GROWTH RECEPTOR BOUND PROTEIN-2 (GRB2) EXPANDS MYELOID CELLS AND INCREASES PROLIFERATION IN CHRONIC MYELOID LEUKEMIA (CML)

A Thesis

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to the Faculty of

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In Partial Fulfillment

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by

Jesse S. Smith

Fall 2016

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DEDICATION

To Sarah

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Dr. David Stachura for his instrumental role in shaping this project and guiding my development as a scientist. It has been an honor to work with you. Your consistent and constructive teaching style along with your commitment to working closely with your students makes you an outstanding advisor and teacher.

Special thanks to Dr. David Keller for your guidance in shaping this project and in helping steer the course of my experiments. Additionally to Dr. Carolynn Arpin for your role in developing the scope of this project, and for your hard work in providing the SH2 antagonists that proved fundamental to the project. Thanks also to Dr. Don Miller for helping me develop the habits and writing styles of a scientist. Thanks to Dr. Mitchell Weiss, Dr. David Keller, Dr. Carolynn Arpin, Dr. Troy Cline and Dr. Gordon Wolfe for providing equipment, reagents, cells and drugs for this project.

I would like to acknowledge my funding sources for their contribution to my research. Thanks to Dr. David Stachura and the Stachura Zebrafish Laboratory at CSUC for being the primary funding source for this project. Thanks also to the CSUC Honors Department, the CSUC Biology Department and the CSUC Rood Scholarship for providing funding for this project. Additional thanks to the entire biology department staff and faculty as well as the CSUC community at large.

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ABSTRACT

GROWTH RECEPTOR BOUND PROTEIN-2 (GRB2) EXPANDS MYELOID CELLS AND INCREASES PROLIFERATION IN CHRONIC MYELOID LEUKEMIA (CML)

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Growth receptor bound protein-2 (GRB2) is an intracellular adapter protein responsible for linking receptor tyrosine kinases to downstream signaling proteins involved in cellular growth and differentiation. GRB2 is overexpressed in certain forms of human cancer, but no research has investigated GRB2 overexpression in chronic myeloid leukemia (CML), a disease involving the over-proliferation of myeloid cells. GRB2 is upstream of several cellular signaling pathways involved in the tyrosine kinase (TK) breakpoint cluster region-Abelson murine leukemia viral oncogene-1 (BCR-ABL). BCR-ABL is the transforming factor in 95% of CML cases. Here we demonstrate that GRB2 is overexpressed in the human CML cell line K562. Furthermore, we demonstrate that GRB2 overexpression increases proliferation of K562 cells. Additionally, we show that *in vivo* overexpression of GRB2 in zebrafish (*Danio rerio*) causes a 2-fold expansion of myeloid cells relative to mock-injected fish at 24 hours post fertilization (hpf). Finally,

we demonstrate that targeted antagonism of GRB2's SH2 binding region blocks proliferation of K562 cells. Our findings demonstrate the role of GRB2 overexpression in the proliferation of both normal and malignant myeloid cells. These findings highlight GRB2 overexpression as a potential biomarker for disease prevention, treatment optimization, and a target for new drug therapies to treat CML.

CHAPTER I

INTRODUCTION

Chronic myeloid leukemia (CML) is a malignant blood disease affecting the normal growth of myeloid cells in the blood and bone marrow. The American Cancer Society estimates that there will be 8220 new cases of CML in the US in 2016. Of those 8220 new cases, 1070 will be fatal. CML is a disease that primarily affects elderly people, with an average age of diagnosis at 64 years old (American Cancer Society, 2016).

CML is caused by an fusion protein called breakpoint cluster region-Abelson murine leukemia viral oncogene-1 (BCR-ABL), which disrupts the normal growth and functionality of myeloid cells in the blood and bone marrow. This disruption leads to an over-proliferation of myeloid cells and an impaired immune system that is fatal if not treated. While important developments have been made in the treatment of CML, the underlying mechanisms of disease onset and progression remain a mystery. This study focuses on determining a key mechanism in CML oncogenesis that will help improve the understanding and treatment of this disease.

Myeloid Development

Myeloid cells are a population of white blood cells that fight pathogenic infections in the body. These cells contribute primarily to the non-adaptive immune response known as the innate immune system. The process of myeloid development is known as myelopoiesis, and is held under strict genetic control throughout life. In mammals, post-fetal myelopoiesis takes place in the bone marrow. Myeloid cells originate from

multipotent stem cells known as hematopoietic stem cells (HSC). Hematopoiesis is a process of restricting development, where cells start as stem cells with potential to mature into any blood cell are assigned a direction of development toward different subsets of the hematopoietic system. As myeloid cells develop from HSCs, complex protein signaling initiates their development into what is known as a common myeloid progenitor (CMP). From here, the cell's fate is assigned to the erythromyeloid lineage. Further assignment leads CMPs to develop into myeloblasts, an immature myeloid cell. In a normal functioning hematopoietic system, myeloblasts are signaled to develop into fully functional cells and migrate out of the bone marrow as older populations die away, or as the immune system demands. The process of a myeloid cell transforming from a CMP to a mature myeloid cell is known as differentiation.

There are a variety of different types of myeloid cells in the body. These include circulatory myeloid cells such as neutrophils, monocytes, eosinophils and basophils, and tissue-residing myeloid cells such as macrophage, dendritic and mast cells. Each cell possesses a specific function in the immune response. During infection, tissue-residing myeloid cells are the first to contact invading pathogens. Their primary role is destruction of pathogens and initiation of the inflammatory response. However, they have complex secondary functions that signal to the adaptive immune response. Adaptive immunity is the process of recognizing antigens and creating antibodies against those antigens, essentially creating a memory of that antigen should it present itself again. Tissue specific myeloid cells are responsible for phagocytosis of these invading antigens, but signal the adaptive immune system through a function known as antigen presenting, where cells display antigens on their exterior for recognition by adaptive immune cells.

In order to fulfill their role within this system, myeloid cells must fully develop cellular elements needed for fighting pathogens. When their growth and maturation is disrupted, these cells never fully develop their anti-pathogenic functionality. Likewise, as these cells develop, growth signaling slows as mature myeloid cells are no longer maturing or dividing. Two important disruptions to myeloid development that are important to leukemia are disrupted differentiation and accelerated growth.

