2012-01-01

JAK3/STAT5 Signaling Cascade Represents A Therapeutic Target To Treat Select Hematologic Malignancies

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Dedication

I dedicate this thesis to my loving mother, Maria Rosado. She was someone that sought knowledge. As a child I would skim through her books on cancer. She had a desire to understand more on her disease, which led me to pursue and embrace cancer research. I hope to leave some results and ideas that could open up doors for future cancer research and studies. Therefore, I dedicate this to my mother, for always helping me seek to expand my knowledge and do something important with the life she has given me.

JAK3/STAT5 SIGNALING CASCADE REPRESENTS A THERAPEUTIC TARGET TO TREAT SELECT HEMATOLOGIC MALIGNANCIES

by

DAMARIS CRYSTAL ROSADO, Bachelors of Science

THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

May 2012

Acknowledgements

I want to thank my Tia Josie and Tia Luisa for their continuous support. They have always supported every decision I have made and I thank them for always being there. I want to thank all of my laboratory members for their enormous support. I particularly want to thank Dr. Jeremy Ross, Dr. Georgialina Rodriguez, and Dr. Robert Kirken. Dr. Ross was a great mentor and supporter on this long road. He always made me feel like there was a light at the end of the tunnel. I thank him for always believing in me and making my tough days in the laboratory a little easier. He is a wonderful person that brought laughter, immense knowledge, and a helping hand into my lab life. I thank him for being the "lab knowledge man". I thank Dr. Rodriguez for being a great friend for many years. She was always the person in the lab to bring light to any situation. I thank her for being the "lab mom". I thank her for her advice in and out of the laboratory and for making my life transitions easier. She is a phenomenal person inside and out and makes any environment more pleasant to be in. I thank Dr. Kirken for his never ceasing continuous support. I came into his laboratory in 2006 after my mothers' passing and have always felt that he has made this laboratory feel like my home. When I lost my way, he was there to help me find it again. He has never questioned my choices, but has instead provided an environment for me to grow and thrive. I thank him for taking me into his lab and making me feel like I can do anything in this world. I thank him for making me feel like I was part of a family. Steven and Blanca, I thank you both for making my laboratory experience a fun one. I will never forget the music and laughter that made my days in the lab more enjoyable and the bad results a little bit easier to bear. Thank you both for adding joy and laughter to my days. Derrick, thank you for all your help with experiments, especially confocal. Thank you for a great friendship that I am sure will last throughout the years.

Abstract

Tyrosine kinases are an essential component of cell signal transduction pathways, many of which promote cellular proliferation. However, when a tyrosine kinase is aberrantly activated or its negative regulation is lost, the result can be malignancy. In humans, 90 tyrosine kinases are present and of these, 51 have been linked to a malignancy through mutation or overexpression. Janus kinase 3 (JAK3) is one such kinase that upon hyperactivation, due to a somatic mutation, has been linked to cancer including its substrate, signal transducer and activator of transcription (STAT5). Few studies have investigated the role of JAK3/STAT5 pathways in hematopoietic cancers such as leukemia and lymphoma, nor whether health disparities exist among different groups with respect to these types of cancer and effectors. This is one of the first studies where multiple signaling molecules were studied in a large cohort of patients with cancer. This study suggests that multiple proteins, including JAK3 and STAT5, are activated in different cancers. Multikinase inhibitors may represent a viable treatment option for patients displaying activation of multiple proteins, and a clinically approved JAK3 inhibitor needs to be developed. Using a peptide library a putative JAK3 consensus peptide substrate was identified. Of the 181 proteins "mined" as possible JAK3 substrates many may also represent a therapeutic target for uncoupling JAK3 dependent cancers. For example, our results implicate reciprocal activation of JAK3 and NPM-ALK in anaplastic large-cell lymphoma. Indeed, many of these proteins require further study and to define their pathways, which many be pivotal in therapeutic intervention in certain hematological malignancies.

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Chapter I: General Introduction

1.1 T-CELL ACTIVATION

The immune system is a dynamic defense against a host of pathogens, and at its center are T-cells. T-cells are activated following antigenic stimulation via antigen presenting cells (APC's). This event triggers a number of processes including T-cell growth, survival, anergy, apoptosis, or differentiation (Smith-Garvin et al., 2009). Antigen is presented via the Major Histocompatibility Complex (MHC) on the APC to the T-cell receptor (TCR) on the T-cell, thus constituting Signal 1 (TCR/CD3). This signal is amplified via various costimulatory molecules (Signal 2) that act cooperatively with Signal 1 to initiate activation of a cascade of enzymatic reactions that include SYK and SRC tyrosine kinase family members ZAP-70 and LCK/FYN, respectively, to promote T-cell differentiation. These signal transduction pathways upregulate key T-cell growth factor (TGCF) (cytokines) genes such as Interleukin-2 (IL-2), which subsequently activate signal transduction pathways referred to as Signal 3 (Kirken & Stepkowski, 2002). The magnitude and duration of this response is due to the type and dose of antigen presented to a T-cell, strength of TCR/CD3 interaction, kinetics and efficiency of the antigen stimulation phase, as explained below (Bluestone, 1998).

1.1.1 T-CELL DIFFERENTIATION: SIGNAL 1 AND 2

Antigen presentation to a T-cell activates two major signals: [1] TCR recognizes the antigen and [2] the T-cell co-stimulatory receptor CD28 and/or cytotoxic lymphocyte-associated molecule-4 (CTLA-4) bind to their respective ligands CD80/CD86 (Slavik, 1999), which activates several intracellular signaling cascades inducing TCGFs such as IL-2, IL-7, IL-9, and IL-15. TCR/CD3 recruitment and accessory molecules form a supramolecular activation cluster (SMAC). All surface molecules necessary for signal transduction are organized in the SMAC (Lin et al., 2005). The immunoreceptor-based tyrosine activation motif (ITAM) is located at the

cytosolic components of the TCR/CD3 complex and is essential for TCR-mediated activation. Once optimal TCR engagement and costimulation occurs, Signal 1 and Signal 2, tyrosine residues within each ITAM are phosphorylated and act as recruitment sites for proteins that contain binding domains (e.g. PTB and SH2).

Two major SRC family kinases, LCK and FYN, mediate ITAM phosphorylation upon which ZAP-70 can then bind to the TCR via its SH2 domains. ZAP-70 tyrosine phosphorylates the adaptor molecule, Linker for the Activation of T-cells (LAT), which recruits to the membrane many signal amplifying proteins. SYK and SRC family members can also phosphorylate tyrosine residues on CD3 receptor chains. These residues also function as docking sites for intracellular second messengers, PLCγ1, PI3K, and Shc/Grb2/SOS/Ras, which become phosphorylated to eventually regulate transcription factors that initiate gene transcription, such as cytokines that are necessary to initiate Signal 3 (Samelson, 2002).

1.1.2 T-cell Proliferation: Signal 3

Cytokines are low-molecular weight regulatory proteins or glycoproteins secreted by a variety of immune and non-immune cells such as lymphocytes, monocytes, neutrophils, and fibroblasts. However, the principle producers of cytokines are T helper (Th) cells, dendritic cells, and macrophages (Aringer, 2002). Cytokines can regulate the function of the same (autocrine) or distal cells (paracrine) (Fitzgerald et al., 2001). Cytokines bind to specific receptors on the membrane of target cells resulting in the activation of various signal transduction pathways essential to regulate T-cell growth and differentiation. These pathways constitute Signal 3 (Kovamen & Leonard, 2004). Once Signal 1, 2, and 3 have been engaged, a T-cell is considered

fully activated **(Figure 1.1).** The current study will focus on the role of Signal 3 and Janus Kinase 3/Signal Transducer and Activator of Transcription 5 (JAK3/STAT5) pathway that can be activated by cytokines such as IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, and IL-21 (Kirken & Stepkowski, 2002). The IL-2 receptor subfamily cytokines bind to receptors that share a γ common chain to activate JAKs and STATs, which are critical for proliferation and survival of T-cells (Ross, 2007).

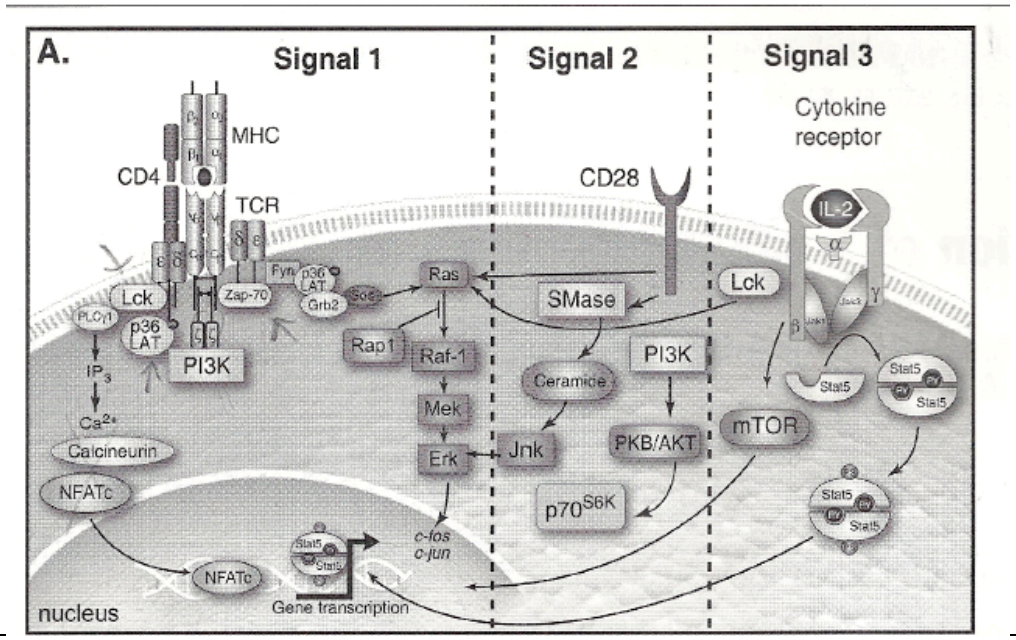


Figure 1.1. T-cell Activation. Full T-cell activation requires 3 sequential signals: [1] TCR recognizes the antigen, [2] the T-cell co-stimulatory receptor CD28 binds to its ligand which induces IL-2 and other cytokine production [3] IL-2 or other gamma chain cytokines, then bind to receptors that share a γ common chain associated with an α–chain for each cytokine or with a β chain (for IL-2 and IL-15) and this will activate the JAK/STAT signal transduction pathway to drive proliferation and survival of T-cells. (Ross et al., 2007)

1.1.3 T-cell proliferation in response to IL-2

Regulation of T-cell apoptosis and survival is controlled by distinct cytokines, such as interferons that promote cell death, while cytokines like IL-2, IL-7, IL-9, and IL-15 promote cell survival (Ross, 2007). IL-2 is a cytokine released by activated T-cells and plays a major role in immune system homeostasis. When IL-2 engages its receptor, several pathways become activated including JAK/STAT, Mitogen Activated Protein Kinase (MAPK), and Phosphatidyl Inositol 3 Kinase/Mammalian Target of Rapamycin (PI3K/AKT/mTOR), which mediate cellular proliferation, survival, apoptosis, and differentiation (Zhao, 2010; Aringer, 2002; Cardoso et al., 2008). Deregulation of these three pathways is associated with a variety of malignancies (Ross, 2007; Kirken & Stepkowski, 2002). Similarly in B-cells, activation of the B-cell receptor (BCR) by antigen activates the MAPK, PI3K/AKT/mTOR, ZAP-70, and other pathways to promote lymphocyte function (Efremov, 2007).

The IL-2 receptor (IL-2R) is composed of three chains denoted α, β, and γ. IL-2Rβ chain contains a serine rich region (S-aa. 267-322) that also contains a box 1 and 2 motif, an acidic region (A-aa.313-382), and H region (aa.392-510) (Nelson, 1998; Hatakeyama et al., 1989b). The IL-2R chains are non-covalently associated and spatially separate in the membrane, but reversibly form the IL-2R once IL-2 binds its α and β chains. Subsequently, the activated IL-2R recruits JAK1 and JAK3 to the box1/box2 region on β chain and γ chain, respectively (Lin, 2000). While JAK1 and JAK3 are both required for IL-2 signaling to occur, studies by Kirken et al. (1995) have revealed a predominant involvement of JAK3 in IL-2R signaling. Upon recruitment to the IL-2R, JAK1 and JAK3 trans-activate each other and promote tyrosine phosphorylation of the IL-2Rβ chain at specific sites including the H region (Y392 and Y510), A

region (Y338/355/358/361), S region, and other tyrosine sites that create docking sites for cytoplasmic-signaling proteins that have SH2 or PTB domains such as STAT5a, STAT5b, SYK, LCK, SHC, PI3K, SHP-2, and SOCS1 (Suppressor of Cytokine Signal-1) (Nelson, 1998; Zhou et al., 2000; Hatakeyama et al., 1991; Leonard, 1996). These signaling molecules link the IL-2/IL-2R to downstream signaling events (e.g. JAK/STAT, MAPK) **(Figure 1.2)**.

Activated JAK3 tyrosine phosphorylates IL-2Rβ at Y392/510 to create docking sites for STAT5a/b, which binds to the phosphorylated residues via their conserved SH2 domains. JAK3 then tyrosine phosphorylates the C terminal STAT5 tyrosine residues (Y694/Y699 on STAT5a and STAT5b, respectively) allowing STAT5 dimers to form via their SH2 domains. The STAT5 dimers then translocate to the nucleus to initiate transcription of genes that promote survival and proliferation in T-cells (Hoey & Grusby, 1999; Friedmann et al., 1996; Lin et al.,1996).

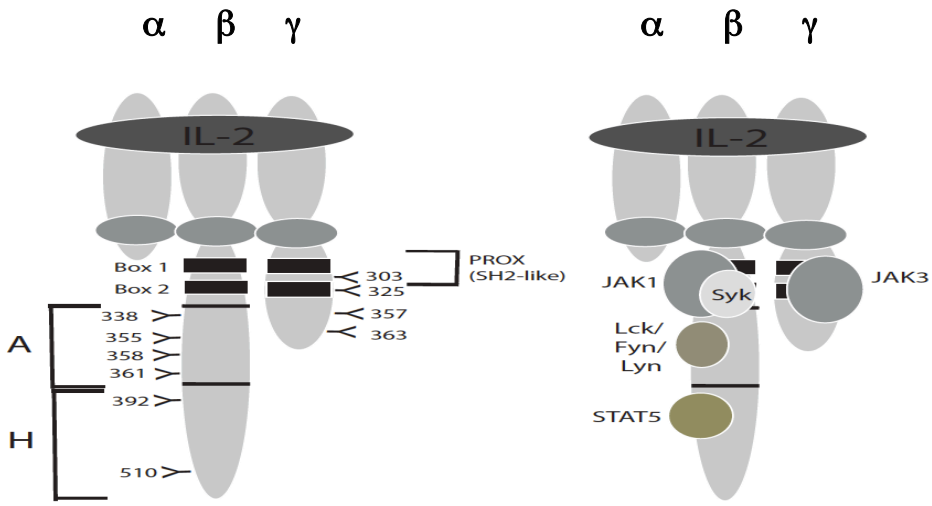


Figure 1.2. IL-2 Activation of IL-2R. Binding of IL-2 to the IL-2R promotes recruitment of JAK1 and JAK3 to beta and gamma chains, respectively. Autoactivation of JAK3 promotes tyrosine phosphorylation of the beta and gamma chains creating docking sites for cytosolic tyrosine kinases such as SYK and PI3K that bind to the IL-2RBeta chain S region (aa. 267-322), SHC and LCK bind to the A region (aa. 313-382), and STAT5 binds to the H region (a.a. 392-510). Tyrosine phosphorylation of these docked cytosolic proteins leads to the activation of multiple downstream signaling events.

1.2 JAKs, STATs, AND THEIR FUNCTION

JAKs are intracellular, cytoplasmic localized, non-receptor associated tyrosine kinase proteins. There are four members of the JAK family: JAK1, JAK2, JAK3, and TYK2. JAK3 is the only JAK expressed in lymphoid tissue while JAK1, JAK2, and TYK2 are ubiquitously expressed (Ross, 2007). JAKs have seven homological (JH) domains **(Figure 1.3).** JAK kinase activity is contained in the JH1 domain, while the JH2 contains a pseudokinase domain that may act as a negative regulator of kinase activity. The JH3-4 domains possess a (SRC homology 2) SH2-like domain, while JH5-7 contain a FERM domain that has been shown to promote JAK binding to its receptor (Wilks, 2008).

There are seven STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STAT proteins contain six domains, including an N-terminal and a coiled coil domain that are important for protein-protein interaction, a DNA binding domain, linker domain, SH2 domain for docking to the receptor or other STAT members, and a trans-activation domain that promotes transcriptional activity **(Figure 1.4)** (Ross, 2007).

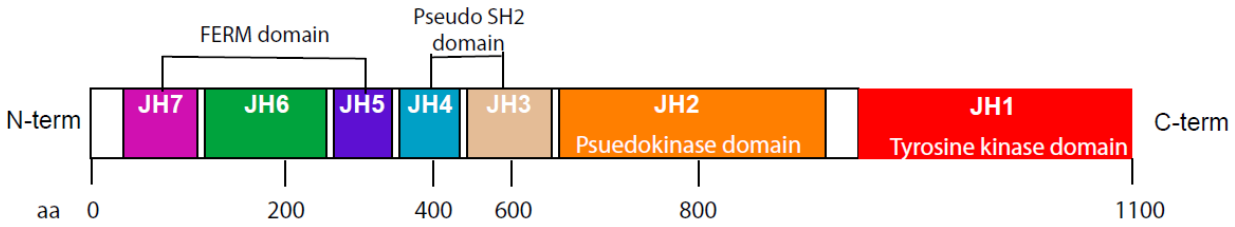


Figure 1.3. Schematic Model of JAK Structure. JAKs share seven homology domains (JH1-JH7). JH1 contains the tyrosine activity and a conserved Tyr-Tyr (Y-Y) motif that is within the autoactivation loop. JH2 is the pseudokinase domain that regulates the kinase domain. JH3-JH7 is critical for association of JAK with its receptor and protein substrates.

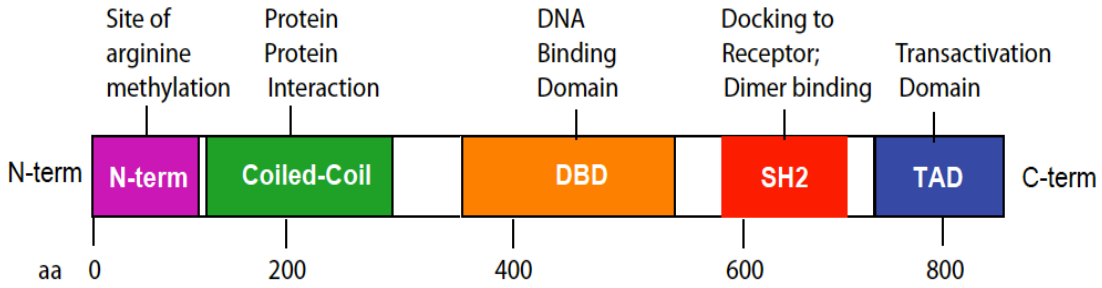


Figure 1.4. Schematic Model of STAT Structure. STATs have five conserved domains. The N-terminal promotes STAT tetramerization. The coiled-coiled domain is important for protein-protein interaction along with the N-term. The SH2 domain allows binding to a phosphorylated Y and the TAD domain promotes transcription.

Murine genetic deletions of JAKs and STATs has taught us much about their function. JAK1 deficient mice have an impairment of cytokine signaling, immune function, early stages of thymocyte maturation, and subsequently die perinatally (Rodig, 1998). A genetic defect in JAK2 is embryonic lethal (Paraganas, 1998). JAK2 regulates erythropoiesis through multiple hematopoietic factors such as erythropoietin, which is critical for red blood cell formation. TYK2 is necessary for IFN α/β and IL-12 signaling in mice and thus is important for pathogen clearance. If TYK2 is genetically deleted, mice display increased pathogen susceptibility (Shimoda, 2000). JAK3 is critical for the development and survival of T- and B-cells. Indeed, JAK3 deficient mice display a Severe Combined Immunodeficiendy (SCID) phenotype due to failure of cytokine signal transduction from γc-containing receptors. Humans and mice display non-functional T-cells and reduced B-cells with such a condition (Thomis, 1997).

STATs are a family of transcription factors that require phosphorylation of specific tyrosine residues to become activated in order to promote gene transcription. STATs are divided into two groups with specialized functions: [1] STAT2, STAT4, and STAT6 are known to be involved in specialized B- and T-cell differentiation, while [2] STAT1, STAT3, STAT5a, and STAT5b are involved in driving cell cycle progression and protecting lymphocytes from apoptosis. Therefore, hyperactivation of STATs 1, 3, 5a, and 5b may promote enhanced proliferation of lymphocytes and other cell types. STAT1 or STAT2 deficient mice can not respond to IFN dependent immune responses and are therefore susceptible to viral infections (Meraz, 1999; Park and Schindler, 2000). Deletion of STAT3 is embryonically lethal (Takeda, 1997). STAT4 deficient mice have revealed its importance in IL-12 signaling due to an impaired Th1 differentiation (Kaplan, 1996). STAT5a knock out mice have shown that STAT5a is a required mediator of mammapoietic signaling, such as prolactin (Liu et al.,1997). Deletion of

STAT5b leads to a loss of responses that are coupled with the sexually dimorphic pattern of pituitary growth hormone secretion (Udy et al., 1997). Importantly, genetic deletions of both STAT5a and STAT5b have established that T-cells are protected from apoptosis through STAT5 mediated transcription of anti-apoptotic genes such as Bcl2 and c-Myc (Lord, 1998). Finally, STAT6 has been shown to be important in Th2 cell differentiation induced by cytokines such as IL-4 and IL-3 (Shimoda, 1996).

1.3 HEMATOLOGIC MALIGNANCIES

Hematologic malignancies are cancers that affect or are derived from the bone marrow, blood cells, or lymphatic system (Cancer Facts & Figures, 2010). Prominent hematologic malignancies include leukemia, lymphoma, and myeloma. Leukemia derives from cells in the bone marrow that become transformed and then enter the blood stream. Leukemia can be subdivided based upon the major blood cell lineages they are derived from to include myeloid or lymphoid and whether they are chronic or acute in nature (**Table 1.1)**. Acute lymphoblastic leukemia (ALL) is the most common leukemia in children (National Institute of Health, 2010). The World Health Organization (WHO), as of 2008, has classified over 30 types of lymphoid neoplasms including precursor B-ALL, precursor T-ALL, acute biphenotypic leukemia, and Burkitt's leukemia (Campo et al., 2011; Swerdlow et al., 2008).

Table 1.1. Leukemia Types and Characteristics. Leukemia is a malignancy that develops when blood cells produced in the bone marrow become deregulated. Leukemia can be classified into four major groups: ALL, CLL, AML, and CML. Leukemia is first classified based on the cell origin (1st column). The second classification is based on the phase (2nd & 3rd column) of the leukemia, acute or chronic. Acute phase leukemia is described by the rapid increase of immature blood cells, while chronic is characterized by the rapid increase of abnormal blood cells.

Cell Type	Acute	Chronic
Lymphocytic Leukemia ("lymphoblastic")	**Acute lymphoblastic leukemia (ALL)**	**Chronic lymphocytic leukemia (CLL)**
Myelogenous Leukemia ("Myeloid" or "nonlymphocytic")	**Acute myelogenous Leukemia (AML)**	**Chronic myelogenous leukemia (CML)**

Lymphoma is a term used for hematologic malignancies that are concentrated in the lymphatic system. In this case, a lymphocyte undergoes a malignant change and at a certain point "pushes" healthy cells out of the lymphatic system. These malignant cells accumulate in the lymph nodes, spleen, liver, or bone marrow. There are two general types of lymphoma: Hodgkin's and Non-Hodgkin's lymphoma. Non-Hodgkin's lymphoma can be subdivided into either B- or T-cell Non-Hodgkin's lymphoma (National Cancer Institute, 2010). Hodgkin's lymphoma is subdivided into classical or nodular lymphocyte-predominant Hodgkin's lymphoma (NLPHL). Classical Hodgkin's lymphoma is further subdivided into lymphocyte depleted, lymphocyte-rich, nodular sclerosis, or mixed cellularity Hodgkin's lymphoma (Küppers, 2009). As for leukemia, the WHO has classified its subtypes based on cell types and pathological profile.

1.4 STAT5 AND JAK3 IN HEMATOLOGIC MALIGNANCIES

1.4.1 STAT5 and Hematologic Malignancies

STAT5 has been shown to be hyperactive in several hematologic malignancies including AML, ALL, CML, and (Human Leukemia Virus Type 1) HTLV-1 induced adult T-cell leukemia. (Wittig & Groner, 2005). In addition, other groups have found STAT5 to be hyperactive in erythroleukemia, megakaryotic leukemia, anaplastic large T-cell lymphoma, and Sezary syndrome. In addition, STAT5 has been shown hyperactive in solid tumors such as breast, head, and neck cancer as shown in **Table 1.2**. One possible mechanism for aberrant STAT5 activation is deregulation of upstream activators such as JAKs.

Table 1.2. Hyperactivation of STAT5 in Cancer. Constitutive activation of STAT5 has been found in HTLV-1 transformed cells, leukemias, lymphomas and several types of solid tumors, such as breast and neck cancer.

Tumor Type	Activated STAT	Reference
Blood Tumors		
Leukemias:		
HTLV-dependent	STAT5,STAT3	Migone et al., 1995; Takemoto et al., 1997
Erythroleukemia	STAT5,STAT1	Carlesso et al., 1996
Acute Myelogenous Leukemia (AML)	STAT5,STAT3,STAT1	Chai et al., 1997; Gouilleux-Gruart et al., 1996; Weber-Nordt et al., 1996; Ferbeyre et al., 2008
Chronic Myelogenous Leukemia(CML)	STAT5	Chai et al., 1997; Carlesso et al., 1996; Kotecha et al., 2008
Acute lymphocytic Leukemia (ALL)	STAT5, STAT1	Gouilleux-Gruart et al., 1996; Weber-Nordt et al., 1996
Chronic Lymphocytic Leukemia(CLL)	STAT5,STAT3,STAT1	Klampfer, L., 2006; Ferbeyre et al., 2010
Megakaryotic leukemia	STAT5	Liu et al., 1999
Lymphomas:		
Anaplastic large T cell lymphoma	STAT5,STAT3	Zhang et al., 1996c
Sezary syndrome	STAT5,STAT3	Zhang et al., 1996c
Solid Tumors		
Breast Cancer	STAT5,STAT3,STAT1	Hua, Y., & Jove, R., 2004; Ferbeyre et al., 2010
Head and Neck Cancer	STAT5,STAT3,STAT1	Hua, Y., & Jove, R., 2004

1.4.2 JAK3 and Hematologic Malignancies

Auto- or trans-phosphorylation of key tyrosine residues within JAK3 is a critical mechanism governing its activation. Phosphorylation of tyrosine residues Y980 and Y981 in the activation loop of its kinase domain positively and negatively regulate its activity, respectively (Leonard and O'Shea, 1998). Y904 and Y939 also positively regulate JAK3 activity and are required for optimal phosphorylation of a substrate, while phosphorylation of Y939 promotes STAT5 activation and binding (Cheng et al., 2008). The pseudokinase domain also interacts with STAT5 and negatively regulates JH1 kinase activity (Cornejo, 2009). Disruption of JAK3 by mutations in the kinase, pseudokinase, or SH2 domain that prevents its phosphorylation, renders lymphocytes unable to proliferate in response to antigens and thus promote a SCID phenotype (Pesu, 2005). On the other hand, hyperactivation of JAK3 has been found in HTLV-1 induced adult T-cell lymphoma/leukemia (ATLL), cutaneous T-cell lymphoma, mantle cell lymphoma (MCL), anaplastic large cell lymphoma (ALCL), ALL, acute megakaryoblastic leukemia (AMKL), and Burkitt's lymphoma (Cornejo, 2009; Mullighan, 2009; Nagy, 2010; Walters, 2006).

