1-Cre+) as well as into bone marrow LDMNCs from WT control littermates ($p110\alpha^{flox/flox}$; Mx1-Cre-) approximately four weeks after polyI:polyC treatment, allowing sufficient time for recombination of the *Pik3ca* gene to occur. Because we hypothesized that p110δ is the crucial mediator of gain-of-function mutant Shp2-induced GM-CSF hypersensitivity, we predicted that genetic deletion of p110α would not cause a significant decrease in mutant Shp2-induced hyperactivation of PI3K-Akt signaling or MAPK signaling, and that, therefore, it would not lead to reduced GM-CSF-stimulated hyper-proliferation (Figure 5.3). However, because of p110α's frequent association with human cancer, and because of our observation that p110α expression was retained, albeit at drastically reduced levels, in *Pik3r1*$^{-/-}$ cells transduced with mutant Shp2 E76K (Figure 3.7), we thought it would be prudent to formerly investigate the role of p110α in gain-of-function mutant Shp2-induced GM-CSF hypersensivity, using a similar genetic approach that we had employed for p110δ.

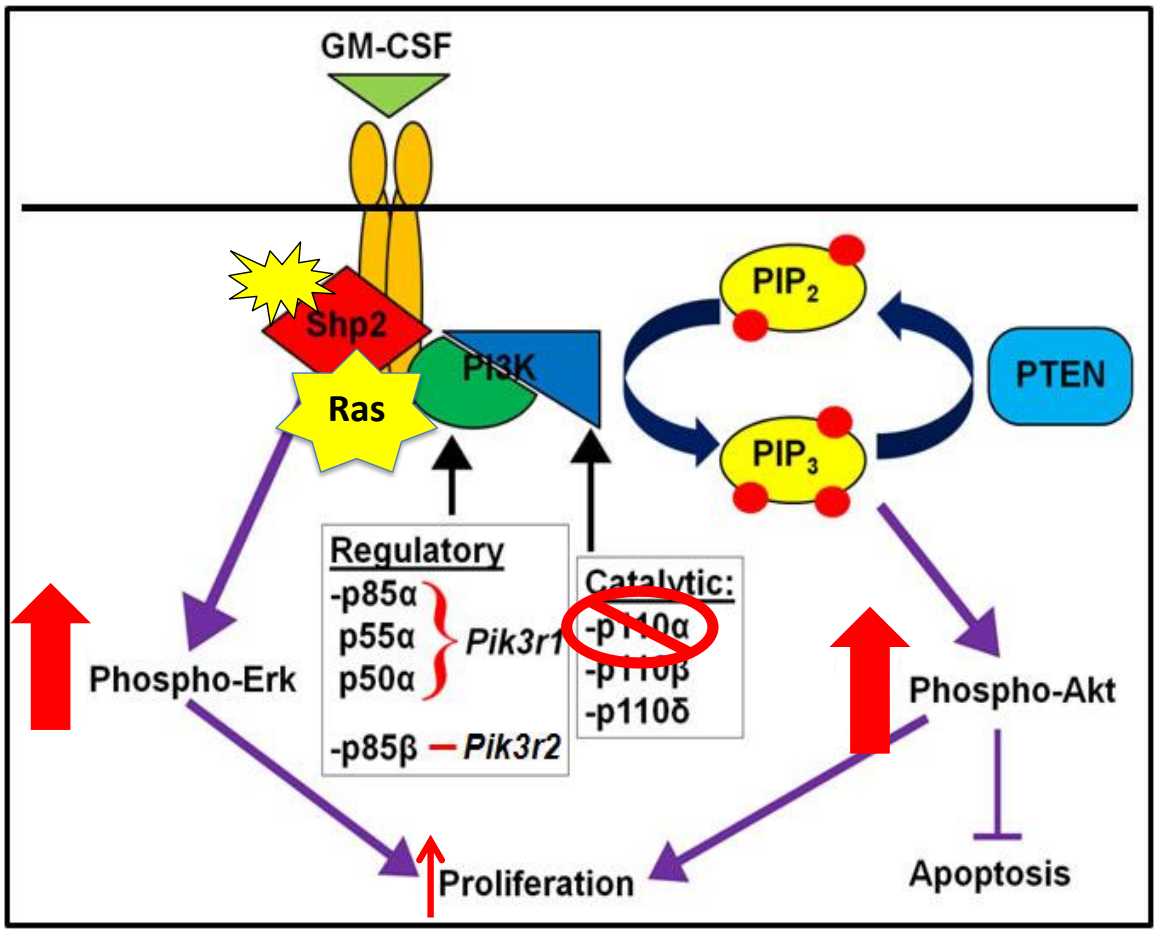



**Figure 5.3: Schematic representation of hypothesized role of the Class IA PI3K catalytic subunit p110α in gain-of-function mutant Shp2-induced GM-CSF hypersensitivity**

We hypothesize that the Class IA PI3K catalytic subunit p110α is not an important mediator of gain-of-function mutant Shp2-induced PI3K hyperactivation, and that, therefore, genetic disruption of the *Pik3ca* gene, which encodes p110α, will have no effect on GM-CSF-stimulated PI3K-Akt signaling, Erk activation, or resulting GM-CSF hypersensitivity.

Similar to our observations with cells bearing genetic interruption of p110δ activity, loss of p110α had no apparent effect on gain-of-function mutant Shp2 E76K-induced GM-CSF hypersensitivity (Figure 5.4A). Again, WT cells expressing Shp2

E76K displayed a proliferative response, which was greater for each increasing concentration of GM-CSF stimulation than that of WT cells expressing WT Shp2. No difference in GM-CSF-stimulated proliferative response was observed between Shp2 E76K-expressing cells lacking p110α versus those with intact p110α expression (Figure 5.4A).

However, in contrast to our observations of $p110\delta^{D910A/D910A}$ cells expressing Shp2 E76K (Figure 5.2B), western blot analyses demonstrated that GM-CSF-stimulated Akt and Erk hyper-phosphorylation was not lower in $p110\alpha^{flox/flox}$; Mx1-Cre+ cells expressing mutant Shp2 E76K compared to WT cells expressing Shp2 E76K (Figure 5.4B, compare lanes 6 and 8), indicating that mutant Shp2-induced hyperactivated PI3K-Akt and MAPK signaling remains intact following the loss of p110α expression, unlike genetic disruption of p110δ activity. Western blots also showed that p110α had been efficiently deleted in cells from the p110α inducible knockout mice, and that there is no apparent compensatory increase in p110δ expression (Figure 5.4B). Thus it appears that genetic disruption of p110α had no appreciable effect on gain-of-function mutant Shp2-induced aberrant signaling or GM-CSF hypersensitivity. As noted above, however, the fact that gain-of-function mutant Shp2 E76K is exogenously overexpressed in these cells is a confounding factor, which may obscure the subtle effects of losing the expression or activity of one or another Class IA PI3K catalytic subunit, and which can only be remedied by assessing their roles in the context of endogenously expressed gain-of-function mutant Shp2.

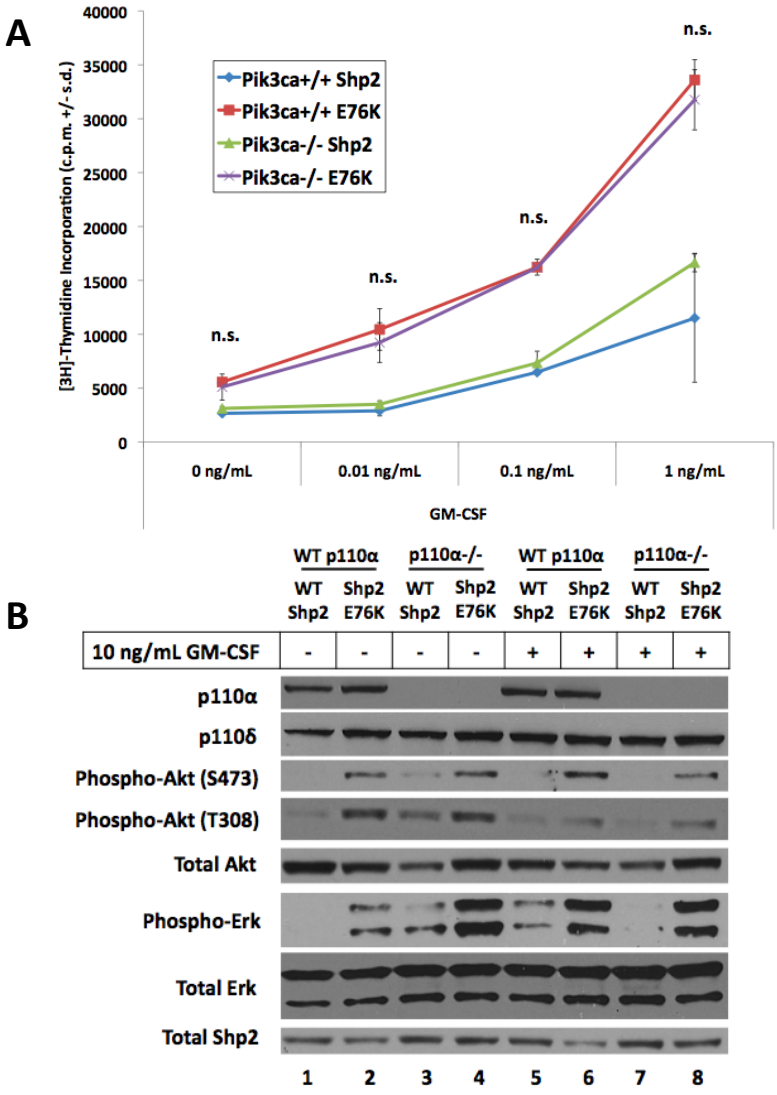



**Figure 5.4: Genetic deletion of p110α does not reduce GM-CSF-stimulated hyper-proliferation or Akt and Erk hyper-phosphorylation in cells exogenously overexpressing gain-of-function mutant Shp2 E76K**

(A) [$^{3}$H]-Thymidine incorporation assay measuring proliferation of bone marrow-derived LDMNCs from WT and p110α$^{flox/flox}$ inducible knockout mice transduced with either WT Shp2 or gain-of-function mutant Shp2 E76K in response to increasing concentrations of GM-CSF (0-1 ng/mL). Data representative of 2 independent experiments; n = 4; no significant (n.s.) differences were found comparing WT cells versus p110α-/- cells transduced with WT Shp2 at any GM-CSF concentration; no significant (n.s.) differences were found comparing WT cells versus p110α-/- cells transduced with Shp2 E76K at any GM-CSF concentration; statistics performed using unpaired, two-tailed student's t-test. (B) Western blot analysis of macrophages cultured from bone marrow-derived LDMNCs from WT and p110α$^{flox/flox}$ inducible knockout mice transduced with WT Shp2 or gain-of-function mutant Shp2 and stimulated for 60 minutes with 0 or 10 ng/mL GM-CSF. Data representative of 2 independent experiments.

***Development of mouse models that allow the investigation of the roles of p110δ and p110α in endogenously expressed gain-of-function mutant Shp2-induced GM-CSF hypersensitivity* in vitro *and MPD development* in vivo**

Because genetic disruption of p110δ activity in cells with exogenously overexpressed mutant Shp2 E76K did not reduce GM-CSF-stimulated hyper-proliferation, despite causing a modest decrease in GM-CSF-stimulated Akt and Erk hyper-phosphorylation, we suspected that the effect of lost p110δ activity on gain-of-function mutant Shp2-induced PI3K hyperactivation and resulting GM-CSF hypersensitivity was not being adequately investigated, and could be better assessed in the more physiologically relevant setting of endogenously expressed mutant Shp2. To accomplish this, we crossed the p110δ D910A kinase-dead constitutive knockin mice with gain-of-function mutant Shp2 D61Y inducible knockin mice (LSL-Shp2$^{D61Y/+}$; Mx1-Cre+) to yield mice with genetically interrupted p110δ D910A in the presence of endogenously expressed mutant Shp2 D61Y (p110δ$^{D910A/D910A}$; LSL-Shp2$^{D61Y/+}$; Mx1-Cre+, "experimental"), which can be compared to mice expressing mutant Shp2 D61Y in the presence of intact p110δ activity (p110δ$^{+/+}$; LSL-Shp2$^{D61Y/+}$; Cre+, "positive control" or "disease control") and mice expressing WT Shp2 in the presence of intact p110δ activity (p110δ$^{+/+}$; LSL-Shp2$^{D61Y/+}$; Cre-, "negative control" or "WT control") (Figure 5.5A). Among these different groups of mice, we predicted that the negative control (WT) mice would not develop myeloproliferative disease (MPD) *in vivo* and would not exhibit GM-CSF hypersensitivity or hyperactivated PI3K or MAPK signaling *in vitro*. In addition, we predicted, as has been reported (Chan, Kalaitzidis et al. 2009), that the positive control (D61Y) mice would develop MPD *in vivo* and would exhibit both GM-

CSF hypersensitivity and hyperactivated PI3K and MAPK signaling *in vitro*. Finally, because we hypothesize that p110δ is the crucial mediator of gain-of-function mutant Shp2-induced GM-CSF hypersensitivity, we predicted that the experimental (D61Y/p110δ) mice would either not develop MPD or would present with a milder MPD phenotype *in vivo*, and that they would exhibit either significantly reduced or entirely corrected GM-CSF hypersensitivity and hyperactivation of PI3K and MAPK signaling *in vivo*.

Similarly, we wanted to assess the role of p110α by crossing p110α inducible knockout mice ($p110\alpha^{flox/flox}$; Mx1-Cre+) with Shp2 D61Y inducible knockin mice. In so doing, we can better determine if p110δ is an indispensable mediator of gain-of-function mutant Shp2-induced PI3K hyperactivation and GM-CSF hypersensitivity, or, rather, if p110α plays an equally crucial role in this process. Answering this question is important, because it will allow us to define the most effective way of targeting mutant Shp2-mediated PI3K hyperactivation; therefore, is targeting p110δ alone sufficient to reduce GM-CSF hypersensitivity or must p110α and p110δ be co-targeted for effective blockade of hyperactivated PI3K signaling?

As with the p110δ kinase-dead mice, we crossed $p110\alpha^{flox/flox}$; Mx1-Cre+ mice with LSL-$Shp2^{D61Y/+}$; Mx1-Cre+ mice to generate mice that express endogenous levels of gain-of-function mutant Shp2 D61Y in the absence of p110α expression ($p110\alpha^{flox/flox}$; LSL-$Shp2^{D61Y/+}$; Mx1-Cre+, "experimental"), which can likewise be compared to mice expressing Shp2 D61Y in the presence of p110α and all other catalytic subunits ($p110\alpha^{+/+}$; LSL-$Shp2^{D61Y/+}$; Mx1-Cre+, "positive control" or "disease control") and mice expressing WT Shp2 in the presence of normal p110α expression ($p110\alpha^{flox/flox}$; LSL-

Shp2$^{D61Y/+}$; Mx1-Cre-, "negative control" or "WT control") (Figure 5.5B). Among these different groups, we again predicted that negative control (WT) mice would not develop MPD *in vivo* and would not exhibit GM-CSF hypersensitivity or hyperactivated PI3K or MAPK signaling *in vitro*, while we predicted that the positive control (D61Y) mice would develop MPD *in vivo* and exhibit both GM-CSF hypersensitivity and hyperactivated PI3K and MAPK signaling *in vitro*. However, because we hypothesized that p110δ uniquely is the principal mediator of gain-of-function mutant Shp2-induced PI3K hyperactivation, and that, p110α's role in contrast is dispensable, we predicted that the p110α experimental mice (p110α$^{flox/flox}$; LSL-Shp2$^{D61Y/+}$; Mx1-Cre+) would develop features of MPD *in vivo* similar to the Shp2 D61Y-expressing positive control mice, and that, *in vitro*, their cells would to exhibit GM-CSF hypersensitivity and hyperactivated PI3K and MAPK signaling, unimpeded by the loss of p110α.