Oncogenesis in CML

Over 95% of CML cases are caused by a single fusion protein known as BCR-ABL. BCR-ABL is an abnormal fusion protein, which functions as a constitutively active tyrosine kinase (TK). TKs respond to a variety of growth signals from inside and outside of the cell. They transmit these signals through the cytoplasm of a cell by linking a variety of adapter proteins and transcription factors that lead ultimately to cellular growth, migration and differentiation. Dysregulation of TKs is a common finding in human cancer (Dutt *et al.,* 2008, Jang *et al.,* 2001*,* Pollock *et al.,* 2007) including acute myeloid leukemia (AML) (Meshinchi *et al.,* 2003)

BCR-ABL emerges from a genetic translocation of chromosomes 9 and 22, which connects the BCR of chromosome 22 to the ABL1 region of chromosome 9. This modified rearrangement leads to translation of a protein that permanently links a breakpoint cluster region (BCR) to the proto-oncogene ABL1, known as BCR-ABL. BCR-ABL associates with GRB2 at tyrosine residue 177 (T-177) on the BCR region. The binding of GRB2 to this region is required for BCR-ABL leukemic transformation (Million, 2000). Mutation of this binding region reduces GRB2 interaction and

subsequent RAS activation (Pendergast *et al.*, 1993). Interestingly, the oncogenic properties of BCR-ABL$^+$ K562 cells are due to BCR-ABL signaling through various pathways in concert (Sonoyama *et al.*, 2002) including the mitogen activated protein kinase pathway (MAPK), which, in response to external cytokine signaling, utilizes GRB2 to connect certain phosphorylated proteins leading to increased cellular growth and proliferation. MAPK signaling is a well known pathway involved in oncogenesis, making GRB2 an important player in this process. Our study focuses primarily on BCR-ABL signaling along the MAPK pathway (Figure 1).

Growth receptor bound protein-2 (GRB2)

Growth receptor bound protein-2 (GRB2) is a membrane associated adapter protein 27kD in size that possesses one SRC homology-2 (SH2) binding region flanked on either side by two SH3 binding regions. GRB2 is responsible for linking receptor tyrosine kinase to MAPK signaling pathways (Lowenstein *et. al,* 1992).

GRB2 binds to a phosphorylated tyrosine site on BCR-ABL, allowing it to upregulate MAPK signaling (Lowenstein *et al.,* 1992, Pendergast *et al.*, 1993). Blocking GRB2 by antagonizing its SH2 region decreases tumor growth (Guibellino *et al.*, 2007) and importantly decreases BCR-ABL signaling as well as decrease viability of CML cell lines (Zhang *et al.*, 2014).

Finally, GRB2 is overexpressed in human breast cancer cell lines and tissue (Daly *et al.*, 1994, Verbeek *et al.*, 1997, Yip *et al.*, 2000). This indicates that GRB2 overexpression is a potential characteristic of cancer, raising the question of whether or not GRB2 might be overexpressed in CML.

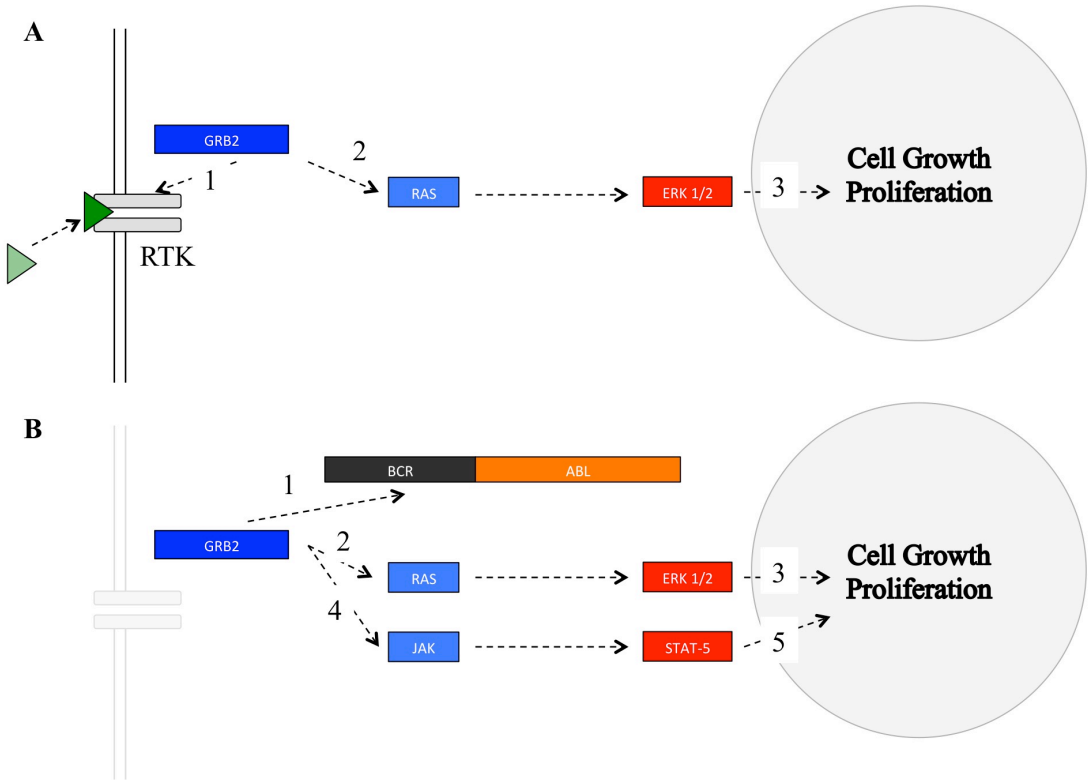

Figure 1: Proposed model whereby GRB2 overexpression increases cell proliferation. (A) The model for GRB2 signaling down the mitogen activated protein kinase (MAPK) pathway. After ligand (green triangle) binding to RTK, GRB2 SH2 domain binds RTK (1). This activates RAS (2), which leads to phosphorylation of extracellular signal-regulated kinase (ERK) (3). (B) Model of GRB2 signaling in BCR-ABL^{+} CML. (1) GRB2 binds BCR region of BCR-ABL in a ligand independent fashion. The GRB2 SH2 domain binds to BCR-ABL, which activates RAS (2), which leads to phosphorylation of ERK and increased cellular growth (3) or GRB2 binding to BCR-ABL activates JAK (4) and STAT-5 leading to cell proliferation (5). Increased expression of GRB2 may activate these pathways simultaneously.

STATEMENT OF PROBLEM

CML is a disease with an insidious onset and an unknown molecular transformation between a chronic phase and a more dangerous acute phase (Druker *et al.*, 1996). Understanding molecular mechanisms in cancer is a critical step in developing better treatments and improving outcomes for patients. Given GRB2's role in BCR-ABL signaling in CML and its overexpression in breast cancer, we hypothesized that GRB2 would be overexpressed in CML, and that its overexpression would increase proliferation of CML myeloid cells.

This study involves *in vitro* and *in vivo* models to elucidate the role of GRB2 overexpression in myeloid development and CML proliferation. To investigate this mechanism *in vivo*, we employed the zebrafish (*Danio rerio*) as an animal model. Zebrafish have unique characteristics that make them an advantageous model for studying hematopoiesis. This allows us to draw correlations to human hematopoiesis and how cancer develops (Amatruda *et al.*, 2002). Zebrafish develop externally, which allows for the investigation of development from the point of fertilization. Additionally, zebrafish are optically transparent, allowing the visualization of internal structures in living models and without the need for dissection. Most importantly, zebrafish are an important tool in modeling of human blood diseases (Langenau *et al.*, 2003, Santoriello *et al.*, 2012). We employed transgenic zebrafish expressing green fluorescent protein (GFP) under the myeloid specific promoter myeloperoxidase (*mpx*), which enabled us to specifically visualize myeloid cell development in our animals.