1.5 OTHER SIGNALING PATHWAYS HYPERACTIVATED IN HEMATOLOGIC MALIGNANCIES

1.5.1 SRC Family Kinase-STAT pathway

The SRC-STAT pathway has been shown to be an important factor in certain malignancies. The direct phosphorylation of STAT5 by c-SRC has been observed *in vitro* (Hayakawa, 2006). More recently in a study performed by Ozawa et al. (2008), AML cell lines K562 and KG-1a cell lines demonstrated constitutively active STAT5 and SRC family kinases (SFK). Moreover, inhibition of SFKs was found to also block STAT5 activation and

proliferation in these cell lines. This evidence suggests the SFK/STAT5 pathway represents a therapeutic target for treating leukemias containing an aberrant SFK. Several genetic aberrations have been elucidated in the mechanism of action of SFK creating constitutive activation of STAT proteins. STAT1, STAT3, and STAT5 activation by SFK has been demonstrated in a v-SRC-transformed myeblastic cell line (Hayakawa, 2006). Thus, STAT5 appears to be a central point where JAKs and SRC tyrosine kinase can drive malignant cell proliferation.

1.5.2 PI3K/AKT/mTOR Pathway

Phosphatidylinositol-3OH-kinase (PI3K) is a lipid kinase that phosphorylates phoshphatidylinositol 4,5-biphosphate (PIP2) to generate phoshphatidylinositol 3,4,5-triphosphate. PI3K is a serine/threonine kinase that regulates cellular proliferation and apoptosis by activating distinct downstream effectors, including the serine/threonine kinase AKT. AKT phosphorylation generates pro-survival signals through activation of mTOR (mammalian target of rapamycin). The PI3K/AKT/mTOR pathway has been shown to be constitutively phosphorylated and activated in T-ALL cell lines. Thus, novel therapeutics inhibiting the PI3K/AKT/mTOR pathway have also become attractive targets for treating T-cell malignancies (Cardoso et al., 2008).

1.5.3 Mitogen-Activated Protein Kinase Pathway (MAPK)

MAPKs are a family of proline-directed Ser-Thr kinases. MAPK is another cascade important for cellular proliferation, differentiation, and survival. Mammalian cells have three MAPK families: [1] extracellular signal-regulated kinase (ERK) within the Ras-Raf-MEK-ERK pathway which preferentially regulates cell growth and differentiation; [2] c-Jun N-terminal kinase (JNK) and [3] p38-MAPK cascades which function in the cellular stress response. Both ERK1/2 and ERK 5 have been reported to contribute to the survival of leukemic T-cells and thus represent a therapeutic target to treat cancer (Zhao, 2010).

1.5.4 Cross-Talk

It is important to note that cellular signaling pathways are highly integrated as shown in **Figure 1.5**. For example, mTOR may also activate STAT5 along with its normal signal transduction pathway activation (Mitra et al, 2012). Cross talk has also been shown to exist between the JAK/STAT and MAPK pathway by which ERK has been shown to activates STAT5a (Pircher et al., 1999). Since JAK3 is one of the first signaling molecules activated upon receptor engagement, many downstream signals are possibly driven by a hyperactivated JAK3. When a kinase is hyperactived, it has the ability to activate effector proteins not normally under its control. Thus significant signaling cross talk can occur in hematological malignancies driven by an oncogenic tyrosine kinase.

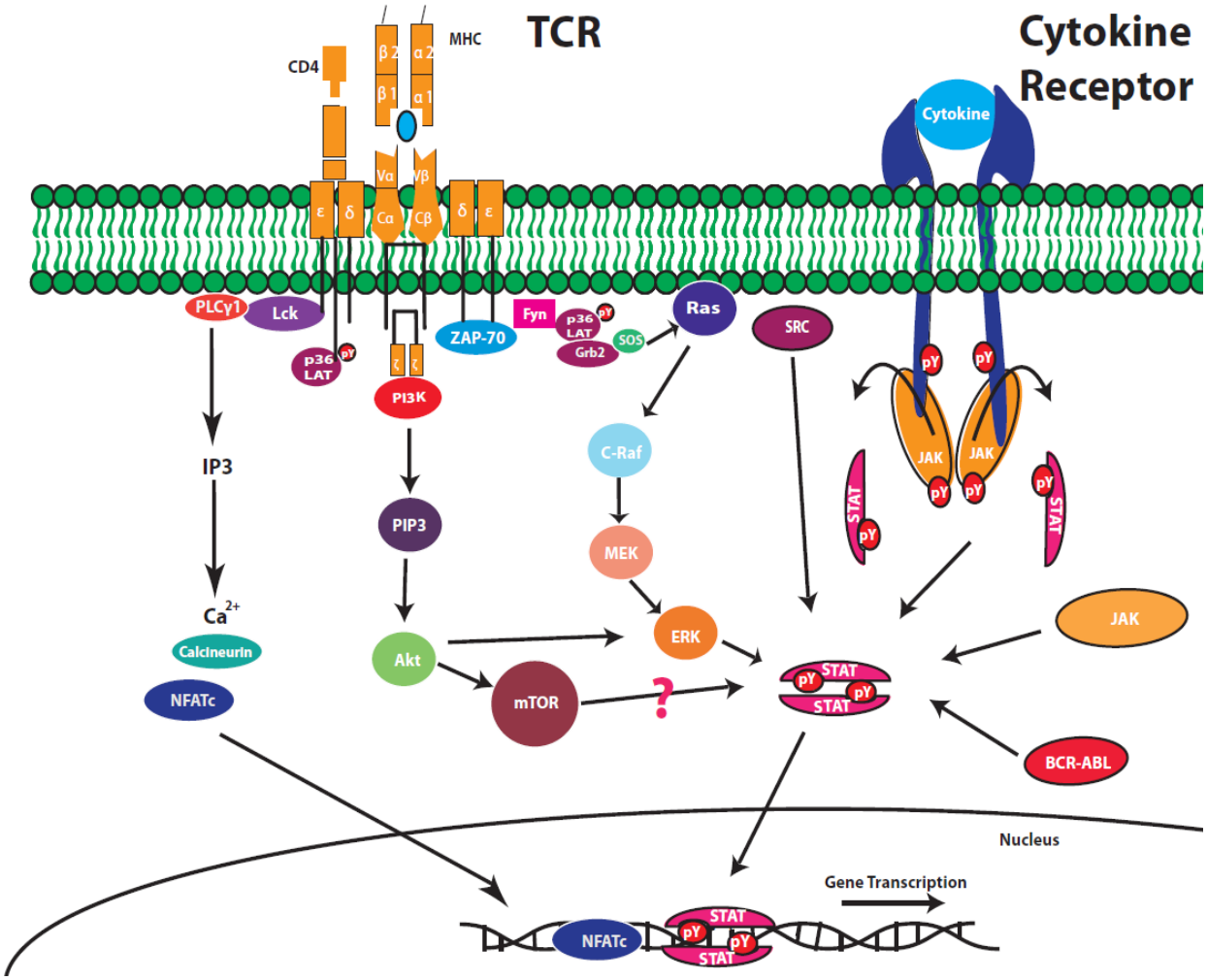


Figure 1.5. Tyrosine Kinase Signaling Cascades and Cross-Talk. The signaling cascades detailed demonstrate signal transduction pathways initiated by TCR and cytokine receptor engagement. These pathways contain multiple proteins that are highly integrated and tightly controlled. Aberrant regulation of these signaling pathways through expression of oncogenic tyrosine kinases (JAK or BCR-ABL) has been shown in multiple hematological malignancies.

1.5 SIGNIFICANCE AND HYPOTHESIS

Activated T-cells play a major role in adaptive immunity. Homeostasis of the adaptive immune response is maintained by complex regulatory mechanisms governing cell proliferation and survival through a variety of signaling molecules, including members of the JAK/STAT pathway (Aringer, 2002). The JAK3 tyrosine kinase is critical for normal T-cell signaling, however aberrant JAK3 activity can cause a number of immune mediated diseases such as SCID, leukemia, lymphoma, and graft versus host disease (Zitvogel et al., 2010; Thomis, 1997; Ross et al., 2007; Kirken & Stepkowski, 2002; Cardoso et al., 2008). FDA approved tyrosine kinase inhibitors have been developed to block signal transduction pathways critical for T-cell activation and proliferation, but none to date target JAK3. The fact that JAK3 is exclusively expressed in active immune cells, but not other tissues, makes it a strong drugable target to treat these malignancies. It is therefore important to **determine the activation status of the JAK3 signaling pathway in hematologic malignancies, which should yield valuable insight into new molecular targets for cancer treatment.**

The Leukemia and Lymphoma Society estimates that one person in the United States (US) is diagnosed with a hematologic cancer approximately every four minutes. Every ten minutes someone dies of this cancer (Leukemia and Lymphoma Society, 2012). In 2011, it is estimated that 140,310 people in the US were diagnosed with leukemia, lymphoma, or myeloma which equates to 9% of all cancers diagnosed in the US. Estimates also suggest 53,010 people died in 2011 from leukemia, lymphoma, or multiple myeloma. For children 0-19 years of age, leukemia, Non-Hodgkin lymphoma, and Hodgkin lymphoma are the most common types of cancer. Indeed, leukemia is known to cause one-third of all cancer deaths in children younger than 15 years of age. Leukemia also shows dramatic health disparities with Hispanic children

under 20 years of age having the highest rates of incidence (Leukemia and Lymphoma Society, 2012). Furthermore, Hispanic women have the second highest rate of lymphoma (National Cancer Institute, 2012).

Within Texas, El Paso county had the highest cancer deaths of children ages 15 years and younger between 2003-2007 (Texas Cancer Registry, 2012). In 2010, the Hispanic population in El Paso increased by 4%, with Hispanics making up 82% of the El Paso county population. (US Census Bureau, 2012). Understanding leukemia and lymphoma in Hispanics is of great importance not only in El Paso, but also in the country as we are experiencing a significant growth in this underrepresented minority population (National Cancer Institute, 2012). **It is therefore essential to identify novel molecular pathways for therapeutic intervention for these hematologic malignancies for Hispanics and others.**

During the last part of the 20th century, a dramatic improvement in survival rates of patients with hematologic malignancies was due largely to chemotherapy and radiation. In addition, newer agents such as tyrosine kinase inhibitors (Gleevec®) have been shown to be effective against certain types of leukemias, while having less side effects because they do not directly interfere with normal cellular processes like traditional chemotherapy. (National Cancer Institute, 2012). With 51 hyperactivated kinases being identified in various cancers, and the clinical success of Gleevec in BCR-ABL positive leukemias, a paradigm-shift in the treatment of cancer has occurred and has fueled interest in tyrosine kinase inhibitors as a new class of promising drug candidates for such tumors (Hunter, 2009**)**. With the discovery of JAK2V617F in myeloproliferative neoplasms, and the clinical success of Jakafi® (Ruxolitinib) in myelofibrosis, focus has now been put on development of inhibitors towards other JAK family member, such as JAK3 (Verstovsek et al., 2012). Therefore, **we hypothesize that the JAK3/STAT5 signaling**

pathway is involved in select hematological cancers and its uncoupling is a viable therapeutic strategy for the treatment of these malignancies.

Chapter II: Determine the Activation Status of JAK3/STAT5 in Primary Hematological Malignancies

2.1 INTRODUCTION

Tyrosine kinases are important effector molecules required for normal cell physiology. These enzymes contain a catalytic subunit that transfers the gamma phosphate from adenosine triphosphate (ATP) to the hydroxyl group of a tyrosine residue. Phosphorylation of a tyrosine residue can regulate protein function and therefore cell signaling by causing conformational changes in the protein. For example, protein kinase C phosphorylates myristoylated analine-rich protein kinase C substrate (MARCKS) causing the protein to convert from an extended structure into a more compact structure (Bubb et al., 1999). This post-translational modification also allows for the recruitment of proteins with structurally conserved domains that bind phosphomotifs (Hanks et al., 1988). For example, LCK and FYN mediate ITAM phosphorylation upon which ZAP-70 can then bind to the TCR via its SH2 domains (Samelson, 2002). When deregulated, tyrosine kinases can be associated with multiple diseases, including hematological malignancies (Sebolt-Leopold & English, 2006; Uckun & Chen, 2004). Tyrosine kinases can become constitutively activated and lead to a neoplastic disease by three mechanisms: 1) chromosomal translocation, 2) overexpression, and 3) activating mutations. When a hyperactivated tyrosine kinase leads to a neoplastic disease, it is then known as an oncogenic tyrosine kinase (OTK) (Skorski, 2002). OTKs drive cell proliferation in the absence of growth factors, and can enable cells to become resistant to anti-neoplastic agents. Clinical success with the tyrosine kinase inhibitor Gleevec, for the treatment of BCR-ABL positive chronic myelogenous leukemia (CML), has produced significant interest in tyrosine kinase

inhibitors for the treatment of neoplastic diseases. However, little focus has been put on JAK3 and its role in hematological malignancies.

Leukemia can arise following mutations of JAK3. In acute megakaryoblastic leukemia (AMKL), a A572V mutation in the kinase domain of JAK3 results in constitutive activation of JAK3 (Walters, 2006). Mice transplanted with bone marrow cells that had been retrovirally transduced with the mutation showed an AMKL phenotype and displayed a marked decrease in survival (Cornejo, 2009). In addition, mutation on the neighboring amino acid, A573V, (De Vita, 2007) as well as V722I in the JH2 domain, and P132T in the JH6 domain have been reported (Walters, 2006). The mutations A572V, V722I, and P132T all transform the IL-3 dependent cell line BaF3 to a cytokine independent state (Constantinescu, 2007). Another study reported the JAK3 mutation M511I in patients with AML, and showed that it possessed transforming ability, which was confirmed both *in vitro* and *in vivo* (Yamashita, 2010). **Figure 2.1** reviews all JAK3 somatic mutations in leukemia known to date. Although multiple mutations have been found within the JAK3 gene, these studies focused on AMLs and little is known about JAK3 mutations within other hematological malignancies. We therefore sought to develop and implement a method to sequence JAK3 positive patient tumor cells to identify new JAK3 mutations in various hematological malignancies.

Studies of malignant T-cells have shown that they rarely arise from a single gene alteration, instead multiple signaling defects are likely present. Indeed, deregulation of PI3K, MAPK, SFK-STAT, and JAK/STAT signaling pathways is commonly found in malignancies (Ross, 2007; Kirken & Stepkowski, 2002). However, no previous study has determined the activation status of multiple signaling pathways in a large cohort of patients. Therefore, the focus

of the present chapter was to determine the oncogenic drivers of select hematologic malignancies, which we hypothesized were primarily the JAK3/STAT5 signaling cascade. This was accomplished by multiplex signaling analysis of a set of primary patient samples using a broad spectrum of signaling molecules to create a unique data bank that will expand the knowledge of aberrant molecular pathways in certain hematologic malignancies.

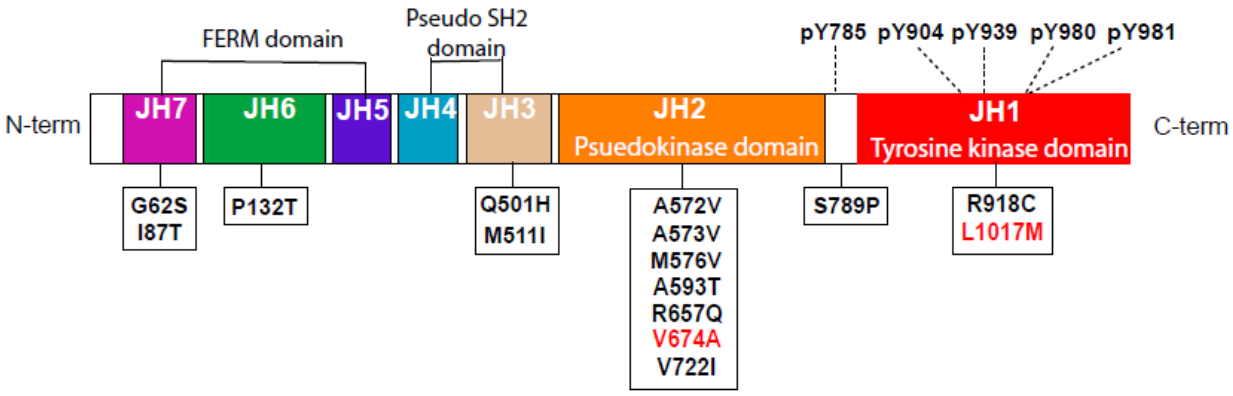


Figure 2.1. Somatic JAK3 Mutations in Leukemia. To date, 15 somatic JAK3 mutations are known to harbor transforming potential. Two of these somatic mutations have not been confirmed in patients, but have been confirmed in cancer cell lines (Red).

2.2 MATERIALS AND METHODS

Sample preparation and PBMC purification:

Primary patient leukemia and lymphoma cells were obtained from de-identified excess diagnostic material (peripheral blood, lymph node or bone marrow biopsies). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using Ficoll-Paque Plus according to the manufacturer's protocol and from bone marrow aspiration using differential centrifugation. After purification, PBMCs were preserved as cell pellets at -80 °C. PBMC pellet freeze down: PBMCs were resuspended at a concentration of $1x10^7$ cells/ml in 1 ml microcentrifuge tubes, centrifuged at 100 x g for 10 min, and pellets stored at -80 °C. PBMC cryopreservation: PBMCs were resuspended at a concentration of $1x10^7$ cells/ml in freezing media (90% filtered FBS and 10% DMSO) and stored in liquid nitrogen.

Cell culture and treatment

Naïve PBMCs were collected from buffy coats obtained from normal donors and purified by isocentrifugation (Ficoll-Hypaque). Naïve PBMCs ($3x10^6$/ml) were activated for 72 hr using phytohemagglutinin (PHA) (10 μg/ml) and then used in multiple assays. Cancer patient PBMCs were seeded at a density of $2x10^5$ in 100 μl in triplicate fashion in 96-well plates in RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals), 2mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin (complete RPMI) with increasing concentrations of NC1153 (JAK3 inhibitor) for 72 hr and cell viability measured by 3-(4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazoilum salt (MTS). Cancer patient PBMCs were also seeded in 6-well plates at a $5x10^6$ in 3ml complete RPMI with increasing concentrations of NC1153 and incubated for 24 hr before lysis and cell signaling analysis.

Viability assay:

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazoilum salt (MTS). reagent (Promega) in triplicate, according to manufacturer's instructions. Error bars represent standard deviation.

Cell lysis, immunoprecipitation, and Western blot analysis:

Cells ($1x10^7$) were lysed using Triton lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 200 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin.) followed by centrifugation at 15,000 x g for 15 min at 4 ºC to pellet unsoluble material. Protein concentration was determined by the bicinchoninic acid method (Pierce). Samples were then 1) run as total cell lysate (10 μg per lane on SDS-PAGE gels) or 2) subjected to immunoprecipitation using 300 μg of protein. For immunoprecipitation reactions, supernatants were rotated end over end with 3 μl of JAK3 antibody for 2 hrs at 4 ^oC. The JAK3 antibody used for immunoprecipitation was raised against a peptide derived from the carboxyl terminus of human JAK3, as previously described by Malabarba et al. (1996). The JAK3 immune complexes were then captured by incubation with protein A-Sepharose beads (Rockland Immunochemicals) for 30 minutes at 4 ^oC. Samples were washed three times using cold lysis buffer and were eluted by boiling for 5 min in 2x SDS sample buffer (50mM Tris-HCL [pH 6.8], 100 mM dithiothreitol, 2%SDS, 0.02% bromophenol blue, 10% glycerol [pH 6.8]). For total cell lysate, 10 μg of protein lysate for each sample was boiled for 5 min in 2x SDS sample buffer. Samples were then resolved by 7.5% SDS-PAGE at 15 mA for 1 hr, transferred to polyvinyl-diflouride (PVDF) membrane at 150 mA for 1 hr

(Amerasham Biosciences), and blocked with 1% bovine serum albumin (BSA) for 1 hr at room temperature.

Western blot analysis was performed with the following primary antibodies diluted in 1% BSA: α-JAK3 C-terminal antibody (Epitomics Inc.) at 1:1000 dilution for 1 hr at 25 °C, α-phospho-tyrosine (PY) (Millipore) at 1:1000 dilution at 4 °C overnight, α- PYSTAT5 (Epitomics Inc.) at 1:1000 dilution at 4°C overnight, or α-GAPDH at a dilution of 1:10000 for 1 hr at 25 °C. Apoptotic cell death was assessed by Western blot detection of caspase mediated PARP cleavage, α-PARP (Millipore) 1:1000. To develop, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG (CalbioChem) at a 1:5000 dilution in 1% BSA and visualized using enhanced chemiluminescence substrate (1M Tris-HCL[pH 8.5], 250 mM Luminal, 90 mM Coumaric acid) and exposed to X-ray film. For reblotting, PVDF membranes were stripped in SDS buffer (2% SDS, 62.5 mM Tris [pH 6.7], Beta-mercaptoethanol is added to a concentration of 100 mM before use) for 30 min at 55 °C, blocked, and then re-probed.

Immunoflourescent confocal microscopy:

Patient PBMCs ($8x10^5$) were cytocentrifuged onto glass slides, fixed with cold methanol and permeabolized with 0.2% Triton X-100 for 5 min. All staining procedures were performed at 25 °C. The slides were blocked in 2% BSA using 1x PBS for 1 hr and incubated with α-JAK3 C-terminal (Epitomics Inc) 1:50 in PBS-T (0.05% Tween 20 in PBS) for 1 hr. Cells were then washed three times with PBS-T and incubated with secondary Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) for 1 hr at a 1:400 dilution and then incubated with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining at a 1:800 dilution for

15 minutes under dark conditions. After three washes in PBS-T and one wash with deionized water, slides were mounted in FlourSave mounting medium (Calbiochem) and imaged with a Zeiss Pascal confocal microscope (UTEP Analytical Cytology Core Facility).

Luminex assay and sensitivity:

MILLIPLEX MAP microbeads conjugated to the indicated protein specific antibodies were incubated with 20 μg of cell lysates, or control samples in 96-well 1.2 mm filter plates according to the manufacturer's instructions (MultiScreen-BV Plate, Millipore). The plates were then incubated overnight on an orbital shaker at 4 °C. The microbeads were washed in 25 μl of Assay 2 buffer (Millipore), followed by the addition of 25 μl phospho-specific biotinylated antibodies (Millipore) and incubated on an orbital shaker for 1 hr under dark conditions at 25 °C. This was then followed by 30 min incubation with 25 μl of streptavidin-phycoerythrein (SAPE) at 25 °C. Samples were then analyzed with the Luminex 200 instrument (LX-200) and xPONENT 3.1 software according to the manufacturer's instructions. The phosphorylation status of the following signaling molecules was assessed: 1) Transcription factors: STAT1, STAT2, STAT3, STAT5A/B, and STAT6 (Cat #48-610); 2) Kinases: LCK, LYN, SRC, YES, FYN, BLK, HCK, FGR, JAK3, MAPK, and mTOR (Cat # 48-650).

To determine the sensitivity of the luminex assay for detection of phospho-protein, PHA activated PBMCs total cell lysate was collected and total protein concentrations assessed. Decreasing protein concentrations were 1) run on a 7.5% SDS-PAGE and immunoblotted for α-pYSTAT5 (Epitomics Inc.) or 2) assayed by the Luminex α-pYSTAT5 bead set (Cat #46-641).

JAK3 Exon Amplification:

Genomic DNA was purified using the Puregene Core Kit A (Qiagen). Purified genomic DNA was brought to a final concentration 100 ng/μl. JAK3 coding exons were sequenced using 23 primer sequence sets (forward and reverse) (Table 2.1), based upon the National Center for Biotechnology Information (NCBI) trace archive (www.ncbi.nlm.nih.gov/Traces/trace.cgi), (Mullighan et al., 2009). JAK3 coding exons within the samples genomic DNA were PCR amplified using the High Fidelity Platinum® Taq DNA Polymerase (Invitrogen). The manufacturer's instructions were followed for the Platinum Taq polymerase PCR protocol using the following reaction buffer (60 mM Tris-SO_4 (pH 8.9), 180 mM $(NH_4)_2SO_4$, 1.2 mM $MgSO_4$, 20 mg/ml BSA). Each PCR reaction contained: 100 ng DNA, reaction buffer, Platinum® Taq DNA Polymerase High Fidelity polymerase, and 10 μM forward and reverse PCR primers in a 50 μl reaction. PCR cycling parameters were as follows: one cycle of 95 ^{o}C for 15 min, 35 cycles of 95 ^{o}C for 20 s, 60 ^{o}C for 30 s and 72 ^{o}C for 1 min, followed by one cycle of 72 ^{o}C for 3 min. The resultant PCR products were gel purified (Qiagen) and sequenced using the indicated primer (Table 2.1).

Sequencing interpretation:

Sequencing results (forward and reverse) were aligned to the JAK3 coding exons using Gene Tools. A nucleotide change in both the forward and reverse sequence constituting an amino acid change were confirmed using an additional round of sequencing.

Table 2.1. JAK3 Sequencing Primers: Human JAK3 contains 23 exons on chromosome 19, therefore 23 different primer sets were used to amplify and sequence the JAK3 gene.

Primer ID	Forward Primer 5'-3'	Reverse Primer 5'-3'
Exon1	TCCAGGCAGGTCTCAAACTCC	CAGCTGTTCCCTTCATGTGC
Exon2	TTTGAGGTATGGAAGGATCTGG	AACCCTGGGATGAAAGTGC
Exon3	TTTTATCATCTCCTTGCATTTCG	CACAGGGAGGGTCAGACG
Exon4	TCAGGTTAACAACAGGGCTTGA	GGGTCATAGGAACACCCTGA
Exon5a	TCCGGTCCTCATACCTGACC	CACATCCCCTACCACTCTC
Exon5b	TCCTGGGTTTGTGTGTGTCC	CAACCCTTCACTCAGTTTGC
Exon6 and7	TAAGGGATAGGGAGTGGATGG	TGAAAACTTGACCCCTGTCC
Exon8	TAAGGATCCCAGGGCTACAGA	CTCCCAAAGTGCTGGGATTA
Exon9	GGACTGAGAAGGAGAGTGTC	CCAGAGGAAGAGCTGAGAGC
Exon10	TGTTGCAGTGAGCTGAGATCG	TCTCATGCTGAATGGTGAGG
Exon11	TGAGGCGATACCTCAGTCTGG	ACGAGGTCTCGCTATGTTGC
Exon12	TTCCCGTATCAGAAAATCATGG	GCTGGATATGGGTGAGAACC
Exon13	TACAGGGCTCAACACCTTCC	TCGAACCCTTACCAAACTCC
Exon14 and15	TGGAGCATGTCTGAGCAGTACC	AATCCCCAACCCAATAGACC
Exon15 and16	TCCTGATCCCACTTTCATTCC	AACCTCACCAGACACACAGG
Exon17p	TTTAGGTTTCCATGGGTCAGG	ATAGAGCTGGGCACCATTCC
Exon17 and18	TGCACAGCAAGTCAACTCAGG	ATCACGTTCCCAGCCTACC
Exon19	TGCAAAACTGAGGTCGAGAGG	TCTGATCCTGAGCCCTAAGC
Exon20	TCAGAACTTCAGTGGAGGATGG	GGCGAGAGCTGAGAGAAGG
Exon21	TGAATCCACCTATCCCACAGC	GTGACCCCATGCTAAAGAGG
Exon22	TACCTTTCTGACCCCTTCACG	CATAGGCACAGGTGTTCAGG
Exon23a	TGATCATGCCATTGCACTCC	TTGGTTCCTTGCTTCTTTGG
Exon23b	TCACGACCCCATTATCTGTCC	CCACCCTGGGTAACAGAGC

2.3 RESULTS

Presence of JAK3 in patient samples

JAK3 gene expression is continuously present in lymphocytes. However, the degree of expression varies with activation and differentiation statuses. JAK3 RNA is able to be detected by RT-PCR in naïve PBMCs, however negligible JAK3 protein is detected by Western blot analysis. JAK3 protein is detectable by Western blot analysis in mature activated PBMCs (Sharfe etal., 1997). Therefore, to determine if patient samples contained aberrant JAK3 protein expression, patient PBMC lysate were separated by 7.5% SDS-PAGE along with naïve PBMCs (lane a) and PHA activated PBMCs (lane b) and subjected to Western blot analysis with anti-JAK3 C-terminal. Patients 64 (lane f), 1 (lane g), 2 (lane h), and 3 (lane i) in **Figure 2.2A** showed JAK3 protein expression along with patients 67 (lane b) and 69 (lane d) in **Figure 2.2B.** Patient 13 also showed JAK3 protein expression by Western blot analysis (data not shown). **Table 2.2** contains patient sample numbers along with each respective diagnosis.