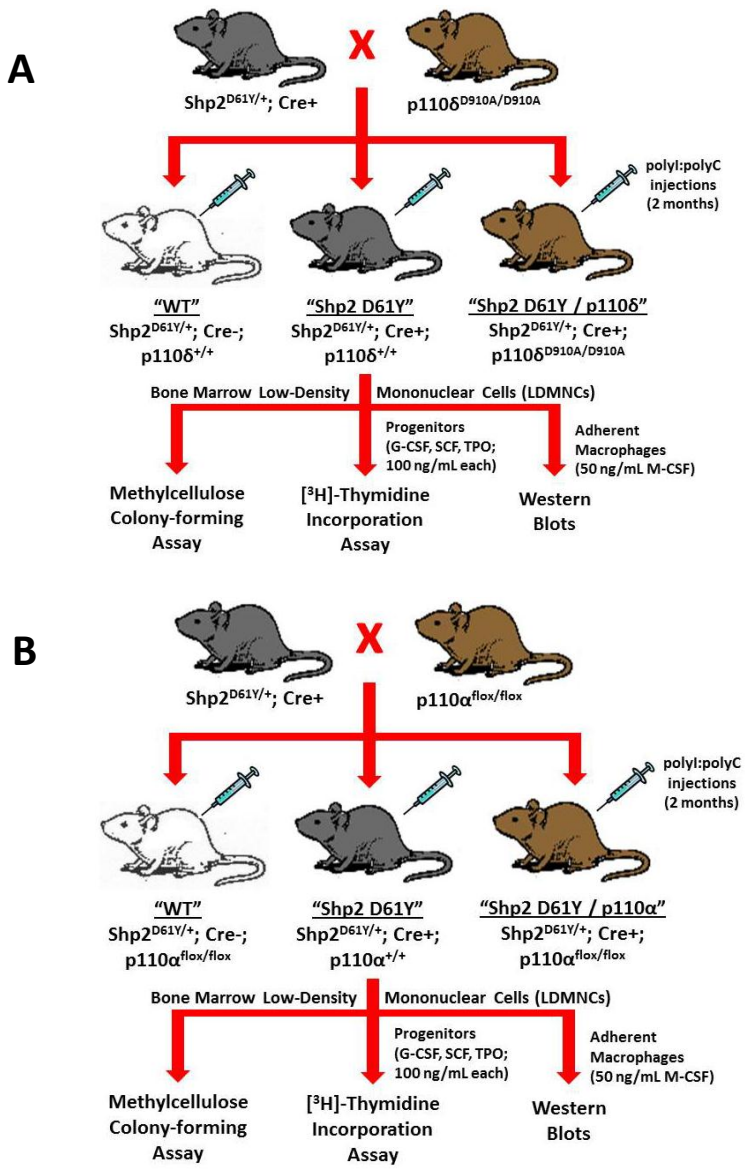



**Figure 5.5: Schematic of strategy for developing mouse models that can be used to investigate the roles of the PI3K catalytic subunits p110δ and p110α in endogenously expressed gain-of-function mutant Shp2-induced GM-CSF hypersensitivity *in vitro* and myeloproliferative disease development *in vivo***

(A) To investigate the role of p110δ in GM-CSF hypersensitivity induced by endogenously expressed gain-of-function mutant Shp2 D61Y *in vitro* and MPD development *in vivo*, mice expressing kinase-dead mutant p110δ D910A were crossed with LSL-Shp2$^{D61Y/+}$; Mx1-Cre+ mice, which can be induced to express endogenous levels of gain-of-function mutant Shp2 D61Y in hematopoietic cells upon polyI:polyC treatment. (B) To investigate the role of p110α in GM-CSF hypersensitivity induced by endogenously expressed gain-of-function mutant Shp2 D61Y *in vitro* and MPD development *in vivo*, p110α-inducible knockout mice (p110α$^{flox/flox}$) mice were crossed with LSL-Shp2$^{D61Y/+}$; Mx1-Cre+ mice.

***Genetic interruption of p110δ, but not p110α, results in significantly reduced GM-CSF-stimulated hyper-proliferation induced by endogenously expressed gain-of-function mutant Shp2 D61Y***

To investigate the effects of the loss of either p110δ activity or of p110α expression on gain-of-function mutant Shp2 D61Y-induced hyper-proliferation in response to GM-CSF stimulation, we performed both [$^3$H]-Thymidine incorporation assays and methylcellulose colony-forming assays using bone marrow-derived low-density mononuclear cells (LDMNCs) isolated from mice representing the different experimental groups described above approximately 8-12 weeks following polyI:polyC treatment, allowing sufficient time for disease onset.

As we have seen before in [$^3$H]-Thymidine incorporation assays, cells expressing gain-of-function mutant Shp2 D61Y in the presence of intact PI3K signaling exhibited a significantly greater proliferative response for each increasing concentration of GM-CSF stimulation compared to cells expressing WT Shp2 (Figure 5.6A, compare red line with blue line). Interestingly, we found that cells expressing kinase-dead p110δ D910A in the presence of mutant Shp2 D61Y showed a significant decrease in GM-CSF-stimulated proliferation compared to those cells expressing Shp2 D61Y in the presence of WT p110δ, but rather had a GM-CSF-stimulated proliferative response similar to that of WT control cells (Figure 5.6A, compare green line with red line and blue line). This suggests that intact p110δ activity is necessary for maintaining gain-of-function mutant Shp2-induced GM-CSF hypersensitivity. In contrast, genetic deletion of p110α had no effect on GM-CSF-stimulated proliferation in Shp2 D61Y-expressing cells, as there was no significant difference in [$^3$H]-Thymidine incorporation between cells from p110α$^{flox/flox}$;

LSL-Shp2$^{D61Y/+}$; Mx1-Cre+ (D61Y/p110α) mice and cells from p110α$^{+/+}$; LSL-Shp2$^{D61Y/+}$; Mx1-Cre+ (D61Y) mice (compare purple line with red line).

Similarly, we performed methylcellulose colony-forming assays in which equal numbers of cells from each group were plated in methylcellulose in the presence of increasing concentrations of GM-CSF (0-10 ng/mL), before colonies were counted 7-10 days later. In this assay, we observed the same pattern as with [$^3$H]-Thymidine incorporation assay. As previously reported, cells expressing Shp2 D61Y have increased colony forming capacity in response to increasing doses of GM-CSF stimulation compared to WT Shp2-expressing cells (Figure 5.6B, compare red and blue lines) (Chan, Kalaitzidis et al. 2009). Shp2 D61Y cells expressing kinase-dead p110δ D910A have significantly reduced colony-forming capacity for each dose of GM-CSF up to 1 ng/mL (Figure 5.6B, compare red and green lines). However, genetic deletion of p110α did not affect mutant Shp2 D61Y-induced enhanced colony formation in response to GM-CSF stimulation (Figure 5.6B, compare red and purple lines), similar to the pattern observed in the [$^3$H]-Thymidine incorporation assay data.

Thus, together these data demonstrate that genetic interruption of p110δ, but not p110α, can significantly reduce GM-CSF-stimulated hyper-proliferation and enhanced colony-forming capacity in cells with activating Shp2 mutations. This provides further support that p110δ is the principal mediator of mutant Shp2-induced GM-CSF hypersensitivity, with p110α playing either a negligible or a dispensable role, and that, therefore, p110δ should be considered a potential therapeutic target for gain-of-function mutant Shp2-associated MPD.

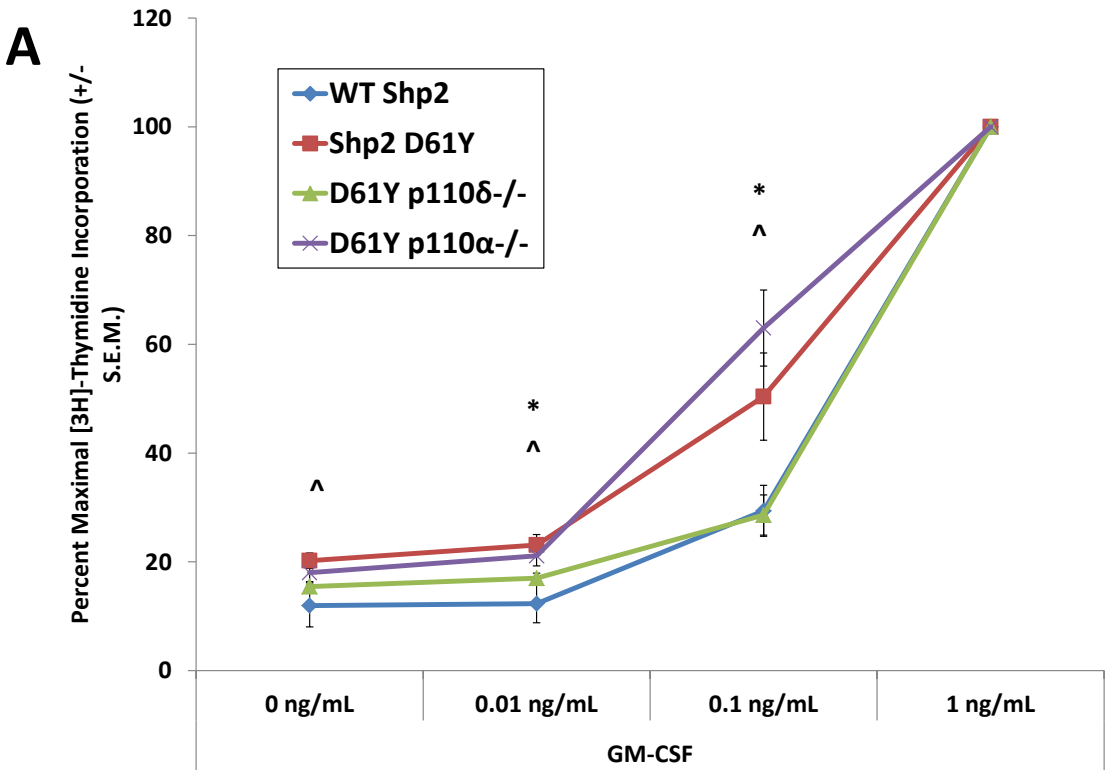



**Figure 5.6: Genetic disruption of p110δ, but not p110α, significantly reduces GM-CSF-stimulated hyper-proliferation induced by endogenously expressed gain-of-function mutant Shp2 D61Y**

(A) Proliferation in response to increasing concentrations of GM-CSF (0-1 ng/mL) as measured by [$^3$H]-Thymidine incorporation and reported as average percent maximal stimulation of bone marrow-derived LDMNCs from WT mice, Shp2 D61Y inducible knockin mice, Shp2 D61Y-inducible knockin mice expressing kinase-dead mutant p110δ D910A, and Shp2 D61Y-inducible knockin mice with genetic deletion of p110α; n=5-8 mice, performed in replicates of 6; *$p < 0.05$ comparing Shp2 D61Y mice with WT Shp2 mice at 0.01 ng/mL and 0.1 ng/mL GM-CSF; ^$p < 0.05$ comparing Shp2 D61Y mice with D61Y p110δ-/- mice at 0 ng/mL, 0.01 ng/mL, and 0.1 ng/mL GM-CSF; no significant difference was found comparing Shp2 D61Y mice with D61Y p110α-/- mice at any GM-CSF concentration; statistics performed using unpaired, two-tailed student's t-test (figure continued on next page).

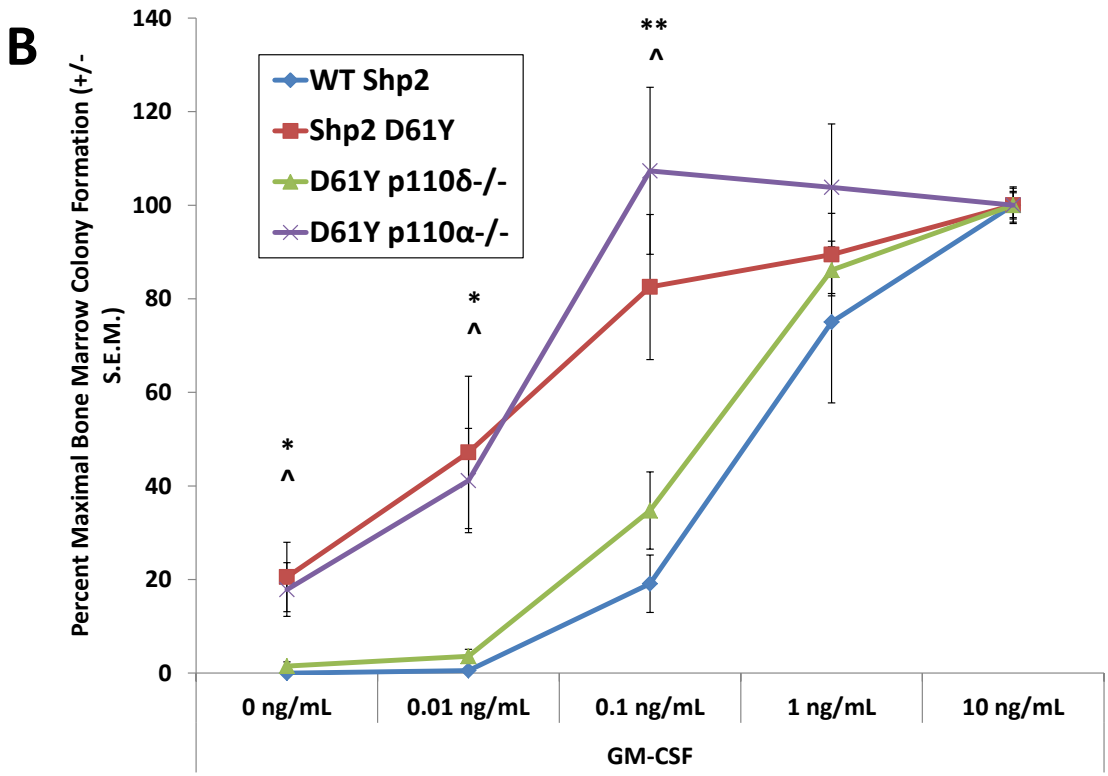



**Figure 5.6: Genetic disruption of p110δ, but not p110α, significantly reduces GM-CSF-stimulated hyper-proliferation induced by endogenously expressed gain-of-function mutant Shp2 D61Y (continued)**

(B) Proliferation in response to increasing concentrations of GM-CSF (0-10 ng/mL) as measured by methylcellulose colony-forming assay and reported as average percent maximal stimulation of bone marrow-derived LDMNCs from WT mice, Shp2 D61Y inducible knockin mice, Shp2 D61Y-inducible knockin mice expressing kinase-dead mutant p110δ D910A, and Shp2 D61Y-inducible knockin mice with genetic deletion of p110α; n=6-8 mice, performed in replicates of 2; *$p < 0.05$ comparing Shp2 D61Y mice with WT Shp2 mice at 0 ng/mL and 0.01 ng/mL GM-CSF; **$p < 0.005$ comparing Shp2 D61Y mice with WT Shp2 mice at 0.1 ng/mL GM-CSF; ^$p < 0.05$ comparing Shp2 D61Y mice with D61Y p110δ-/- mice at 0 ng/mL, 0.01 ng/mL, and 0.1 ng/mL GM-CSF; no significant difference was found comparing Shp2 D61Y mice with D61Y p110α-/- mice at any GM-CSF concentration; statistics performed using unpaired, two-tailed student's t-test.

***Genetic interruption of p110δ, but not p110α, results in decreased GM-CSF-stimulated hyper-phosphorylation of Akt, Erk, and Shp2***

To evaluate the consequences of genetic interruption of p110δ and p110α on mutant Shp2-induced hyperactivated PI3K-Akt and MAPK-Erk signaling pathways, western blot analyses were performed on adherent macrophages cultured from bone marrow-derived LDMNCs from mice endogenously expressing gain-of-function mutant Shp2 D61Y in the presence of genetically inactivated p110δ D910A or genetically deleted p110α. When we transduced cells from $p110\delta^{D910A/D910A}$ mice with gain-of-function mutant Shp2 E76K, we observed modest decreases in GM-CSF-stimulated hyper-phosphorylation of both Akt and Erk, suggesting that loss of p110δ activity was having some effect on mutant Shp2-induced aberrant signaling, though it was not sufficient to decrease the hyper-proliferative phenotype. We hypothesized that in the presence of endogenously expressed mutant Shp2 (rather than exogenously overexpressed mutant Shp2) the effects on hyperactivated signaling would be more pronounced, especially since there was a significant reduction in GM-CSF-stimulated hyper-proliferation and colony-forming capacity in the Shp2 D61Y-expressing cells lacking p110δ activity.

Comparing macrophages derived from two mice expressing Shp2 D61Y in the presence of kinase-dead p110δ D910A with macrophages derived from two mice expressing Shp2 D61Y in the presence of intact p110δ activity, we observed that mutant Shp2-induced PI3K hyperactivation had been significantly reduced, as GM-CSF-stimulated Akt hyper-phosphorylation at both Serine 473 and Threonine 308 had been substantially decreased (Figure 5.7A, compare lanes 5 and 6 with lanes 7 and 8).

Furthermore, we also found that Erk hyper-phosphorylation was also greatly reduced in keeping with our previous observations that PI3K positively regulates Erk in the context mutant Shp2 (Figure 5.7A, compare lanes 5 and 6 with lanes 7 and 8). Finally, we were also surprised to find that Shp2 phosphorylation at Tyrosine 580 was also reduced, indicating that gain-of-function mutant Shp2 hyperactivation was also decreased in the absence of p110δ activity (Figure 5.7A, compare lanes 5 and 6 with lanes 7 and 8). Finally, we observed that there was no evidence of upregulation of the other two Class IA PI3K catalytic subunits, p110α and p110β, to compensate for the lost p110δ activity.