For *in vitro* study of GRB2 overexpression, we used the human myelogenous leukemia cell line K562. This cell line is an immortalized line of BCR-ABL$^+$ CML in

blast crisis, the terminal stage of CML progression. To study the effects of GRB2 overexpression, we used a lipid-based transfection regent to transfect our GRB2-containing plasmid construct into K562 cells. Using antibiotic selection, we selected for cells containing our plasmid to create a line of cells that stably overexpressed GRB2. These cells were assayed to assess the effect of GRB2 overexpression on growth and proliferation.

Our findings show that GRB2 is overexpressed in K562 cells. Furthermore, our findings show that additional GRB2 overexpression increases proliferation of K562 cells *in vitro.* Protein analysis of cell signaling pathways indicates that GRB2 induced proliferation is achieved in part through activation of the MAPK pathway. We also demonstrate that GRB2 inhibition with SH2 antagonists inhibits K562 proliferation. Finally, we show that GRB2 overexpression expands myeloid cells in 24 and 48hpf zebrafish. Our findings indicate that GRB2 has an expansive and proliferative role in myeloid development and growth.

Impact

Our findings indicate that GRB2 overexpression is playing a role in CML proliferation. These findings provide additional understanding of the molecular mechanisms in CML and myelopoiesis. Increasingly, targeting and enhanced therapies for cancer rely on understanding the workings of molecular signaling pathways in cellular growth and survival. As specific disease-causing molecular mechanisms are elucidated, it allows researchers to develop therapies that target these mechanisms and improve treatments for patients suffering from CML.

CHAPTER II

MATERIALS AND METHODS

GRB2 cloning

Human GRB2 was cloned by first generating RNA from HEK293FT cells. From this, complimentary DNA (cDNA) was generated (Bio-Rad iScript cDNA Synthesis Kit) and PCR was used to expand *GRB2* using primers:

Forward: 5'GCCACCATGGAAGCCATCGCCAAATA3'

Reverse: 5'TTAGACGTTCCGGTTCACTGGGGTGA3'

GRB2 was then cloned into a Topo-TA vector (Invitrogen TOPO TA Cloning Kit). Resulting constructs were used to transform competent Top10F' *E. coli* cells. Plasmid constructs were confirmed via Mini-Prep (Thermo Scientific) and restriction enzyme digestion to confirm proper insertion and orientation. Properly inserted plasmids were amplified and purified using Midi-Prep (Invitrogen) and further verified using restriction enzyme digestion.

GRB2 was sub-cloned into specific vectors based on their intended use. For transfections, *GRB2* was cloned into a pcDNA3-FLAG vector containing Neomycin (G418) resistance cassette. *GRB2* was also cloned into a pCS2+ vector for mRNA *in vitro* generation.

Drugs

Neomycin (G418) (ThermoFisher) was used for selection of stable transfections. G418 was re-suspended in tissue culture pure water at 50mg/mL, aliquoted at 1mL and stored

at -20^{o}C. Imatinib (Gleevec, Cayman Chemical) was dissolved in water and stored at -20^{o}C. Compounds 2-92, 2-107, 2-15 and 2-114 were synthesized and provided by Dr. Carolynn Arpin, a faculty member in the Chemistry Department at California State University, Chico. Compounds were dissolved in DMSO and stored at 4^{o}C.

Zebrafish stocks and embryos

Zebrafish were mated, staged, raised and maintained in accordance with California State University, Chico IACUC guidelines. All experiments were approved by CSU Chico IACUC before being performed.

Cell culture

The chronic myeloid leukemia cell line K562 was obtained from Dr. Mitchell Weiss, chair of hematology at St. Jude's Children's Hospital. Cells were maintained in RPMI 1640 media with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine (referred to as "K562 media"). Cells were grown at 37^{o}C, 5% CO_2. HEK293FT cells were provided by Dr. David Keller at California State University, Chico. HEK293FT cells were maintained in Dulbecco's modified eagle media (DMEM) with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine (referred to as "HEK media"). Cells were grown at 37^{o}C, 5% CO_2.

Transfections

K562 cells were plated at 2.0-5.0 x 10^5 cells per well and chemically transfected with 2.5ug of pcDNA-FLAG vector, or pcDNA-*Grb2* according to established protocol for

Lipofectamine 3000 transfection reagent (ThermoFisher). All cells were transfected with 2.5ug of a green fluorescent protein (GFP) containing plasmid (pMAX-*GFP*) for fluorescent monitoring of transfection efficiency. All transfections were checked 48 hours post transfection. Images were acquired via fluorescent inverted microscope and transfection efficiency was determined by the number of GFP labeled cells divided by total cells. Transfection efficiencies were determined visually using acquired images and confirmed by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences). At 72 hours post transfection, cells were selected with antibiotics.

Establishing stably expressing cell lines

Transfected K562 cells at 72 hours post transfection were removed from wells and expanded in T-75 tissue culture flasks in fresh K562 media containing 0.7 mg/mL Neomycin (G418) and allowed to grow for 1 week. At one week, cells were monitored for GFP expression and overall cell viability both visually and via flow cytometry using untransfected K562 cells as a positive control for G418 effectiveness. Stably expressing K562 cells were maintained in K562 media containing 0.7mg/mL of G418. Cells were split every 2-3 days, replacing media and G418 at each split.

Western blotting

Protein isolated from cell lysate was run on 4-20% acrylamide gels (Mini-Protean GTX, BioRad). Protein was transferred to PVDF blotting paper and blocked with milk for 1 hour. Gels were stained post-transfer using Coomassie stain to visualize loaded protein levels. Blots were probed for GRB2 using anti-GRB2 antibodies (Abcam, Ab32037).

Anti-GAPDH antibodies (Abcam, Ab181602) were used as a loading control. Levels of pBCR-ABL, pSTAT5, pAKT, and pERK 1/2 were detected using antibody cocktails (Pathscan Western Cocktail I, Pathscan Western Cocktail III and Pathscan Bcr/Abl cocktail, Cell Signal). Blots were stained with Ponceau stain to confirm loading amounts. Blot images were adjusted for quality and clarity using Photoshop. All adjustments were made equally across all blots.

Proliferation and differentiation assays

K562 cells stably overexpressing GRB2 were plated in a T-75 tissue culture flask at $2.5x10^5$ cells per mL in serum free K562 media for 12-18 hours. At that time, cells were spun down and replaced in K562 media in a T-75 flask and allowed to grow for 48 hours grown at 37^oC, 5% CO_2. At 48 hours, an absolute cell count was obtained via hemacytometer and recorded as total cell numbers. At that time, 100uL of cell solution was plated in triplicate in a 96-well plate. Cell Titer 96 proliferation assays were used to assess cellular growth at 48 hours according to manufacturer's specifications. Cell Titer 96 assays measure the conversion of a tetrazolium salt compound to formazin by growing cells. Data is recorded as absorbance at 570nm. Cell Titer 96 assays were optimized prior to experimentation for K562 cells. Remaining cells were spun down, washed with sterile phosphate buffered saline (PBS) and prepared for protein isolation.

mRNA injections

Human GRB2 mRNA was generated *in vitro* (Invitrogen mMachine RNA kit). 1ng of PBS or *GRB2* mRNA was injected into transgenic zebrafish expressing green fluorescent

protein (GFP) under the myeloid specific promoter myeloperoxidase (*mpx*) at the single cell stage. *mpx*:GFP transgenic fish have GFP labeled myeloid cells, and were developed as a model for monitoring neutrophil activity (Renshaw, 2006). Embryos were allowed to incubate post injection for 48 hours. At 24 hours, the fish were enzymatically removed from their chorion, anesthetized using tricane and visualized using fluorescent microscopy. Visual counts were repeated at 48 hours.