Samples from the first screen that contained JAK3 expression were then subjected to JAK3 immunoprecipitation using 300 μg of protein from each patient sample, and then separated by 7.5% SDS-PAGE. Western blot analysis for α-pY was performed to detect levels of tyrosine phosphorylated JAK3 (activated JAK3). As shown in **Figure 2.3A,** all five patient samples contained tyrosine phosphorylated JAK3. Molluscum contagiosum (Pt1) and Castleman's (Pt4) were used as positive controls alongside Hodgkin's Lymphoma (Pt2), Non-Hodgkin's Lymphoma (Pt3), B-cell lymphoma (Pt5), T-ALL (Pt6), T-ALL Gleevec failure (Pt6G). The membrane was then stripped and reblotted with α-JAK3 C terminal antibody to confirm for equal loading. **Figure 2.3B** shows that all seven patients contain equal amounts of total JAK3.

Table 2.2. Patient Sample Number and Diagnosis. 70 patient samples were used in this study.

Patient sample number	Diagnosis	Patient sample number	Diagnosis
1	Molluscum contagiosum	35	Pre-B ALL
2	Hodgkin's Lymphoma	36	Essential thrombocytemia
3	Non Hodgkin's Lymphoma	37	AML/CML
4	Castleman's	38	Hairy Cell Leukemia
5	B-cell Lymphoma	39	Relapsed ALL
6	T-ALL	40	JMML
6A	T-ALL	41	Non-Hodgkin’s lymphoma
6G	T-ALL Gleevec Resistant	42	No Diagnosis
7	AML/CML	43	CML
8	B-ALL relapse	45	No Diagnosis
9	AML	46	Myeloproliferative disorder
10	AMoL	47	Adult AML
11	AMoL	48	APL
12	CML	49	New onset ALL
13	AML/CML	50	No Diagnosis
14	T-cell lymphoma	51	No Diagnosis
15	AMML	52	ALL
16	No Diagnosis	53	No Diagnosis
17	No Diagnosis	54	Essential thrombocytemia
18	CML	55	AML
19	Multiple Myeloma	56	AMML
20	No Diagnosis	57	JMML
21	PH-CML	58	No Diagnosis
22	B-ALL	59	Congenital leukemia
23	CML	60	AMML
24	CML	61	JMML
25	No Diagnosis	62	AMoL
26	Hodgkin's Lymphoma	63	Castleman's
27	AMoL	64	Castleman’s
28	B-ALL	65	JMML to AMML
29	No Diagnosis	66	Multiple Myeloma
30	APL	67	T-cell lymphoma
31	B-cell lymphoma	68	AML relapse
32	Acute Leukemia	69	No Diagnosis
33	AMoL relapse	70	Acute biphenotypic leukemia
34	B-ALL		

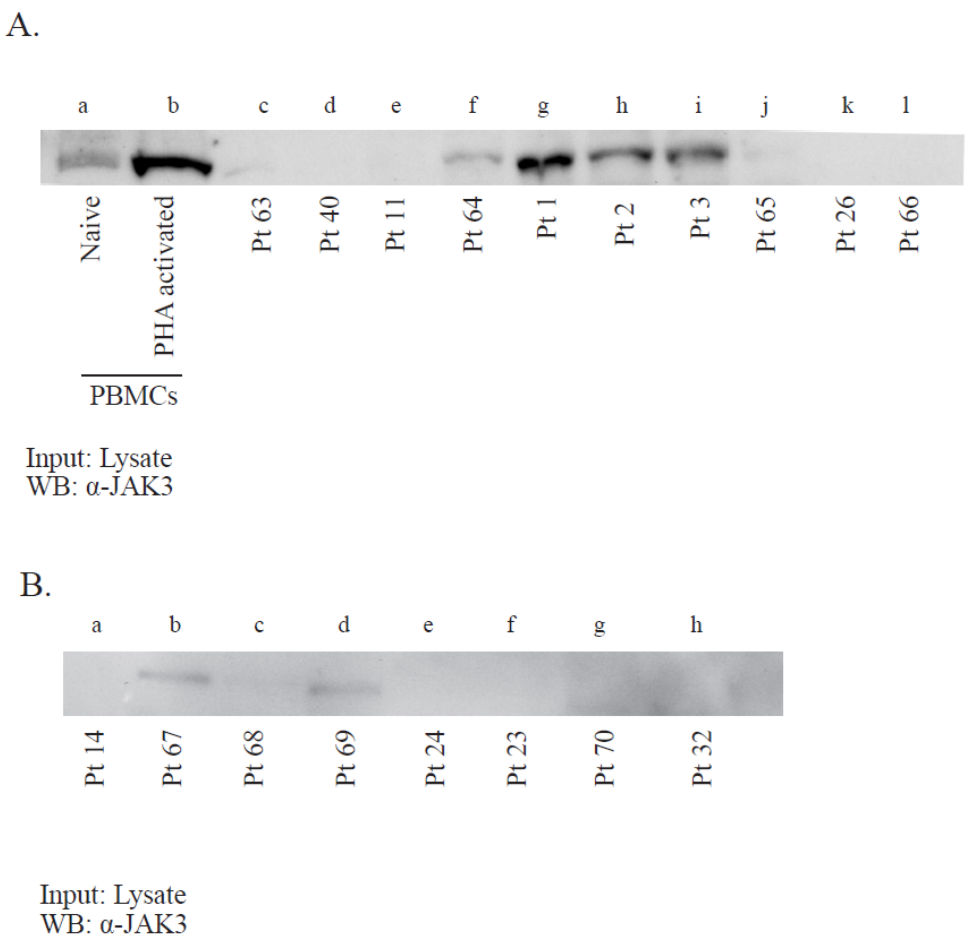


Figure 2.2. Presence of JAK3 in Patient Samples. Patient and control cell lysate (10 µg) were separated by 7.5% SDS-PAGE and Western blot analysis performed using anti-JAK3 C-terminal (1:1000). Patient sample diagnosis can be found in Table 2.2. Naïve PBMCs and PHA activated PBMCs were used as negative and positive controls, respectively.

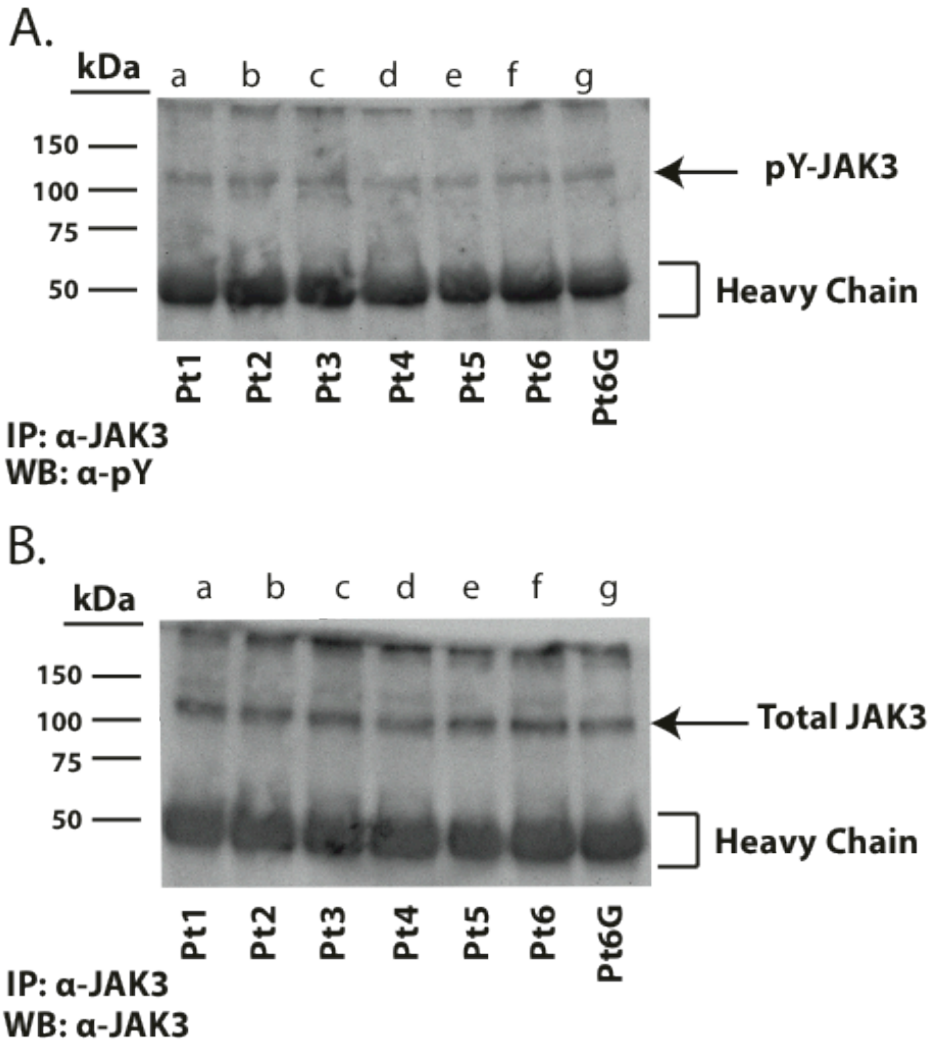
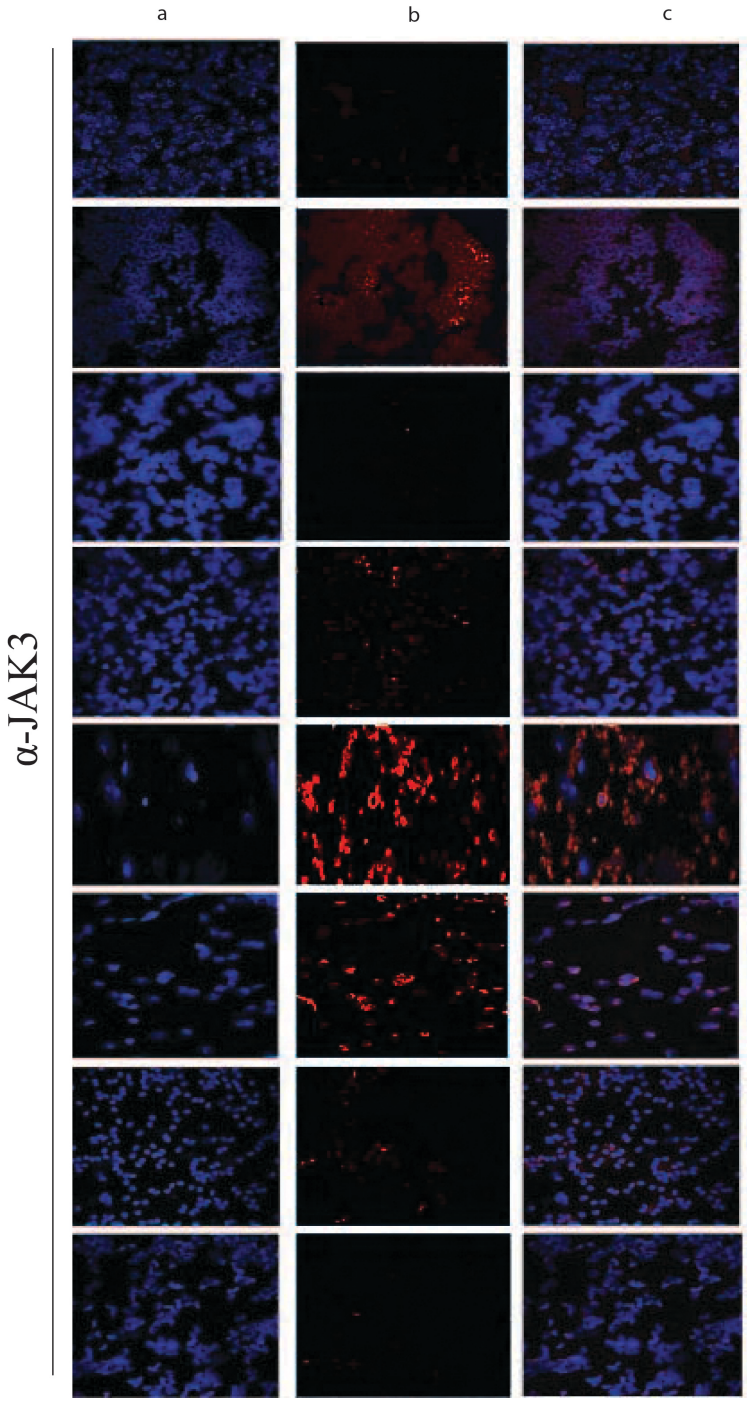


Figure 2.3. JAK3 Activation Status in Patients With Hematologic Malignancies. Five hematologic malignancies along with two positive controls were immunoprecipitated for JAK3, 300 μg of protein separated by 7.5% SDS-PAGE, and Western blotted with the antibodies indicated: A) anti-pY (1:1000) and B) anti-JAK3 (1:1000). Molluscum contagiosum (lane a) and Castleman's (lane d) were used as positive controls and were ran alongside the patient samples. Lanes b, c, and e contain patient samples diagnosed with Hodgkin's Lymphoma, Non-Hodgkin's Lymphoma, and B-cell lymphoma, respectively. Lanes f and g contain a patient diagnosed with T-ALL at different points of treatment.

Due to limited number of patient tumor cells, the presence of JAK3 protein expression was also determined using immunofluorescent confocal microscopy (**Figure 2.4A and Figure 2.4B).** Patient samples were compared to naïve PBMCs that express negligible amount of JAK3 protein. Indeed, patients 3, 6, 11, 27, 22, 26, 23, 20, and 40 contained JAK3 protein expression in the cytoplasm at a greater level than patient 7 which also showed expression but to a lesser extent (lane b). JAK3 was not detected in samples 37, 28, and 13 (lane b).

To investigate the use of a high-throughput screening methodology for JAK3 activation (pYJAK3) in patient samples, patient PBMCs and naïve PBMCs (negative control) were analyzed using the Luminex multiplex system (**Figure 2.5)**. The use of a phospho-peptide standard curve allowed us to measure pYJAK3 levels quantitatively (ng/ml). Patients 4, 10, 13, 14, 17, 21, 22, 23 demonstrated between 1-1.5 fold increase, while patients 6, 11, 27, 37 demonstrated a 1.5-2 fold increase compared to the negative control. Patients 3 and 7 demonstrated a 3-3.5 fold increase, patients 19, 26, and 30 between 4-4.5 fold increase, and patients 25, 38, 39, and 42 showed the greatest expression of pYJAK3, having between a 6-24 fold increase in the presence pYJAK3.

A.



Naive PBMCs

Patient Samples

Pt6 T-ALL

Pt28 B-ALL

Pt22 B-ALL

Pt26 Hodgkin's Lymphoma

Pt23 CML

Pt20 No Diagnosis

Pt7 AML/CML

B.

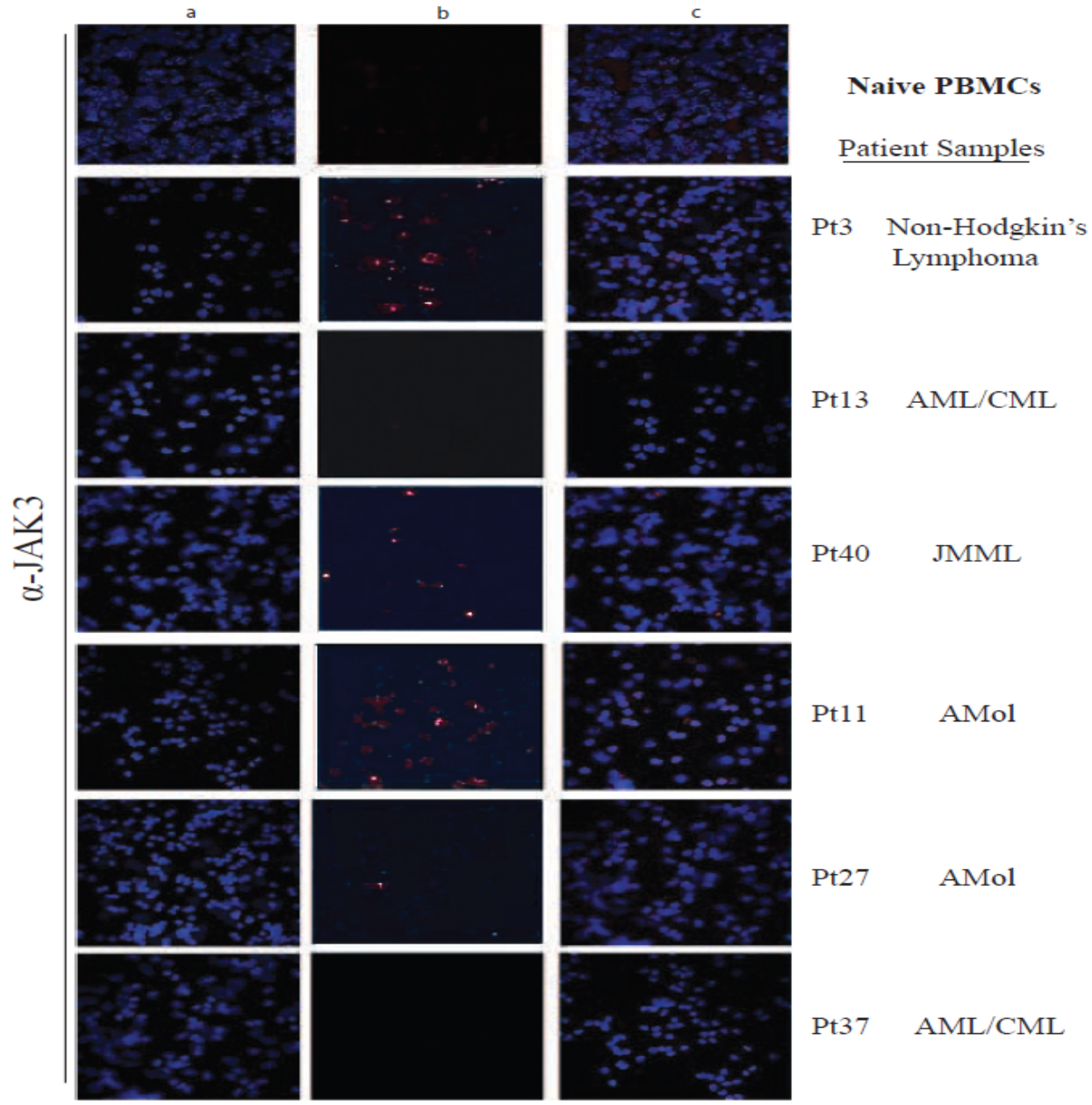


Figure 2.4. JAK3 Expression in Patient Samples. A and B) Immunoflourescent confocal microscopy was utilized to examine the expression of JAK3 expression in primary tumor cells isolated from patients with hematological malignancies. To the right side of each panel is the Pt number, along with its respective diagnosis. Naïve PBMCs served as negative controls. Immunofluorescent images were captured using PASCAL software on a Zeiss LSM 510 Meta confocal microscope at 20X magnification. Lanes a, b, and c represent DAPI (1:800), anti-JAK3 (1:50), and overlay stains, respectively.

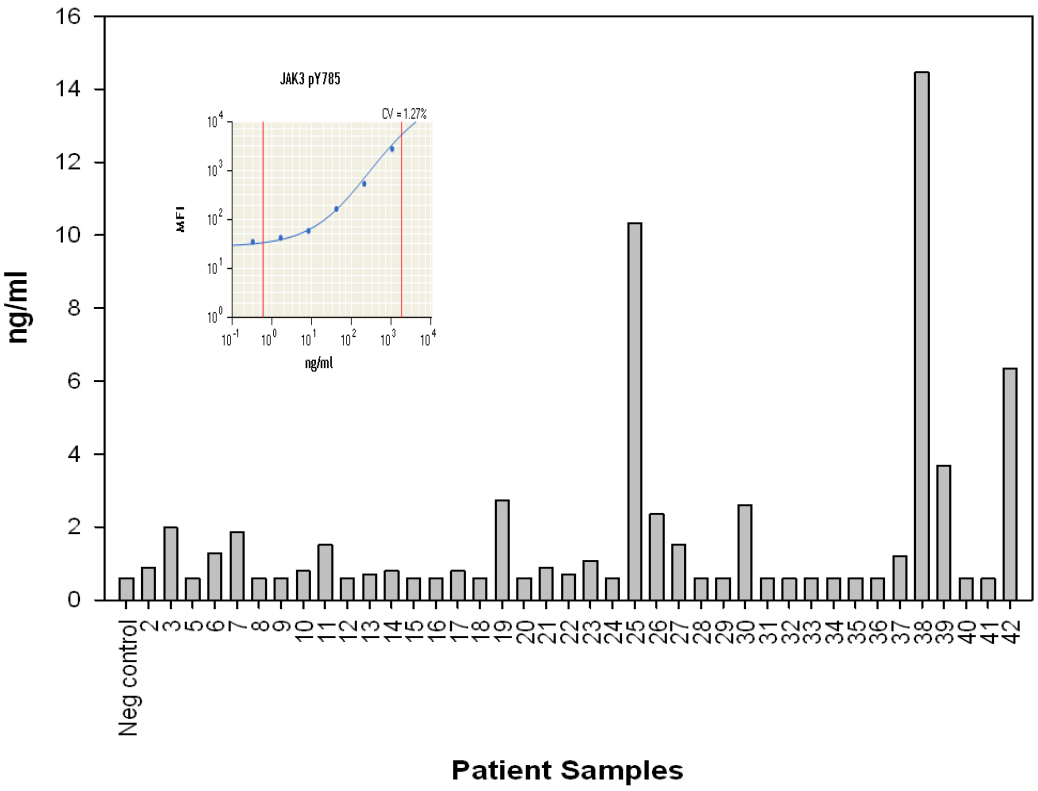


Figure 2.5. Activated JAK3 Levels in Patient Samples. Tyrosine phosphorylated JAK3 levels were determined by multiplex analysis using 20 µg of patient and naïve PBMCs (negative control). Inserted into the graph is the phospho-peptide standard curve, which allows pYJAK3 levels to be measured quantitatively (ng/ml). (n=1)

T-ALL Patient Contained JAK3/pYSTAT5 and Blockade of JAK3 Decreases Cellular Proliferation

One T-ALL sample was received multiple times. The intial sample (Pt6) was received after chemotherapy relapse and a second sample was received after the patient became resistant to Gleevec (Pt6G) . To determine whether JAK3 expression and pYSTAT5 changed in this patient during the treatment course, patient PBMC lysate was separated by 7.5% SDS-PAGE along with naïve PBMCs as a negative control (lane a) and subjected to Western blot analysis with anti-JAK3 (C-terminal), pYSTAT5, and GAPDH (loading control) (**Figure 2.6)**. The chemotherapy relapse sample tumor cells (Pt6) displayed aberrant JAK3 expression and STAT5 activation (lane b). After Gleevec resistance (Pt6G), JAK3 and pYSTAT5 levels increased (lane c). Therefore, it is possible that increased hyperactivation of the JAK3/STAT5 pathway plays a role in the Gleevec resistance of the tumor cells.

Previous studies have shown that NC1153-mediated JAK3 blockade induces apoptosis and uncouples the activation of the JAK3/STAT5 pathway in certain leukemia/lymphoma cell lines (Nagy et al., 2010). To determine if NC1153 can uncouple the activation of the JAK3/STAT5 pathway, PBMCs from Pt6 (T-ALL) and naïve PBMCs were treated with increasing concentrations of NC1153 (JAK3 inhibitor) for 72 hrs and then cell viability measured by MTS. Naïve PBMCs were not affected following treatments of increasing doses of NC1153 but the T-ALL patient that displayed elevated pYJAK3 levels did experience a decrease in cell viability **(Figure 2.7A).** T-ALL PBMCs were then treated for 24 hrs with increasing concentrations of NC1153 and total cell lysate then separated by 7.5% SDS-PAGE and Western blotted for pYSTAT5, JAK3, and total STAT5 to ensure equal loading. NC1153 (IC50=5 μM) treatment resulted in a dose-dependent reduction in STAT5 tyrosine phosphorylation. Total STAT5 and JAK3 expression did not decrease or degrade during increasing NC1153 treatment **(Figure**

2.7B). To determine if NC1153 can induce apoptosis in this primary tumor sample, naïve PBMCs and T-ALL patient PBMCs (Pt6) were treated with increasing concentrations of NC1153 for 24 hrs, total cell lysate separated by SDS-PAGE, and Western blot analysis performed using anti-PARP. Naïve PBMCs did not show PARP cleavage with increasing NC1153 concentrations, but Pt6 (T-ALL) did display dose-dependent PARP cleavage with increasing NC1153 concentrations **(Figure 2.7C).** Therefore, NC1153 can cause apoptotic cell death of cells containing an aberrant JAK3, but not in naïve PBMCs that do not contain an aberrant JAK3.

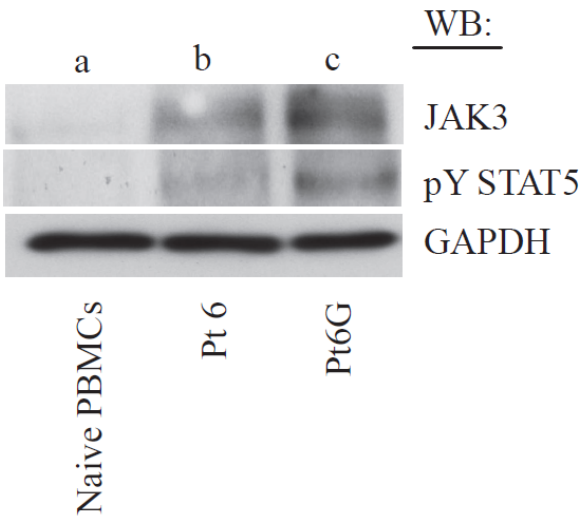


Figure 2.6. T-ALL Patient Contained JAK3 and pYSTAT5. Patient and control cell lysate (20 μ g) were separated by 7.5% SDS-PAGE and Western blot analysis performed using anti-JAK3 C-terminal (1:1000), anti-pYSTAT5 (1:1000), and anti-GAPDH (1:10000). Lane a contains naïve PBMCs that were used as a negative control. Lanes b and c contain a T-ALL patient prior to Gleevec treatment and resistant to Gleevec treatment, respectively.

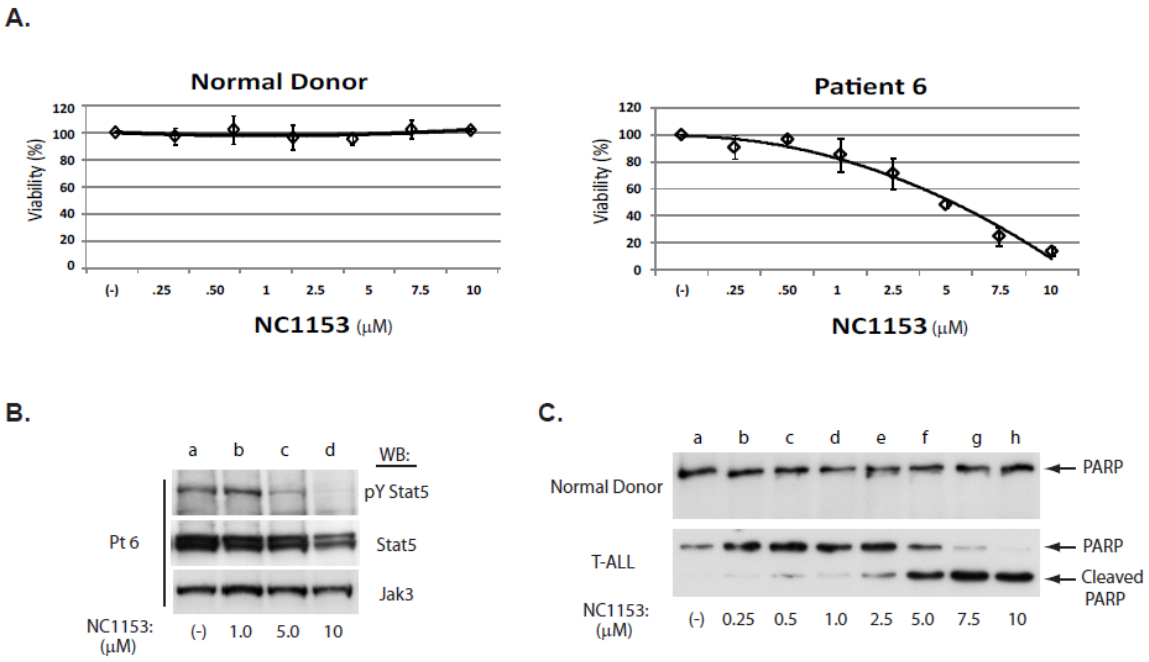


Figure 2.7. NC1153 Diminishes pYSTAT5 in T-ALL Patient and Induces Apoptosis. A) Naïve PBMCs and Pt6 (T-ALL) were treated with medium (lane a) or increasing concentrations of NC1153 as indicated for 72 hrs and cell viability measured by MTS. B) Pt 6 (T-ALL) PBMCs were treated with medium (lane a) or increasing concentrations of NC1153 (lanes b-d) for 24 hrs and Western blotted with anti-pYSTAT5 (1:1000), total anti-STAT5 (1:1000), and total anti-JAK3 (1:1000). C) Naïve (top row) and Pt 6 (T-ALL) PBMCs were treated for 24 hrs with mediuem (lane a) or increasing concentrations of NC1153 (lane b-h) and a Western blot done with anti-PARP (1:1000) to determine PARP cleavage.