In contrast, deletion of p110α had no apparent effect on mutant Shp2 D61Y-induced hyperactivation of PI3K and MAPK signaling pathways downstream of GM-CSF stimulation. First, we observed that p110α had been efficiently deleted and that there were no compensatory upregulation of the other two Class IA PI3K catalytic subunits, p110β and p110δ (Figure 5.7B, compare lanes 7 and 8 with lane 6, and lanes 3 and 4 with lane 2). Secondly, unlike genetic interruption of p110δ activity, deletion of p110α did not negatively affect mutant Shp2 D61Y-induced PI3K hyperactivity, as the levels of Akt phosphorylation at both Serine 473 and Threonine 308 remained as high in the cells from mice expressing Shp2 D61Y in the absence of p110α as in the cells from mice expressing Shp2 D61Y in the presence of p110α (Figure 5.7B, compare lanes 7 and 8 with lane 6). Similarly, mutant Shp2 D61Y-induced hyperactivation of Erk as well as Shp2 itself was also unaffected by the deletion of p110α (Figure 5.7B, compare lanes 7 and 8 with lane 6).

Thus, the results of the biochemistry assays correlate with the observations from the proliferation assays, in which genetic inactivation of p110δ substantially reduced both

Akt and Erk hyper-phosphorylation as well as GM-CSF-stimulated hyper-proliferation and enhanced colony-forming capacity, suggesting that intact p110δ activity is necessary to maintain gain-of-function mutant Shp2-induced hyperactivation of PI3K-Akt and Erk signaling, which in turn promote the phenotype of an enhanced proliferative response following GM-CSF stimulation. The role of p110α, on the other hand, is either negligible or dispensable, as genetic deletion of p110α had no effect on mutant Shp2 D61Y-induced Akt or Erk hyperactivation, and thus, not surprisingly, caused no reduction in GM-CSF-stimulated hyper-proliferation or enhanced colony-forming capacity.

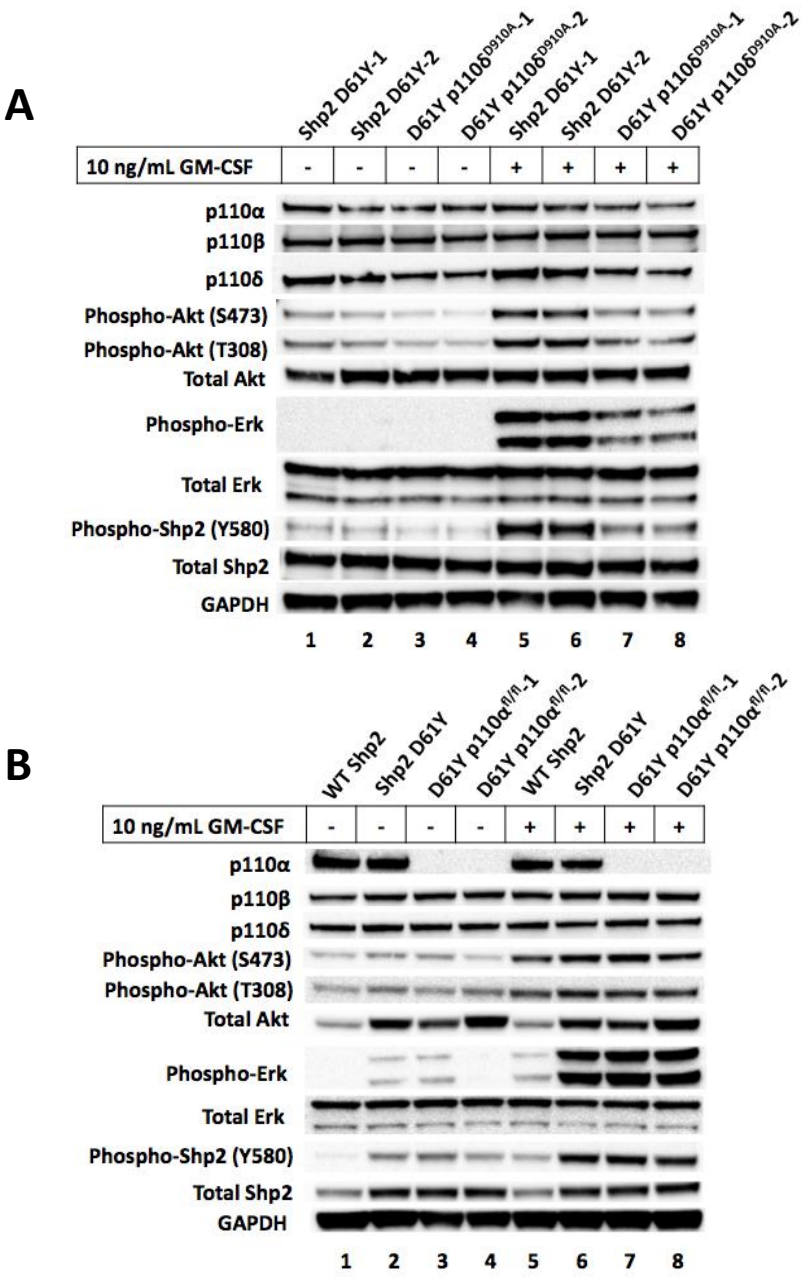



**Figure 5.7: Genetic disruption of p110δ, but not p110α, significantly reduces GM-CSF-stimulated hyper-phosphorylation of Akt, Erk, and Shp2 in cells endogenously expressing gain-of-function mutant Shp2 D61Y**

(A) Western blot analyses of macrophages cultured from bone marrow-derived LDMNCs from two Shp2 D61Y inducible-knockin mice expressing WT p110δ and two Shp2 D61Y-inducible knockin mice expressing kinase-dead mutant p110δ D910A stimulated for 60 minutes with 0 or 10 ng/mL GM-CSF. (B) Western blot analysis of macrophages cultured from one WT Shp2-expressing mouse, one Shp2 D61Y inducible knockin mouse with intact PI3K signaling, and two Shp2 D61Y inducible knockin mice with deleted p110α stimulated for 60 minutes with 0 or 10 ng/mL GM-CSF.

***Genetic disruption of p110δ activity, but not p110α expression, results in a significant reduction mutant Shp2 D61Y-induced splenomegaly***

As has been previously reported, LSL-Shp2$^{D61Y/+}$; Mx-Cre+ mice develop enlarged spleens (Chan, Kalaitzidis et al. 2009), recapitulating a common clinical feature of Juvenile Myelomonocytic Leukemia. At approximately 8-12 weeks following polyI:polyC injections, we measured the spleen weights, animal body weights, and the number of ficoll gradient-isolated splenocytes, and we evaluated relative spleen sizes by calculating the ratio of spleen weight to body weight (Figure 5.8). We also determined the total number of ficoll gradient-isolated splenocytes and determined the splenocyte cellularity by dividing the total number of ficoll gradient-isolated splenocytes by the spleen weight (Figure 5.9).

As expected, we found that mice expressing gain-of-function mutant Shp2 D61Y in the presence of intact PI3K signaling had significantly larger relative spleen sizes compared to WT control mice (Figure 5.8A). Interestingly, we found that Shp2 D61Y-expressing mice with genetically inactivated p110δ D910A had significantly reduced spleen sizes compared to Shp2 D61Y mice (Figure 5.8A), suggesting that loss of p110δ activity delayed the onset of or reduced the severity of mutant Shp2-induced MPD development. This is consistent with our previous observations that p110δ is a crucial mediator of mutant Shp2-induced PI3K hyperactivation and GM-CSF hypersensitivity, which is thought to contribute to the pathogenesis of JMML. However, in agreement with our *in vitro* data, loss of p110α did not significantly lower average relative spleen, though we did observe a lot of variation in spleen size in this group (Figure 5.8A). Indeed, if p110α's role in mutant Shp2-induced GM-CSF hypersensitivity was negligible

or dispensable as our *in vitro* data suggest, then we could expect that loss of p110α would probably have a minimal impact on MPD development. In Figure 5.8B can be seen representative spleens showing that spleens from mice expressing Shp2 D61Y in the presence of intact PI3K signaling are larger than those of WT control mice. Here it can be seen that spleens from Shp2 D61Y mice with genetically inactivated p110δ are smaller, while those from Shp2 D61Y mice with deleted p110α are not appreciably different in size from the Shp2 D61Y mice with intact PI3K signaling.

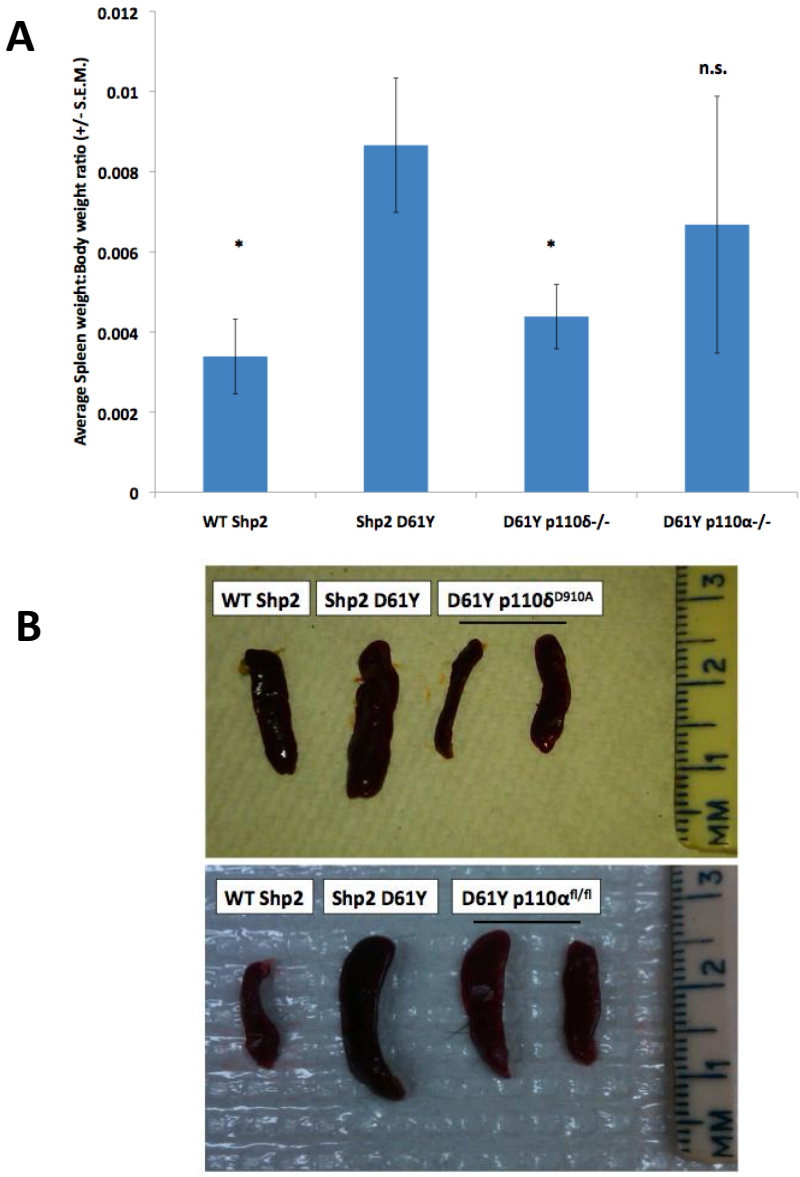



**Figure 5.8: Genetic disruption of p110δ, but not p110α, reduces gain-of-function mutant Shp2 D61Y-induced splenomegaly**
(A) Average relative spleen weight, calculated as the ratio of spleen weight to body weight 8-12 weeks following polyI:polyC treatment, which induces expression of Shp2 D61Y and deletion of p110α. Relative spleen weights were calculated for WT mice (n = 6), Shp2 D61Y mice (n = 8), Shp2 D61Y mice expressing kinase-dead mutant p110δ D910A (n=9), and Shp2 D61Y mice with genetic deletion of p110α (n=6); *$p < 0.05$ comparing average relative spleen weights of Shp2 D61Y mice to WT mice or Shp2 D61Y $p110\delta^{D910A}$ mice; no significant difference was found comparing Shp2 D61Y mice to Shp2 D61Y $p110\alpha^{fl/fl}$ mice; statistics were performed using unpaired, two-tailed student's t-test. (B) Photograph showing representative spleens of the various experimental groups.

The total number of splenocytes tended to reflect that relative spleen sizes such that mice expressing gain-of-function mutant Shp2 D61Y in the setting of intact PI3K signaling had a significantly greater total number of ficoll gradient-isolated splenocytes compared to WT control mice (Figure 5.9A). Shp2 D61Y mice expressing kinase-dead p110δ D910A had significantly less splenocytes on average than Shp2 D61Y mice, correlating with their smaller average overall spleen sizes, while the total number of ficoll gradient-isolated splenocytes was not significantly different between Shp2 D61Y mice with deleted p110α and Shp2 D61Y mice with intact PI3K signaling (Figure 5.9A). We also calculated splenocyte cellularity as the total number ficoll gradient-isolated splenocytes divided by spleen weight to approximate the percentage of immature myeloid cells, or infiltrating leukemia cells, occupying the spleen, and we found the same essential pattern. The splenocyte cellularity of the Shp2 D61Y-expressing mice with intact PI3K signaling trended to be greater than that of the WT control mice ($p = 0.07$) (Figure 5.9B). The splenocyte cellularity of the Shp2 D61Y mice with genetically inactivated p110δ was significantly lower than that of the Shp2 D61Y mice, while again, there was no difference between the Shp2 D61Y mice lacking p110α expression and Shp2 D61Y mice with intact PI3K signaling (Figure 5.9B).

Together, the spleen data—relative spleen weight, total number of ficoll gradient-isolated splenocytes, and splenocyte cellularity—can be used to assess MPD development in Shp2 D61Y-expressing mice, and it is a clinically relevant metric as JMML patients frequently suffer from splenomegaly (Loh 2011). We found that genetic inactivation of p110δ significantly reduced mutant Shp2-induced splenomegaly *in vivo*, while genetic deletion of p110α had no measurable effect, suggesting that p110δ is not

only a crucial mediator of GM-CSF hypersensitivity *in vitro*, but of MPD development *in vivo*, while p110α's role again appears to be negligible or dispensable. This provides further support that targeting p110δ pharmacologically represents a potentially effective therapeutic strategy for the treatment of mutant Shp2-associated MPD.

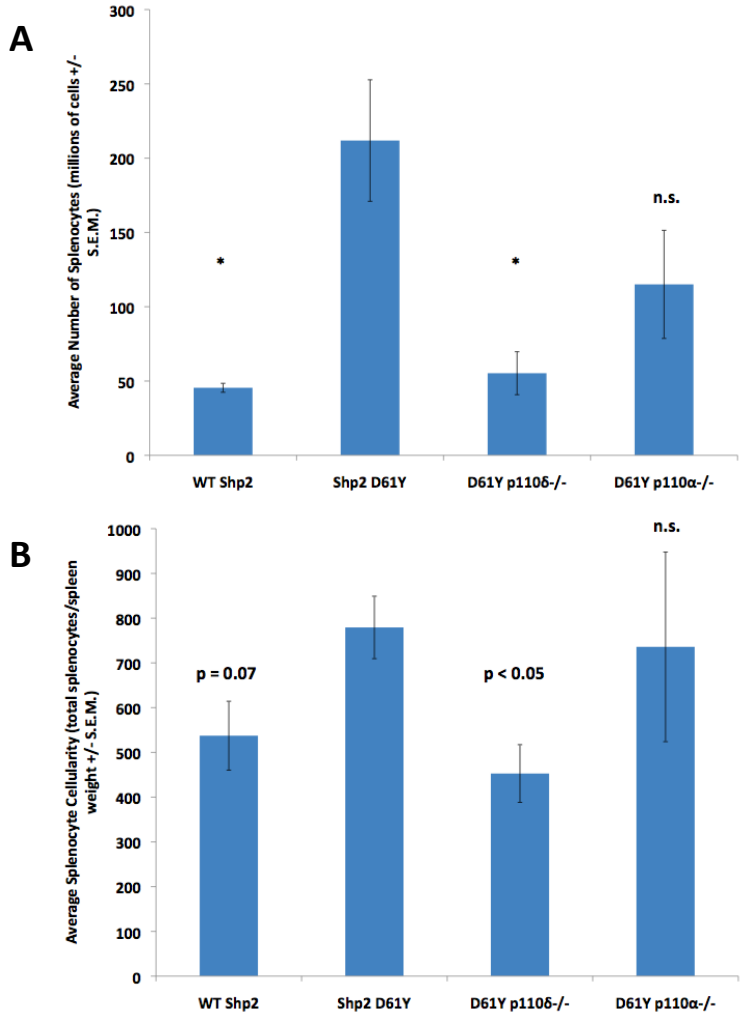



**Figure 5.9: Genetic deletion of p110δ, but not p110α, significantly reduces gain-of-function mutant Shp2 D61Y-induced splenic hyper-cellularity**
(A) Average numbers of ficoll gradient-isolated splenocytes from WT mice (n=5), Shp2 D61Y inducible knockin mice (n=5), Shp2 D61Y inducible knockin mice expressing kinase-dead mutant p110δ D910A (n=4), and Shp2 D61Y inducible mice with genetic deletion of p110α (n=5); *$p < 0.05$ comparing splenocyte numbers of Shp2 D61Y mice with those of WT Shp2 mice and Shp2 D61Y $p110\delta^{D910A}$ mice; no significant (n.s.) difference was found comparing splenocyte numbers of Shp2 D61Y mice with that of Shp2 D61Y $p110\alpha^{fl/fl}$ mice; statistics performed using unpaired, two-tailed student's t-test. (B) Average splenocyte cellularity for each mouse was calculated by dividing the total number of ficoll gradient-isolated splenocytes by the spleen weight for WT mice (n=5), Shp2 D61Y inducible knockin mice (n=5), Shp2 D61Y inducible knockin mice expressing kinase-dead mutant p110δ D910A (n=4), and Shp2 D61Y inducible mice with genetic deletion of p110α (n=5); $p = 0.07$ comparing splenocyte cellularity of Shp2 D61Y mice with that of WT Shp2 mice; $p < 0.05$ comparing splenocyte cellularity of Shp2 D61Y mice with that of Shp2 D61Y $p110\delta^{D910A}$ mice; no significant (n.s.) difference was found comparing splenocyte celluarity of Shp2 D61Y mice with that Shp2 D61Y $p110\alpha^{fl/fl}$ mice; statistics performed using unpaired, two-tailed student's t-test.