Enumeration of myeloid cells

Zebrafish were anesthetized using tricane and placed under a fluorescence microscope. Still images were obtained using at Leica FireCam fluorescent microscope camera and GFP^+ cells were counted manually at 24 and 48 hpf and recorded as total cell counts. Total cell counts were represented in graphs as total cell counts, with error bars indicating standard deviation from the mean.

Flow cytometry

Transfection efficiency was assessed using an Accuri C6 (BD Biosciences) flow cytometer to monitor GFP expression and cell viability after transfection.

Statistics

Statistical significance was analyzed using a two-tailed heteroscedastic t-test for two sample arrays. * = <0.05, *** = <0.0001.

CHAPTER III

FINDINGS AND RESULTS

GRB2 is overexpressed in K562 cells

To determine the expression level of GRB2 in K562 cells we compared the levels of GRB2 in K562 cells to that of a non-malignant cell line. A western blot from protein obtained from cell lysate of K562 cells and human embryonic kidney cells (HEK293FT) showed elevated expression of GRB2 in K562 cells relative to HEK293FT cells (Figure 2a). Data obtained from the Cancer Cell Line Encyclopedia (CCLE) in conjunction with the Broad Institute and Novartis looking at mRNA expression levels in a variety of human cancer cell lines indicates relatively high levels of expression in leukemia cell lines including CML (Figure 2b). These findings indicate that GRB2 overexpression is a feature of CML.

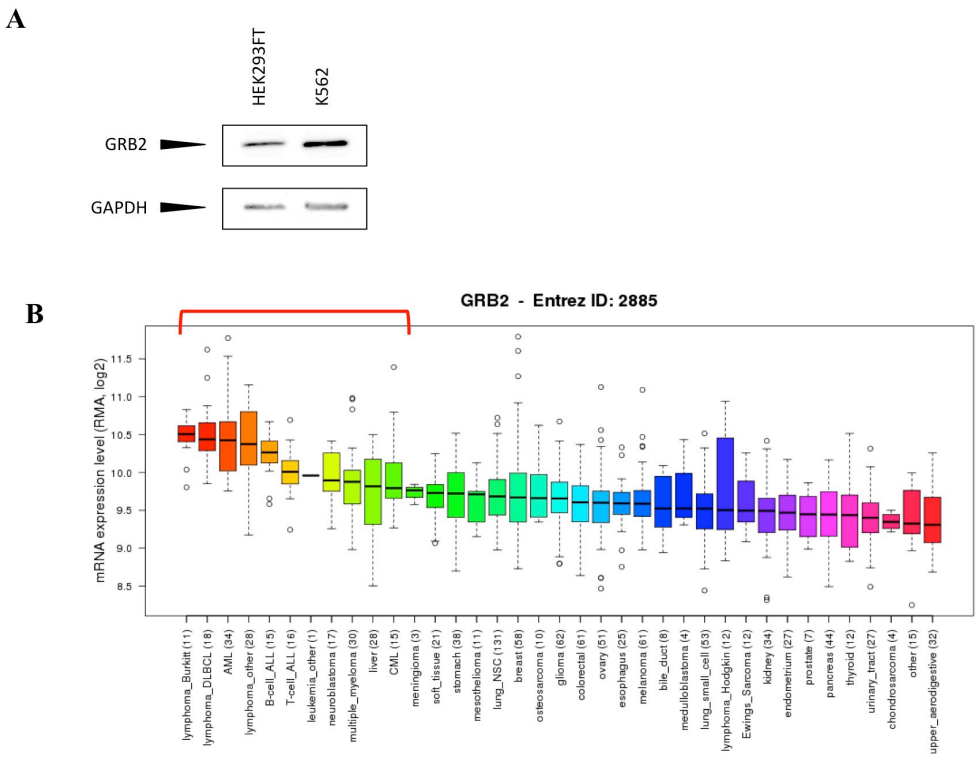

Figure 2: GRB2 is overexpressed in K562 cells. (A) Western blot of protein obtained from HEK293FT and K562 cells probed for anti-GRB2 antibodies (Abcam) showing increased expression of GRB2 in K562 cells relative to HEK293FT. GAPDH is shown as a loading control. (B) mRNA expression levels across various human cancer cell lines. Red brackets showing elevated GRB2 expression levels in various leukemia cells lines including chronic and acute myeloid leukemia. Data and image obtained from the Cancer Cell Line Encyclopedia (CCLE, Broad Institute-Novartis).

GRB2 overexpression causes proliferation of K562 cells

Next we investigated the effect of additional GRB2 overexpression in K562 cells. Growth assays of transfected K562 cells stably overexpressing GRB2 show a 22%

increase in total cell numbers at 48 hours (Figure 3a). Western blot of protein isolated at 48 hours indicates that upregulation of phospho-ERK 1/2 was associated with GRB2 overexpression induced proliferation (Figure 3b). There was no discernable upregulation of phosphorylation of protein kinase B (PKB, otherwise known as pAKT) indicating that the growth measured in this assay correlates GRB2 overexpression with upregulation of MAPK signaling, but not PI3-K signaling, which is a signaling pathway that has been shown to be active in BCR-ABL$^+$ CML (Sonoyama *et al.*, 2002).

GRB2 overexpression causes myeloid expansion in 24 hpf zebrafish

Finally we investigated the effect of GRB2 overexpression in myeloid cell development. Injection of transgenic *mpx*:GFP zebrafish with *GRB2* mRNA at the single cell stage expanded myeloid cells compared to PBS-injected embryos (Figure 4b-e). Enumeration of GFP$^+$ myeloid cells show a roughly 2-fold expansion in myeloid cells at 24hpf (Figure 4f) The same population of injected fish were monitored at 48hpf to investigate the role of GRB2 overexpression on myeloid expansion. At 48hpf fish injected with *GRB2* mRNA maintained a significantly higher level of GFP$^+$ myeloid cells (Figure 5a-d). Enumeration of GFP$^+$ myeloid cells shows a continued expansion of myeloid cells at 48hpf (Figure 5e). These findings demonstrate that GRB2 overexpression expands myeloid cells early in development.