Activation of multiple signaling proteins in patient samples

Previous studies in patient samples have involved a limited number of signaling pathway proteins. To effectively screen a large cohort of primary patient samples for activated signaling proteins the Luminex Multiplex System was employed. To determine the sensitivity of the Luminex assay for detection of pYSTAT5 protein, PHA activated PBMC total cell lysate was utilized. Decreasing protein concentrations were either 1) run on a 7.5% SDS-PAGE and immunoblotted for α-pYSTAT5 or 2) assayed by the Luminex α-pYSTAT5 bead set. The α-pYSTAT5 signal was detected at 1.6 μg (lane f) of protein by Western blot analysis **(Figure 2.8B).** However, when assayed using the Luminex system, pYSTAT5 was detectable down to 0.25 μg of protein **(Figure 2.8A).** This finding demonstrates that the Luminex system is more sensitive and is preferable for the screening of patient samples when protein amount is limited.

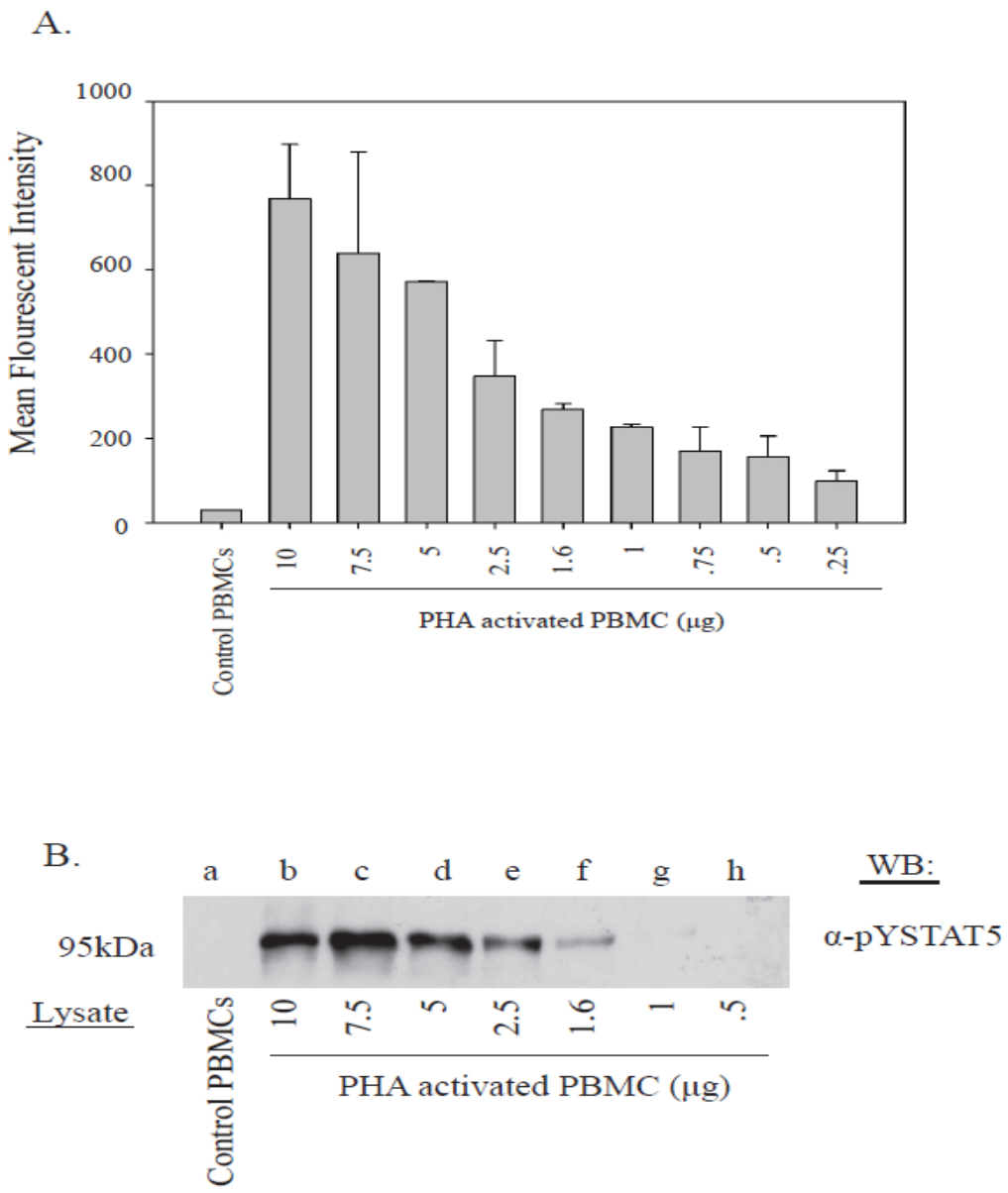


Figure 2.8. Multiplex sensitivity. PHA activated PBMCs were assayed for the presence of activated STAT5 (anti-pYSTAT5) using two different methods**:** A) Control PBMCs (negative control) and decreasing concentrations of PHA activated PBMCs were assayed by the Luminex multiplex system using the anti-pYSTAT5 bead set. pYSTAT5 intensity was measured via mean fluorescent intensity. Error bars represent the standard deviation (n=3). B) Control PBMCs (lane a) and decreasing concentrations of PHA activated PBMCs (lanes b-h) were separated by 7.5% SDS PAGE and Western blot performed with anti-pYSTAT5 (1:1000).

The Luminex multiplex analysis was further utilized to determine the activation status of 13 signaling proteins in patient samples. These signaling molecules were divided into two panels: 1) Transcription factors: STAT1, STAT2, STAT3, STAT5A/B, and STAT6 and 2) SRC Family Kinases: LCK, LYN, SRC, YES, FYN, BLK, HCK, and FGR. Patient samples were normalized to naïve PBMCs and graphed as fold induction **(Figures 2.9-2.12).** The two panels were divided into their individual signaling molecules for further analysis **(Figures 2.11-2.12)**.

In **Figure 2.11A** patients 45 and 50 showed greater than a 6 fold induction in STAT1 activation as compared to naïve PBMCs. Patients 38 and 50 showed greater than 2 fold induction in STAT2 activation **(Figure 2.11B),** while patients 38, 45 and 50 also showed greater than 4 fold induction in STAT3 activation **(Figure 2.11C)**. Patients 38, 40, 43 and 50 showed greater than a 4 fold induction in STAT5 activation **(Figure 2.11D)**. It is important to note that Pt 6, which was shown to possess pYSTAT5 by Western blot **(Figure 2.6)** is identified as having 1.6 fold induction of STAT5 activation, thus confirming the presence of pYSTAT5 in this sample by Luminex. Patients 40, 43, and 50 had greater than 3 fold induction in STAT6 activation **(Figure 2.11E)**. Finally, patients 38, 40, 43, 45, and 50 contained more than a 2 fold induction in activation of more than one STAT family member.

In **Figure 2.12A,** patients 38, 50, and 59 contained greater than 3 fold induction in BLK activation and **Figure 2.12B** showed greater than a 2 fold induction for FGR activation in patients 38, 50, 52, 59, 60, 61, and 62. Patients 38, 59, 60, and 61 indicated a 3 fold induction in FYN activation **(Figure 2.12C),** while patients 11, 40, 43, 45, 47, 50, 52, 57, 59, 60, 61, and 62 showed more than 2 fold induction in HCK activation **(Figure 2.12D)**. In **Figure 2.12E,** patients 6G, 38, 40, and 59 indicated greater than 2 fold induction in LCK activation and patients 59, 60, 61, and 62 had greater than 2 fold induction in LYN activation **(Figure 2.12F).** Patients 46 and

54 displayed greater than a 1 fold induction in SRC activation **(Figure 2.12G)** and patients 38, 50, and 59 showed greater than a 4 fold induction in YES activation **(Figure 2.12H)**. Taken together, patients 38, 40, 50, 52, 59, 60, 61, and 62 contained at least a 2 fold induction activation of multiple SRC family kinases. Also, patients 38, 40, 43, 45, and 50 contain a 2 fold induction or greater in activation of both a STAT and SRC family member **(Figures 2.9-2.12)**.

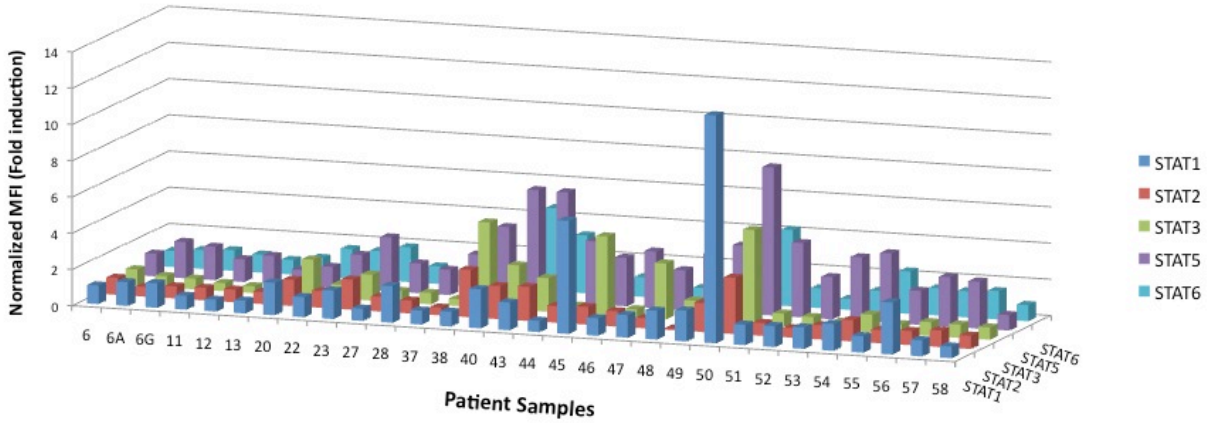


Figure 2.9. STAT Panel Activation in Patient Samples. Tyrosine phosphorylated STATs were detected using the multiplex analysis in patient primary tumor cells using 20 μg of total cell lysate. Samples were normalized to naïve PBMCs and signal intensity measured via fold induction of mean fluorescent intensity (MFI). Tyrosine phosphorylation of the following STATs was measured: STAT1, STAT2, STAT3, STAT5, and STAT6. (n=1)

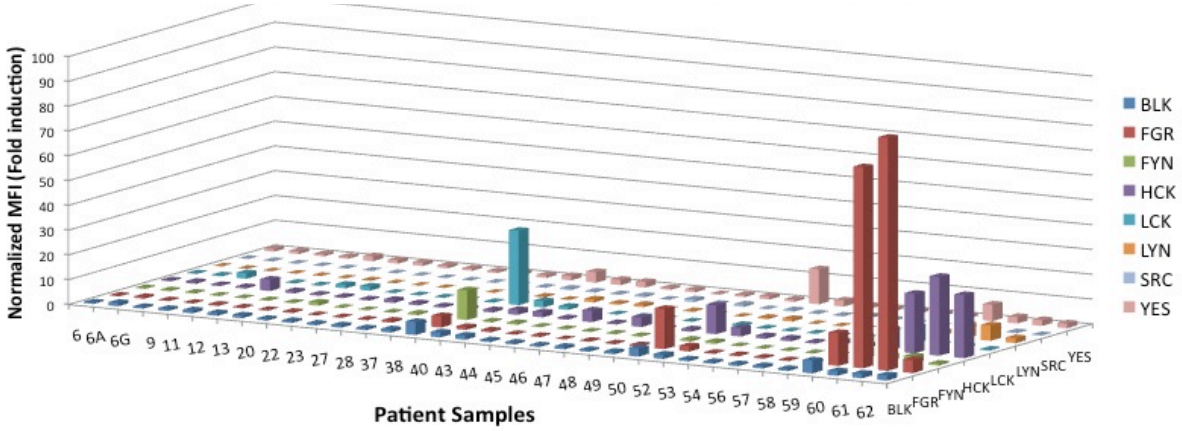


Figure 2.10. SRC Panel Activation in Patient Samples. Tyrosine phosphorylated SRCs were detected using the multiplex analysis in patient primary tumor cells using 20 µg of total cell lysate. Samples were normalized to naïve PBMCs and signal intensity measured via fold induction of mean fluorescent intensity (MFI). Tyrosine phosphorylation of the following SRCs was measured: BLK, FGR, FYN, HCK, LCK, LYN, SRC, and YES. (n=1)

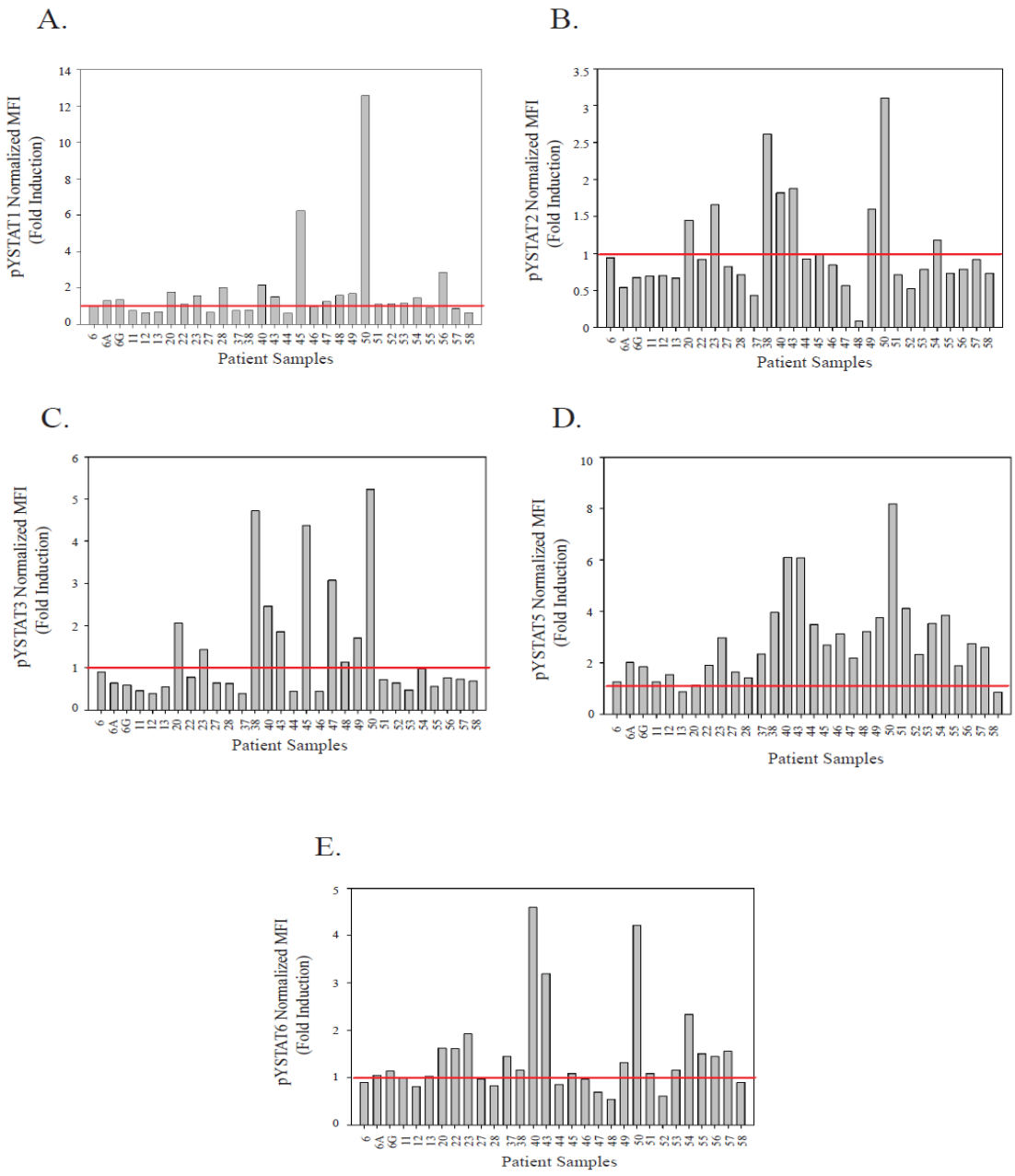


Figure 2.11. STAT Panel Activation in Patient Samples. Tyrosine phosphorylated STATs were detected using the multiplex analysis in patient primary tumor cells using 20 µg of total cell lysate. Samples were normalized to naïve PBMCs and signal intensity measured via fold induction of mean fluorescent intensity (MFI). The following graphs were taken from data in **Figure 2.9.** The STAT panel contains the family members: A)pYSTAT1, b)pYSTAT2, C)pYSTAT3, D)pYSTAT5, and E)pYSTAT6. (n=1)

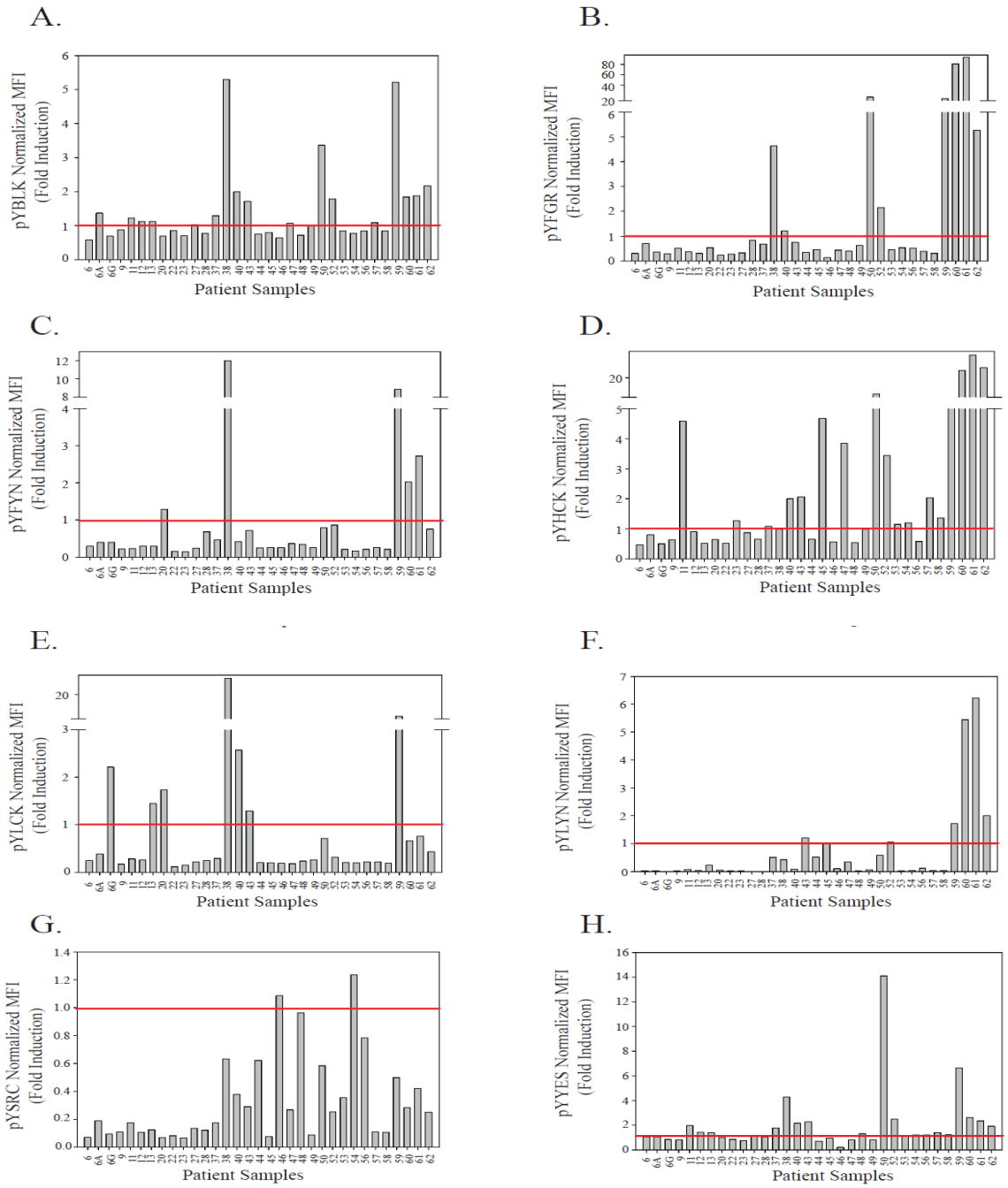


Figure 2.12. SRC Panel Activation in Patient Samples. Tyrosine phosphorylated SRCs were detected using the multiplex analysis in patient primary tumor cells using 20 µg of total cell lysate. Samples were normalized to naïve PBMCs and signal intensity measured via fold induction of mean fluorescent intensity (MFI). The following graphs were taken from data in **Figure 2.10.** The SRC panel contains the family members: A)pYBLK, b)pYFGR, C)pYFYN, D)pYHCK, E)pYLCK, F)pYLYN, G)pYSRC, and H)pYYES. (n=1)

JAK3 Sequencing to Detect Somatic Mutations

In order to determine whether JAK3 hyperactivation in Pt6 (T-ALL) **(Figure 2.3)** was due to an activating mutation, a protocol was established to amplify and sequence the 23 exons present in the JAK3 gene. Naive PBMCs **(Figure 2.13A)** were employed for protocol optimization of JAK3 gene amplification using 23 primer sequences **(Table 2.2).** Different cycle parameters, polymerases, and buffers were optimized for the amplification of the 23 exons. The optimal PCR reaction contained: 100 ng DNA, reaction buffer (60 mM Tris-SO_4 (pH 8.9), 180 mM $(NH_4)_2SO_4$, 1.2 mM $MgSO_4$, 20 mg/ml BSA) , 10 mM dNTP, 2.5 units Platinum® Taq DNA Polymerase High Fidelity polymerase, and 10 μM forward and reverse PCR primers in a 50 μl reaction volume. We also optimized the primers (**Table 2.2)** that were essential in amplifying the JAK3 exons. With successful PCR amplification of the JAK3 gene using naïve PBMCs genomic DNA as a template **(Figure 2.13A),** the T-ALL patient (Pt6) genomic DNA was amplified and JAK3 exons subsequently sequenced **(Figure 2.13B).** Alignments for Pt6 sequencing can be found in the **Appendix.** Pt6 did not contain any somatic mutations in the JAK3 gene. To ensure accuracy of sequencing the JAK3 Kinase Domain in KCL-22 cell line, previously shown to contain an L1017M somatic mutation (Yamashita et al., 2010), was amplified and sequenced **(Figure 2.14)**. Indeed, nucleotide C was shown to be mutated to A, thus resulting in the amino acid mutation of L to M at residue 1017.

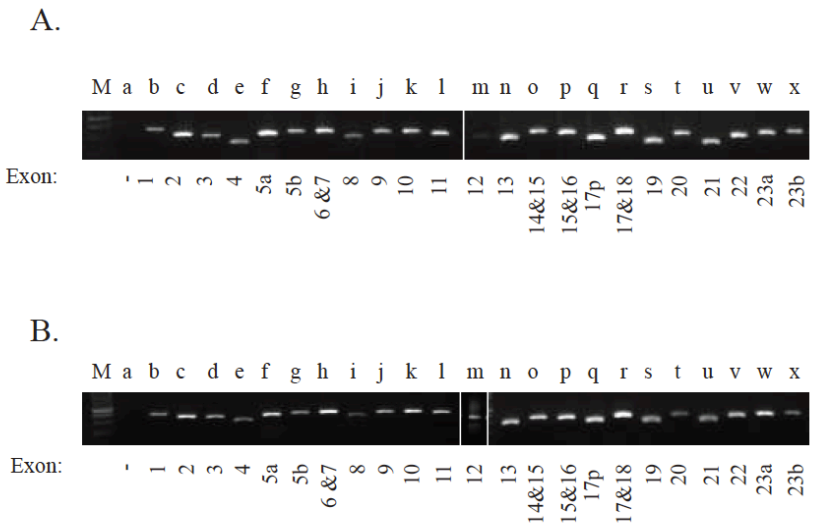


Figure 2.13. PCR Amplification of JAK3. Panels A and B both contain a 1 kb DNA ladder (M), a negative control (lane a), and the 23 JAK3 exons amplified (lanes b-x). A) The parameters for amplification of the 23 exons encoding the JAK3 gene were optimized using naïve PBMCs and 23 primer sets. B) All 23 exons of the JAK3 gene were amplified and subsequently gel purified and sequenced (Pt6).