**Conclusions**

Our previous genetic and pharmacologic studies have demonstrated that hyperactivated PI3K, particularly that mediated by the catalytic subunit p110δ, plays a crucial role in mutant Shp2-induced GM-CSF hypersensitivity, which is thought to be an important contributing factor to the development of Juvenile Myelomonocytic Leukemia. Our studies using cells from *Pik3r1*$^{-/-}$ mice transduced with gain-of-function mutant Shp2 E76K indicated that activating Shp2 mutations promote hyperactivation of Class IA PI3K signaling, which then enhances the activity of one or more downstream effectors that cause cells to have an exaggerated proliferative response following GM-CSF stimulation. These studies also implicated p110δ as potentially playing an important role in this process.

In an effort to increase the clinical relevance of our work, we then wanted to investigate the question of whether just one of the Class IA PI3K catalytic subunits—p110α, p110β, or p110δ—was primarily responsible for mediating this mutant Shp2-induced PI3K hyperactivity, which would then allow us to take advantage of the newer classes of catalytic subunit-specific PI3K inhibitors. This pharmacologic strategy makes it possible to target only the aberrant PI3K activity directly involved in the leukemia process, which we hypothesized to be mediated by a single catalytic subunit (i.e. p110δ), while sparing as much as possible the normal PI3K activity involved in other normal physiologic processes, which would be mediated primarily by the other catalytic subunits, thereby reducing the risk of side effects. We particularly wanted to focus on p110δ, because its expression was preferentially stabilized—though at substantially reduced levels—in *Pik3r1*$^{-/-}$ cells transduced with mutant Shp2 E76K compared to

*Pik3r1*$^{-/-}$ cells transduced with WT Shp2. Furthermore, p110δ's hematopoietic-specific expression profile, in contrast to the ubiquitously expressed p110α and p110β, makes it a particularly attractive target for hematologic malignancies. However, we also wanted to investigate p110α at the same time, because of its frequent association with human cancer.

Accordingly, we initiated studies with three inhibitors that target p110δ: IC87114, which is a highly specific, but not very potent, inhibitor of p110δ; GDC-0941, which is clinical-grade compound that potently inhibits p110δ, but also inhibits p110α activity very efficiently; and GS-9820, a highly potent, clinical grade p110δ-specific inhibitor. We found that these inhibitors significantly reduced GM-CSF hypersensitivity and PI3K hyperactivation in cells exogenously overexpressing gain-of-function mutant Shp2 E76K, as well as in cells from Shp2 D61Y-inducible knockin mice, which provide a more physiologically relevant model of endogenously expressed mutant Shp2. The results of these studies provided more evidence that p110δ could be the principal mediator of gain-of-function mutant Shp2-induced PI3K hyperactivity and an important contributor to GM-CSF hypersensitivity.

To examine the role of p110δ more thoroughly, we used a genetic approach, which would circumvent some of the potential ambiguities posed by the use of small molecule inhibitors. We began these studies by transducing cells from mice expressing genetically inactivated p110δ D910A and p110α inducible knockout mice with gain-of-function mutant Shp2 E76K. In both cases, neither loss of p110δ activity nor loss of p110α expression had a significant effect on mutant Shp2 E76K-induced hyper-proliferation in response to GM-CSF stimulation, suggesting that these two catalytic

subunits might be playing redundant roles in mediating mutant Shp2-induced PI3K hyperactivation. However, we did observe that p110δ$^{D910A/D910A}$ cells—but not p110α$^{flox/flox}$; Mx1-Cre+ cells—exogenously overexpressing Shp2 E76K showed modest decreases in Akt and Erk hyper-phosphorylation, suggesting that loss of p110δ activity was negatively impacting mutant Shp2-induced PI3K hyperactivation, but apparently not enough to affect the hyper-proliferative phenotype. We suspected that we might be missing the true importance of p110δ in mutant Shp2-induced PI3K hyperactivation, as overexpression of an oncogene like Shp2 E76K might artificially distort mitogenic signaling beyond what might typically occur in the more physiologically relevant context of endogenously expressed gain-of-function mutant Shp2.

Accordingly, we crossed the PI3K catalytic subunit-targeted transgenic mice form the initial studies with Shp2 D61Y-inducible knockin mice to more accurately evaluate the consequences of loss of p110δ activity or p110α expression on endogenously expressed mutant Shp2 D61Y-induced PI3K hyperactivation and GM-CSF hypersensitivity *in vitro*. Specifically, we crossed LSL-Shp2$^{D61Y/+}$; Mx1-Cre+ mice with p110δ$^{D910A/D910A}$ mice or with p110α$^{flox/flox}$; Mx1-Cre+ mice to generate p110δ$^{D910A/D910A}$; LSL-Shp2$^{D61Y/+}$; Mx1-Cre+ mice, which express mutant Shp2 D61Y in the absence of p110δ activity, and p110α$^{flox/flox}$; LSL-Shp2$^{D61Y/+}$; Cre+, which express mutant Shp2 D61Y in the absence of p110α expression. These cells were compared with those of Shp2 D61Y-expressing mice with intact PI3K activity and with those of WT control mice.

We found that loss p110δ activity significantly reduced Shp2 D61Y-induced GM-CSF hypersensitivity, both in terms of GM-CSF-stimulated proliferation as measured by

[$^{3}$H]-Thymdine incorporation assays and in terms of GM-CSF-stimulated colony-forming capacity. Loss of p110α expression, in contrast, had no measurable effect on mutant Shp2-induced GM-CSF hypersensitivity. These functional results correlated well with our biochemical analyses, in which we found substantial decreases in mutant Shp2 D61Y-induced hyper-phosphorylation of Akt at both Serine 473 and Threonine 308 as well as Erk and Shp2 itself, suggesting that genetic interruption of p110δ significantly abrogated mutant Shp2 D61Y-induced hyperactivated PI3K and MAPK signaling. Genetic deletion of p110α, on the other hand, had no effect on Akt or Erk hyperactivation in Shp2 D61Y-expressing cells, just as it did not impact GM-CSF-stimulated hyper-proliferation or enhanced colony-forming capacity. Together, these results implicate p110δ as the principal mediator of mutant Shp2-induced PI3K hyperactivation and resulting GM-CSF hypersensitivity, while suggesting that p110α's role is negligible or dispensable.

Additionally, from the mouse models that we have developed, we have evidence suggesting that p110δ-mediated PI3K hyperactivation is crucial for mutant Shp2-induced MPD development, as mice expressing Shp2 D61Y in the presence of kinase-dead p110δ D910A demonstrate substantial improvement in splenomegaly, a common clinical feature of JMML, whereas mice expressing Shp2 D61Y in the absence of p110α expression had no reduction in relative spleen sizes, total number of ficoll gradient-isolated splenocytes, or splenocyte cellularity, in comparison to mice expressing Shp2 D61Y in the presence of intact PI3K signaling. This suggests that p110δ not only plays a crucial role in mutant Shp2-induced GM-CSF hypersensitivity, but that its activity is also necessary for the development of mutant Shp2-induced leukemogenesis. Therefore, on the basis of these

genetic *in vitro* and *in vivo* studies, we believe that p110δ represents a promising therapeutic target for the treatment of JMML.

# CHAPTER SIX

# GAIN-OF-FUNCTION MUTANT SHP2'S FUNCTIONAL DOMAINS ARE REQUIRED FOR FULL HYPERACTIVATION OF PI3K

## Introduction

In our previous studies, we have shown that gain-of-function mutant Shp2 promotes GM-CSF-stimulated hyperactivation of PI3K, which is principally mediated by the hematopoietic-specific Class IA PI3K catalytic subunit p110δ, and which contributes significantly to the phenotype of GM-CSF-stimulated hyper-proliferation. We also wanted to know the mechanisms by which gain-of-function mutant Shp2 promotes hyperactivation of PI3K with the hope of identifying additional therapeutic targets for the treatment of myeloproliferative disease (MPD) associated with activating Shp2 mutations. For instance, gain-of-function mutant Shp2 is thought to induce hyperactivated pro-growth and pro-survival signaling primarily, if not exclusively, through promoting hyperactivation of Ras. By using the farnesyl-transferase inhibitor, tipifarnib, as a pharmacologic means of interrupting Ras activity, we demonstrated that gain-of-function mutant Shp2 can, at least in part, promote hyperactivation of PI3K independently of Ras, even though all PI3K catalytic subunits contain Ras-binding domains and, accordingly, have been demonstrated to be direct effectors of Ras activity. This is particularly significant in light of our observations, that p110δ appears to be the principal mediator of mutant Shp2-induced PI3K hyperactivaiton, since it has been reported that p110δ, unlike the other Class PI3K catalytic subunits, can oncogenically transform cells independent of Ras (Denley, Kang et al. 2008). This finding suggests that gain-of-function mutant Shp2 can promote PI3K hyperactivation independently of Ras,

and that, therefore, PI3K most likely must be targeted as an independent and parallel pathway in addition to pharmacologically targeting the MAPK pathway for complete abrogation of mutant Shp2-induced aberrant mitogenic signaling.

One possible strategy to pharmacologically interrupt mutant Shp2-induced hyperactivated signaling is to target mutant Shp2 itself. Shp2 is thought to regulate signaling both through its enzymatic function, mediated by it C-terminal phosphatase domain, and through its scaffolding function, mediated by its two SH2 domains—N-SH2 and C-SH2 (Figure 6.1). Regarding Shp2's scaffolding function, it has been shown to interact with a variety of receptors, including the $\beta_c$ subunit of the GM-CSF receptor at Tyrosine 577 and Tyrosine 612 as well as several pertinent signaling molecules, including p85α and the Ras GEF Sos, most likely via interactions with other scaffolding molecules such as Gab2 and Grb2 (Neel, Gu et al. 2003). Genetic inactivation of both the N-SH2 and C-SH2 domains have been shown to reduce GM-CSF hypersensitivity, suggesting that Shp2's role as a scaffolding molecule might be important for this process. On the other hand, the substrates of Shp2's phosphatase activity, particularly those most relevant for promoting human diseases such as hematologic malignancies and Noonan Syndrome, have yet to be clearly defined, though it has been shown that this phosphatase activity is required for full activation of Ras, and that genetic inactivation of Shp2's phosphatase domain significantly reduces GM-CSF-stimulated hyper-proliferation. Interestingly, it has been reported that genetic inactivation of Shp2 E76K's phosphatase domain does not affect PI3K hyperactivation in response to IL-3 stimulation (Yu, Daino et al. 2006). The IL-3 receptor shares the $\beta_c$ subunit with the GM-CSF receptor, and its signaling mechanisms are thought to be very similar; additionally, activating Shp2

mutations have been shown to impart IL-3 hypersensitivity as well as GM-CSF hypersensitivity. This would suggest that, perhaps, Shp2's phosphatase activity might not be necessary for promoting PI3K hyperactivation in response to GM-CSF stimulation, though it has been demonstrated that this phosphatase activity is necessary for GM-CSF hypersensitivity (Mohi, Williams et al. 2005). Investigating this point is of particular interest in light of recent work to develop and evaluate the efficacy of a compound that inhibits Shp2's phosphatase activity (Zhang, He et al. 2010). Accordingly, we wanted to investigate which of mutant Shp2's functional domains were necessary for promoting full GM-CSF-stimulated hyperactivation of PI3K, in order to determine which of Shp2's function—enzymatic, scaffolding, or both—were required for its regulation of PI3K activity.

## Results

### *Genetic interruption Shp2's three functional domains—N-SH2, C-SH2, and phosphatase—all reduce gain-of-function mutant Shp2 E76K-induced hyper-proliferation in response to GM-CSF stimulation*

To investigate whether mutant Shp2 promotes hyperactivation of PI3K through its scaffolding function, its phosphatase function, or both, we used site-directed mutagenesis to create constructs of mutant Shp2 E76K with an additional loss-of-function mutation targeting one of Shp2's three functional domains: N-SH2, C-SH2, and phosphatase. Specifically, we interrupted the two SH2 domains' ability to bind phosphorylated tyrosine residues by mutating a conserved arginine residue to a lysine residue to create Shp2 double-mutant constructs that impaired Shp2 E76K's scaffolding function: Shp2

E76K/R32K (interrupting the N-SH2 domain) and Shp2 E76K/R138K (interrupting the C-SH2 domain) (Figure 6.1). To genetically inactivate Shp2 E76K's phosphatase activity, we introduced a mutation in the phosphatase domain, converting a conserved cysteine residue to alanine, thus generating the Shp2 E76K phosphatase-dead construct, Shp2 E76K/C463A (Figure 6.1).

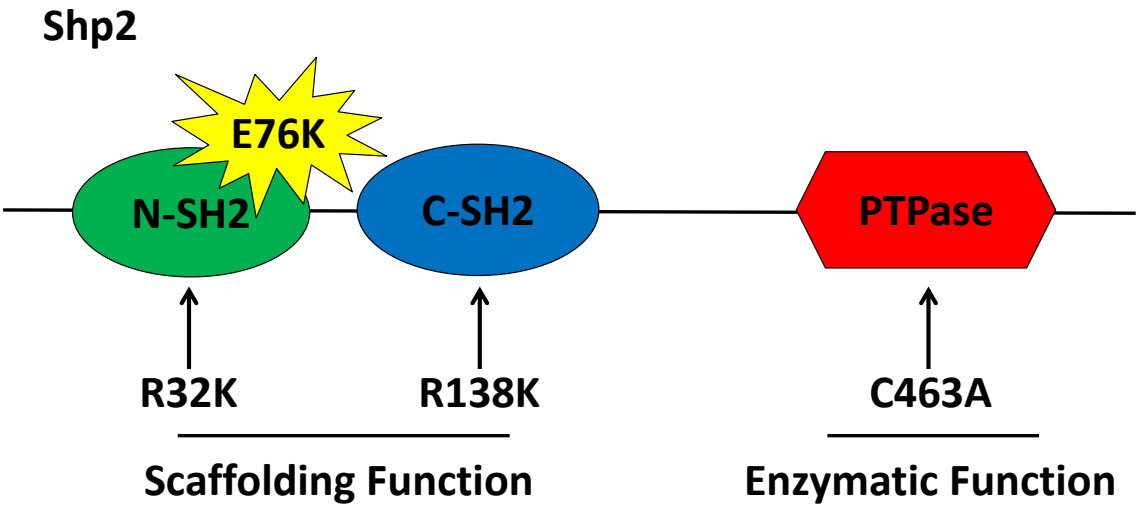



**Figure 6.1: Schematic of Shp2 structure with loss-of-function mutations targeting gain-of-function mutant Shp2 E76K's N-SH2, C-SH2, and phosphatase domains**

Site-directed mutagenesis was used to introduce loss-of-function point mutations in each of Shp2 E76K's domains—N-SH2 (R32K), C-SH2 (R138K), and phosphatase (PTPase) (C463A)—thereby disrupting their function. In the N-SH2 and C-SH2 domain, conserved arginine residues were converted to lysine residues, which prevent effective binding to phosphorylated tyrosine residues, thereby abrogating Shp2 E76K's scaffolding function. In the phosphatase domain, a conserved cysteine residue was converted to an alanine residue, thereby abrogating Shp2 E76K's enzymatic function.