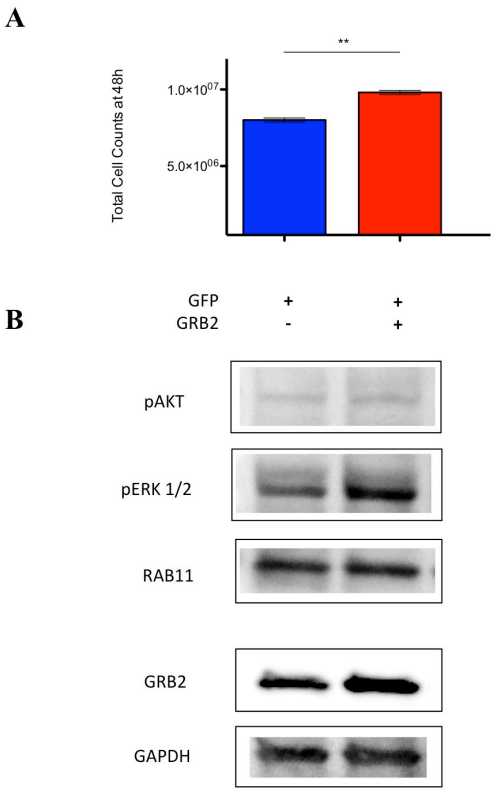

Figure 3: GRB2 overexpression increases proliferation of K562 cells and increases phosphorylation of ERK. (A) Total cell counts at 48h showing increased proliferation indicating increased proliferation of GRB2 overexpressing K562 cells. (B) Western blot of transfected K562 cellular protein at 48h probed with anti-GRB2 antibodies (Abcam) showing increased GRB2 expression in cells transfected with a plasmid construct containing *GRB2* (pcDNA-*GRB2)* and reporter plasmid containing GFP (pMAX-GFP*)* as compared to cells transfected with pMAX-GFP only. Protein probed for pERK and pAKT show upregulation of pERK correlated with GRB2 overexpression. (*=$p<0.05$)

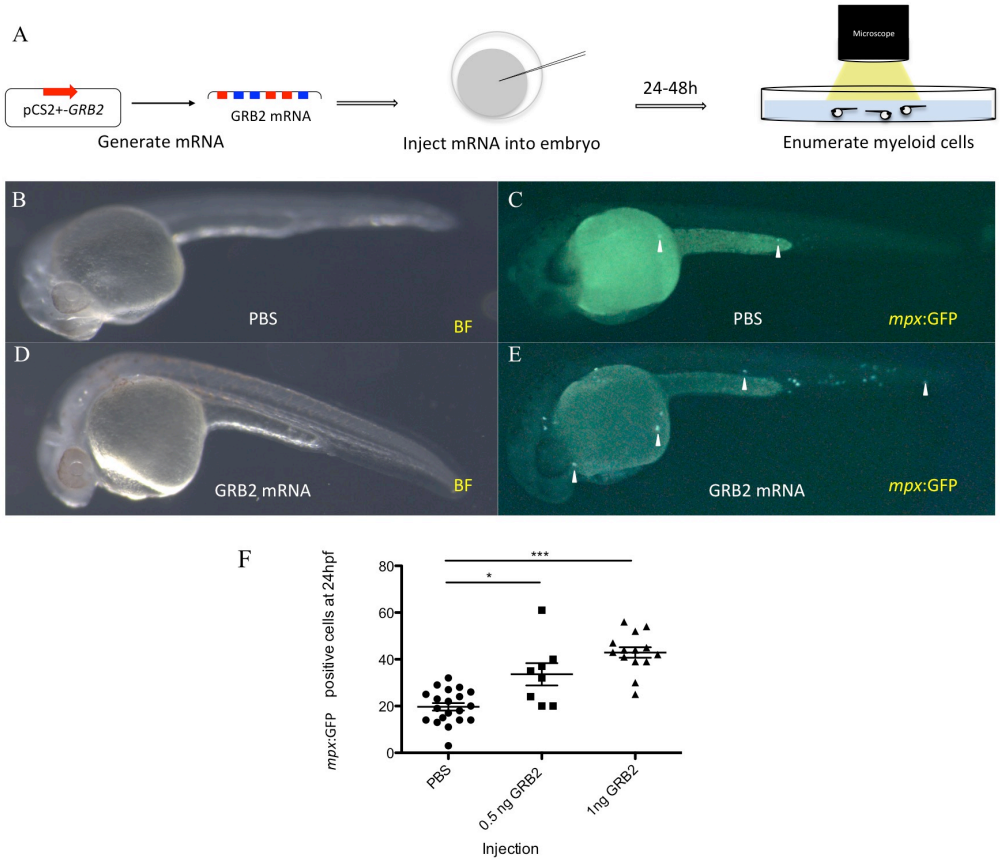

Figure 4: GRB2 overexpression expands myeloid cells in 24hpf zebrafish. (A) Diagram of GRB2 mRNA injection and myeloid enumeration process procedure. (B, D) Brightfield (BF) images of 24hpf *mpx*:GFP zebrafish injected with PBS (B) or GRB2 mRNA(D). (C, E) Fluorescent images of 24hpf *mpx*:GFP zebrafish injected with PBS (C) or GRB2 mRNA (E). White arrowheads represent examples of GFP+ myeloid cells. (F) Graphic representation of GFP^{+} cells from 24hpf *mpx*:GFP zebrafish injected with PBS, 0.5ng of GRB2 mRNA or 1ng of GRB2 mRNA. Circles, squares and triangles represent counts from individual fish. Horizontal lines represent mean and error bars represent standard deviation from the mean. (* = $p<0.05$, ***=$p<0.0001$)