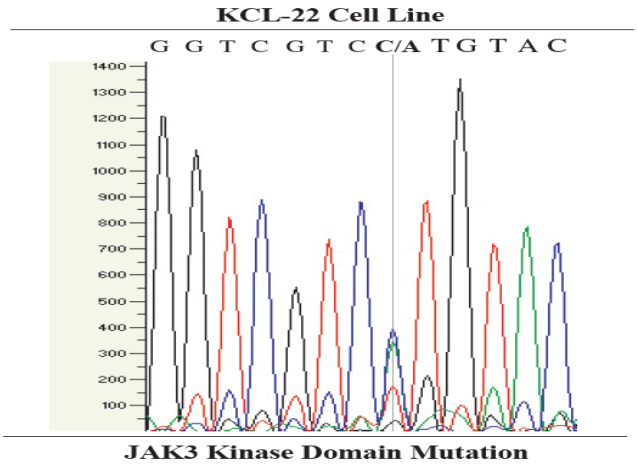


Figure 2.14. Sequencing of JAK3 Kinase Domain in KCL-22 Cell Line. The JAK3 Kinase Domain was amplified using primer set 21 **(Table 2.1)** and sequenced. The chromatogram shown for the alignment of KCL-22, exon 21, with JAK3 exon 21 shows an amino acid change C/A. The solid line from C/A down denotes the nucleotide change in the chromatogram

2.4 DISCUSSION

Multiplex analysis of pYJAK3, pYSTAT, and pYSRC family members was performed on 34 patient samples from various hematological malignancies. Results from these analyses showed that 8 patients (24%) had more than one SRC family member active and within these eight patients, four of them also had a STAT family member activated, and one of them had JAK3 activated **(Figures 2.9-2.12)**. This illustrates that multiple pathways are activated in these hematological malignancies. Interestingly, every hematological malignancy analyzed had a unique set of proteins that were activated. **This data therefore, suggests that multikinase inhibitors could be a form of treatment for patients displaying activation of multiple proteins, or a cocktail of inhibitors specifically set to inhibit a panel of kinases specific to the patient profile.**

During this study 12 out of 40 patients (30%) that were analyzed for pYJAK3 by Luminex showed 1.7 ng/ml of activated protein or greater **(Figure 2.5).** This data indicates that certain hematological malignancies do contain a hyperactivated JAK3. Morever, direct evidence that patient primary tumor cells with hyperactivated JAK3 can be treated with a JAK3 inhibitor (NC1153) and have decreased activation of STAT5 and decreased cellular proliferation was shown in **Figure 2.7.** This suggests that certain hematological malignancies with an overactive JAK3 can be treated with a specific JAK3 inhibitor that not only causes a decrease in cellular proliferation, but also induces apoptotic death of cells harboring an overactive JAK3. **This indicates that certain hematological malignancies contain a hyperactivated JAK3 and uncoupling its activation is a viable treatment option for these malignancies.**

Somatic mutations can lead to hyperactivation of JAK3. It was therefore important to set up a protocol to sequence patient samples containing a hyperactivated JAK3. A successful

protocol is now in place for amplification and sequencing of the JAK3 gene, however sequencing of Pt6 that contained hyperactivated JAK3 led to no somatic mutation being identified **(Figure 2.13)**. This suggests that hyperactivation of JAK3 in this patient was caused by other means, such as overexpression of the JAK3 protein or loss of negative regulation. **The query to find new JAK3 somatic mutations should not be discontinued, but investigation into new possibilities of how JAK3 can become hyperactivated and drive an oncogenic signal should also be considered. Recent published results indicate JAK3 mutations are present in hematological malignancies (Walters, 2006).**

In an effort to determine the best method to detect total JAK3 expression, activated JAK3, and activated STAT5, multiple methods were tested. It was determined that confocal microscopy is more sensitive to detect JAK3 expression than by total cell lysate. Samples that did not show JAK3 expression by Western blot analysis, did in fact show expression by confocal microscopy, this could be due to the single cell detection capabilities of confocal microscopy. Activated JAK3 expression (pYJAK3) was detectable by both immunoprecipation of JAK3 in patient samples and by quantitative Luminex analysis. However, JAK3 immunoprecipitations require more sample than Luminex and is not quantitative, therefore, Luminex analysis will be the method of choice for detecting activation of JAK3 in future patient samples. This method allows for a high throughput system to detect in small sample amounts and is quantitative. **We have therefore utilized multiple approaches to detect the presence of total JAK3 and activated JAK3 and STAT5 in patient samples. Each technique has its own advantages and can all be used together to screen patients for the presence and activation of these proteins.**

Chapter III: Identification of a JAK3 Consensus Phosphorylation Sequence and Putative Substrates

3.1 INTRODUCTION

Protein phosphorylation is a critical post-translational modification for controlling cellular signal transduction. The human proteome contains ~700,000 potential phosphorylation sites (8.5% Ser, 5.7% Thr, 3.0% Tyr). To ensure signaling accuracy, kinases must be able to discriminate amongst all potential phosphorylation sites (Ubersax & Ferrel, 2007). A consensus phosphorylation sequence is one of the most important mechanisms that allows for substrate specificity. This consensus sequence is complementary to the sequence found on the active site of the kinase. Within this consensus sequence, the amino acids situated closest to the phosphorylation site (N-terminally and C-terminally) will be the most pivotal in kinase-substrate recognition. Indeed, the four amino acids on either side of the phosphorylation site are most important for this interaction (Mok, Kim, et. al, 2010).

The introduction of orientated peptide library screens and the Spot array have been pivotal in identifying potential consensus phosphorylation sequences of kinases. Using a custom synthesized Spot array on a cellulose membrane support (Kinexus Inc.), we have identified a putative consensus phosphorylation sequence for JAK3. Utilizing this sequence, possible substrates for JAK3 were identified and further tested in tumor T-cell lines. By identifying possible JAK3 substrates, this has allowed us to understand how JAK3 drives a proliferative signal through previously unrecognized signaling pathways.

3.2 MATERIALS AND METHODS

Spot array:

A spot array was performed by Kinexus Inc. using an 11mer peptide in an amino acid (AA) cluster format. The peptides produced contained the same format for each run: a central tyrosine residue with 5 AA on each flanking side (XXXXX-Y-XXXXX). The approach consisted of five rounds, where each round the AA's flanking the Y were varied beginning with the AA's most proximal to the Y and moving more distal until the ultimate sequence was determined. The screening proceeded as follows: Round 1 consisted of changing positions -1 (B1) and +1 (B2) with each fixed AA cluster (Table 3.1) (eleven possible) and each X position was all possible AA (XXXX-B1-Y-B2-XXXX), this created 121 peptides that were spotted on a nitrocellulose membrane for each fixed AA at B1 and B2, therefore, 121 peptides were spotted in each square in **Figure 3.2** using the following single and amino acid clusters**:** Single Amino Aids: C, H, M, P; Amino Acid Clusters: a= A, G; d= D, E; f= F, W; i= I, L, V; k= K, R; n= N, Q; s= S, T. The amino acid clusters were formed based on physiochemical/structural similarities of the amino acids. There are four unique amino acids that do not fall into any cluster, C, H, M, P. Together the amino acid clusters and unique amino acids form eleven unique "amino acid clusters" that can be positioned at each position.

Once spotted, the membrane was moistened in 10 ml of ethanol and then blocked in 10 ml of Buffer 2 (10 mM MOPS (pH 7.0), 0.3 mM EDTA, 0.001% Triton, 0.5% Glycerol, 0.01% 2-mercaptoethanol, 100 mM NaCl, 0.2 mg/ml BSA) overnight at 25 ^{o}C. The membrane was then incubated in 10 ml of Buffer 3 (10 mM MOPS (pH 7.0), 0.3 mM EDTA, 0.001% Triton, 0.5% Glycerol, 0.01% 2-mercaptoethanol, 100 mM NaCl, 1 mg/ml BSA, 10 mM $MgCl_2$, 50 µM ATP)

for 1 hr at 30 ^{o}C. The membrane was then incubated in Kinase Assay Buffer (10 mM MOPS (pH 7.0), 0.3 mM EDTA, 0.001% Triton, 0.5% Glycerol, 0.01% 2-mercaptoethanol, 0.1 mg/ml BSA, 10 mM $MgCl_2$, and 100 μM ATP) along with 470 ng/ml JAK3 (Millipore #14-629) for 2 hr at 30 ^{o}C. The membrane was washed in T-TBS (0.05% Tween-20) following the kinase reaction. The membrane was then blocked overnight in blocking buffer (5% sucrose, 4% skim milk in T-TBS) at 25 ^{o}C and washed again. The primary antibody, α-pY (4G10), was added at a concentration of 1:1000 in blocking buffer and incubated for 3 hr at 25 ^{o}C. After washing, the membrane was incubated with secondary antibody-HRP that was diluted 1:5000 in blocking buffer for 2hr at 25 ^{o}C. The membrane was developed using DSI (100 mg NaCl in 2.5 ml 200 mM Tris-HCl pH 7.4 and 5.8 ml water) and DSII (5 mg 4-chloro-1-naphthol in 1.7 ml methanol) in a 1:1 ratio with the addition of 5 μl 30% H_2O_2 at the time of use.

At the end of each round, densitometry was performed on the membrane image. Densitometry results were taken into account, along with the physiochemical properties of the AAs present in the peptide, when deciding what AA to select for each position of the consensus peptide. At the end of the fifth round, a peptide was generated that contained the AA clusters (B) at each specific position that were important for its ability to be phosphorylated (B9-B7-B5-B3-B1-Y-B2-B4-B6-B8-B10). Once round 5 was completed, the consensus peptide sequence was elucidated by taking the peptide cluster sequence from this round and specifying the AA's from each respective cluster at each position until the final sequence was elucidated (e.g. a-d-f-i-k-Y-k-i-f-d-a → A-D-F-I-K-Y-L-W-E-G).

Potential JAK3 substrate recognition:

Upon elucidation of the JAK3 cluster consensus sequence, all potential peptides were subjected to BLAST analysis using the human NCBI non-redundant protein sequence database. Upon completion of the analysis, extracellular proteins, or proteins not containing a tyrosine at the required position were removed from the final protein population. Ten proteins did not meet the required criteria and were removed from the final compilation. The required criteria proteins had to meet to be included as putative JAK3 substrate included, being an intracellular protein, contain a tyrosine, and not contain amino acids C, M, K, or R at position -1 or P, K, or R at position +1. This criteria is required so that JAK3 may have the possibility of phosphorylating the protein.

Tyrosine kinase assay:

The final JAK3 consensus sequence (P-A-D-P-D-Y-F-N-V-T-C) was used to perform an in vitro tyrosine kinase assay with JAK2 (Sigma-Aldrich, Cat # SRP0170) and JAK3 (Genscript). Both kinases were used at a stock concentration of 100 ng/ul. The manufacturer's instructions were followed for the tyrosine kinase reaction (Upstate, Cat #17-315). The *in vitro* kinase reaction was performed in 50 µl reaction volume for each reaction. Each kinase reaction set consisted of six reactions: - (no kinase), 100 ng, 250 ng, 500 ng, 750 ng, and 1000 ng of kinase. For each 50 µl reaction volume, the following was added in a microcentrifuge tube: 10 µl tyrosine kinase reaction buffer (Cat #20-278), 1 µl sodium orthovanadate 50 mM, purified enzyme (varied), 10 µl Luminex microbeads covalently conjugated to the JAK3 consensus sequence, and sterile water (varied to reach final volume of 50 µl). The reactions were incubated for 30 min at 30 ^{o}C. Immediately after the reaction the reaction volumes were moved to 96-well

1.2 mm filter plates (MultiScreen-BV Plate, Millipore). The wells were then washed two times with Assay 2 buffer, followed by the addition of 25 µl phospho-specific biotinylated antibodies (Millipore) and incubated on an orbital shaker for 1 hr under dark conditions at 25 °C. This was then followed by a 30 min incubation with 25 µl of streptavidin-phycoerythrein (SAPE). Samples were then analyzed with the LX-200 and xPONENT 3.1 software according to the manufacturer's instructions.

Cell culture, activation, and treatments:

The IL-2 dependent human T-cell line Kit225 (leukemia) (Hori et al., 1987) was maintained in RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals), 2mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin (complete RPMI) plus 10 IU/ml recombinant IL-2. The human cell lines YT (lymphoma) (Yodoi et al., 1985) and SUP-M2 (anaplastic lymphoma kinase (ALK)-positive anaplastic large-cell lymphoma) (Morgan et al., 1989) were maintained in complete RPMI. To investigate possible JAK3 substrates, YT and Kit225 cell lines were quieted for 24 hr in RPMI 1640 supplemented with 1% FBS (Atlanta Biologicals), 2 mM L-glutamine, 50 IU/ml penicillin, 50 mg/ml streptomycin, and then stimulated with 10,000 IU of recombinant IL-2 at 37 ^{o}C for the following time course: 0, 5, 10, 15, 30, and 60 min.

Kit225 and SUP-M2 cell lines were seeded at a density of $1x10^{7}$ in 5 mls of complete RPMI in 6-well plates and treated with PF-2341066 (Crizotinib), an ALK inhibitor, for 6 and 16 hrs, respectively. Kit225 and SUP-M2 cell line viability was determined in triplicate fashion in 96-well plates with a density of 7,500 cells per well with PF-2341066 and CP-690550 treatment for 72 hrs.

Immunoprecipitation, cell lysis, and Western blot analysis:

TFII-I (Cell Signaling) and LIMK1 (Millipore) antibodies were used to immunoprecipitate cell lysate as previously described (Chapter II). Immunoprecipitations were performed on both YT and Kit225 IL-2 stimulation time courses. JAK3 (Malabarba, 1996) and ALK antibodies were used to IP SUP-M2 and Kit225 cell line treatments. Western blot analysis was performed as previously described in Chapter I using the following antibodies: α-PY (Millipore) at 1:1000, α-TFII-1 (Cell Signaling) at 1:1000, α-ALK (Cell Signaling) at 1:1000, and α-LIMK1(Millipore) 1:1000, α-JAK3 C terminal (Epitomics Inc.) at 1:1000. Apoptotic cell death was assessed by Western blot detection of caspase mediated PARP cleavage, α-PARP (Millipore) 1:1000.

Viability assay:

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazoilum salt (MTS) reagent (Promega) in triplicate, according to manufacturer's instructions. Error bars represent standard deviation.

3.3 RESULTS

Identification of JAK3 Consensus Sequence

A spot array was performed (Kinexus Inc.) to determine the final 11mer consensus peptide for JAK3 in an amino acid (AA) cluster format. Six total rounds were performed to elucidate the final JAK3 conesus peptide, P-A-D-P-D-Y-F-N-V-T-C **(Figure 3.1-3.4)**. Each round was performed in the same manner that round 1 was performed **(Figure 3.2)**. Round 1 elucidated the preferred AA clusters for positions B1 and B2 (X_4-B1-Y-B2-X_4) were B1 was d and B2 was f. These two clusters were selected based on densitometry of the spot array in

conjuction with the physiochemical properties of these two clusters of AA. Upon differentiation of the final JAK3 consensus sequence it was found that amino acids D or E at position -1 and F or W at +1 are important for optimal phosphorylation. In addition, phosphorylation was prohibited if amino acids C, M, K, or R are present at position -1 or if P, K, or R are at position +1 **(Figure 3.4)**. Upon identification of the final JAK3 consensus sequence from the cluster AA sequence **(Figure 3.3)**, a tyrosine kinase assay was performed using purified recombinant JAK2 and JAK3 in increasing concentrations to determine if JAK3 and/or JAK2 could phosphorylate the sequence determined from the spot array. The tyrosine kinase assay determined that both JAK3 and JAK2 were able to phosphorylate the final JAK3 consensus sequence **(Figure 3.5)**.

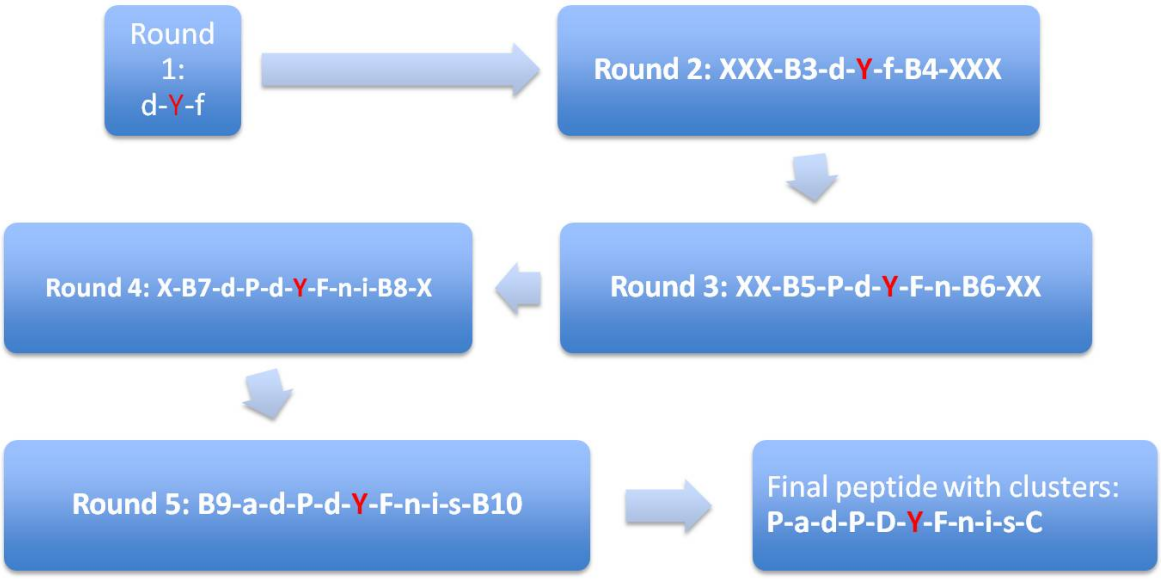


Figure 3.1. Schematic of Amino Acid Cluster Approach to Determine the JAK3 Consensus Phosphorylation Sequence. At the end of each round, the AA clusters with the combined best densitometry and physiochemical properties were chosen to continue the screen. During the screen, certain positions, such as B2, were differentiated. At each position, certain AA clusters were also found to be unfavorable. The final cluster peptide was P-a-d-P-D-Y-F-n-i-s-C. The cluster peptide was used for the last round of screening to find the best final peptide **(Figure 3.3).** **Amino acid legend:** Single Amino Acids: C, H, M, P; Amino Acid Clusters: a= A, G; d= D, E; f= F, W; i= I, L, V; k= K, R; n= N, Q; s= S, T.

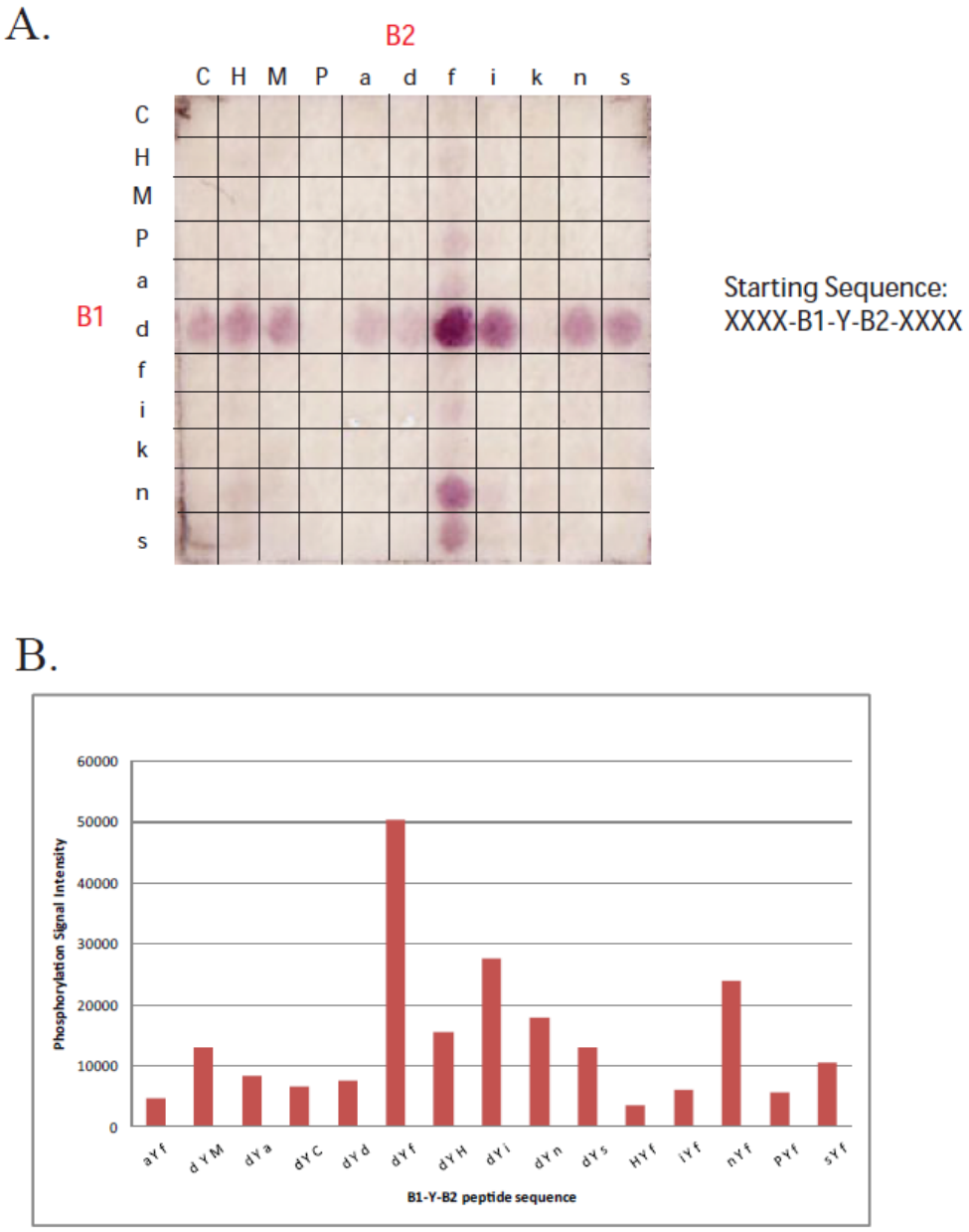
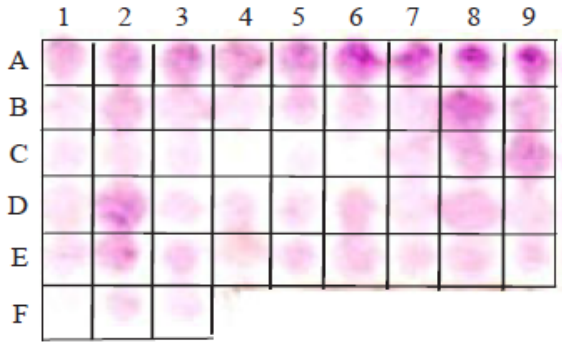


Figure 3.2. Round 1 JAK3 Consensus Sequence Spot Array. A) To perform the spot array 121 peptides were spotted onto each square on the membrane followed by a kinase reaction and signal intensity visualized via colorimetric detection. B) Densitometry was performed on the spot array membrane and phosphorylation signal intensity for the best peptide combinations (B1-Y-B2) was graphed. **Amino acid legend:** Single Amino Acids: C, H, M, P; Amino Acid Clusters: a= A, G; d= D, E; f= F, W; i= I, L, V; k= K, R; n= N, Q; s= S, T.



Key:

Position	Sequence	Position	Sequence
A 1	PADPDYFNISC	C 7	PGDPDYFNISC
A 2	PADPDYFNLSC	C8	PGDPDYFNLSC
A 3	PADPDYFNVSC	C9	PGDPDYFNVSC
A 4	PADPDYFNITC	D1	PGDPDYFNITC
A5	PADPDYFNLTC	D2	PGDPDYFNLTC
A6	**PADPDYFNVTC**	D3	PGDPDYFNVTC
A7	PADPDYFQISC	D4	PGDPDYFQISC
A8	PADPDYFQLSC	D5	PGDPDYFQLSC
A9	PADPDYFQVSC	D6	PGDPDYFQVSC
B1	PADPDYFQITC	D7	PGDPDYFQITC
B2	PADPDYFQLTC	D8	PGDPDYFQLTC
B3	PADPDYFQVTC	D9	PGDPDYFQVTC
B4	PAEPDYFNISC	E1	PGEPDYFNISC
B5	PAEPDYFNLSC	E2	PGEPDYFNLSC
B6	PAEPDYFNVSC	E3	PGEPDYFNVSC
B7	PAEPDYFNITC	E4	PGEPDYFNITC
B8	PAEPDYFNLTC	E5	PGEPDYFNLTC
B9	PAEPDYFNVTC	E6	PGEPDYFNVTC
C1	PAEPDYFQISC	E7	PGEPDYFQISC
C2	PAEPDYFQLSC	E8	PGEPDYFQLSC
C3	PAEPDYFQVSC	E9	PGEPDYFQVSC
C4	PAEPDYFQITC	F1	PGEPDYFQITC
C5	PAEPDYFQLTC	F2	PGEPDYFQLTC
C6	PAEPDYFQVTC	F3	PGEPDYFQVTC

Figure 3.3. Differentiation of Final JAK3 Consensus Sequence From the Final Peptide Cluster. Each square represents a specific peptide that is spotted at that position. The peptide spotted at each position can be found in the **Key.** Densitometry and physiochemical relationships between the AA were considered. **A6 (bolded)** was chosen as the final JAK3 consensus sequence.

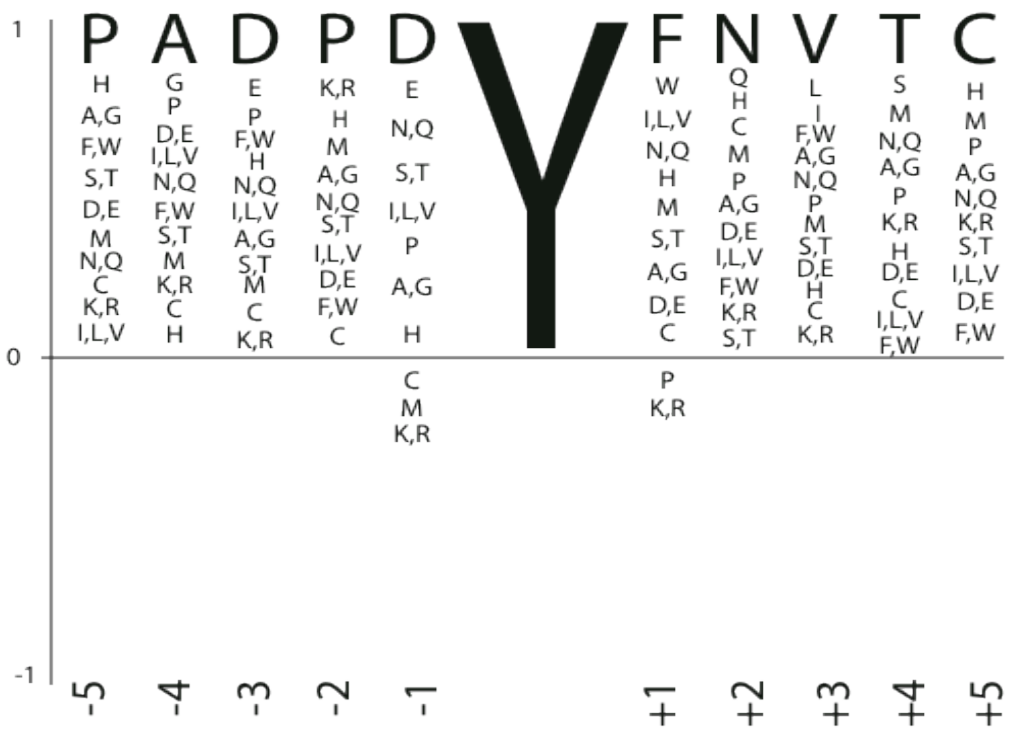
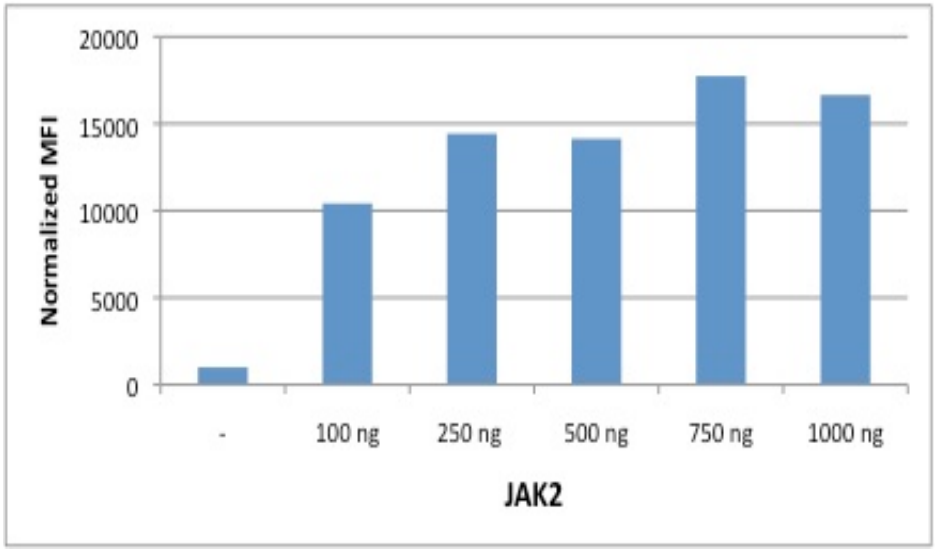


Figure 3.4. Final JAK3 Consensus Sequence. The Y-axis represents the intensity of the signal when the given amino acid is at that position. The closer to 1 the amino acid is, the better the signal. The farther from 1, the less possibility that a signal will occur. However, the likelihood that a signal will occur also depends on the amino acids that are present next to position. Therefore, this is a representation of the likelihood of a signal occurring, but is not absolute. If an amino acid falls below the 0 X-axis, then it is definite that a signal will not occur if these amino acids are present at these positions. The X-axis represents the position of the amino acids relevant to the tyrosine.

A.



B.