We then transduced WT bone marrow-derived low-density mononuclear cells (LDMNCs) with these Shp2 double-mutant constructs as well as with WT Shp2 and mutant Shp2 E76K with all three functional domains intact, as controls. When we measured GM-CSF-stimulated proliferation in these cells by [$^3$H]-Thymidine incorporation, we found, as had been previously reported, that genetic disruption of any of the functional domains of gain-of-function mutant Shp2 E76K significantly reduced proliferation in response to GM-CSF stimulation (Figure 6.2). We observed, however, that genetic disruption of the phosphatase domain seemed to result in the greatest reduction in GM-CSF-stimulated hyper-proliferation, particularly at GM-CSF concentrations of 0.1 and 1 ng/mL. This suggests that mutant Shp2 promotes GM-CSF hypersensitivity via both its scaffolding function and its enzymatic function, but perhaps its enzymatic function may be more important for mediating the aberrant signaling that results in GM-CSF hypersensitivity.

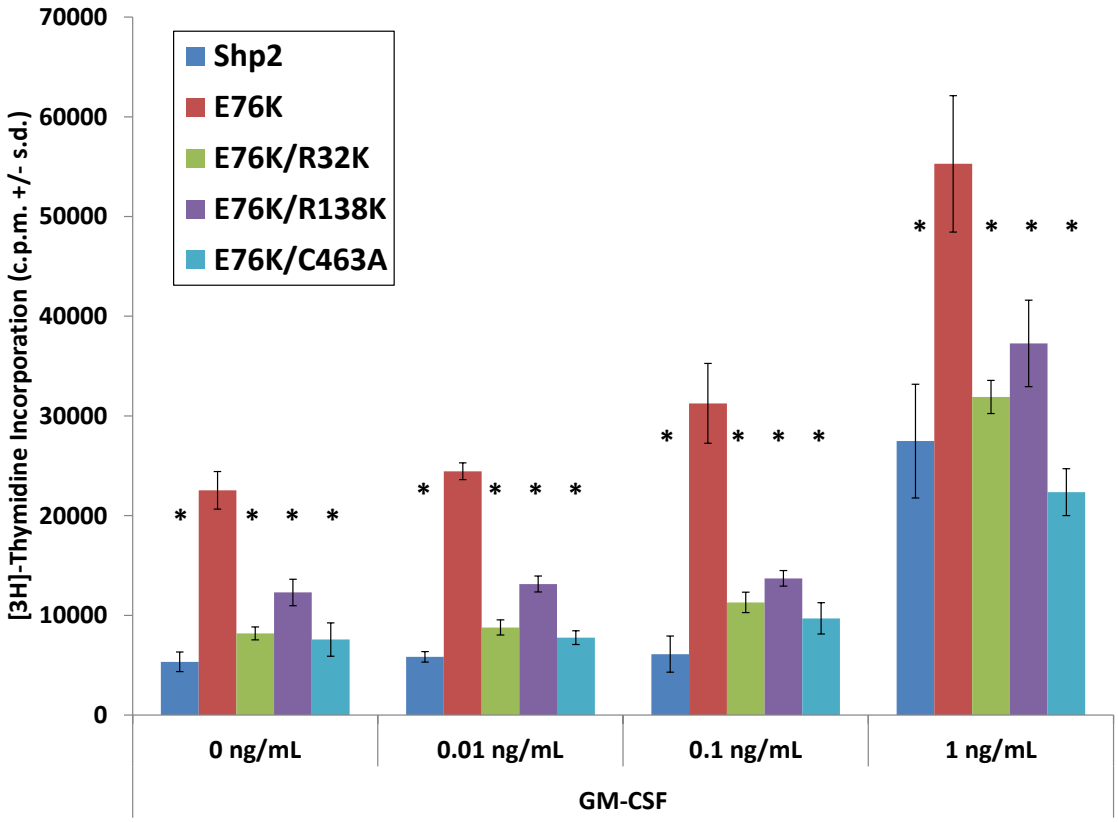



**Figure 6.2: Genetic disruption of Shp2 E76K's three functional domains—N-SH2, C-SH2, and phosphatase—all significantly reduce GM-CSF-stimulated hyper-proliferation**

[$^3$H]-Thymidine incorporation assay measuring proliferation in response to increasing concentrations of GM-CSF (0-1 ng/mL) in WT bone marrow-derived LDMNCs transduced with WT Shp2, gain-of-function mutant Shp2 E76K with all three functional domains intact, Shp2 E76K with genetic interruption of the N-SH2 domain (R32K), Shp2 E76K with genetic interruption of the C-SH2 domain (R138K), and Shp2 E76K with genetic interruption of the phosphatase domain (C463A). Representative data of two independent experiments in replicates of 4-6; *$p < 0.001$ comparing [$^3$H]-Thymidine incorporation of Shp2 E76K-expressing cells with WT Shp2-, Shp2 E76K/R32K-, Shp2 E76K/R138K-, and Shp2 E76K/C463A-expressing cells at 0 ng/mL, 0.01 ng/mL, 0.1 ng/mL, and 1 ng/mL GM-CSF; statistics performed using unpaired, two-tailed student's t-test.

***Genetic interruption Shp2's three functional domains—N-SH2, C-SH2, and phosphatase—all reduce gain-of-function mutant Shp2 E76K-induced hyperactivation of PI3K and MAPK signaling***

To investigate the consequences of genetic interruption of Shp2's three functional domains—N-SH2, C-SH2, and phosphatase—on mutant Shp2 E76K-induced hyperactivation of PI3K and MAPK signaling, we cultured bone marrow-derived LDMNCs transduced with one of the three double mutant Shp2 E76K constructs described above as well as with WT Shp2 and mutant Shp2 E76K with intact functional domains as controls, into adherent macrophages for biochemistry. We found that genetic interruption of either one of mutant Shp2 E76K's SH2 domains results in slight reductions in Akt hyper-phosphorylation at both Serine 473 and Threonine 308, as well as modest reductions in Erk hyper-phosphorylation, indicating that mutant Shp2 E76K's scaffolding function plays a role in promoting hyperactivation of PI3K and MAPK signaling (Figure 6.3, compare lanes 3 and 4 with lane 2, and lanes 8 and 9 with lane 7). Genetic interruption of the phosphatase domain, however, resulted in substantially greater reductions in mutant Shp2 E76K-induced hyper-phosphorylation of Akt at both Serine 473 and Threonine 308 and of Erk (Figure 6.3, compare lane 5 with lane 2, and lane 10 with lane 7), which correlates with the more pronounced reductions in GM-CSF-stimulated hyper-proliferation observed in the cells expressing Shp2 E76K with the genetically inactivated phosphatase domain (Figure 6.2). These results suggest that gain-of-function mutant Shp2 causes dysregulated PI3K and MAPK signaling through both its scaffolding function and its phosphatase function.

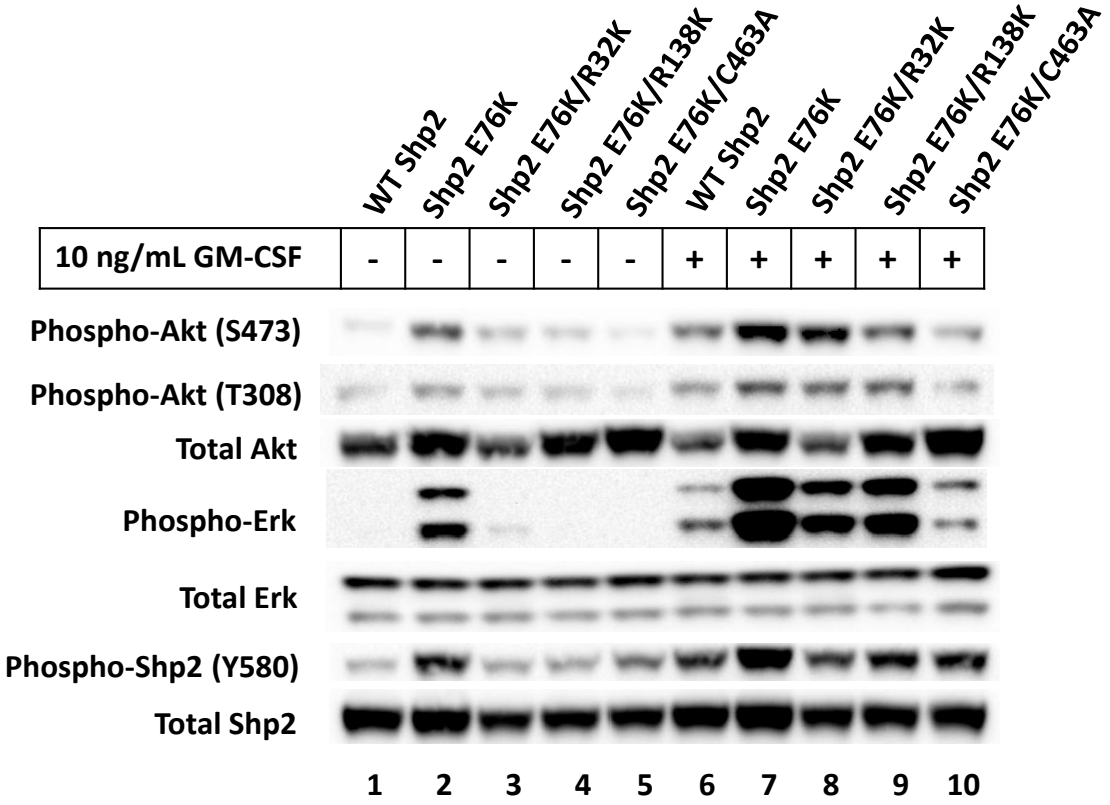



**Figure 6.3: Genetic disruption of Shp2 E76K's three functional domains—N-SH2, C-SH2, and phosphatase—significantly reduce GM-CSF-stimulated hyper-phosphorylation of Akt, Erk, and Shp2**

Western blot analysis of adherent macrophages cultured from WT bone marrow-derived LDMNCs transduced with WT Shp2, gain-of-function mutant Shp2 E76K with all three functional domains intact, Shp2 E76K with genetic interruption of the N-SH2 domain (R32K), Shp2 E76K with genetic interruption of the C-SH2 domain (R138K), and Shp2 E76K with genetic interruption of the phosphatase domain (C463A) stimulated for 60 minutes with either 0 ng/mL GM-CSF or 10 ng/mL GM-CSF. Data representative of two independent experiments.

**Conclusions**

Previous studies have investigated the mechanisms by which Shp2 regulates mitogenic signaling, especially in the context of Shp2 bearing disease-causing mutations (Yu, Daino et al. 2006). These studies have identified two major signal-regulating functions of Shp2: a scaffolding function mediated by Shp2's two SH2 domains, which promote interactions between Shp2 and phosphorylated tyrosine residues on other scaffolding molecules such as Gab2 and Grb2 and activated receptors such as the GM-CSF receptor; and its enzymatic function mediated by its C-terminal phosphatase domain, though the substrates of this activity still remain to be definitively elucidated. Previous studies have shown that both the scaffolding function and the phosphatase function are necessary for promoting gain-of-function mutant Shp2-induced hyper-proliferation in response to GM-CSF stimulation.

We wanted to know more specifically the roles of these functions of Shp2 in regulating particular signaling pathways, especially PI3K, since we have demonstrated its importance in promoting GM-CSF hypersensitivity *in vitro* and myeloproliferative disease *in vivo*. However, previous studies using Ba/F3 cells transduced with activating mutant Shp2 constructs bearing genetic disruption of the three functional domains suggested that, while the scaffolding function of mutant Shp2 was necessary for promoting hyperactivation of PI3K, interruption of the phosphatase domain did not reduce Akt hyper-phosphorylation in response to stimulation with IL-3, whose receptor shares the $\beta_c$ subunit with the GM-CSF receptor (Yu, Daino et al. 2006). In other words, mutant Shp2-induced PI3K hyperactivation can apparently remain intact in the absence of Shp2's phosphatase function. This conflicts with a previous study, which reports that

Shp2's phosphatase function is required for promoting GM-CSF hypersensitivity (Mohi, Williams et al. 2005) and our observation that PI3K contributes to gain-of-function mutant Shp2-induced GM-CSF hypersensitivity. We hoped to resolve this discrepancy by evaluating the roles of mutant Shp2's functional domains in PI3K hyperactivation and GM-CSF hypersensitivity in the more physiologically relevant setting of primary murine hematopoietic progenitor cells. We found in this setting that not only did genetic interruption of mutant Shp2's phosphatase domain reduce Akt hyper-phosphorylation, but that it did so more dramatically than the Shp2 E76K constructs with mutations in the SH2 domains, which correlated with the more robust reductions in GM-CSF-stimulated hyperproliferation observed in cells expressing Shp2 E76K bearing phosphatase-dead mutations. Together, these results show that mutant Shp2 promotes hyperactivation of PI3K and resulting GM-CSF hypersesnsitivity via both its scaffolding function and its phosphatase functions.

However, it is difficult to fully compare the consequences of mutating the SH2 domains with that of the phosphatase mutations, since the cells transduced with Shp2 E76K bearing loss-of-function domain mutations still retain endogenous WT Shp2 expression. It can be expected that the phosphatase-dead Shp2 E76K mutants might exhibit a dominant-negative effect on the endogenous WT Shp2, because this construct's SH2 domains are functionally intact and can therefore still interact with potential phosphorylated substrates for Shp2 phosphatase activity, thereby blocking access to the still-functioning endogenous WT Shp2. On the other hand, in those cells transduced with mutant Shp2 E76K constructs bearing genetic interruption of one of the SH2 domains, one could expect that the endogenous WT Shp2 might be able to interact preferentially

with its phosphorylated substrates, because its SH2 domains are functionally intact. In other words, the phosphatase-dead Shp2 E76K mutants might interfere more with endogenous WT Shp2 activity than the Shp2 E76K mutants bearing genetically interrupted SH2 domains, thereby enhancing the apparent effect of the loss of mutant Shp2's phosphatase activity in comparison to the loss of its scaffolding function. Regardless of their relative importance, we are still able to conclude that mutant Shp2 requires both of its SH2 domains as well as its phosphatase function to promote aberrant PI3K and MAPK signaling, which contribute to GM-CSF hypersensitivity. This is significant, because it suggests that small molecules that might be able to interrupt either one of these functions might represent potential therapeutic strategies for the treatment of mutant Shp2-induced MPD.

# CHAPTER SEVEN

# DISCUSSION

Although, Juvenile Myelomonocytic Leukemia (JMML) is rare, it remains extremely challenging to treat. Unlike other pediatric hematologic malignancies such ALL, JMML does not respond to standard chemotherapy, and, therefore, the only curative therapy at this time is allogeneic hematopoietic stem cell transplantation, which has an unacceptably low survival rate of approximately 50% (Loh 2011). Thus, much more effective therapeutic options are needed for this disease. Hyperactivated RAS signaling appears to be a common molecular feature in the pathogenesis of JMML, and this can result from gain-of-function mutations in *RAS* itself, loss-of-function mutations in *NF1* or *CBL*, but the most commonly mutated gene in JMML are activating mutations in *PTPN11*, which encodes the protein tyrosine phosphatase Shp2 (Figure 1.1).

To date much of the work in JMML research has focused on the hyperactivation of the RAF-MEK-ERK (MAPK) pathway in the presence of JMML-causing mutations, and likewise, this pathway has been studied extensively as a potential therapeutic target. Indeed, the MEK inhibitor PD0325901 has shown great promise in mouse models of JMML bearing gain-of-function *Kras* mutations and genetic deletion of *Nf1* (Lyubynska, Gorman et al. 2011; Chang, Krisman et al. 2013). However, the PI3K-AKT pathway is also known to be positively regulated by RAS activity, and is frequently found to contribute to the pathogenesis of wide array of human malignancies. Furthermore, Shp2 is known to form complexes with the Class IA p85 regulatory subunit of PI3K (Yu, Daino et al. 2006), suggesting it may directly promote its activation, and our lab has also shown that Akt is hyper-phosphorylated in cells expressing gain-of-function mutant Shp2

following GM-CSF-stimulation, suggesting that mutant Shp2 promotes PI3K hyperactivation, which may contribute to GM-CSF hypersensitivity and leukemogenesis (Figure 1.5) (Yang, Li et al. 2008). Thus, we wanted to know if PI3K signaling might also be important mutant SHP2-induced MPD development, and therefore a potential therapeutic target for JMML.