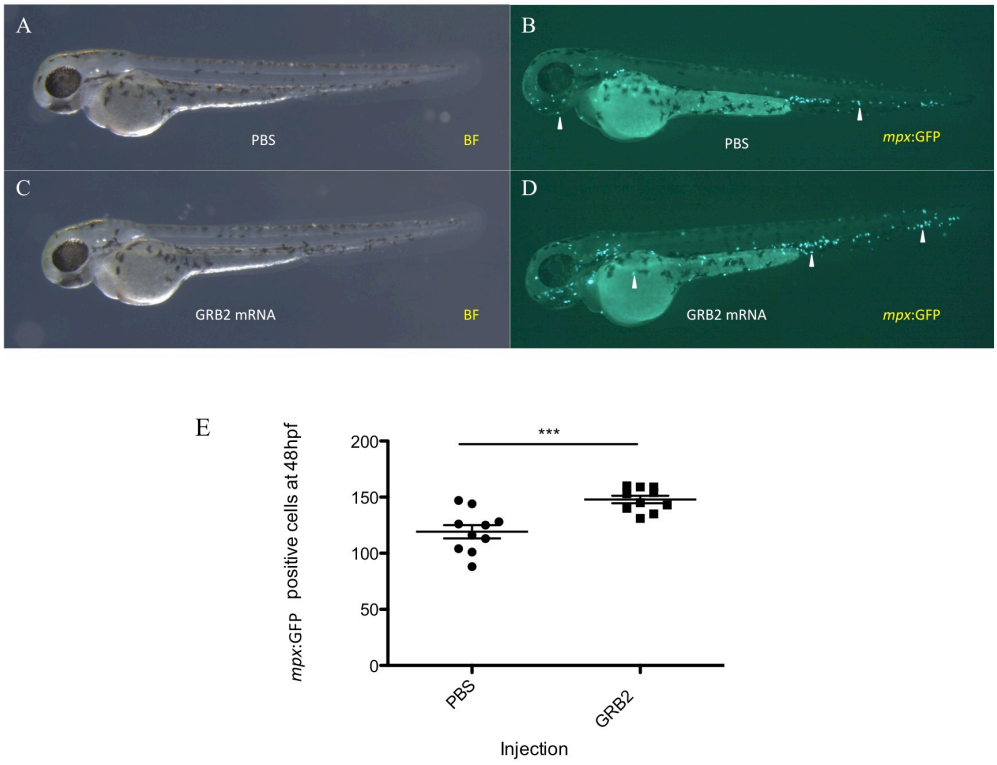

Figure 5: GRB2 overexpression expands myeloid cells in 48hpf zebrafish. (A,C) Brightfield (BF) images of 48hpf *mpx*:GFP zebrafish injected with PBS (A) or GRB2 mRNA (C). (B, D) Fluorescent images of 48hpf *mpx*:GFP zebrafish injected with PBS (B) or GRB2 mRNA (D). White arrowheads represent examples of GFP^+ myeloid cells. (E) Graphic representaiton of GFP^+ cells from 24hpf *mpx*:GFP zebrafish injected with PBS or 1ng of GRB2 mRNA. Circles and squares represent counts from individual fish. Broad lines represent mean and error bars represent standard deviation from the mean. (***=$p<0.0001$)

Designed SH2 antagonists inhibit growth of K562 cells

Inhibition of K562 cells can be achieved through SH2 antagonism (Zhang *et al.*, 2014). We tested four novel SH2 antagonists against K562 cells. These flat heterocyclic compounds were generated by a novel cyclization reaction to bind to SH2 regions on proteins. We found growth reduction of K562 cell growth across a range of concentrations in all four compounds (Figure 6a-d). When treated at a concentration of 62.5uM, we saw reduced growth of K562 cells with all four compounds, with three of the four compounds showing a more than 50% reduction in growth over 48h (Figure 6e). Western blot of protein isolated at 48 hours showed a varied range of effects on cellular signaling pathways. Compound 2-92 and 2-15 both showed significant reduction in phosphorylated BCR-ABL (pBCR-ABL), which correlated with a more that 50% reduction in cell growth. Compound 2-107 showed no effect on pBCR-ABL levels, but demonstrated a reduction in phosphorylated ERK 1/2 (pERK 1/2) indicating a reduction in MAPK signaling. Compound 2-15 showed a reduction in phosphorylated BCR-ABL and STAT-5 (pSTAT-5) levels as compared to untreated K562 cells, with a marked upregulation of pERK 1/2 (Figure 6f). Our findings demonstrate that blocking GRB2's SH2 binding regions decreases K562 cell growth.

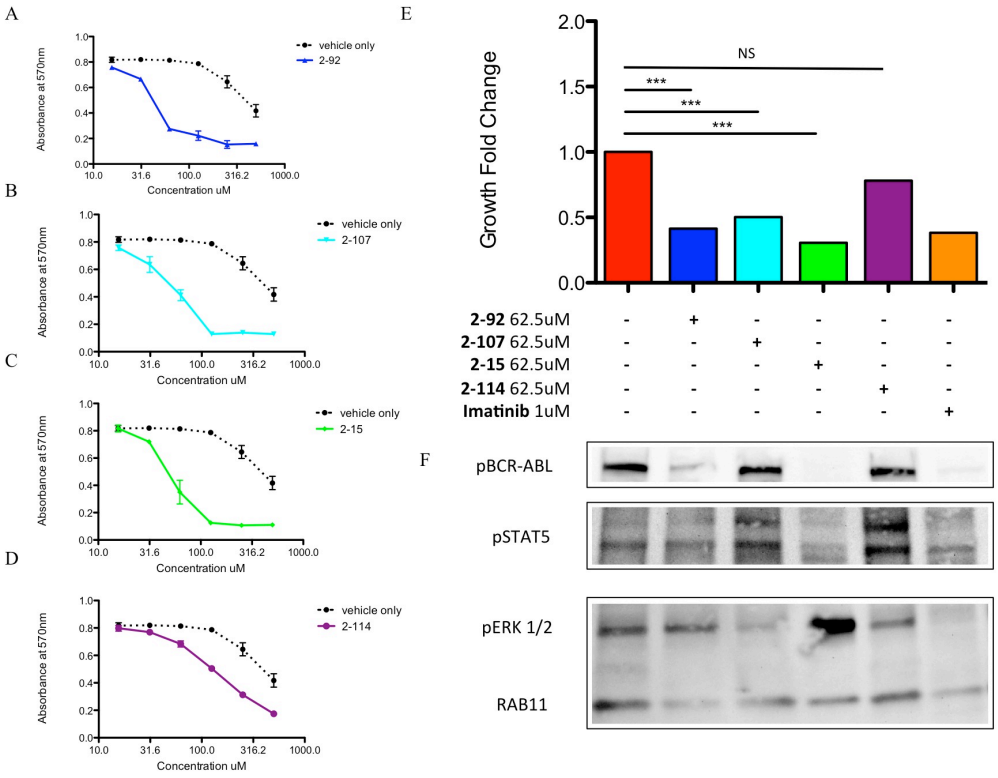

Figure 6: Designed SH2 antagonists block K562 proliferation. (A-D) Graphs of K562 cells grown in the presence of SH2 inhibitors (2-92, 2-107, 2-15, 2-114) showing the effect of SH2 antagonism on cell proliferation at 48h. Imatinib was used as a positive control. Proliferation is represented as absorbance at 570nm after Cell Titer 96 assay. Dashed line indicates effect of drug vehicle (DMSO) only at noted concentrations. Error bars indicated standard deviation from the mean. (E) Graph of K562 cell proliferation represented as fold change (relative to untreated K562 cells) at 48h. (F) Western blots of protein isolated at 48h from K562 cells and K562 cells grown in the presence of SH2 antagonists. Compound list and dosing correlates with panel E columns and corresponding blots in panel F. 48h protein was probed with anti-pBCR-ABL, pSTAT5, pERK 1/2 and anti-RAB11 (loading control) antibodies (Cell Signal, Pathscan BCR-ABL WB Cocktail, Pathscan WB Cocktail I). (*** = $p < 0.0001$)