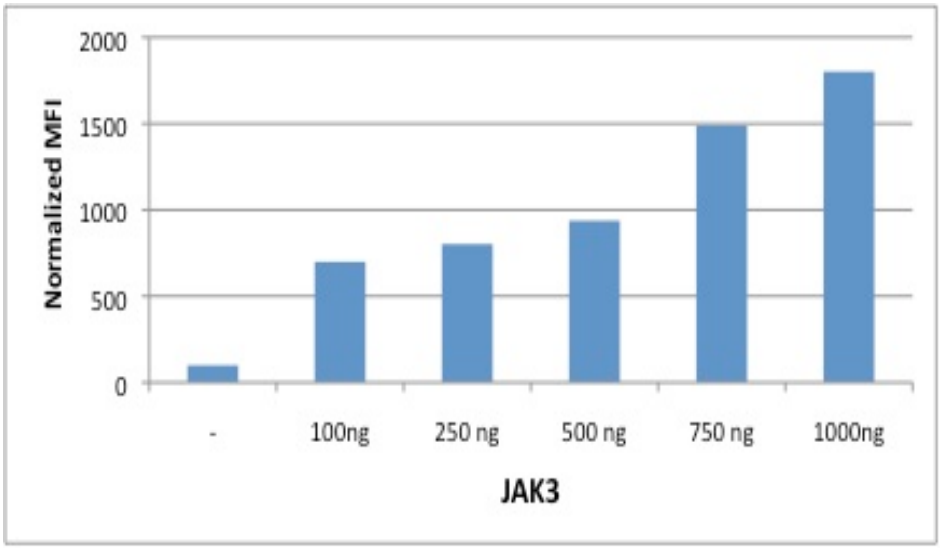


Figure 3.5. JAK2 and JAK3 Phosphorylate the Identified Consensus Sequence. Increasing amounts of purified JAK2 (A) and JAK3 (B) were incubated with microsphere beads coupled to the JAK3 consensus sequence for 30 min at 30^{o}C. The ability of either purified JAK3 or JAK2 to phosphorylate the consensus sequence was then measured using Luminex and samples normalized to a negative control containing no kinase. Tyrosine phosphorylation of the consensus sequence using either kinase was measured via normalized mean fluorescent intensity. (n=1)

Identification of Putative JAK3 Substrates

The final cluster peptide (P-a-d-P-D-Y-F-n-i-s-C) was used to "mine" for putative JAK3 substrates. By interchanging the AA clusters at each position in the final cluster peptide, 48 final peptide sequences are possible. In order to prevent the loss of possible JAK3 substrates, these 48 peptides were subjected to BLAST analysis using the human NCBI non-redundant protein sequence database (Altschul et al., 1990). The BLAST analysis identified 191 proteins as putative JAK3 substrates, however this was reduced to 181 possible substrates **(Appendix, Table 1)** once extracellular proteins, proteins containing C, M, K, or R at position -1 and P, K, or R at position +1, and proteins not containing a tyrosine were removed from the query. The proteins were then categorized using the Ingenuity IPA software creating nine categories: 1) DNA repair and remodeling, 2) Signal Transduction, 3) Matrix, cell adhesion, and cytoskeleton, 4) Metabolism, 5) Transcription, 6) Translation, 7) Transport, 8) Ubiquitination, and 9) Unknown **(Figure 3.6)**. Most of the proteins mined as JAK3 substrates fell into two of the nine categories: unknown and signal transduction **(Appendix, Table 1).**

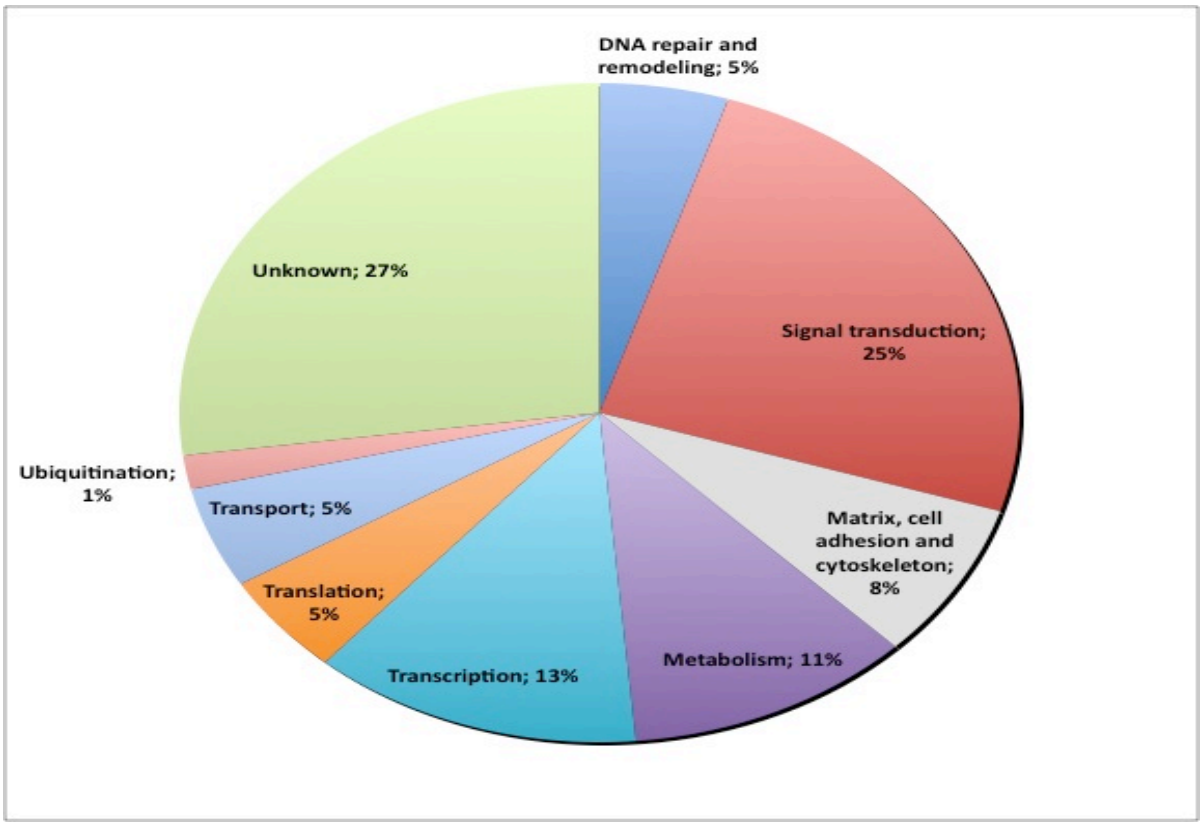


Figure 3.6. JAK3 Putative Substrate Categorization. JAK3 putative substrates that were "mined" using NCBI Blast were categorized into nine categories using Ingenuity IPA software. The 181 proteins "mined" were categorized into the following categories: 1) DNA repair and remodeling, 2) Signal Transduction, 3) Matrix, cell adhesion, and cytoskeleton, 4) Metabolism, 5) Transcription, 6) Translation, 7) Transport, 8) Ubiquitination, and 9) Unknown proteins. Most JAK3 putative substrates "mined" consisted of signal transduction proteins (25%) and unknown proteins (27%).

Reciprocal Activation of JAK3 and ALK

Focus was set on investigating the possible JAK3 substrates that fell into the signal transduction category **(Appendix, Table 1),** included in this category was anaplastic lymphoma kinase (ALK). ALK is expressed as the constitutively active chimeric fusion protein, NPM-ALK, in anaplastic large-cell lymphoma (ALCL) and promotes tumorigenesis (Kinney et al., 2011). Previous studies have shown that JAK3 and NPM-ALK coimmunoprecipitate (Amin et al., 2003). To determine if JAK3 and ALK could be reciprocally activated, the presence of JAK3 protein expression was first determined in the anaplastic lymphoma kinase (ALK)-positive anaplastic large-cell lymphoma cell line, SUP-M2. It was determined that SUP-M2 expressed a greater amount of JAK3 than Kit225 cells **(Figure 3.7)**. To determine the activation status of JAK3 in ALCL, immunoprecipitation of JAK3 was performed on both Kit225 and SUP-M2. Kit225 (lane a) and SUP-M2 (lane b) cells contained constitutively active JAK3 (pYJAK3), as well as a coimmunoprecipitating protein at ~70 kDa **(Figure 3.8A)**.

The fusion protein NPM-ALK has an apparent molecular weight of 75 kDa (Bischof et al., 1997), therefore further studies were performed to confirm the identification of this protein. We stripped and reblotted the membrane with anti-ALK to confirm the identification of this protein at ~70 kDa. Upon immunoblotting for anti-ALK, it was discovered that this protein in SUP-M2 (lane b) was the NPM-ALK fusion protein **(Figure 3.8C).** Kit225 (lane a), which is a cell line that does not contain the NPM-ALK fusion protein, and therefore serves as a negative control for this experiment, did not contain a band at ~70kDa when immunoblotted with anti-ALK. To ensure equal loading, the membrane stripped and reblotted with anti-JAK3 (lanes a and b) **(Figure 3.8B)**. Taken together, these results confirm that JAK3 co-IPs with NPM-ALK in the ALCL cell line SUP-M2.

Previous studies have shown that inhibition of JAK3 decreases NPM-ALK kinase activity (Amin et al., 2003; Lai et al., 2005). Therefore, to confirm this in SUP-M2 cells, increasing concentrations of CP-690550 (Pan-JAK inhibitor) (Karaman et al., 2008) were incubated with these cells for 16 hrs, a JAK3 immunoprecipitation was performed, samples were separated by 7.5% SDS-PAGE, and a Western blot performed against anti-pY. SUP-M2 cells displayed a decrease in both a ~125 kDa and ~70 kDa band, which we previously established **(Figure 3.7 & 3.8)** as JAK3 and NPM-ALK, respectively. Treatment of SUP-M2 with CP-690550 dose-dependently decreased tyrosine phosphorylation of both JAK3 and NPM-ALK. JAK3 tyrosine phosphorylation dose-dependently decreases until CP-690550 reaches a concentration of 100 nM, at which point tyrosine phosphorylation of JAK3 is not detected. NPM-ALK tyrosine phosphorylation also dose-dependently decreased, but was almost obsolete at 50 nM concentration of CP-690550 **(Figure 3.9A)** No significant changes were detected in total levels of JAK3 **(Figure 3.9B).**

In agreement with previous studies, we have shown that inhibition of JAK3 can decrease NPM-ALK kinase activity **(Figure 3.9)**, however, it is not known if inhibition of NPM-ALK will decrease JAK3 kinase activity. Therefore, SUP-M2 cells were treated with PF-02341066 (Crizotinib), an established ALK inhibitor currently FDA approved for the treatment of ALK (+) ALCL (Cui et al., 2011). To determine the IC50 of PF-02341066 (PF), a 72 hr viability assay was performed with increasing concentration of PF. An IC50 of 50 nM was determined **(Figure 3.10)**, which corresponds to that seen in previous literature (Ou, 2011). To determine the effect of PF on JAK3 activation, SUP-M2 were treated with increasing concentrations of PF-02341066 for 16 hrs, a JAK3 immunoprecipitation was performed, samples separated by 7.5% SDS-PAGE, and then immunoblotted with anti-pY. This resulted in a decrease of pYJAK3 and pYNPM-

ALK. A noticeable decrease is seen in pYJAK3 and pYNPM-ALK between 10 (lane a) and 50 nM (lane b) of PF treatment. JAK3 tyrosine phosphorylation dose-dependently decreased until a concentration of 250 nM of PF, where it is not detected (lane e), while NPM-ALK tyrosine phosphorylation displays a slightly protracted dose-dependent decrease until reaching maximum inhibition at 500 nM **(Figure 3.11A)**. A reblot of total JAK3 confirmed equal loading **(Figure 3.11B)**, while reblot of ALK showed a decrease in total NPM-ALK protein **(Figure 3.11C).** To ensure that NPM-ALK was not being degraded during PF treatment, total lysate (10 μg) from the same SUP-M2 PF treatments **(Figure 3.11A)** were separated by 7.5% SDS-PAGE, and then immunoblotted with anti-ALK. This blot showed no degradation of total NPM-ALK and equal loading **(Figure 3.11D)**. This suggests that inhibition of NPM-ALK upon PF treatment results in loss of NPM-ALK association with JAK3 and JAK3 kinase activity.

To ensure that decrease in phosphorylation was not due to apoptotic cell death from the PF treatment, total cell lysate of SUP-M2 with increasing amounts of PF were separated out by SDS-PAGE and blotted with anti-PARP. PF-02341066 did not cause significant apoptotic death in SUP-M2, while a small amount of PARP cleavage is noticed around 250 (lane e) and 500 nM (lane f) **(Figure 3.12)**.

To test the possibility that PF is a direct inhibitor of JAK3 kinase activity, Kit225 cells were treated with increasing concentrations of PF for 6 hrs and JAK3 tyrosine phosphorylation analyzed by Western blot. Interestingly, increasing concentrations of PF did not decrease JAK3 tyrosine phosphorylation **(Figure 3.13)**. JAK3 reblot confirmed equal loading. In addition, PF treatment had minimal effect on Kit225 cell viability **(Figure 3.14)**. Taken together, this data suggests that NPM-ALK and JAK3 can reciprocally activate each other to create aberrant cell signaling in ALCL.

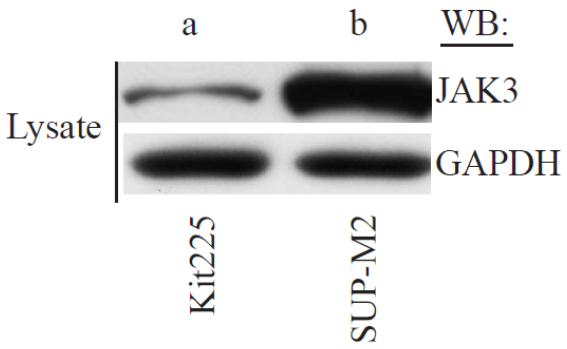


Figure 3.7. JAK3 expression in SUP-M2 and Kit225. Kit225 (lane a) and SUP-M2 (lane b) total cell lysate (10µg) was separated by 7.5% SDS-PAGE and Western blotted with anti-JAK3 (1:1000) for the presence of JAK3 and then reblotted with anti-GAPDH (1:10000) to ensure equal loading.

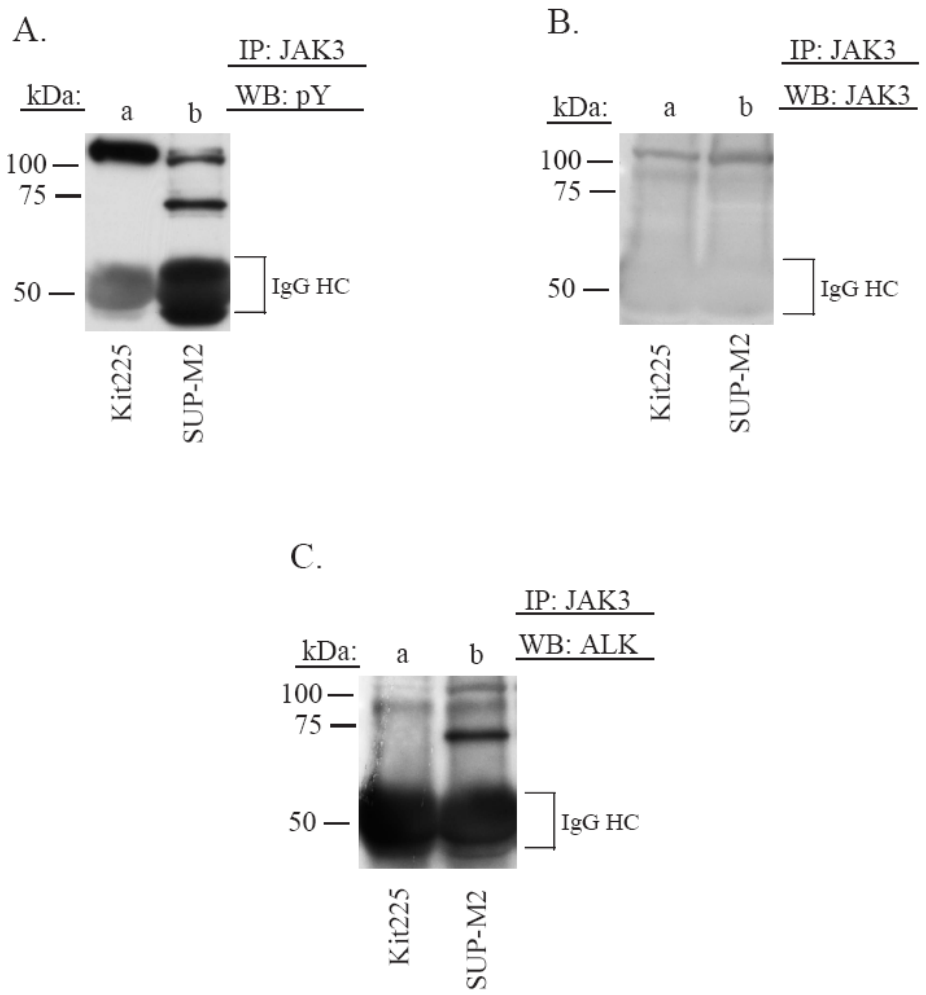


Figure 3.8. ALK coimmunoprecipitates with JAK3. A) Kit225 (lane a) and SUP-M2 (lane b) cell lysates were immunoprecipitated with anti-JAK3, separated by 7.5% SDS-PAGE, and Western blotted with anti-pY (1:1000). A protein ~70 kDa was pulled down along with JAK3 during immunoprecipitation (lane b). B) The membrane was stripped and reblotted with anti-JAK3 (C-terminal) (1:1000) to ensure equal loading. C) The membrane was then stripped and reblotted with anti-ALK (1:1000) to identify the ~70 kDa band that coimmunoprecipitated with JAK3 in panel A. IgG HC in all panels denotes the IgG Heavy Chain. Molecular weight markers (kDa) are shown to the left of each panel.

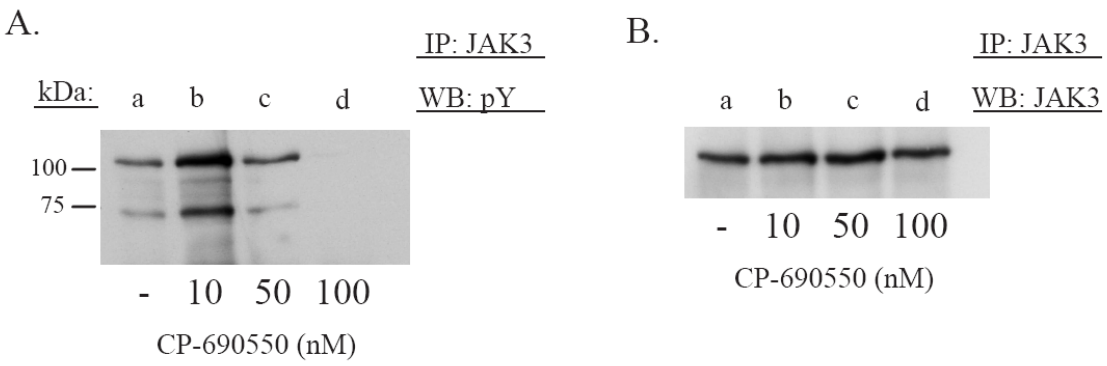


Figure 3.9. CP-690550 Dose-dependently Decreases pYJAK3 and pYNPM-ALK in ALCL Cell Line. SUP-M2 cell line was treated with increasing concentrations of CP-690550 (Pan-JAK inhibitor) in both panels A and B. A) SUP-M2 cells were treated with media only (lane a) or increasing concentrations of CP-690550 (lanes b-d), immunoprecipitated with JAK3, and Western blotted for pY (1:1000). Molecular weight markers (kDa) are shown to the left. B) The membrane was then reblotted for anti-JAK3 (1:1000).

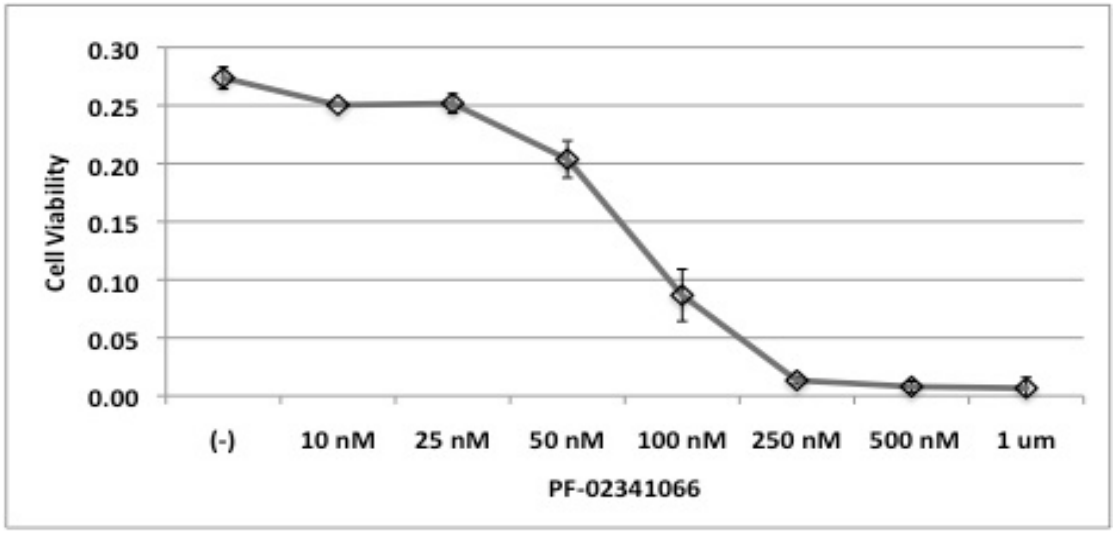


Figure 3.10. IC50 of PF-02341066 in SUP-M2 Cells. SUP-M2 ($7x10^3$ cells/well) were treated with media alone (-) or increasing concentrations of PF-02341066 for 72 hrs at 37ºC and cell viability was measured by MTS. Error bars represent standard deviation (n=3).

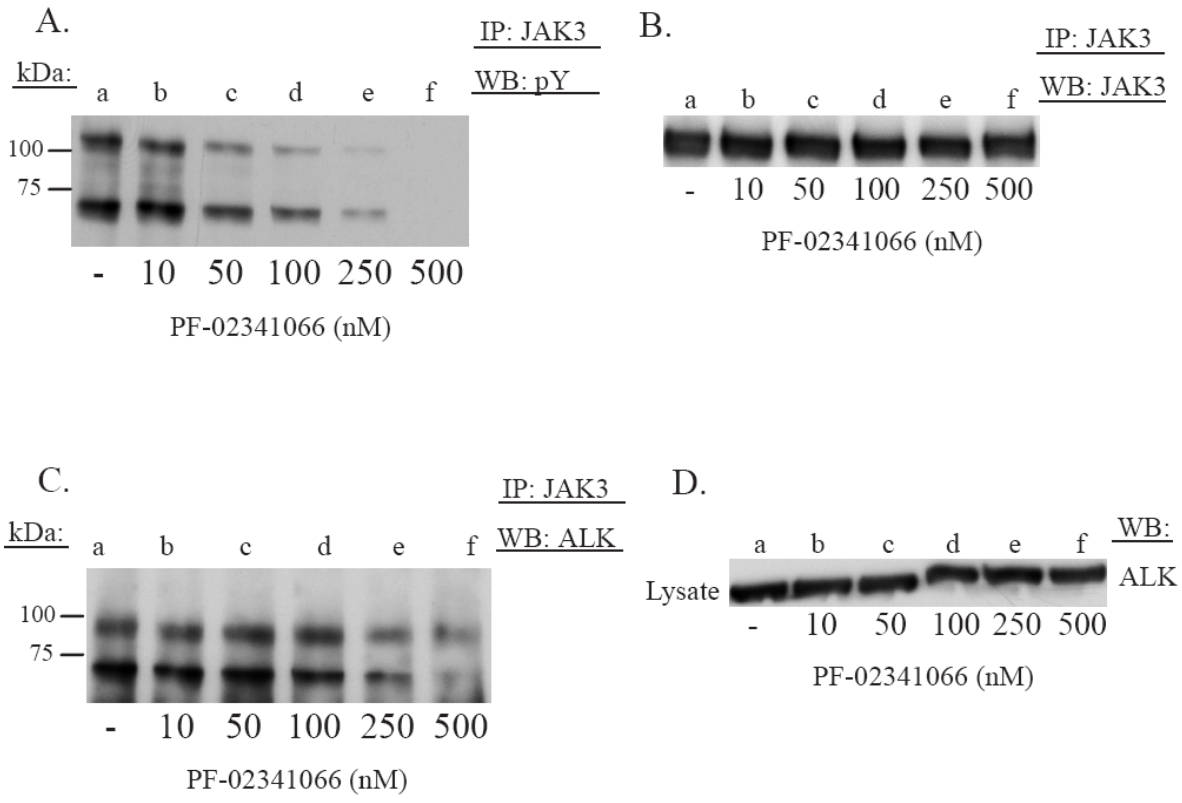


Figure 3.11. PF-02341066 Decrease pYJAK3 and pYNPM-ALK A) SUP-M2 ($1x10^7$ cells/treatment) were treated with media alone (lane a) or with increasing concentrations of PF-02341066 (lanes b-f) for 16 hrs at 37ºC, a JAK3 immunoprecipitation performed, samples separated by 7.5% SDS-PAGE, and then immunoblotted with anti-pY (1:1000). Molecular weight markers (kDa) are shown to the left. B) The membrane was then stripped and reblotted with anti-JAK3 (1:1000) to ensure equal loading. C) The membrane was once again stripped and reblotted with anti-ALK (1:1000). D) SUP-M2 total cell lysate (10µg) from the same treatment performed in panel A was separated by 7.5% SDS-PAGE, and then immunoblotted with anti-ALK (1:1000) to ensure no protein degradation.

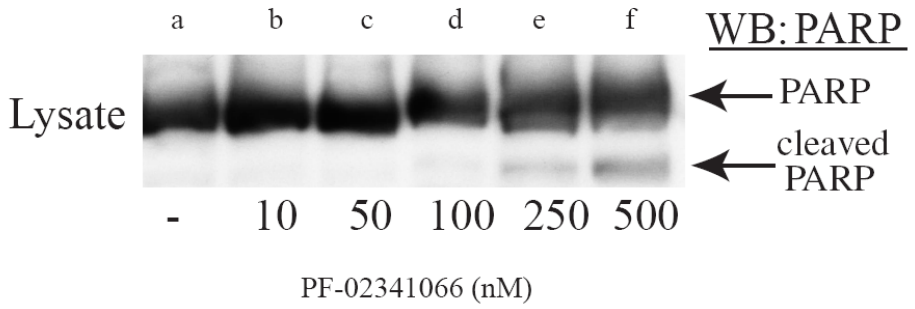


Figure 3.12. PF-02341066 Treatment of SUP-M2 Does Not Cause Significant Apoptotic Cell Death. SUP-M2 ($1x10^7$ cells/treatment) were treated with media alone (lane a) or increasing concentrations of PF-02341066 (lanes b-f) for 16 hrs at 37ºC. Samples (10µg) were then separated by 7.5% SDS-PAGE and Western blotted with anti-PARP (1:1000).

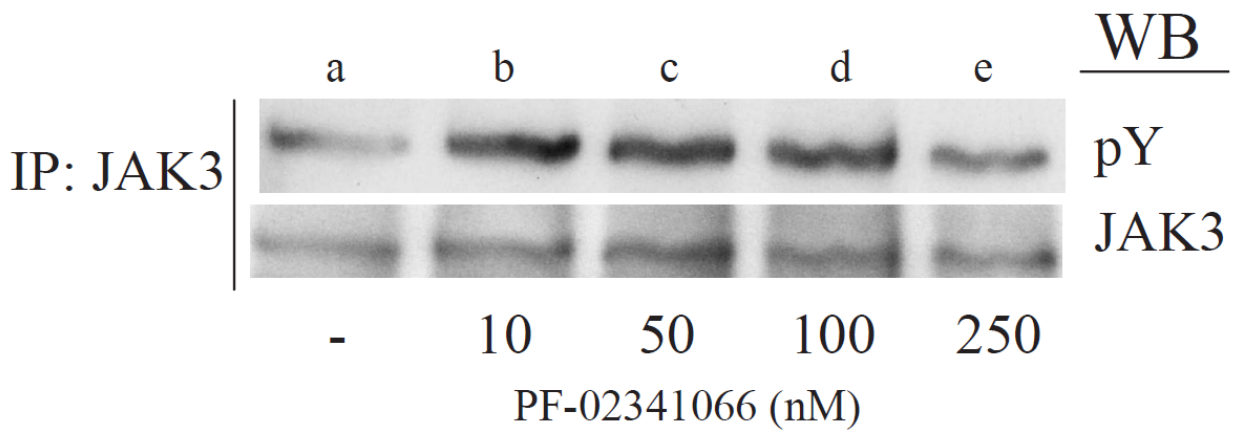


Figure 3.13. PF-02341066 Does Not Decrease JAK3 Tyrosine Phosphorylation in Kit225. Kit225 ($1x10^7$ cells/treatment) were treated with media alone (lane a) or increasing concentrations of PF-02341066 (lanes b-f) for 6 hrs at 37ºC. A JAK3 immunoprecipitation was then performed and samples separated by 7.5% SDS-PAGE. Western blot was carried out with anti-pY (1:1000), and then for anti-JAK3 (1:1000).