We began by first confirming that gain-of-function mutant Shp2 can promote PI3K hyperactivation in response to GM-CSF stimulation by performing an *in vitro* PI3K activity assay (Figure 3.1). We also used pharmacologic and genetic approaches to demonstrate that mutant Shp2-induced hyperactivtion of PI3K contributes to GM-CSF hypersensitivity. We found that the pan-PI3K inhibitor LY294002 and genetic deletion of *Pik3r1*, which encodes the major Class IA PI3K regulatory subunit p85α and its splice variants, p55α and p50α, both reduced GM-CSF-stimulated hyper-proliferation and Akt hyper-phosphorylation (Figure 3.2 and 3.4). We also made the interesting observation that mutant Shp2-induced Erk hyper-phosphorylation was also reduced in the absence of the *Pik3r1*-encoded regulatory subunits, an observation that we consistently observed as we targeted different components of Class IA PI3K in mutant Shp2-expressing cells using both pharmacologic and genetic approaches (Figure 3.4). The fact that genetic deletion of p85α and its splice variants did not result in a complete correction of mutant Shp2-induced GM-CSF hypersensitivity led us to suspect that there was residual GM-CSF-inducible PI3K activity. We found that this residual PI3K activity was likely mediated by p110α and/or p110δ, as there was continued expression of these two PI3K catalytic subunits in the absence of the *Pik3r1*-encoded regulatory subunits, albeit at substantially lower levels. Surprisingly, we found that p110δ's expression was

preferentially stabilized in *Pik3r1*$^{-/-}$ cells expressing gain-of-function mutant Shp2 E76K, suggesting that p110δ might be a critical mediator of mutant Shp2-induced PI3K hyperactivation (Figure 3.6).

In order to increase the translational potential of these findings we next wanted to explore individual contributions of the three Class IA PI3K Catalytic subunits to gain-of-function mutant Shp2-induced GM-CSF hypersensitivity. To do this, we first took advantage of the newer classes of catalytic subunit-specific PI3K inhibitors that have been recently developed (Table 4.1). We began by focusing on two commercially available inhibitors: IC87114, a highly specific, but not very potent p110δ-specific inhibitor, and GDC-0941, a clinical-grade pan-PI3K inhibitor that very potently targets p110δ and p110α. We found that both inhibitors reduced GM-CSF-stimulated hyper-proliferation (Figure 4.1 and Figure 4.5) and Akt and Erk hyper-phosphorylation (Figure 4.2 and Figure 4.5) in cells expressing gain-of-function mutant Shp2. We found these results to be consistent for cells expressing either exogenously overexpressed mutant Shp2 or endogenously expressed mutant Shp2. In addition to reducing GM-CSF-stimulated proliferation, we also found that both of these inhibitors selectively increased apoptosis in cells expressing mutant Shp2 E76K, suggesting that pharmacologically targeting p110δ in mutant Shp2-induced MPD could have the dual beneficial effect of both killing leukemia cells and reducing their proliferation. Next, we collaborated with Gilead Sciences to assess the efficacy of the clinical-grade p110δ-specific inhibitor GS-9820 and found that it efficiently decreased both GM-CSF-stimulated hyper-proliferation and GM-CSF-stimulated Akt and Erk hyper-phosphorylation in Shp2 D61Y-expressing cells at clinically relevant, nanomolar doses.

Together, this pharmacologic data suggested that p110δ might be an effective target for reducing mutant Shp2-induced GM-CSF hypersensitivity, but we believed using a genetic approach would provide the most conclusive demonstration of the roles of individual catalytic subunits in gain-of-function mutant Shp2-induced GM-CSF hypersensitivity and MPD development. To do this, we used two available mouse models: (1) a mouse constitutively expressing kinase-dead p110δ D910A (Okkenhaug, Bilancio et al. 2002); and, (2) a $p110\alpha^{flox/flox}$ inducible knockout mouse (Graupera, Guillermet-Guibert et al. 2008). Our initial studies with these mice, which relied on exogenously overexpressing gain-of-function mutant Shp2 E76K, were inconclusive, as genetic interruption of either p110δ or p110α did not reduce GM-CSF-stimulated hyper-proliferation, but genetic interruption of p110δ, but not p110α, caused modest reductions in GM-CSF-stimulated Akt and Erk hyper-phosphorylation (Figure 5.2 and Figure 5.4). To investigate this question in a more physiologically relevant manner, we crossed these mice— $p110\delta^{D910A/D910A}$ and $p110\alpha^{flox/flox}$—with mice inducibly expressing gain-of-function mutant Shp2 D61Y under its endogenous promoter: LSL-$Shp2^{D61Y/+}$; Mx1-Cre+ (Chan, Kalaitzidis et al. 2009) (Figure 5.5). We found that genetic interruption of p110δ, but not p110α, reduced GM-CSF-stimulated hyper-proliferation and enhanced colony-forming capacity in Shp2 D61Y-expressing cells (Figure 5.6), and that, likewise, genetic disruption of p110δ, but not p110α, reduced GM-CSF-stimulated hyper-phosphorylation of Akt, Erk, and Shp2 (Figure 5.7). Furthermore, mice expressing the presence of kinase-dead mutant p110δ D910A significantly improved mutant Shp2 D61Y-induced splenomegaly, while genetic deletion of p110α had no effect, suggesting that p110δ is a crucial mediator of gain-of-function mutant Shp2-induced GM-CSF hypersensitivity *in*

*vitro* and MPD development *in vivo*, while the role of p110α in these processes is negligible or dispensable. As a result of these genetic studies, p110δ would appear to be a potentially effective target for MPD associated with activating SHP2 mutations.

In addition to demonstrating the role of PI3K in gain-of-function mutant Shp2-induced GM-CSF hypersensitivity, we also wanted to explore the mechanisms by which PI3K becomes hyperactivated, as it might reveal additional therapeutic targets. SHP2, like all proteins found to be mutated in JMML, are known to regulate RAS activity, which serves a signaling node for a number of pathways that regulate proliferation, growth, survival, and other important cellular functions. Two of the most important RAS effector signaling pathways are the RAF-MEK-ERK pathway and the PI3K-AKT pathway. Not surprisingly, in the presence of JMML-causing mutations, including gain-of-function mutations in *PTPN11*, RAS becomes hyperactivated, as do the downstream signaling pathways that it regulates, contributing to the enhanced proliferation and survival that gives rise to MPD. In the case of PI3K, all four Class I PI3K catalytic subunits have a conserved RAS-binding domain, through which RAS directly promotes activation of PI3K.

Our data suggest that p110δ is the principal mediator of gain-of-function mutant Shp2-induced PI3K hyperactivation, but it has been reported that p110δ with a mutated Ras-binding domain can oncogenically transform cells (Denley, Kang et al. 2008), suggesting that p110δ, unlike the other PI3K catalytic subunits, can conduct its signaling activity without input from Ras. Intrigued by this, we wanted to know if gain-of-function mutant Shp2 could promote hyperactivation of p110δ in a Ras-independent manner. This would be the first demonstration of gain-of-function mutant Shp2 promoting

hyperactivation of an oncogenic signaling pathway independently or Ras, and would imply that gain-of-function mutant Shp2 is not necessarily functionally equivalent to hyperactivated Ras. An important implication of this model is that efforts to target hyperactivated RAS in the context of activating SHP2 mutations would likely have no effect on hyperactivated p110δ-mediated PI3K signaling, which would have to be targeted as an independent and parallel pathway (Figure 1.7).

To investigate this question, we used a pharmacologic approach to inhibiting Ras activity with the farnesyl-transferase inhibitor tipifarnib, which blocks the addition of a hydrophobic farnesyl group to mature Ras proteins, thereby preventing their targeting to the plasma membrane where they conduct their signaling function (Epling-Burnette and Loughran 2010; Liu, Sabnis et al. 2012). We found that treatment of *Pik3r1*$^{-/-}$ cells expressing gain-of-function mutant Shp2 E76K with tipifarnib only modestly reduced GM-CSF-stimulated proliferation and had no effect on Akt phosphorylation, indicating that the residual PI3K activity in Shp2 E76K-expressing *Pik3r1*$^{-/-}$ cells was largely Ras-independent (Figure 3.7). This is significant because we postulated that the residual PI3K activity in these cells was largely due to the retained expression and preferential stabilization of p110δ, which was expressed at significantly higher levels in *Pik3r1*$^{-/-}$ cells transduced with Shp2 E76K than *Pik3r1*$^{-/-}$ cells transduced with WT Shp2 (Figure 3.6). Furthermore, when we added PI3K inhibition with GDC-0941 to mutant Shp2-expressing cells treated with tipifarnib, we found that GDC-0941 was able to produce a concentration-dependent decrease in GM-CSF-stimulated proliferation and Akt phosphorylation beyond the reduction achieved by tipifarnib, indicating the existence of

GM-CSF-stimulated Ras-independent PI3K activity in mutant Shp2-expressing cells (Figure 4.3 and Figure 4.5).

In addition to its role in RAS regulation, SHP2 is thought to have two functions by which it can regulate signal transduction processes. The first of these is its phosphatase function, through which it can regulate the activity of signal transduction proteins by promoting their dephosphorylation. The putative substrates of this Shp2 phosphatase activity remain to be definitively identified, but it has been demonstrated that this activity is necessary for full activation of Ras, and that abrogation of this activity can significantly impair mutant Shp2-induced GM-CSF hypersensitivity (Neel, Gu et al. 2003; Mohi, Williams et al. 2005). Secondly, it is thought that Shp2 can promote signal transduction events by acting as a scaffolding molecule, bringing together different components of signal transduction machinery by binding their phosphorylated tyrosine residues with its two SH2 domains. Specifically, interactions that are pertinent for our studies, have been demonstrated with the GM-CSF receptor, the p85 subunit of PI3K, and other scaffolding molecules like Gab2 and Grb2, which then form a complex with the Ras GEF Sos, a positive regulator of Ras activity (Yu, Luo et al. 2006; Yu, Daino et al. 2006; Mohi and Neel 2007).

We next wanted to know if gain-of-function mutant Shp2 promotes hyperactivation of PI3K via its phosphatase function, its scaffolding function, or both. We were particularly interested in this question because of a previous study that showed that Shp2 E76K's phosphatase function was not needed to promote hyperactivation of PI3K when stimulated with IL-3 (Yu, Daino et al. 2006), a cytokine which can promote a hyper-proliferative response to similar to GM-CSF in mutant Shp2-expressing cells and

whose receptor is structurally similar to the GM-CSF receptor. We found that genetic interruption of Shp2 E76K's SH2 domains as well as its phosphatase domain caused a significant decrease in both GM-CSF-stimulated proliferation and Akt hyper-phosphorylation (Figure 6.2 and Figure 6.3), suggesting that mutant Shp2 promotes enhanced PI3K activity through both its scaffolding and its enzymatic function. Furthermore, disruption of the phosphatase domain caused a much more significant decrease in GM-CSF-stimulated hyper-proliferation and Akt hyperactivation then did either of the SH2 domain mutations. Thus, it appears that Shp2's phosphatase function is critically important for regulating PI3K activity particular in the presence of MPD-inducing mutations. This suggests that compounds that can inhibit Shp2's phosphatase activity should significantly reduce PI3K signaling, and thus result in decreased GM-CSF hypersensitivity.

**Overall Conclusions and Significance**

*PTPN11*, which encodes the protein tyrosine phosphatase SHP2, is the most commonly mutated gene found in Juvenile Myelomonocytic Leukemia (JMML), accounting for more than a third of cases. These mutations, which cause enhanced SHP2 phosphatase activity, promote dysregulated mitogenic signaling, particularly following stimulation of growth factor and cytokine receptors, thereby resulting in a growth factor or cytokine hypersensitivity response. The best characterized of these hypersensitivity responses involve the cytokine Granulocyte Macrophage-Colony-Stimulating Factor (GM-CSF), which is known to induce significant hyper-proliferation in hematopoietic progenitors from patients with JMML. It is thought that gain-of-function mutant SHP2 promotes dysregulation of these MPD-inducing mitogenic signaling pathways by causing hyperactivation of RAS, a signaling node that regulates several pro-growth and survival pathways, most notably the MAPK (RAF-MEK-ERK) and the PI3K-AKT pathways.

Until now, much of the focus in JMML research has been on the MAPK pathway as the primary mediator of GM-CSF hypersensitivity, and therefore as a potential therapeutic target. Indeed, the MEK inhibitor PD0325901 has shown great efficacy in murine JMML models induced by gain-of-function mutant *Kras* expression or *Nf1* deletion (Lyubynska, Gorman et al. 2011; Chang, Krisman et al. 2013). Likewise, we show that PD0325901 effectively inhibits GM-CSF hypersensivity induced by gain-of-function mutant Shp2 D61Y, suggesting that it may be a potential therapeutic option for JMML patients harboring a variety of mutations. However, because MEK is ubiquitously expressed and is important for multiple normal physiologic processes, pharmacologic inhibition of its activity carries significant risk of toxic off-target effects. Furthermore,

targeting a single pathway introduces the risk of developing resistance analogous to Imatinib resistance that develops in patients with CML (Reddy and Aggarwal 2012). Thus, it is important to identify additional targets in JMML that can reduce the risks of off-target side effects and the development of inhibitor resistance.

Our studies suggest that PI3K represents one such potential additional target for JMML, as they provide the first evidence demonstrating that mutant Shp2-induced hyperactivation of the PI3K pathway also contributes significantly to GM-CSF hypersensitivity *in vitro* and development of splenomegaly *in vivo*. Interestingly, we found that this mutant Shp2-induced hyperactivated PI3K positively regulates or reinforces the hyperactivated MAPK signaling as well, since we observed that genetic or pharmacologic interruption of PI3K signaling consistently reduced not only Akt hyperactivation, as expected, but Erk hyperactivation as well. Thus, targeting the PI3K hyperactivation in the presence of activating Shp2 mutations has the added advantaged of reducing oncogenic MAPK signaling as well. The MEK inhibitor PD0325901 had no analogous effect on PI3K signaling, as Akt hyper-phosphorylation remained unchanged.

Furthermore, our results are significant because they show specificity in the PI3K hyperactivity, in that p110δ appears to be the principal mediator of this hyperactivated PI3K signaling. Therefore, by taking advantage of newer classes of catalytic subunit inhibitors with high specificity for p110δ, we can expect to block only the PI3K activity participating in oncogenic signaling, and not the PI3K signaling mediated by other catalytic subunits. This conclusion is particularly significant because p110δ is expressed almost exclusively in hematopoietic cells, making it an ideal target for MPD or other diseases of the hematopoietic system. As a result, because p110δ expression is either

absent or negligible in other tissue types, it can be expected that pharmacologic inhibition of p110δ will carry greatly reduced risk of toxic side effects. Thus, targeting p110δ has two advantages over targeting MEK: (1) it positively regulates two major mitogenic pathways—PI3K and MAPK—contributing to MPD development, while MEK only regulates the MAPK pathway; and, (2) p110δ's expression is hematopoietic-specific, while MEK is ubiquitous.

In summary, we believe these studies implicate p110δ as a potentially effective target for gain-of-function mutant Shp2-induced MPD, such as JMML. The p110δ catalytic subunit is a particularly interesting target because of its hematopoietic-specific expression profile, its ability to regulate not only PI3K-Akt signaling, but MAPK signaling as well, and because of its apparent Ras-independence. It would be interesting to see if other JMML-inducing mutations such as gain-of-function mutations in *RAS* or loss-of-function mutations in *NF1* or *CBL* also promote hyperactivation of p110δ, and thus could also be effectively inhibited with p110δ-specific compounds, or if this is a feature unique to activating Shp2 mutations.

To this end, one important potential future direction for this work would include more thoroughly investigating the role of PI3K, and in particular specific Class IA catalytic subunits in promoting aberrant signaling, GM-CSF hypersensitivity, and leukemogenesis *in vivo* in mouse models of other JMML-associated mutations, including gain-of-fucntion *Kras* inducible knockin mice, and *Nf1* inducible knockout mice. A series of *in vitro* pharmacologic studies, similar to those presented in this dissertation, could be performed using these mouse models, to expand the potential utility of PI3K inhibitors in the treatment of JMML, regardless of the underlying genetic lesion.

Likewise, genetic studies could also be performed by crossing these additional JMML mouse models with the kinase-dead p110δ D910A constitutive knockin mouse or the $p110\alpha^{flox/flox}$ inducible knockout mouse to achieve this same goal. Certainly, because JMML is such a rare disease, translating this research into clinical trials would be very challenging from a patient enrollment standpoint. This challenge could be eased somewhat if it could be demonstrated that hyperactvation of PI3K in general, and p110δ in particular, were a common molecular feature of all JMML, and not just JMML associated with activating *PTPN11* mutations.