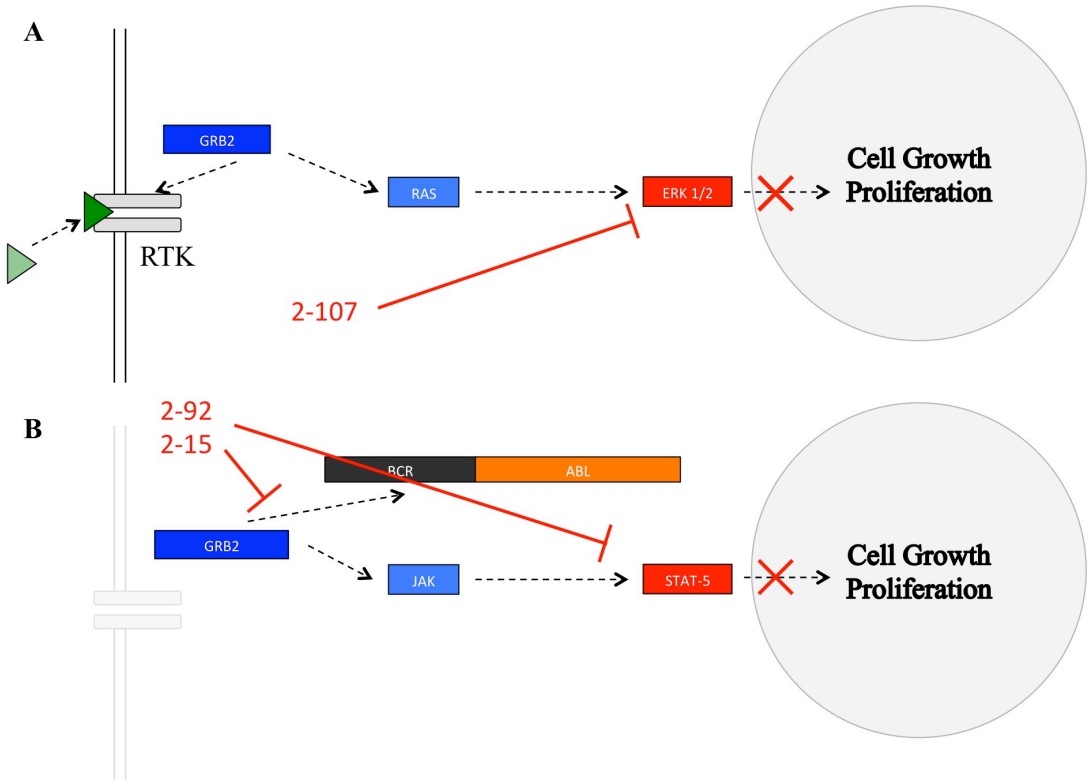

Figure 7: Proposed model for SH2 antagonist effect on K562 growth. Proposed cell signaling model for SH2 antagonists compounds 2-92, 2-107 and 2-15 effect on K562 cell growth. (A) Compound 2-107 blocks GRB2 binding to RTK, decreasing phosphorylation of ERK (B) Compounds 2-92 and 2-15 decrease phosphorylation of BCR-ABL and subsequently decreasing phosphorylation of STAT-5. Compound 2-114 appeared to have no discernable effect on these pathways and was not included in this figure.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Our study demonstrated that GRB2 is overexpressed in K562 cells relative to HEK293FT cells. This data correlates with established data showing that GRB2 is overexpressed in breast cancer cell lines (Daly *et al.*, 1994) as well as in breast cancer tissue (Verbeek *et al.*, 1997, Yip *et al.*, 2000). To date, the only evidence of GRB2 overexpression has been found in solid tumor cancers. The expression level of GRB2 in liquid tumors such as leukemia has yet to be investigated. Given that different cancers share common cell signaling pathway activation, including those that involve GRB2 activity, we hypothesized that GRB2 would be overexpressed in leukemic cells.

Our goal was to compare GRB2 expression levels to a non-malignant cell line. However, some limitations to our choice of control cells exist. HEK293FT cells come from a different region of the body, and may be expressing different relative levels of GRB2 with respect to the receptors and ligands involved. A better approach would be to compare GRB2 expression in K562 cells to a non-malignant population of myeloblasts, or more optimally to compare bone marrow samples of CML patients to normal patients to get a clearer picture of whether GRB2 is overexpressed in these cases of CML. This would be an important next step in this investigation. Regardless, our findings indicate a clear overexpression of GRB2 in a CML cell line as compared to non-malignant cell line, which indicates that this is a potential feature of CML.

We also demonstrated that additional GRB2 overexpression increased proliferation of K562 cells. The mechanism of proliferation appeared to be through upregulation of MAPK as indicated by elevated levels of pERK 1/2. Phosphorylation of ERK correlates with the established role of GRB2 in MAPK signaling, and GRB2 overexpression appears to enhance this signaling pathway. It is necessary to perform a loss of function assay to knock down ERK 1/2 to determine this role completely.

It is important to note that while MAPK is an important pathway in cellular growth and proliferation, there are number of other pathways that could be involved in GRB2 overexpression induced proliferation of malignant cells. Our study found that GRB2 overexpression did not induce any discernable upregulation of PI3K signaling as seen by levels of pAKT. Any future work should investigate additional growth pathways that may be upregulated by GRB2 overexpression.

GRB2 has also been shown to associate with GRB2-associated binding protein-2 (GAB2) to phosphorylate PI3K and AKT in BCR-ABL^{+} cells (O'Hare, 2011). Our protein analysis show little to no phosphorylation of AKT, and no discernable difference between GRB2 overexpressing cells and control cells indicating a possible preference for other pathways, or an inhibitory loop that downregulates PI3K.

It should be noted that the resulting proliferation varied somewhat between different assays. This indicates our cells are changing their internal signaling environment continuously in response to unknown factors. To get a clear correlation between GRB2 expression levels and cell proliferation, we isolated protein at the same time that cell proliferation was measured. This allowed us to more accurately correlate GRB2 expression with K562 proliferation.

Through *in vivo* study of zebrafish hematopoiesis, we demonstrated that GRB2 overexpression expands myeloid cells at 24hpf and 48hpf. This was achieved through injection of exogenous human GRB2 mRNA. Zebrafish have two variants of *GRB2*, G*rb2a* and *Grb2b*. While there are genetically similar, there may be some variation in their binding behavior as compared to the human version. An important next step in this study would be to clone the variants of zebrafish G*rb2a* and *Grb2b* and determine the effect of the native variants of GRB2 on zebrafish myelopoiesis. However, for the purposes of modeling a human disease, the use of GRB2 is appropriate.

One limitation to this assay was our inability to experimentally establish whether mRNA was actually translated into a functioning GRB2 protein in the developing fish. Performing western blots to measure GRB2 levels would be limited in one important way. Injection of mRNA at the single cell stage overexpresses GRB2 in all cells of the fish, not just the hematopoietic system or myeloid cells specifically. Isolating protein from the whole fish and probing for GRB2 levels would likely show an elevated level of GRB2, but there would be no way of knowing that GRB2 was overexpressed in myeloid cells specifically. To accomplish this would require fluorescent activated cell sorting (FACS) technology to isolate GFP^+ cells and isolate protein from that population. Sorting GFP^+ myeloid cells and analyzing protein isolated from them would be an important addition to these findings. FACS would also allow for more comprehensive enumeration of myeloid cells because it would accurately measure any cell expressing GFP in a transgenic fish carrying GFP under a myeloid specific promoter such as *mpx*. While zebrafish are particularly advantageous for this kind of visual enumeration, FACS data would be a strong supportive addition to the experiment.