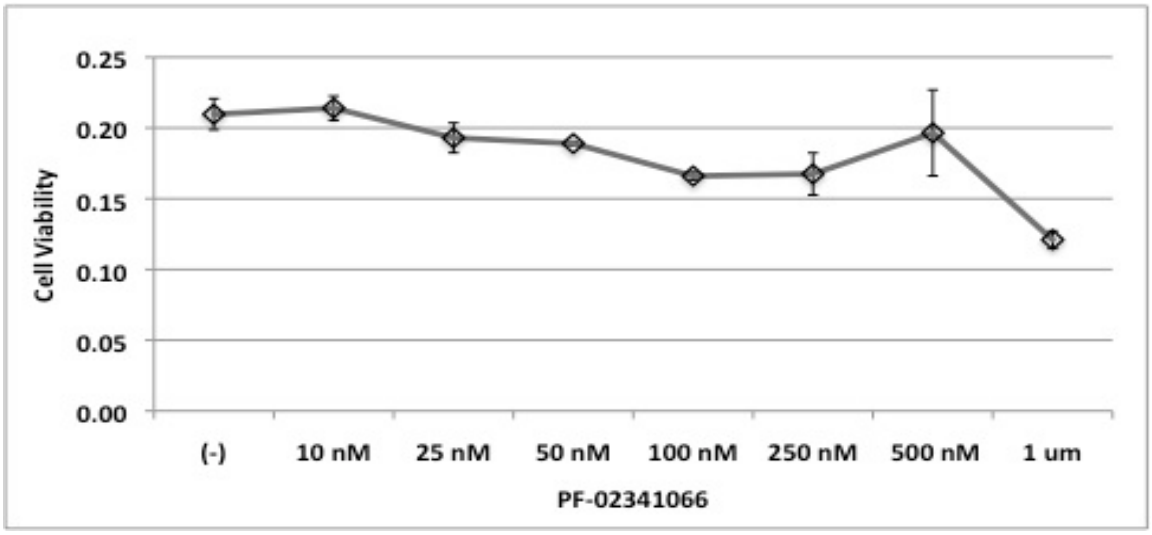


Figure 3.14. PF-02341066 Had Minimal Effect on Kit225 Cell Viability. Kit225 ($7x10^3$ cells/well) were treated with media alone (-) or increasing concentrations of PF-02341066 for 72 hrs at 37ºC and cell viability measured by MTS. Error bars represent standard deviation (n=3).

3.4 DISCUSSION

In an effort to gain insight into previously unrecognized signaling pathways through which JAK3 can drive an oncogenic signal, a novel JAK3 consensus phosphorylation signal was discovered **(Figure 3.4).** Using the final peptide cluster sequence **(Figure 3.2)**, 181 proteins were identified as possible JAK3 substrates. Further investigation of these 181 proteins could lead to unrecognized signaling pathways that are important in normal cell signaling and oncogenic cell signaling. It is important to note that a significant amount of proteins found to be possible JAK3 substrates are cell signaling proteins **(Figure 3.6).**

One of the proteins found to be a substrate by BLAST analysis and in vitro analysis was ALK. Indeed, the oncogenic fusion protein, NPM-ALK coimmunoprecipitated with activated JAK3 in the SUP-M2 cell line **(Figure 3.11).** This data suggests a possible reciprocal activation mechanism. When SUP-M2 cells were treated with an ALK directed inhibitor, a decrease in the activation of both ALK and JAK3 is detected. When SUP-M2 are treated with a pan-JAK inhibitor, a decrease in the activation of both NPM-ALK and JAK is seen as well. Taken together this data suggests that a reciprocal activation exists between NPM-ALK and JAK3. It is still not clearly demonstrated how these proteins drive each others activation, but JAK3 could be a new target for the treatment of anaplastic large-cell lymphoma. Because JAK3 is only expressed in lymphoid tissue while NPM-ALK is more ubiquitously expressed, it may be a superior target in anaplastic large-cell lymphomas. This discovery suggests that JAK3 could contribute to oncogenesis via unrecognized pathways, and the 181 proteins "mined" should be studied further for other possible substrate interactions. These pathways could provide valuable evidence for novel therapeutic intervention in certain hematological malignancies.

Chapter IV: Overview

4.1 OVERVIEW

Due to Gleevec's success in treating CML, tyrosine kinases have become new targets of interest for cancer therapy. Moreover, tyrosine kinases are of interest because they are central in regulating T-cell activation, proliferation, and differentiation, which when deregulated have shown to lead to cancer, immunodeficiency, and autoimmunity. Currently, nine FDA tyrosine kinase inhibitors exist with multikinase inhibition ability, however it is not well understood which kinases these inhibitors act upon besides their main targets. Therefore, there is a critical need to characterize which proteins and signal transduction pathways are overactive in hematological malignancies so that new and rational strategies to detect and effectively control T-cell mediated malignancies can be accomplished. It is also vital to characterize novel signal transduction pathways that mediate T-cell activation to have new targets to create tyrosine kinase inhibitors against.

The first objective of this thesis was to identify a high throughput method to detect overactive JAK3 expression in hematological malignancies. Confocal microscopy and multiplex analysis were identified as the two methods to detect the activation of multiple proteins in patient samples **(Figure 2.2-2.4 and 2.8-2.12). Using these methods, we discovered that each hematological malignancy was unique in its activation of signaling proteins.** When considering their activation profile, no two patient samples were alike, even if they were both the same diagnostic subtype. Considering this and the multikinase inhibition profiles that current FDA approved tyrosine kinase inhibitors hold, these drugs may hold great promise for treatment of cancers where standard chemotherapy has failed. Their multikinase inhibition allows for the inhibition of multiple pathways, and with cross-talk so evident in cell signaling, this additive

effect can help decrease aberrant cell signaling. **However, it is important to understand and uncover the signaling pathways through which the individual oncogenic phenotype developed. The 181 proteins that were "mined" as putative JAK3 substrates should be further studied for the potential to discover novel normal and oncogenic cell signaling pathways.**

In Chapter III, it was determined that NPM-ALK and JAK3 work in concert to create an oncogenic signal transduction pathway in anaplastic large-cell lymphoma **(Figure 3.11). This could lead to a new target in ALCL with NPM-ALK fusion protein.** Since JAK3 is a tyrosine kinase that is located focally in hematopoietic cells, it can serve as a better treatment target than other kinases, such as ALK that is diffusely localized in multiple cell types, including cells in the human brain (Souttou et al., 2000). Treatment of NPM-ALK (+) ALCL with Crizotinib leads to drug resistance (Ryohei et al., 2011). Therefore, JAK3 may serve as a secondary treatment option for NPM-ALK (+) ALCL that have become resistant to Crizotinib. In Chapter III, the JAK3 consensus phosphorylation sequence (P-A-D-P-D-Y-F-N-V-T-C) was also determined**. The JAK3 consensus phosphorylation sequence discovered during these studies can be further analyzed to create an inhibitor of JAK3 activation.** The JAK3 consensus phosphorylation sequence was phosphorylated by both JAK2 and JAK3, therefore, further studies need to be done to differentiate amino acids in the JAK3 consensus sequence that are important for phosphorylation by JAK3 and not JAK2. The JAK2 and JAK3 consensus sequence phosphorylation sequences must be compared and analyzed so as to make each sequence specific for each kinase. The tyrosine kinase inhibitors available to date, all work by binding to the ATP binding pocket and blocking the phosphorylation of the putative substrate. Because all tyrosine kinases contain this ATP binding pocket, specificity of tyrosine kinase inhibition is difficult

(Hartmann et al., 2009). **As previously stated, it is important to target the multiple kinases that are active in a patient, but it is also imperative to specifically inhibit the active kinases so that side effects are not so wide spread. By utilizing the previously described peptide sequence, this type of inhibition could potentially be attained for JAK3 aberrant activity.**

Taken together, the results from this thesis indicate that high throughput screening for activated proteins in patients is crucial for the personalization of treatment and that novel pathways driven by JAK3 should be further investigated for the development of new tyrosine kinase inhibitors for the treatment of select hematological malignancies, such as anaplastic large-cell lymphoma. **Therefore, JAK3 is not only an important target for JAK3 driven oncogenesis, but could also be a target in other cancers that contain an established oncogene, like NPM-ALK. This increases the need to determine if JAK3 is present and hyperactivated in hematological malignancies and to develop an FDA approved JAK3 inhibitor.**

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Glossary

- Anaplastic large cell lymphoma: ALCL
- Acute lymphoblastic leukemia: ALL
- Acute myelogenous leukemia: AML
- Acute megakaryoblastic leukemia: AMKL
- Antigen Presenting Cell: APC
- Adult T cell lymphoma/leukemia: ATLL
- B lymphocyte receptor: BCR
- C-Jun N-terminal kinase: JNK
- Chronic lymphoblastic leukemia: CLL
- Chronic myelogenous leukemia: CML
- Cytotoxic lymphocyte-associated molecule-4: CTLA-4
- Extracellular signal-regulated kinase: ERK
- Human T Cell Leukemia Virus Type I: HTLV-1
- Immunoreceptor-based tyrosine activation motif: ITAM
- Interleukin: IL
- Janus kinase: JAK
- Linker for the activation of T cells: LAT
- Major Histocompatibility Complex: MHC
- Mammalian Target of Rapamycin : mTOR
- Mantle cell lymphoma: MCL
- Mitogen activated protein kinase: MAPK
- Oncogenic tyrosine kinase: OTK
- Peripheral blood mononuclear cell: PBMC
- Phosphatidyl Inositol 3 Kinase: PI3K
- Phosphotyrosine-binding protein: PTB
- Protein inhibitor of activated STATs: PIAS
- Severe Combined Immunodeficiency: SCID
- Signal transducer and activator of transcription: STAT
- SRC family kinases: SFK
- SRC Homology 2: SH2
- Standard Operating Procedure: SOP
- Suppressor of Cytokine Signal: SOCS
- Supramolecular activation cluster: SMAC
- T cell growth factors: TGCF: cytokines
- T lymphocyte receptor: TCR
- Tyrosine: Y
- Tyrosine Kinase 2: TYK2
- White blood cell: WBC
- World Health Organization: WHO

Appendix

Table 1: JAK3 Putative Substrates

DNA Repair and Remodeling

Protein Name	Protein Type
bromodomain adjacent to zinc finger domain, 1A	chromatin remodeling
cat eye syndrome chromosome region, candidate 2	chromatin remodeling
aprataxin and PNKP like factor	DNA repair
excision repair cross-complementing rodent repair deficiency, complementation group 4	DNA repair
Fanconi anemia, complementation group L	DNA repair
mutS homolog 2, colon cancer, nonpolyposis type 1	DNA repair
poly (ADP-ribose) polymerase family, member 14	DNA repair
replication protein A1, 70kDa	DNA repair
protection of telomeres 1 homolog (S. pombe)	DNA repair

Signal Transduction

Protein Name	Protein Type
activin A receptor, type I	kinase
anaplastic lymphoma receptor tyrosine kinase	kinase
cyclin-dependent kinase 5, regulatory subunit 1 (p35)	kinase
casein kinase 2, beta polypeptide	kinase
guanylate cyclase 2C (heat stable enterotoxin receptor)	kinase
guanylate cyclase 2C (heat stable enterotoxin receptor)	kinase
insulin receptor	kinase
inositol hexakisphosphate kinase 3	kinase
kinase suppressor of ras 2	kinase
LIM domain kinase 1	kinase
mitogen-activated protein kinase kinase kinase 15	kinase
neurotrophic tyrosine kinase, receptor, type 2	kinase
obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	kinase
p21 protein (Cdc42/Rac)-activated kinase 4	kinase
phosphoinositide-3-kinase, regulatory subunit 6	kinase
protein kinase C, beta	kinase
testis-specific kinase 1	kinase

transient receptor potential cation channel, subfamily M, member 7	kinase
TTK protein kinase	kinase
unc-51-like kinase 2	kinase
MAP3K12 binding inhibitory protein 1	kinase
inositol polyphosphate-5-phosphatase, 72 kDa	phosphatase
protein tyrosine phosphatase, non-receptor type 23	phosphatase
guanine nucleotide binding protein (G protein), alpha 13	Ga protein
brain-specific angiogenesis inhibitor 2	G-protein coupled receptor
calcitonin receptor-like	G-protein coupled receptor
corticotropin releasing hormone receptor 2	G-protein coupled receptor
G protein-coupled receptor 113	G-protein coupled receptor
latrophilin 2	G-protein coupled receptor
trace amine associated receptor 2	G-protein coupled receptor
Rho guanine nucleotide exchange factor (GEF) 11	GEF
dedicator of cytokinesis 1	GEF
adenylate cyclase 5	adenylate cycase
TBC1 domain family, member 25	GAP
TBC1 domain family, member 8 (with GRAM domain)	GAP
B-cell scaffold protein with ankyrin repeats 1	scaffolding protein
linker for activation of T cells family, member 2	scaffolding protein
arrestin, beta 2	scaffolding protein
T cell immunoreceptor with Ig and ITIM domains	transmembrane receptor
CD86 molecule	transmembrane receptor
C-type lectin domain family 4, member E	transmembrane receptor
deleted in colorectal carcinoma	transmembrane receptor
low density lipoprotein receptor-related protein 1B	transmembrane receptor
tumor necrosis factor receptor superfamily, member 8	transmembrane receptor
phospholipase C, gamma 1	phospholipase
patatin-like phospholipase domain containing 6	phospholipase

Transcription

Protein Name	Protein Type
calmodulin binding transcription activator 1	transcription regulator
ankyrin repeat and SOCS box containing 12	transcription regulator
CREB binding protein	transcription regulator
ecdysoneless homolog (Drosophila)	transcription regulator
EF-hand calcium binding domain 6	transcription regulator
E1A binding protein p300	transcription regulator
general transcription factor IIi	transcription regulator
INO80 complex subunit C	transcription regulator
interferon regulatory factor 9	transcription regulator
nuclear receptor corepressor 2	transcription regulator
nuclear transcription factor, X-box binding 1	transcription regulator
nuclear receptor binding SET domain protein 1	transcription regulator
PR domain containing 10	transcription regulator
pancreas specific transcription factor, 1a	transcription regulator
staphylococcal nuclease and tudor domain containing 1	transcription regulator
ventral anterior homeobox 1	transcription regulator
zinc finger protein, multitype 1	transcription regulator
zinc finger protein 205	transcription regulator
zinc finger, ZZ-type containing 3	transcription regulator
programmed cell death 4 (neoplastic transformation inhibitor)	transcriptional regulator
zinc finger, BED-type containing 6	transcriptional regulator
mediator complex subunit 13-like	transcriptional regulator
metastasis suppressor 1	transcriptional regulator

Translation

Protein Name	Protein Type
cysteinyl-tRNA synthetase	translation regulator
ribosomal protein L22-like 1	translation regulator
iron-responsive element binding protein 2	translation regulator
poly(A) binding protein interacting protein 1	translation regulator
DnaJ (Hsp40) homolog, subfamily C, member 18	Chaperone
senataxin	helicase
DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57	helicase
PRP3 pre-mRNA processing factor 3 homolog	mRNA processing
SON DNA binding protein	mRNA processing

Matrix, Cell Adhesion, and Cytoskeletion

Protein name	Protein type
coiled-coil domain containing 80	adhesion
cadherin 19, type 2	adhesion
cell adhesion molecule with homology to L1CAM	adhesion
contactin associated protein 1	adhesion
integrin, beta 8	adhesion
protocadherin 17	adhesion
protocadherin gamma subfamily B, 1	adhesion
erythrocyte membrane protein band 4.1-like 1	cytoskeletal
filamin A, alpha	cytoskeletal
kinesin family member 26A	cytoskeletal
La ribonucleoprotein domain family, member 1	cytoskeletal
myosin IB	cytoskeletal
SPRY domain containing 3	cytoskeletal
tight junction protein 1 (zona occludens 1)	adherin

Cell Metabolism

Protein Name	Protein Type
ATP/GTP binding protein-like 3	peptidase
calmegin	peptidase
N-acetylated alpha-linked acidic dipeptidase-like 2	peptidase
aminopeptidase puromycin sensitive	peptidase
ovochymase 2 (gene/pseudogene)	peptidase
ubiquitin specific peptidase 8	peptidase
ubiquitin specific peptidase 9, X-linked	peptidase
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	Sialyltransferase
inter-alpha (globulin) inhibitor H5-like	serine-type endopeptidase inhibitor activity
fucosyltransferase 9 (alpha (1,3) fucosyltransferase)	metabolic protein
methylsterol monooxygenase 1	metabolic protein
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	metabolic protein
glutaminase	metabolic protein
monoacylglycerol O-acyltransferase 2	metabolic protein
methylenetetrahydrofolate reductase (NAD(P)H)	metabolic protein
phosphatidylglycerophosphate synthase 1	metabolic protein

sphingomyelin synthase 2	metabolic protein
acyl-CoA synthetase medium-chain family member 3	metabolic protein
asparagine-linked glycosylation 13 homolog (S. cerevisiae)	Glycosyltransferase
catalase	catalase

Transport

Protein Name	Protein Type
calcium channel, voltage-dependent, alpha 2/delta subunit 3	ion channel
potassium channel tetramerisation domain containing 8	ion channel
sodium channel, voltage-gated, type IX, alpha subunit	ion channel
adaptor-related protein complex 3, beta 2 subunit	transporter
component of oligomeric golgi complex 8	transporter
solute carrier family 15 (oligopeptide transporter), member 1	transporter
sorting nexin 13	transporter
transmembrane 9 superfamily member 1	transporter
zinc finger, FYVE domain containing 16	transporter
calcium channel, voltage-dependent, alpha 2/delta subunit 3	ion channel
potassium channel tetramerisation domain containing 8	ion channel
sodium channel, voltage-gated, type IX, alpha subunit	ion channel
adaptor-related protein complex 3, beta 2 subunit	transporter
component of oligomeric golgi complex 8	transporter
solute carrier family 15 (oligopeptide transporter), member 1	transporter
sorting nexin 13	transporter
transmembrane 9 superfamily member 1	transporter

Ubiquitination

Protein Name	Protein Type
ubiquitin protein ligase E3A	Ubiquitin Ligase
ubiquitin protein ligase E3 component n-recognin 3 (putative)	Ubiquitin Ligase
F-box protein 25	Nucleus

Unknown

Protein Name	Protein Type
chromosome 10 open reading frame 140	unknown
chromosome 12 open reading frame 63	unknown
chromosome 18 open reading frame 34	unknown
chromosome 19 open reading frame 56	unknown

chromosome 19 open reading frame 59	unknown
chromosome 6 open reading frame 204	unknown
CUB and Sushi multiple domains 3	unknown
cell wall biogenesis 43 C-terminal homolog	unknown
disabled homolog 2, mitogen-responsive phosphoprotein	unknown
family with sequence similarity 135, member B	unknown
family with sequence similarity 164, member A	unknown
family with sequence similarity 187, member B	unknown
family with sequence similarity 188, member A	unknown
fer-1-like 6 (C. elegans)	unknown
FERM domain containing 7	unknown
hydrocephalus inducing homolog (mouse)	unknown
KIAA1324-like	unknown
lactamase, beta 2	unknown
leucine rich repeat containing 8 family, member C	unknown
matrix-remodelling associated 5	unknown
nanos homolog 2 (Drosophila)	unknown
neurobeachin-like 1	unknown
NLR family, pyrin domain containing 4	unknown
oxysterol binding protein-like 1A	unknown
par-3 partitioning defective 3 homolog B (C. elegans)	unknown
PHD finger protein 14	unknown
prostate stem cell antigen	unknown
patched domain containing 3	unknown
prostaglandin F2 receptor negative regulator	unknown
retinoic acid induced 2	unknown
retinitis pigmentosa GTPase regulator	unknown
sterile alpha motif domain containing 9	unknown
spermidine/spermine N1-acetyl transferase-like 1	unknown
SET binding factor 2	unknown
scratch homolog 2, zinc finger protein (Drosophila)	unknown
sel-1 suppressor of lin-12-like (C. elegans)	unknown
seizure related 6 homolog (mouse)-like	unknown
SLIT and NTRK-like family, member 4	unknown
syntaxin binding protein 5-like	unknown
transmembrane channel-like 5	unknown
transmembrane protein 69	unknown
tripartite motif containing 4	unknown
vestigial like 3 (Drosophila)	unknown
zinc finger, DBF-type containing 2	unknown
zinc finger, MIZ-type containing 1	unknown
estrogen receptor binding site associated, antigen, 9	unknown
schlafen family member 11	unknown
galactosylceramidase	unknown