Regardless of the mouse model, it would also be helpful to have *in vivo* murine inhibitor studies of GS-9820 and/or GDC-0941, either alone or in combination with PD0325901. We have made some attempts at such studies, but the results were inconclusive (data not shown), largely as a consequence of our mouse model's prolonged disease course and inconsistent phenotype (Chan, Kalaitzidis et al. 2009). Thus, conclusive *in vivo* inhibitor studies could be more easily accomplished, if a mouse model of activating mutant Shp2-induced MPD with a more robust disease phenotype could be developed. We have attempted to address this issue by developing new mouse models, but so far with little success. We attempted to develop a syngeneic mouse model by transducing 32D cells with mutant Shp2 E76K, which were then transplanted into C3H/Hej mice, but we were unable to achieve engraftment of the transplanted cells, let alone the onset of MPD (data not shown). Currently, we are crossing Shp2 D61Y inducible knockin mice with the Vav1-Cre recombinase mouse, which should induce expression of the leukemia-causing Shp2 D61Y allele during embryonic development. We hope that by turning on the gene in a juvenile stage of development (rather than in

adulthood, as with the polyI:polyC injections of the Mx1-Cre-driven model), we can better recapitulate this pediatric disease, and achieve a more robust disease phenotype that would permit more conclusive *in vivo* pharmacologic studies.

Additionally, more work needs to be done in addressing the question of whether mutant Shp2 can promote hyperactivation of p110δ independently of Ras. Admittedly, a purely pharmacologic approach is limited, particularly considering the indirect mechanism of action of farnesyl-transferase inhibitors, such as tipifarnib. Certainly, a genetic approach would be much more conclusive. To that end we are currently performing studies in which we introduce into bone marrow-derived LDMNCs from $p110\delta^{D910A/D910A}$; Shp2 D61Y, Mx1-Cre+ by retroviral transduction either WT p110δ or a form of p110δ bearing a point mutation in the Ras-binding domain (K223E), such that Ras is unable to bind and promote activation of the exogenously expressed p110δ. A similar p110δ construct was used to demonstrate the apparent Ras-independence of p110δ-induced oncogenic transformation (Denley, Kang et al. 2008). As we have seen, expression of the kinase-dead p110δ D910A in the presence of gain-of-function mutant Shp2 D61Y significantly reduced GM-CSF hyper-proliferation and Akt and Erk hyper-phosphorylation (Figure 5.6 and Figure 5.7). We predict that introduction of WT p110δ should restore both hyper-proliferation and Akt and Erk hyper-phosphorylation upon GM-CSF stimulation. If mutant Shp2 is able to promote hyperactivation of p110δ independently of Ras, than exogenous expression of the Ras-binding domain mutant p110δ K223E, should also be to restore GM-CSF-stimulated hyper-proliferation and Akt and Erk hyper-phosphorylation. In addition, we could also address the question of Ras-independent p110δ hyperactivion using models of pure Ras hyperactivation-induced GM-

CSF hypersensitivity. We are working with collaborators to acquire bone marrow cells from the gain-of-function mutant *Kras* inducible knockin mouse and the *Nf1* inducible knockout mouse, both of which exhibit dysregulated mitogenic signaling as a consequence of pure Ras hyperactivation. We can take advantage of these models to explore mechanisms of p110δ activation using a combination of Ras and PI3K inhibitors. For instance, we noticed that treatment of mutant Shp2 expressing cells demonstrated great sensitivity to the PI3K inhibitor GDC-0941 in presence of a range of concentrations of tipifarnib, suggesting that a component of mutant Shp2-induced PI3K hyperactivation was Ras-independent (Figure 4.3 and Figure 4.5C). It would be interesting to see if cells bearing either gain-of-function mutations in *Kras* or loss of *Nf1* expression would exhibit similar sensitivity to PI3K inhibition in the presence of tipifarnib, since in these cells, hyperactivated Ras would provide the only mechanism of promoting PI3K hyperactivation.

Combining the results of our studies with the results of other studies on targeting aberrant signaling in JMML, it appears that the best therapeutic option might be a dual inhibitor approach combining a MEK inhibitor such as PD0325901 and a p110δ-specific inhibitor such GS-1101. Combining both drugs would presumably allow lower dosages of each to be used, particularly the MEK inhibitor, since targeting p110δ will bring a concomitant reduction in ERK hyperactivation. We are currently performing studies using combinations of PD0325901 and GS-9820 to confirm this hypothesis. Clinically, this approach would reduce the risk of toxic side effects, and the combined inhibition of two important oncogenic signaling pathways would decrease the risk of developing resistance to one or the other inhibitor. In addition, this dual inhibitor approach may also

be successfully applied to a wide array of other human malignancies, beyond Juvenile Myelomonocytic Leukemia.

# REFERENCES


Alessi, D. R., S. R. James, et al. (1997). "Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha." Curr Biol **7**(4): 261-269.

Bentires-Alj, M., J. G. Paez, et al. (2004). "Activating mutations of the noonan syndrome-associated SHP2/PTPN11 gene in human solid tumors and adult acute myelogenous leukemia." Cancer Res **64**(24): 8816-8820.

Bergstraesser, E., H. Hasle, et al. (2007). "Non-hematopoietic stem cell transplantation treatment of juvenile myelomonocytic leukemia: a retrospective analysis and definition of response criteria." Pediatr Blood Cancer **49**(5): 629-633.

Berndt, A., S. Miller, et al. (2010). "The p110delta structure: mechanisms for selectivity and potency of new PI(3)K inhibitors." Nat Chem Biol **6**(3): 244.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science **296**(5573): 1655-1657.

Castillo, J. J., M. Furman, et al. (2012). "CAL-101: a phosphatidylinositol-3-kinase p110-delta inhibitor for the treatment of lymphoid malignancies." Expert Opin Investig Drugs **21**(1): 15-22.

Chan, G., D. Kalaitzidis, et al. (2008). "The tyrosine phosphatase Shp2 (PTPN11) in cancer." Cancer Metastasis Rev **27**(2): 179-192.

Chan, G., D. Kalaitzidis, et al. (2009). "Leukemogenic Ptpn11 causes fatal myeloproliferative disorder via cell-autonomous effects on multiple stages of hematopoiesis." Blood **113**(18): 4414-4424.

Chan, R. J., T. Cooper, et al. (2009). "Juvenile myelomonocytic leukemia: a report from the 2nd International JMML Symposium." Leuk Res **33**(3): 355-362.

Chan, R. J. and G. S. Feng (2007). "PTPN11 is the first identified proto-oncogene that encodes a tyrosine phosphatase." Blood **109**(3): 862-867.

Chan, R. J., M. B. Leedy, et al. (2005). "Human somatic PTPN11 mutations induce hematopoietic-cell hypersensitivity to granulocyte-macrophage colony-stimulating factor." Blood **105**(9): 3737-3742.

Chan, T. O., U. Rodeck, et al. (2002). "Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit." Cancer Cell **1**(2): 181-191.

Chang, T., K. Krisman, et al. (2013). "Sustained MEK inhibition abrogates myeloproliferative disease in Nf1 mutant mice." J Clin Invest **123**(1): 335-339.

Corvera, S. and M. P. Czech (1998). "Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction." Trends Cell Biol **8**(11): 442-446.

Cushing, T. D., D. P. Metz, et al. (2012). "PI3Kdelta and PI3Kgamma as targets for autoimmune and inflammatory diseases." J Med Chem **55**(20): 8559-8581.

Denley, A., S. Kang, et al. (2008). "Oncogenic signaling of class I PI3K isoforms." Oncogene **27**(18): 2561-2574.

Edouard, T., J. P. Combier, et al. (2010). "Functional effects of PTPN11 (SHP2) mutations causing LEOPARD syndrome on epidermal growth factor-induced phosphoinositide 3-kinase/AKT/glycogen synthase kinase 3beta signaling." Mol Cell Biol **30**(10): 2498-2507.

Emanuel, P. D. (2008). "Juvenile myelomonocytic leukemia and chronic myelomonocytic leukemia." Leukemia **22**(7): 1335-1342.
Emanuel, P. D., L. J. Bates, et al. (1991). "Selective hypersensitivity to granulocyte-macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors." Blood **77**(5): 925-929.
Emanuel, P. D., R. C. Snyder, et al. (2000). "Inhibition of juvenile myelomonocytic leukemia cell growth in vitro by farnesyltransferase inhibitors." Blood **95**(2): 639-645.
Epling-Burnette, P. K. and T. P. Loughran, Jr. (2010). "Suppression of farnesyltransferase activity in acute myeloid leukemia and myelodysplastic syndrome: current understanding and recommended use of tipifarnib." Expert Opin Investig Drugs **19**(5): 689-698.
Feng, G. S., C. C. Hui, et al. (1993). "SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases." Science **259**(5101): 1607-1611.
Folkes, A. J., K. Ahmadi, et al. (2008). "The identification of 2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-t hieno[3,2-d]pyrimidine (GDC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer." J Med Chem **51**(18): 5522-5532.
Freeman, R. M., Jr., J. Plutzky, et al. (1992). "Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of Drosophila corkscrew." Proc Natl Acad Sci U S A **89**(23): 11239-11243.
Fruman, D. A. (2010). "Regulatory subunits of class IA PI3K." Curr Top Microbiol Immunol **346**: 225-244.
Fruman, D. A., F. Mauvais-Jarvis, et al. (2000). "Hypoglycaemia, liver necrosis and perinatal death in mice lacking all isoforms of phosphoinositide 3-kinase p85 alpha." Nat Genet **26**(3): 379-382.
Fruman, D. A., S. B. Snapper, et al. (1999). "Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha." Science **283**(5400): 393-397.
Goodwin, C. B., Z. Yang, et al. (2012). "Genetic disruption of the PI3K regulatory subunits, p85alpha, p55alpha, and p50alpha, normalizes mutant PTPN11-induced hypersensitivity to GM-CSF." Haematologica **97**(7): 1042-1047.
Graupera, M., J. Guillermet-Guibert, et al. (2008). "Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration." Nature **453**(7195): 662-666.
Gysin, S., M. Salt, et al. (2011). "Therapeutic strategies for targeting ras proteins." Genes Cancer **2**(3): 359-372.
Herman, S. E., A. L. Gordon, et al. (2010). "Phosphatidylinositol 3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals." Blood **116**(12): 2078-2088.
Hickey, F. B. and T. G. Cotter (2006). "BCR-ABL regulates phosphatidylinositol 3-kinase-p110gamma transcription and activation and is required for proliferation and drug resistance." J Biol Chem **281**(5): 2441-2450.
Ikeda, H., T. Hideshima, et al. (2010). "PI3K/p110{delta} is a novel therapeutic target in multiple myeloma." Blood **116**(9): 1460-1468.

Itoh, T., R. Liu, et al. (1998). "Definition of the role of tyrosine residues of the common beta subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor." Mol Cell Biol **18**(2): 742-752.

Kalra, R., D. C. Paderanga, et al. (1994). "Genetic analysis is consistent with the hypothesis that NF1 limits myeloid cell growth through p21ras." Blood **84**(10): 3435-3439.

Knight, Z. A. (2010). "Small molecule inhibitors of the PI3-kinase family." Curr Top Microbiol Immunol **347**: 263-278.

Kontaridis, M. I., K. D. Swanson, et al. (2006). "PTPN11 (Shp2) mutations in LEOPARD syndrome have dominant negative, not activating, effects." J Biol Chem **281**(10): 6785-6792.

Kontaridis, M. I., W. Yang, et al. (2008). "Deletion of Ptpn11 (Shp2) in cardiomyocytes causes dilated cardiomyopathy via effects on the extracellular signal-regulated kinase/mitogen-activated protein kinase and RhoA signaling pathways." Circulation **117**(11): 1423-1435.

Kratz, C. P., C. M. Niemeyer, et al. (2005). "The mutational spectrum of PTPN11 in juvenile myelomonocytic leukemia and Noonan syndrome/myeloproliferative disease." Blood **106**(6): 2183-2185.

Lannutti, B. J., S. A. Meadows, et al. (2011). "CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability." Blood **117**(2): 591-594.

Liu, X., H. Sabnis, et al. (2012). "Molecular targets for the treatment of juvenile myelomonocytic leukemia." Adv Hematol **2012**: 308252.

Locatelli, F., P. Nollke, et al. (2005). "Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial." Blood **105**(1): 410-419.

Loh, M. L. (2011). "Recent advances in the pathogenesis and treatment of juvenile myelomonocytic leukaemia." Br J Haematol **152**(6): 677-687.

Loh, M. L., S. Martinelli, et al. (2005). "Acquired PTPN11 mutations occur rarely in adult patients with myelodysplastic syndromes and chronic myelomonocytic leukemia." Leuk Res **29**(4): 459-462.

Loh, M. L., M. G. Reynolds, et al. (2004). "PTPN11 mutations in pediatric patients with acute myeloid leukemia: results from the Children's Cancer Group." Leukemia **18**(11): 1831-1834.

Loh, M. L., D. S. Sakai, et al. (2009). "Mutations in CBL occur frequently in juvenile myelomonocytic leukemia." Blood **114**(9): 1859-1863.

Loh, M. L., S. Vattikuti, et al. (2004). "Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis." Blood **103**(6): 2325-2331.

Luo, J. and L. C. Cantley (2005). "The negative regulation of phosphoinositide 3-kinase signaling by p85 and it's implication in cancer." Cell Cycle **4**(10): 1309-1312.

Luo, J., S. J. Field, et al. (2005). "The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex." J Cell Biol **170**(3): 455-464.

Luo, J., B. D. Manning, et al. (2003). "Targeting the PI3K-Akt pathway in human cancer: rationale and promise." Cancer Cell **4**(4): 257-262.

Lyubynska, N., M. F. Gorman, et al. (2011). "A MEK inhibitor abrogates myeloproliferative disease in Kras mutant mice." Sci Transl Med **3**(76): 76ra27.
Mali, R. S., P. Ma, et al. (2012). "Role of SHP2 phosphatase in KIT-induced transformation: identification of SHP2 as a druggable target in diseases involving oncogenic KIT." Blood **120**(13): 2669-2678.
Marin, T. M., K. Keith, et al. (2011). "Rapamycin reverses hypertrophic cardiomyopathy in a mouse model of LEOPARD syndrome-associated PTPN11 mutation." J Clin Invest **121**(3): 1026-1043.
Marone, R., V. Cmiljanovic, et al. (2008). "Targeting phosphoinositide 3-kinase: moving towards therapy." Biochim Biophys Acta **1784**(1): 159-185.
Martinelli, S., C. Carta, et al. (2006). "Activating PTPN11 mutations play a minor role in pediatric and adult solid tumors." Cancer Genet Cytogenet **166**(2): 124-129.
Mattoon, D. R., B. Lamothe, et al. (2004). "The docking protein Gab1 is the primary mediator of EGF-stimulated activation of the PI-3K/Akt cell survival pathway." BMC Biol **2**: 24.
Miyauchi, J., M. Asada, et al. (1994). "Mutations of the N-ras gene in juvenile chronic myelogenous leukemia." Blood **83**(8): 2248-2254.
Mohi, M. G. and B. G. Neel (2007). "The role of Shp2 (PTPN11) in cancer." Curr Opin Genet Dev **17**(1): 23-30.
Mohi, M. G., I. R. Williams, et al. (2005). "Prognostic, therapeutic, and mechanistic implications of a mouse model of leukemia evoked by Shp2 (PTPN11) mutations." Cancer Cell **7**(2): 179-191.
Nabinger, S. C. and R. J. Chan (2012). "Shp2 function in hematopoietic stem cell biology and leukemogenesis." Curr Opin Hematol **19**(4): 273-279.
Nabinger, S. C., X. J. Li, et al. (2013). "The protein tyrosine phosphatase, Shp2, positively contributes to FLT3-ITD-induced hematopoietic progenitor hyperproliferation and malignant disease in vivo." Leukemia **27**(2): 398-408.
Neel, B. G., H. Gu, et al. (2003). "The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling." Trends Biochem Sci **28**(6): 284-293.
Niemeyer, C. M. and C. P. Kratz (2008). "Paediatric myelodysplastic syndromes and juvenile myelomonocytic leukaemia: molecular classification and treatment options." Br J Haematol **140**(6): 610-624.
Okkenhaug, K., A. Bilancio, et al. (2002). "Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice." Science **297**(5583): 1031-1034.
Pacold, M. E., S. Suire, et al. (2000). "Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma." Cell **103**(6): 931-943.
Papakonstanti, E. A., O. Zwaenepoel, et al. (2008). "Distinct roles of class IA PI3K isoforms in primary and immortalised macrophages." J Cell Sci **121**(Pt 24): 4124-4133.
Perugini, M., A. L. Brown, et al. (2010). "Alternative modes of GM-CSF receptor activation revealed using activated mutants of the common beta-subunit." Blood **115**(16): 3346-3353.
Prentice, M. J. (1979). "On the Problem of m Incomplete Rankings." Biometrika **66**: 167-170.
Reddy, E. P. and A. K. Aggarwal (2012). "The ins and outs of bcr-abl inhibition." Genes Cancer **3**(5-6): 447-454.