One important finding in our *in vivo* assays was the difference in myeloid expansion between 24hpf and 48hpf. Our findings demonstrated a nearly 2-fold expansion at 24hpf, but that difference reduced to a roughly 20% expansion by 48hpf. This raises interesting questions regarding the development of myeloid cells and which signaling pathways are activated at given time points. Our findings indicate that GRB2 has a more potent role in myeloid development earlier and perhaps this effect decreases as the fish develop. This could be explained by the decline in injected GRB2 mRNA levels in the fish as time passes. This could also be explained by GRB2 having a more potent role in primitive myelopoiesis, and less effect on definitive hematopoiesis in which myeloid cells arise specifically from HSCs.

Development of the innate immune system is one of the earliest events in hematopoiesis outside of erythropoiesis. GRB2 may have a more prominent role in the growth and differentiation of progenitor cells as opposed to more mature pre-mitotic myeloid cells. A logical next step for this study would be to investigate the role of GRB2 overexpression on early myeloid progenitors or HSCs to see if the effects of GRB2 are acting on myeloid cells derived from HSCs. This could be easily achieved by utilizing embryos from transgenic fish with fluorescent labels for HSCs or progenitors. Transgenic fish expressing fluorescent markers under promoters such as *c-myb* or *runx1* would be appropriate for labeling of these blood cell populations (Bertrand *et al.*, 2008, Lam *et al.*, 2008). Additionally, not all myeloid cells are derived from HSCs early in development. Primitive myelopoiesis is responsible for generation of the first wave of the innate immune system. The expansion of myeloid cells at 24hpf may also indicate the GRB2 plays a role in emergence of these primitive myeloid cells from the mesoderm.

Along these lines, our choice of transgenic zebrafish (*mpx*:GFP) is somewhat limited in assessing overall myeloid development since it primarily labels definitive neutrophils and macrophage (Renshaw *et al.*, 2006, Stachura *et al.,* 2013). Using a transgenic fish that labels primitive myeloid cells would be important in elucidating this. It would be prudent to investigate other populations of myeloid cells by using different transgenic fish to see if the expansive effect of GRB2 is equal across all myeloid populations, or if it pushes development toward the neutrophil population and away from other populations. A natural final progression of this study would be to create a transgenic fish that overexpresses GRB2 under a myeloid specific promoter to determine the role of GRB2 overexpression throughout the life of the fish. This would allow us to assess whether disease transformation occurs in GRB2 overexpression. Regardless, our findings have shed light on the expansive role of GRB2 on early myeloid cells in zebrafish. This is an important finding because this early development phase is critical for malignant transformation in leukemia.

Finally, we investigated four novel SH2 antagonists and their role in K562 cell growth. These compounds are designed to inhibit the SH2 binding region of GRB2 to block its ability to bind to receptors such as RTKs and BCR-ABL, a tyrosine kinase. We first performed limiting dilutions to determine a concentration curve that would allow us to find an effective concentration. The results of this assay showed varied and interesting results across different compounds. We chose to investigate both MAPK and STAT signaling as these are both established targets of BCR-ABL (Pendergast *et al.*, 1993, Sonoyama *et al.*, 2002). Both compound 2-92 and 2-15 showed significant effects in blocking pBCR-ABL. GRB2 binding to BCR-ABL is a requirement for BCR-ABL

phosphorylation and leukemogenesis (Million *et al.*, 2000). Both of these compounds reduced phosphorylation of STAT-5, a downstream target of BCR-ABL. This is concomitant with a decrease in BCR-ABL activity in these cells that correlates with decreased growth.

GRB2 also associates with GAB2 to bind RTKs in order to signal along the JAK/STAT pathway (O'Hare *et al.*, 2011), so it will be important to investigate the activity of RTKs that utilize the GRB2/GAB2 complex to see if these compounds have an effect on SH2 domains upstream of STAT-5 to see if this effect contributes to the decrease in growth.

Interestingly, compounds 2-107 and 2-114 both showed no effect on BCR-ABL activity. Both compounds 2-107 and 2-114 showed increased levels of pSTAT-5 and a mild reduction of pERK, indicating that these compounds may have non-specific SH-2 antagonistic activity. Phosphorylation of BCR-ABL, ERK 1/2 and STAT-5 require adapter or scaffolding proteins that possess SH2 domains. The variation in expression of pBCR-ABL, pERK 1/2 and pSTAT-5 indicate that all four of these compounds may have different specificity of SH2 domains within the cytoplasm.

One particularly interesting finding was the marked elevation in pERK 1/2 in the cells treated with 62.5uM of 2-15. These cells showed BCR-ABL inhibition as seen by decreased levels of pBCR-ABL and pSTAT5, which explains the growth inhibition we recorded. However, the highly elevated levels of pERK 1/2 indicate that MAPK, or perhaps another signaling loop has been activated despite the decrease in growth. One explanation for this may be the onset of a protective cell signaling pathway that may lead to resistance to this compound. A mechanism for CML resistance to Gleevec (imatinib,

STI-571) via paracrine FGF2 signaling has been identified, which is a strong upregulator of ERK1/2 (Traer *et al.*, 2014).

These compounds, while they were designed for a specific target, appear to have vastly different effects on signaling pathways within the cell. Binding specific assays and investigation of phosphorylation events immediately downstream of adapter and scaffolding proteins that have SH2 domains would more clearly demonstrate the direct effect of these individual compounds.

Small molecules that inhibit SH2 binding have been investigated as potential therapy enhancers or stand-alone drug therapies for leukemia (Zhang *et al.*, 2014). It would be interesting to investigate whether any of these drugs work synergistically with common treatment compounds in CML. One GRB2 inhibiting SH2 antagonist used in conjunction with imatinib was found to synergistically inhibit K562 cells (Zhang *et al.*, 2014). This indicates that there is strong potential to synergistically improve established therapies for CML through small molecule SH2 antagonists. Determining the specificity of our compounds for SH2 containing proteins would be an important step in understanding their specific effect on K562 cell growth. Knowing this would allow us to analyze specific cell signaling pathways and determine their specific effect.

Our findings demonstrate a role of GRB2 overexpression in CML. We found that GRB2 is overexpressed in K562 cells. Given that the transformative process in CML from chronic to acute phases is still unknown, this finding may aid in furthering our understanding of this process. This is supported by our findings that GRB2 overexpression increases cell proliferation in K562 cells. Additionally, GRB2 overexpression upregulates phosphorylation of ERK 1/2 in K562 cells. This demonstrates

that GRB2 overexpression is playing a role in the proliferative nature of myeloblasts. K562 cells are an immortalized cell line of CML in blast crisis. If GRB2 overexpression is a factor in transformation from chronic or acute phase into the blast crisis phase, then GRB2 could potentially be targeted by drug therapies to arrest CML progression. It would be an important study to look at GRB2 levels in CML patients in chronic, acute and blast crisis phase to elucidate any difference in GRB2 expression. Regardless, our findings shed light on an important feature and mechanism in a disease that affects thousands of people every year.

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