Roberts, A. E., J. E. Allanson, et al. (2013). "Noonan syndrome." Lancet **381**(9863): 333-342.
Rodriguez-Viciana, P., P. H. Warne, et al. (1994). "Phosphatidylinositol-3-OH kinase as a direct target of Ras." Nature **370**(6490): 527-532.
Rodriguez-Viciana, P., P. H. Warne, et al. (1996). "Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation." EMBO J **15**(10): 2442-2451.
Rommel, C. (2010). "Taking PI3Kdelta and PI3Kgamma one step ahead: dual active PI3Kdelta/gamma inhibitors for the treatment of immune-mediated inflammatory diseases." Curr Top Microbiol Immunol **346**: 279-299.
Salmena, L., A. Carracedo, et al. (2008). "Tenets of PTEN tumor suppression." Cell **133**(3): 403-414.
Samuels, Y., Z. Wang, et al. (2004). "High frequency of mutations of the PIK3CA gene in human cancers." Science **304**(5670): 554.
Sarbassov, D. D., D. A. Guertin, et al. (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science **307**(5712): 1098-1101.
Schubbert, S., K. Lieuw, et al. (2005). "Functional analysis of leukemia-associated PTPN11 mutations in primary hematopoietic cells." Blood **106**(1): 311-317.
Shannon, K. M., P. O'Connell, et al. (1994). "Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders." N Engl J Med **330**(9): 597-601.
Skorski, T., A. Bellacosa, et al. (1997). "Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway." EMBO J **16**(20): 6151-6161.
Sujobert, P., V. Bardet, et al. (2005). "Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia." Blood **106**(3): 1063-1066.
Tartaglia, M., S. Martinelli, et al. (2004). "Genetic evidence for lineage-related and differentiation stage-related contribution of somatic PTPN11 mutations to leukemogenesis in childhood acute leukemia." Blood **104**(2): 307-313.
Tartaglia, M., C. M. Niemeyer, et al. (2003). "Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia." Nat Genet **34**(2): 148-150.
Vivanco, I. and C. L. Sawyers (2002). "The phosphatidylinositol 3-Kinase AKT pathway in human cancer." Nat Rev Cancer **2**(7): 489-501.
Walker, E. H., O. Perisic, et al. (1999). "Structural insights into phosphoinositide 3-kinase catalysis and signalling." Nature **402**(6759): 313-320.
Yang, Z., Y. Li, et al. (2008). "Activating PTPN11 mutants promote hematopoietic progenitor cell-cycle progression and survival." Exp Hematol **36**(10): 1285-1296.
Yoshimi, A., M. Mohamed, et al. (2007). "Second allogeneic hematopoietic stem cell transplantation (HSCT) results in outcome similar to that of first HSCT for patients with juvenile myelomonocytic leukemia." Leukemia **21**(3): 556-560.
Yu, J., Y. Zhang, et al. (1998). "Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit." Mol Cell Biol **18**(3): 1379-1387.

Yu, M., J. Luo, et al. (2006). "The scaffolding adapter Gab2, via Shp-2, regulates kit-evoked mast cell proliferation by activating the Rac/JNK pathway." J Biol Chem **281**(39): 28615-28626.

Yu, W. M., H. Daino, et al. (2006). "Effects of a leukemia-associated gain-of-function mutation of SHP-2 phosphatase on interleukin-3 signaling." J Biol Chem **281**(9): 5426-5434.

Zhang, S. Q., W. G. Tsiaras, et al. (2002). "Receptor-specific regulation of phosphatidylinositol 3'-kinase activation by the protein tyrosine phosphatase Shp2." Mol Cell Biol **22**(12): 4062-4072.

Zhang, X., Y. He, et al. (2010). "Salicylic acid based small molecule inhibitor for the oncogenic Src homology-2 domain containing protein tyrosine phosphatase-2 (SHP2)." J Med Chem **53**(6): 2482-2493.

Zhao, L. and P. K. Vogt (2008). "Class I PI3K in oncogenic cellular transformation." Oncogene **27**(41): 5486-5496.

Zhao, L. and P. K. Vogt (2008). "Helical domain and kinase domain mutations in p110alpha of phosphatidylinositol 3-kinase induce gain of function by different mechanisms." Proc Natl Acad Sci U S A **105**(7): 2652-2657.

CURRICULUM VITAE

Charles B. Goodwin

## Education

| | |
|---|---|
| 2013 | PhD, Medical and Molecular Genetics<br>Medical Scientist Training Program (MD/PhD)<br>Indiana University, Indianapolis, Indiana<br>Dissertation: PI3K in Juvenile Myelomonocytic Leukemia<br>Advisor: Rebecca J. Chan, MD, PhD |
| 2004 | AB, Linguistics (with Honors)<br>University of Chicago, Chicago, Illinois |

## Honors and Awards

| | |
|---|---|
| 2012 | Outstanding Poster Award, 10th Annual Midwest Blood Club Symposium, Indianapolis, Indiana. March 15-16, 2012 |
| 2011 | 3rd Place, Sigma Xi Biomedical Graduate Research Competition, Indiana University School of Medicine. |
| 2011 | 3rd Place Poster Award (Basic Science-Graduate Student), IU Simon Cancer Center Research Day. |
| 2010 | Travel Award, Protein Phosphorylation and Cell Signaling. Salk Institute for Biological Sciences, La Jolla, California. August 18-22, 2010. |
| 2004 | Elected to Phi Beta Kappa |
| 2003 | Student Marshal, University of Chicago |

## Publications

**Goodwin CB**, Yang Z, Yin F, Yu M, Chan RJ. Genetic disruption of the PI3K regulatory subunits, p85α, p55α, p50α normalizes mutant *PTPN11*-induced hypersensitivity to GM-CSF (2012). Haematologica. 2012 Jul;97(7):1042-7.

Farrow EG, Yu X, Summers LJ, Davis SI, Fleet JC, Allen MR, Robling AG, Stayrook KR, Jideonwo V, Magers MJ, Garringer HJ, Vidal R, Chan RJ, **Goodwin CB**, Hui SL, Peacock M, White KE. Iron deficiency drives and autosomal dominant hypophosphatemic rickets (ADHR) phenotype in fibroblast growth factor-23 (Fgf23) knock-in mice. Proceedings of the National Academy of Science. 2011 Nov 15;108(46): E1146-55.

Mali RS, Ramdas B, Ma P, Shi J, Munugalavadla V, Sims E, Wei L, Vemula S, Nabinger SC, **Goodwin CB**, Chan RJ, Traina F, Visconte V, Tiu RV, Lewis TA, Stern AM, Wen Q, Crispino JD, Boswell HS, Kapur R. Rho Kinase regulates the survival and transformation of cells bearing oncogenic forms of KIT, FLT3, and BCR-ABL. Cancer Cell. 2011 Sept; 20: 357-369.

Alonso M, **Goodwin C**, Liao X, Ortiga-Cavalho T, Machado DS, Wondisford FE, Refetoff S, Weiss RE. In vivo interaction of steroid receptor coactivator (SRC)-1 and the activation function-2 domain of the thyroid hormone receptor (TR) β in TRβ E457A knock-in and SRC-1 knockout mice. Endocrinology. 2009 Aug; 150 (8): 3927-34.

Alonso M, **Goodwin C**, Liao X, Page D, Refetoff S, Weiss RE. Effects of maternal levels of thyroid hormone (TH) on the hypothalamus-pituitary-thyroid set point: studies in TH receptor beta knockout mice. Endocrinology. 2007 Nov; 148 (11): 5305-12.

Moeller LC, Alonso M, Liao X, Broach V, Dumitrescu A, Van Sande J, Montanelli L, Skjei S, **Goodwin C**, Grasberger H, Refetoff S, Weiss RE. Pituitary-thyroid setpoint and thyrotropin receptor expression in consomic rats. Endocrinology. 2007 Oct; 148 (10): 4227-33.

## Oral Presentations

**Goodwin CB**, Yang Z, Chan RJ. Genetic disruption of the PI3K regulatory subunits, p85α, p55α, p50α partially normalizes gain-of-function *PTPN11*-induced hypersensitivity to GM-CSF in hematopoietic progenitors. JMML Working Group Meeting. Orlando, Florida. December 2-3, 2010.

## Abstracts for Oral Presentations

**Goodwin CB**, Gearinger RL, Mali R, Cerabona D, Chan G, Neel BG, Kapur R, Chan RJ. The PI3K catalytic subunit p110 delta is a crucial mediator of leukemia-associated mutant PTPN11-induced GM-CSF hypersensitivity. 6th International Symposium on MDS and Bone Marrow Failure Syndromes in Childhood. Prague, Czech Republic. November 7-9, 2012.

**Goodwin CB**, Gearinger RL, Mali R, Chan G, Neel BG, Kapur R, Chan RJ. The PI3K catalytic subunit p110δ is a crucial mediator of leukemia-associated mutant PTPN11-induced GM-CSF hypersensitivity. 1st Annual South-Central Regional MD/PhD Conference. Louisville, Kentucky. September 8, 2012.

**Goodwin CB**, Yang Z, Vemula S, Yin F, Kapur R, Chan RJ. Genetic disruption of the PI3K regulatory subunit, p85α, partially normalizes gain-of-function *PTPN11*-induced hypersensitivity to GM-CSF in Hematopoietic Progenitors. 8th Annual Midwest Blood Club Symposium. Indianapolis, Indiana. May 6-7, 2010.

**Abstracts for Poster Presentations**

Farlow J, **Goodwin CB**, Piron C, Sevilla-Martir J, Ribera A, Loftus A, Kirchhoff S. The IU Student Outreach Clinic: A Model for Community-Based Interprofessional Education. Association of American Medical Colleges Central Group on Education Affairs (CGEA) Spring Conference: Professionalism and Professional Identity. Cincinnati, Ohio. March 21-23, 2013.

**Goodwin CB**, Gearinger RL, Mali R, Chan G, Neel BG, Kapur R, Chan RJ. The PI3K catalytic subunit p110δ is a crucial mediator of leukemia-associated mutant PTPN11-induced GM-CSF hypersensitivity. Ninth International Workshop on Molecular Aspects of Myeloid Stem Cell Development and Leukemia. Cincinnati, Ohio. May 6-9, 2012.

**Goodwin CB**, Gearinger RL, Mali R, Chan G, Neel BG, Kapur R, Chan RJ. The PI3K catalytic subunit p110δ is a crucial mediator of leukemia-associated mutant PTPN11-induced GM-CSF hypersensitivity. The American Society of Clinical Investigation/Association of American Physicians/American Physician Scientists Association 2012 Joint Meeting. Chicago, Illinois. April 27-29, 2012.

Li XJ, Yang Z, **Goodwin CB**, Feng GS, Chan RJ. The protein tyrosine phosphatase Shp2 positively contributes to ROS generation in macrophages. 10th Annual Midwest Blood Club Symposium. Indianapolis, IN. March 15-16, 2012.

**Goodwin CB**, Gearinger RL, Mali R, Chan G, Neel BG, Kapur R, Chan RJ. The PI3K catalytic subunit p110δ is a crucial mediator of leukemia-associated mutant PTPN11-induced GM-CSF hypersensitivity. 10th Annual Midwest Blood Club Symposium. Indianapolis, IN. March 15-16, 2012.

**Goodwin CB**, Chan RJ. The PI3K catalytic subunits, p110 alpha and p110 delta, serve redundant functions in activating *PTPN11*-induced hematopoietic progenitor hypersensitivity to GM-CSF. International Meeting on Genetic Syndromes of the Ras/MAPK Pathway. Chicago, Illinois. July 29-31, 2011.

**Goodwin CB**, Chan RJ. The PI3K catalytic subunits, p110 alpha and p110 delta, serve redundant functions in activating *PTPN11*-induced hematopoietic progenitor hypersensitivity to GM-CSF. 26th Annual National MD/PhD Student Conference. Keystone, Colorado. July 15-17, 2011.

**Goodwin CB**, Chan RJ. The PI3K catalytic subunits, p110 alpha and p110 delta, serve redundant functions in activating *PTPN11*-induced hematopoietic progenitor hypersensitivity to GM-CSF. 9th Annual Midwest Blood Club Symposium. Cincinnati, Ohio. April 21-22, 2011.

**Goodwin CB**, Vemula S, Kapur R, Chan RJ. FAK Overexpression and Hyperphosphorylation Contribute to Activating PTPN11-Induced Hypersensitivity to GM-CSF. 9th Annual Midwest Blood Club Symposium. Cincinnati, Ohio. April 21-22, 2011.

Yang Z, **Goodwin CB**, Hood D, Carlesso N, Chan RJ. Hematopoietic Cells Expressing Activating *PTPN11* Mutants Demonstrate Enhanced Response to SDF-1 alpha and Increased Stromal Cell-Supported Survival. 9th Annual Midwest Blood Club Symposium. Cincinnati, Ohio. April 21-22, 2011.

**Goodwin CB**, Chan RJ. The PI3K catalytic subunits, p110α and p110δ, serve redundant functions in activating *PTPN11*-induced hematopoietic progenitor hypersensitivity to GM-CSF. The American Society of Clinical Investigation/Association of American Physicians/American Physician Scientists Association 2011 Joint Meeting. Chicago, Illinois. April 15-17, 2011.

**Goodwin CB**, Yang Z, Yin F, Chan RJ. Genetic disruption of the PI3K regulatory subunits, p85α, p55α, p50α partially normalizes gain-of-function *PTPN11*-induced hypersensitivity to GM-CSF in hematopoietic progenitors. Protein Phosphorylation and Cell Signaling. Salk Institute for Biological Sciences, La Jolla, California. August 18-22, 2010.

**Goodwin CB**, Yang Z, Vemula S, Yin F, Kapur R, Chan RJ. Genetic disruption of the PI3K regulatory subunit, p85α, partially normalizes gain-of-function *PTPN11*-induced hypersensitivity to GM-CSF in hematopoietic progenitors. The American Society of Clinical Investigation/Association of American Physicians/American Physician Scientist Association 2010 Joint Meeting. Chicago, Illinois. April 23-25, 2010.

**Goodwin CB**, Yang Z, Vemula S, Yin F, Kapur R, Chan RJ. Genetic disruption of the PI3K regulatory subunit, p85α, partially normalizes gain-of-function *PTPN11*-induced hypersensitivity to GM-CSF in hematopoietic progenitors. 2009 Annual Meeting of the American Society of Hematology, New Orleans, Louisiana. December 5-8, 2009.

Alonso M, **Goodwin CB**, Liao X, Refetoff S, and Weiss RE. Accommodation of maternal thyroid hormone (TH) levels to fetal genotype/phenotype: studies in pregnant TH receptor beta knockout mice. 89th Annual Meeting of the Endocrine Society. Toronto, Canada. June 2-5, 2007.

Alonso M, **Goodwin CB**, Liao X, Refetoff S, and Weiss RE. Intrauterine thyroid hormone (TH) milieu and pituitary-thyroid setpoint: studies in TH receptor beta knockout mice. 89th Annual Meeting of the Endocrine Society. Toronto, Canada. June 2-5, 2007.

Alonso M, **Goodwin CB**, Liao X, Ortiga-Cavalho T, Machado D, Wondisford FE, Weiss RE. In vivo interaction of SRC-1 and AF-2 domain of the thyroid hormone receptor beta. 77th Annual Meeting of the American Thyroid Associaition. Phoenix, Arizona. October 11-15, 2006.

Alonso M, Liao X, Page D, **Goodwin CB**, Refetoff S, Weiss RE. Effect of maternal levels of thyroid hormone (TH) on newborn thyroid function in TH receptor beta knockout mice. 7th International Workshop on Resistance to Thyroid Hormone. Lyon, France. September 19-21, 2005.

## Grants and Scholarships

| | |
|---|---|
| 2010-2015 | NIH/NHLBI, National Research Service Award F30 HL104867<br>(Goodwin – PI; Chan – Sponsor)<br>PI3K Signaling in Juvenile Myelomonocytic Leukemia. |
| 2011 | Marilyn Hester Scholarship for Pediatric Hematology/Oncology Research, IU Simon Cancer Center, 2011. |