calcineurin-like phosphoesterase domain containing 1	unknown

Pt6 JAK3 sequencing attached after Vita

```
 Consensus NNNNNNNNAANNNNGGCCAGTCCAGGCAGGTCTCAAACTCCT 42
Exon1templ ---------------------------------------- 0
        1F ---------------------------------------- 0
        1R NNNNNNNNAANNNNGGCCAGTCCAGGCAGGTCTCAAACTCCT 42

 Consensus GACCT----------------C-CGGCCTCCC-AAATGCTGT 84
Exon1templ ---------------------------------------- 0
        1F -----GNNNNNNNNNNNNNNNCNCGGCCTCCCNAAATGCTGT 37
        1R GACCTCAAGTGATCCTCCCGCCTCGGCCTCCCAAAATGCTGT 84

 Consensus GATTACAGGCATAAGCCACCGCACCCGGCCTCCAGCACTCCT 126
Exon1templ ---------------------------------------- 0
        1F GATTACAGGCATAAGCCACCGCACCCGGCCTCCAGCACTCCT 79
        1R GATTACAGGCATAAGCCACCGCACCCGGCCTCCAGCACTCCT 126

 Consensus TTCCATGCCCTCCCTGCTCAGAAGTCCAATCCCCTCTGACCA 168
Exon1templ ---------------------------------------- 0
        1F TTCCATGCCCTCCCTGCTCAGAAGTCCAATCCCCTCTGACCA 121
        1R TTCCATGCCCTCCCTGCTCAGAAGTCCAATCCCCTCTGACCA 168

 Consensus GGACTGAGGGGCTTTTTCTCTCTGTGCCCCAGGCAAGTTGCA 210
Exon1templ ---------------------------------------- 0
        1F GGACTGAGGGGCTTTTTCTCTCTGTGCCCCAGGCAAGTTGCA 163
        1R GGACTGAGGGGCTTTTTCTCTCTGTGCCCCAGGCAAGTTGCA 210

 Consensus CTCATGGCACCTCCAAGTGAAGAGACGCCCCTGATCCCTCAG 252
Exon1templ ---ATGGCACCTCCAAGTGAAGAGACGCCCCTGATCCCTCAG 39
        1F CTCATGGCACCTCCAAGTGAAGAGACGCCCCTGATCCCTCAG 205
        1R CTCATGGCACCTCCAAGTGAAGAGACGCCCCTGATCCCTCAG 252

 Consensus CGTTCATGCAGCCTCTTGTCCACGGAGGCTGGTGCCCTGCAT 294
Exon1templ CGTTCATGCAGCCTCTTGTCCACGGAGGCTGGTGCCCTGCAT 81
        1F CGTTCATGCAGCCTCTTGTCCACGGAGGCTGGTGCCCTGCAT 247
        1R CGTTCATGCAGCCTCTTGTCCACGGAGGCTGGTGCCCTGCAT 294

 Consensus GTGCTGCTGCCCGCTCGGGGCCCCGGGCCCCCCCAGCGCCTA 336
Exon1templ GTGCTGCTGCCCGCTCGGGGCCCCGGGCCCCCCCAGCGCCTA 123
        1F GTGCTGCTGCCCGCTCGGGGCCCCGGGCCCCCCCAGCGCCTA 289
        1R GTGCTGCTGCCCGCTCGGGGCCCCGGGCCCCCCCAGCGCCTA 336
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 Consensus TCTTTCTCCTTTGGGGACCACTTGGCTGAGGACCTGTGCGTG 378
Exon1templ TCTTTCTCCTTTGGGGACCACTTGGCTGAGGACCTGTGCGTG 165
        1F TCTTTCTCCTTTGGGGACCACTTGGCTGAGGACCTGTGCGTG 331
        1R TCTTTCTCCTTTGGGGACCACTTGGCTGAGGACCTGTGCGTG 378

 Consensus CAGGCTGCCAAGGCCAGCGGTGAGTGCATCCCTAGTGGATCG 420
Exon1templ CAGGCTGCCAAGGCCAGCG---------------------- 184
        1F CAGGCTGCCAAGGCCAGCGGTGAGTGCATCCCTAGTGGATCG 373
        1R CAGGCTGCCAAGGCCAGCGGTGAGTGCATCCCTAGTGGATCG 420

 Consensus GGCCAGAGGGAAGGAN-GGGCTGTGTGGGGCCAAGATTGGAA 462
Exon1templ ---------------------------------------- 184
        1F GGCCAGAGGGAAGGANNGGGCTGTGTGGGGCCAAGATTGGAA 415
        1R GGCCAGAGGGAAGGANGGGGCTGTGTGGGGCCAAGATTGGAA 462

 Consensus GCTGGAATAGTTGCCTGCAGAAGTCAGCATCGGAGCTGGGGC 504
Exon1templ ---------------------------------------- 184
        1F GCTGGAATAGTTGCCTGCAGAAGTCAGCATCGGAGCTGGGGC 457
        1R GCTGGAATAGTTGCCTGCAGAAGTCAGCATCGGAGCTGGGGC 504

 Consensus TTTGGGGGATGAGTAGGAGTTTTGTAATGGAGAAGGGTGTGC 546
Exon1templ ---------------------------------------- 184
        1F TTTGGGGGATGAGTAGGAGTTTTGTAATGGAGAAGGGTGTGC 499
        1R TTTGGGGGATGAGTAGGAGTTTTGTAATGGAGAAGGGTGTGC 546

 Consensus AGGGTTGGCTTC-GAGGCAGA-GGAA--GC------------ 588
Exon1templ ---------------------------------------- 184
        1F AGGGTTGGCTTCTGAGGCAGAGGGAATGGCCTGTGCAGACGG 541
        1R AGGGTTGGCTTCNGAGGCAGANGGAANNGCNNNNNNNNNNNN 588

 Consensus -----TGTGACGGCACATGAAGGGAACAGCTGGGTCATAGNT 630
Exon1templ ---------------------------------------- 184
        1F AGAGGTGTGACGGCACATGAAGGGAACAGCTGGGTCATAGNT 583
        1R NNNNN----------------------------------- 593

 Consensus GTTTCNNN                                  638
Exon1templ --------                                  184
        1F GTTTCNNN                                  591
        1R --------                                  593
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 Consensus TTNTGTNAAANGACGGCCAGTTTGAGGTATGGAAGGATCTGG 42
Exon2templ ---------------------------------------- 0
        2F ---------------------------------------- 0
        2R TTNTGTNAAANGACGGCCAGTTTGAGGTATGGAAGGATCTGG 42

 Consensus ACG------------C-GG---TCCT---GG-CACAGATGG 84
Exon2templ ---------------------------------------- 0
        2F ---NNNNNNNNNNNNNCNGGNNNTCCTNNNGG-CACAGATGG 38
        2R ACGGTTGGGTATGATGCTGGCACTCCTGAAGGGCACAGATGG 84

 Consensus GGTGACTCAGGAGGGAGCTGATGGGACCATCCCCTGTAGGCA 126
Exon2templ ----------------------------------GCA 3
        2F GGTGACTCAGGAGGGAGCTGATGGGACCATCCCCTGTAGGCA 80
        2R GGTGACTCAGGAGGGAGCTGATGGGACCATCCCCTGTAGGCA 126

 Consensus TCCTGCCTGTGTACCACTCCCTCTTTGCTCTGGCCACGGAGG 168
Exon2templ TCCTGCCTGTGTACCACTCCCTCTTTGCTCTGGCCACGGAGG 45
        2F TCCTGCCTGTGTACCACTCCCTCTTTGCTCTGGCCACGGAGG 122
        2R TCCTGCCTGTGTACCACTCCCTCTTTGCTCTGGCCACGGAGG 168

 Consensus ACCTGTCCTGCTGGTTCCCCCCGAGCCACATCTTCTCCGTGG 210
Exon2templ ACCTGTCCTGCTGGTTCCCCCCGAGCCACATCTTCTCCGTGG 87
        2F ACCTGTCCTGCTGGTTCCCCCCGAGCCACATCTTCTCCGTGG 164
        2R ACCTGTCCTGCTGGTTCCCCCCGAGCCACATCTTCTCCGTGG 210

 Consensus AGGATGCCAGCACCCAAGTCCTGCTGTACAGGATTCGGTAGG 252
Exon2templ AGGATGCCAGCACCCAAGTCCTGCTGTACAGGATTCG----- 124
        2F AGGATGCCAGCACCCAAGTCCTGCTGTACAGGATTCGGTAGG 206
        2R AGGATGCCAGCACCCAAGTCCTGCTGTACAGGATTCGGTAGG 252

 Consensus AAGTGCCCCCCAGCCCCCAGGGATTGTACAATTTTATCATCT 294
Exon2templ ---------------------------------------- 124
        2F AAGTGCCCCCCAGCCCCCAGGGATTGTACAATTTTATCATCT 248
        2R AAGTGCCCCCCAGCCCCCAGGGATTGTACAATTTTATCATCT 294

 Consensus CCTTGCATTTCGAGGTGCCCACACCCCTGCCCCAGGGAGGTA 336
Exon2templ ---------------------------------------- 124
        2F CCTTGCATTTCGAGGTGCCCACACCCCTGCCCCAGGGAGGTA 290
        2R CCTTGCATTTCGAGGTGCCCACACCCCTGCCCCAGGGAGGTA 336
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```
 Consensus TGGTCACTACCCATTTCTCAGATGAGGAAACAGACCAGAGAG 378
Exon2templ ---------------------------------------- 124
        2F TGGTCACTACCCATTTCTCAGATGAGGAAACAGACCAGAGAG 332
        2R TGGTCACTACCCATTTCTCAGATGAGGAAACAGACCAGAGAG 378

 Consensus GGTGGGTCACTTGCCCAAGGTCACACAGCAAGTTAAAGGTAC 420
Exon2templ ---------------------------------------- 124
        2F GGTGGGTCACTTGCCCAAGGTCACACAGCAAGTTAAAGGTAC 374
        2R GGTGGGTCACTTGCCCAAGGTCACACAGCAAGTTAAAGGTAC 420

 Consensus AAGCTGGGCTCTGTGAGGCCTCCGCAGAATCT-TCCCTCGCC 462
Exon2templ ---------------------------------------- 124
        2F AAGCTGGGCTCTGTGAGGCCTCCGCAGAATCTGTCCCTCGCC 416
        2R AAGCTGGGCTCTGTGAGGCCTCCGCAGAATCTNTCCCTCGCC 462

 Consensus CCCACCA-A--------------------GGTTGCACTTTC 504
Exon2templ ---------------------------------------- 124
        2F CCCACCATAATGTCACTCCTACTGAGGCTGGGTTGCACTTTC 458
        2R CCCACCANANNNNNNNNNNNNNNNNNNNN------------ 492

 Consensus ATCCCAGGGTTGGTCATANNNNNNNNNNN             534
Exon2templ ----------------------------             124
        2F ATCCCAGGGTTGGTCATANNNNNNNNNNN             488
        2R ----------------------------             492
```

```
 Consensus NTNNNNAAAACGACGGCCAGTTTTATCATCTCCTTGCATTTC 42
Exon3templ ----------------------------------------- 0
        3F ----------------------------------------- 0
        3R NTNNNNAAAACGACGGCCAGTTTTATCATCTCCTTGCATTTC 42

 Consensus GAGG----------C-----CC-GGG--GT-TGGTCACTACC 84
Exon3templ ----------------------------------------- 0
        3F ----NNNNNNNNNNCNNNNNCCNGGGNNGTNTGGTCACTACC 38
        3R GAGGTGCCCACACCCCTGCCCCAGGGAGGTATGGTCACTACC 84

 Consensus CATTTCTCAGATGAGGAAACAGACCAGAGAGGGTGGGTCACT 126
Exon3templ ----------------------------------------- 0
        3F CATTTCTCAGATGAGGAAACAGACCAGAGAGGGTGGGTCACT 80
        3R CATTTCTCAGATGAGGAAACAGACCAGAGAGGGTGGGTCACT 126

 Consensus TGCCCAAGGTCACACAGCAAGTTAAAGGTACAAGCTGGGCTC 168
Exon3templ ----------------------------------------- 0
        3F TGCCCAAGGTCACACAGCAAGTTAAAGGTACAAGCTGGGCTC 122
        3R TGCCCAAGGTCACACAGCAAGTTAAAGGTACAAGCTGGGCTC 168

 Consensus TGTGAGGCCTCCGCAGAATCTGTCCCTCGCCCCCACCATAAT 210
Exon3templ ----------------------------------------- 0
        3F TGTGAGGCCTCCGCAGAATCTGTCCCTCGCCCCCACCATAAT 164
        3R TGTGAGGCCTCCGCAGAATCTGTCCCTCGCCCCCACCATAAT 210

 Consensus GTCACTCCTACTGAGGCTGGGTTGCACTTTCATCCCAGGGTT 252
Exon3templ ----------------------------------------- 0
        3F GTCACTCCTACTGAGGCTGGGTTGCACTTTCATCCCAGGGTT 206
        3R GTCACTCCTACTGAGGCTGGGTTGCACTTTCATCCCAGGGTT 252

 Consensus CTCTCCTCTCCTCACAGCTTTTACTTCCCCAATTGGTTTGGG 294
Exon3templ ----------------CTTTTACTTCCCCAATTGGTTTGGG 25
        3F CTCTCCTCTCCTCACAGCTTTTACTTCCCCAATTGGTTTGGG 248
        3R CTCTCCTCTCCTCACAGCTTTTACTTCCCCAATTGGTTTGGG 294

 Consensus CTGGAGAAGTGCCACCGCTTCGGGCTACGCAAGGATTTGGCC 336
Exon3templ CTGGAGAAGTGCCACCGCTTCGGGCTACGCAAGGATTTGGCC 67
        3F CTGGAGAAGTGCCACCGCTTCGGGCTACGCAAGGATTTGGCC 290
        3R CTGGAGAAGTGCCACCGCTTCGGGCTACGCAAGGATTTGGCC 336
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 Consensus AGTGCTATCCTTGACCTGCCAGTCCTGGAGCACCTCTTTGCC 378
Exon3templ AGTGCTATCCTTGACCTGCCAGTCCTGGAGCACCTCTTTGCC 109
        3F AGTGCTATCCTTGACCTGCCAGTCCTGGAGCACCTCTTTGCC 332
        3R AGTGCTATCCTTGACCTGCCAGTCCTGGAGCACCTCTTTGCC 378

 Consensus CAGGTGGGGTTCTGCCTGGGGTTTGACCCAGGGGGTTGGGGG 420
Exon3templ CAG-------------------------------------- 112
        3F CAGGTGGGGTTCTGCCTGGGGTTTGACCCAGGGGGTTGGGGG 374
        3R CAGGTGGGGTTCTGCCTGGGGTTTGACCCAGGGGGTTGGGGG 420

 Consensus TCCAAGGGGCAACA-GAGG----------------TGGGGC 462
Exon3templ ---------------------------------------- 112
        3F TCCAAGGGGCAACATGAGGACTGGCATGCAATCAGGTGGGGC 416
        3R TCCAAGGGGCAACANGAGGNNNNNNNNNNNNNNNN------ 456

 Consensus CTCGTCTGACCCTCCCTGTGGGTCATAGCTGTTTCNNG     500
Exon3templ ------------------------------------     112
        3F CTCGTCTGACCCTCCCTGTGGGTCATAGCTGTTTCNNG     454
        3R ------------------------------------     456
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 Consensus NNNTNGNNNNNNNNGNNNGTNGTGNNNGNNTNCNNGNNACAG 42
Exon4templ ----------------------------------------- 0
        4F ----------------------------------------- 0
        4R NNNTNGNNNNNNNNGNNNGTNGTGNNNGNNTNCNNGNNACAG 42

 Consensus ANGNNGNNANNNNNNNGAAAGNGNNNGNNATTTTNNNACANG 84
Exon4templ ----------------------------------------- 0
        4F ----------------------------------------- 0
        4R ANGNNGNNANNNNNNNGAAAGNGNNNGNNATTTTNNNACANG 84

 Consensus GNNNGNNNNNANNNANNNGNGTNGGNGNNNNNNNNNAANNTG 126
Exon4templ ----------------------------------------- 0
        4F ----------------------------------------- 0
        4R GNNNGNNNNNANNNANNNGNGTNGGNGNNNNNNNNNAANNTG 126

 Consensus TAAAACGACGGCCAGTCAGGTTAACAACAGGGCTTGAAGTTG 168
Exon4templ ----------------------------------------- 0
        4F ----------------------------------------- 0
        4R TAAAACGACGGCCAGTCAGGTTAACAACAGGGCTTGAAGTTG 168

 Consensus ----------------GCTC----TGGCGGCCCCCC-GCACC 210
Exon4templ -----------------------------------CACC 4
        4F NNNNNNNNNNNNNNNNGCTCNNN-TGGCGGCCCCCCNGCACC 41
        4R GGTGGCCTCAGCTGATGCTCCCTGTGGCGGCCCCCCAGCACC 210

 Consensus GCAGTGACCTGGTGAGTGGGCGCCTCCCCGTGGGCCTCAGTC 252
Exon4templ GCAGTGACCTGGTGAGTGGGCGCCTCCCCGTGGGCCTCAGTC 46
        4F GCAGTGACCTGGTGAGTGGGCGCCTCCCCGTGGGCCTCAGTC 83
        4R GCAGTGACCTGGTGAGTGGGCGCCTCCCCGTGGGCCTCAGTC 252

 Consensus TCAAGGAGCAGGGTGAGTGTCTCAGCCTGGCCGTGTTGGACC 294
Exon4templ TCAAGGAGCAGGGTGAGTGTCTCAGCCTGGCCGTGTTGGACC 88
        4F TCAAGGAGCAGGGTGAGTGTCTCAGCCTGGCCGTGTTGGACC 125
        4R TCAAGGAGCAGGGTGAGTGTCTCAGCCTGGCCGTGTTGGACC 294

 Consensus TGGCCCGGATGGCGCGAGAGCAGGCCCAGCGGCCGGGAGAGC 336
Exon4templ TGGCCCGGATGGCGCGAGAGCAGGCCCAGCGGCCGGGAGAGC 130
        4F TGGCCCGGATGGCGCGAGAGCAGGCCCAGCGGCCGGGAGAGC 167
        4R TGGCCCGGATGGCGCGAGAGCAGGCCCAGCGGCCGGGAGAGC 336
```

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 Consensus TGCTGAAGACTGTCAGGTGAGAGCCACCAGGCTGTGGGGACG 378
Exon4templ TGCTGAAGACTGTCAG------------------------- 146
        4F TGCTGAAGACTGTCAGGTGAGAGCCACCAGGCTGTGGGGACG 209
        4R TGCTGAAGACTGTCAGGTGAGAGCCACCAGGCTGTGGGGACG 378

 Consensus GCCTCTGCTTGGGAGTGAGCAACGTGGGCTCCATCGGGGCTT 420
Exon4templ ---------------------------------------- 146
        4F GCCTCTGCTTGGGAGTGAGCAACGTGGGCTCCATCGGGGCTT 251
        4R GCCTCTGCTTGGGAGTGAGCAACGTGGGCTCCATCGGGGCTT 420

 Consensus -GCCGGGCTCCC-CC--G----------------TTTCAGGG 462
Exon4templ ---------------------------------------- 146
        4F TGCCGGGCTCCCACCATGGAGTTCTCCTGCAAGCTTTCAGGG 293
        4R -GCCGGGCTCCCNCCNNGNNNNNNNNNNNNNNN-------- 453

 Consensus TGTTCCTATGACCCGGTCATAGCTGTTTCCTGNNN        497
Exon4templ ----------------------------------        146
        4F TGTTCCTATGACCCGGTCATAGCTGTTTCCTGNNN        328
        4R ----------------------------------        453
```

```
 Consensus TGTAAAANGACGGCCAGTCCGGTCCTCATACCTGACCCTGAA 42
Exon5templ ---------------------------------------- 0
       5aF ---------------------------------------- 0
       5aR TGTAAAANGACGGCCAGTCCGGTCCTCATACCTGACCCTGAA 42
       5bF ---------------------------------------- 0
       5bR ---------------------------------------- 0

 Consensus TG---------------C--G-G-C--A-CTAGGGCC----- 84
Exon5templ ---------------------------------------- 0
       5aF --NNNNNNNNNNNNNNGCNNGNGNCNNA-CTAGGGCCGCACC 39
       5aR TGAGAGTCTGTGTGTGCCTGGTGCCCCAACTAGGGCCGCACC 84
       5bF ---------------------------------------- 0
       5bR -----------------------------------NTTNN 5

 Consensus --A----C-GG-C--A--CCTGGGTTTGTGTGTGTCCCCGCG 126
Exon5templ ---------------------------------------- 0
       5aF CCAGCCCCTGGGCTAAAGCCTGGGTTTGTGTGTGTCCCCGCG 81
       5aR CCAGCCCCTGGGCTAAAGCCTGGGTTTGTGTGTGTCCCCGCG 126
       5bF ---------------------------------------- 0
       5bR NNAANNNC-GGCC--AGTCCTGGGTTTGTGTGTGTCCCCGCG 44

 Consensus GGG------------CT---GG-CGGCTCCCTCCCCTCC-A 168
Exon5templ ---------------------------------------- 0
       5aF GGGACCCCTCCCGACGCTGAGGGCCGGCTCCCTCCCCTCCAA 123
       5aR GGGACCCCTCCCGACGCTGAGGGCCGGCTCCCTCCCCTCCAA 168
       5bF ---NNNNNNNNNNNNNCTNNNGGNCGGCTCCCTCCCCTCCNA 39
       5bR GGGACCCCTCCCGACGCTGAGGGCCGGCTCCCTCCCCTCCAA 86

 Consensus CCCCTGCAGCTACAAGGCCTGCCTACCCCCAAGCCTGCGCGA 210
Exon5templ ---------CTACAAGGCCTGCCTACCCCCAAGCCTGCGCGA 33
       5aF CCCCTGCAGCTACAAGGCCTGCCTACCCCCAAGCCTGCGCGA 165
       5aR CCCCTGCAGCTACAAGGCCTGCCTACCCCCAAGCCTGCGCGA 210
       5bF CCCCTGCAGCTACAAGGCCTGCCTACCCCCAAGCCTGCGCGA 81
       5bR CCCCTGCAGCTACAAGGCCTGCCTACCCCCAAGCCTGCGCGA 128

 Consensus CCTGATCCAGGGCCTGAGCTTCGTGACGCGGAGGCGTATTCG 252
Exon5templ CCTGATCCAGGGCCTGAGCTTCGTGACGCGGAGGCGTATTCG 75
       5aF CCTGATCCAGGGCCTGAGCTTCGTGACGCGGAGGCGTATTCG 207
       5aR CCTGATCCAGGGCCTGAGCTTCGTGACGCGGAGGCGTATTCG 252
       5bF CCTGATCCAGGGCCTGAGCTTCGTGACGCGGAGGCGTATTCG 123
       5bR CCTGATCCAGGGCCTGAGCTTCGTGACGCGGAGGCGTATTCG 170
```

```
 Consensus GAGGACGGTGCGCAGAGCCCTGCGCCGCGTGGCCGCCTGCCA 294
Exon5templ GAGGACGGTGCGCAGAGCCCTGCGCCGCGTGGCCGCCTGCCA 117
       5aF GAGGACGGTGCGCAGAGCCCTGCGCCGCGTGGCCGCCTGCCA 249
       5aR GAGGACGGTGCGCAGAGCCCTGCGCCGCGTGGCCGCCTGCCA 294
       5bF GAGGACGGTGCGCAGAGCCCTGCGCCGCGTGGCCGCCTGCCA 165
       5bR GAGGACGGTGCGCAGAGCCCTGCGCCGCGTGGCCGCCTGCCA 212

 Consensus GGCAGACCGGCACTCGCTCATGGCCAAGTACATCATGGACCT 336
Exon5templ GGCAGACCGGCACTCGCTCATGGCCAAGTACATCATGGACCT 159
       5aF GGCAGACCGGCACTCGCTCATGGCCAAGTACATCATGGACCT 291
       5aR GGCAGACCGGCACTCGCTCATGGCCAAGTACATCATGGACCT 336
       5bF GGCAGACCGGCACTCGCTCATGGCCAAGTACATCATGGACCT 207
       5bR GGCAGACCGGCACTCGCTCATGGCCAAGTACATCATGGACCT 254

 Consensus GGAGCGGCTGGATCCAGCCGGGGCCGCCGAGACCTTCCACGT 378
Exon5templ GGAGCGGCTGGATCCAGCCGGGGCCGCCGAGACCTTCCACGT 201
       5aF GGAGCGGCTGGATCCAGCCGGGGCCGCCGAGACCTTCCACGT 333
       5aR GGAGCGGCTGGATCCAGCCGGGGCCGCCGAGACCTTCCACGT 378
       5bF GGAGCGGCTGGATCCAGCCGGGGCCGCCGAGACCTTCCACGT 249
       5bR GGAGCGGCTGGATCCAGCCGGGGCCGCCGAGACCTTCCACGT 296

 Consensus GGGCCTCCCTGGGGCCCTTGGTGGCCACGACGGGCTGGGGCT 420
Exon5templ GGGCCTCCCTGGGGCCCTTGGTGGCCACGACGGGCTGGGGCT 243
       5aF GGGCCTCCCTGGGGCCCTTGGTGGCCACGACGGGCTGGGGCT 375
       5aR GGGCCTCCCTGGGGCCCTTGGTGGCCACGACGGGCTGGGGCT 420
       5bF GGGCCTCCCTGGGGCCCTTGGTGGCCACGACGGGCTGGGGCT 291
       5bR GGGCCTCCCTGGGGCCCTTGGTGGCCACGACGGGCTGGGGCT 338

 Consensus GCTCCGCGTGGCTGGTGACGGCGGCATCGCCTGGACCCAGGG 462
Exon5templ GCTCCGCGTGGCTGGTGACGGCGGCATCGCCTGGACCCAGGG 285
       5aF GCTCCGCGTGGCTGGTGACGGCGGCATCGCCTGGACCCAGGG 417
       5aR GCTCCGCGTGGCTGGTGACGGCGGCATCGCCTGGACCCAGGG 462
       5bF GCTCCGCGTGGCTGGTGACGGCGGCATCGCCTGGACCCAGGG 333
       5bR GCTCCGCGTGGCTGGTGACGGCGGCATCGCCTGGACCCAGGG 380

 Consensus AGAACAGGAGGTGAGGGCGGACTCCCCCGCTGGGCGGGGCCA 504
Exon5templ AGAACAGGAG------------------------------ 295
       5aF AGAACAGGAGGTGAGGGCGGACTCCCCCGCTGGGCGGGGCCA 459
       5aR AGAACAGGAGGTGAGGGCGGACTCCCCCGCTGGGCGGGGCCA 504
       5bF AGAACAGGAGGTGAGGGCGGACTCCCCCGCTGGGCGGGGCCA 375
       5bR AGAACAGGAGGTGAGGGCGGACTCCCCCGCTGGGCGGGGCCA 422
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 Consensus --G------------------GGGGCCGGAGAGTGGTAGGG 546
Exon5templ ---------------------------------------- 295
       5aF ACGTGGGGGCGGGGCTCGGGGAGGGGCCGGAGAGTGGTAGGG 501
       5aR NNGNNNNNNNNNNNNNNNNN------------------- 526
       5bF ACGTGGGGGCGGGGCTCGGGGAGGGGCCGGAGAGTGGTAGGG 417
       5bR ACGTGGGGGCGGGGCTCGGGGAGGGGCCGGAGAGTGGTAGGG 464

 Consensus GATGTGGG-C--AGC-----C---AGACTTGGGGAAGTGGGC 588
Exon5templ ---------------------------------------- 295
       5aF GATGTGGGTCATAGCTGTTTCCTN----------------- 525
       5aR ---------------------------------------- 526
       5bF GATGTGGGGCGGAGCCAAAACGAAAGACTTGGGGAAGTGGGC 459
       5bR GATGTGGGGCGGAGCCAAAACGAAAGACTTGGGGAAGTGGGC 506

 Consensus GAGGCTTAATGAGGGGCGGGGCT-AG--------G------- 630
Exon5templ ---------------------------------------- 295
       5aF ---------------------------------------- 525
       5aR ---------------------------------------- 526
       5bF GAGGCTTAATGAGGGGCGGGGCTTAGTGAGGGAGGAGACTGC 501
       5bR GAGGCTTAATGAGGGGCGGGGCTNAGNNNNNNNNGNNNNNNN 548

 Consensus ------GGGAGGGGCAAACTGAGTGAAGGGTTGGGTCATAGC 672
Exon5templ ---------------------------------------- 295
       5aF ---------------------------------------- 525
       5aR ---------------------------------------- 526
       5bF GGGAATGGGAGGGGCAAACTGAGTGAAGGGTTGGGTCATAGC 543
       5bR NNNNNN---------------------------------- 554

 Consensus TGTTTCNNG                                681
Exon5templ ---------                                295
       5aF ---------                                525
       5aR ---------                                526
       5bF TGTTTCNNG                                552
       5bR ---------                                554
```

```
 Consensus NNTTTTTTTTTNNNNAACGACGNCCAGTAAGGGATAGGGAGT 42
Exon6templ ---------------------------------------- 0
        6F ---------------------------------------- 0
        6R NNTTTTTTTTTNNNNAACGACGNCCAGTAAGGGATAGGGAGT 42

 Consensus GGATGGTGTGGCT--------------GGGCG-GGTTTGGC 84
Exon6templ ---------------------------------------- 0
        6F ------------NNNNNNNNNNNNNNNGGGCGNGGTTTGGC 29
        6R GGATGGTGTGGCTTGGGGGTGGGTTCATGGGCGTGGTTTGGC 84

 Consensus GGGGTCC-GCTGGGCCCCCACTTCGGTACTCCCCCTCCTTCC 126
Exon6templ ---------------------------------------- 0
        6F GGGGTCCNGCTGGGCCCCCACTTCGGTACTCCCCCTCCTTCC 71
        6R GGGGTCCAGCTGGGCCCCCACTTCGGTACTCCCCCTCCTTCC 126

 Consensus CAGGTCCTCCAGCCCTTCTGCGACTTTCCAGAAATCGTAGAC 168
Exon6templ ---GTCCTCCAGCCCTTCTGCGACTTTCCAGAAATCGTAGAC 39
        6F CAGGTCCTCCAGCCCTTCTGCGACTTTCCAGAAATCGTAGAC 113
        6R CAGGTCCTCCAGCCCTTCTGCGACTTTCCAGAAATCGTAGAC 168

 Consensus ATTAGCATCAAGCAGGCCCCGCGCGTTGGCCCGGCCGGAGAG 210
Exon6templ ATTAGCATCAAGCAGGCCCCGCGCGTTGGCCCGGCCGGAGAG 81
        6F ATTAGCATCAAGCAGGCCCCGCGCGTTGGCCCGGCCGGAGAG 155
        6R ATTAGCATCAAGCAGGCCCCGCGCGTTGGCCCGGCCGGAGAG 210

 Consensus CACCGCCTGGTCACTGTTACCAGGACAGACAACCAGATTTTA 252
Exon6templ CACCGCCTGGTCACTGTTACCAGGACAGACAACCAGATTTTA 123
        6F CACCGCCTGGTCACTGTTACCAGGACAGACAACCAGATTTTA 197
        6R CACCGCCTGGTCACTGTTACCAGGACAGACAACCAGATTTTA 252

 Consensus GTGGGTGCAGGATTCCCCTCCCCTTCAGCCTTACCCCGAGGG 294
Exon6templ ---------------------------------------- 123
        6F GTGGGTGCAGGATTCCCCTCCCCTTCAGCCTTACCCCGAGGG 239
        6R GTGGGTGCAGGATTCCCCTCCCCTTCAGCCTTACCCCGAGGG 294

 Consensus CGGGACCGGCACCCTCGGGTTTCACTGGGCTCTGACGCTTGT 336
Exon6templ ---------------------------------------- 123
        6F CGGGACCGGCACCCTCGGGTTTCACTGGGCTCTGACGCTTGT 281
        6R CGGGACCGGCACCCTCGGGTTTCACTGGGCTCTGACGCTTGT 336
```

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 Consensus CCCTCGCAGGAGGCCGAGTTCCCAGGGCTGCCCGAGGCTCTG 378
Exon6templ ----------------------------------------- 123
        6F CCCTCGCAGGAGGCCGAGTTCCCAGGGCTGCCCGAGGCTCTG 323
        6R CCCTCGCAGGAGGCCGAGTTCCCAGGGCTGCCCGAGGCTCTG 378

 Consensus TCGTTCGTGGCGCTCGTGGACGGCTACTTCCGGCTGACCACG 420
Exon6templ ----------------------------------------- 123
        6F TCGTTCGTGGCGCTCGTGGACGGCTACTTCCGGCTGACCACG 365
        6R TCGTTCGTGGCGCTCGTGGACGGCTACTTCCGGCTGACCACG 420

 Consensus GACTCCCAGCACTTCTTCTGCAAGGAGGTGGCACCGCCGAGG 462
Exon6templ ----------------------------------------- 123
        6F GACTCCCAGCACTTCTTCTGCAAGGAGGTGGCACCGCCGAGG 407
        6R GACTCCCAGCACTTCTTCTGCAAGGAGGTGGCACCGCCGAGG 462

 Consensus CTGCTGGAGGAAGTGGCCGAGCAGTGCCACGGCCCCATCACG 504
Exon6templ ----------------------------------------- 123
        6F CTGCTGGAGGAAGTGGCCGAGCAGTGCCACGGCCCCATCACG 449
        6R CTGCTGGAGGAAGTGGCCGAGCAGTGCCACGGCCCCATCACG 504

 Consensus TAAGGACCTG-CCCCCATTCCCGGCCTCTGTGGCCACTCAGG 546
Exon6templ ----------------------------------------- 123
        6F TAAGGACCTGTCCCCCATTCCCGGCCTCTGTGGCCACTCAGG 491
        6R TAAGGACCTGNCCCCCATTCCCGGCCTCTGTGGCCACTCAGG 546

 Consensus GCCCCTCCCCTTCTCTA-GC---------------------- 588
Exon6templ ----------------------------------------- 123
        6F GCCCCTCCCCTTCTCTATGCCTCAGTGTCCTCACCTTCCAGG 533
        6R GCCCCTCCCCTTCTCTANGCNNNNNNNNNNNNNNNNNNNNN 588

 Consensus -GCCCTGGACAGGGGTCAAGTTTTCAGGTCATAGCTGNNNNN 630
Exon6templ ----------------------------------------- 123
        6F AGCCCTGGACAGGGGTCAAGTTTTCAGGTCATAGCTGNNNNN 575
        6R N----------------------------------------- 589

 Consensus NNNNN                                      635
Exon6templ -----                                      123
        6F NNNNN                                      580
        6R -----                                      589
```

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 Consensus NNTTTTTTTTTNNNNAACGACGNCCAGTAAGGGATAGGGAGT 42
Exon7templ ---------------------------------------- 0
        7F ---------------------------------------- 0
        7R NNTTTTTTTTTNNNNAACGACGNCCAGTAAGGGATAGGGAGT 42

 Consensus GGATGGTGTGGCT--------------GGGCG-GGTTTGGC 84
Exon7templ ---------------------------------------- 0
        7F -------------NNNNNNNNNNNNNNNGGGCGNGGTTTGGC 29
        7R GGATGGTGTGGCTTGGGGGTGGGTTCATGGGCGTGGTTTGGC 84

 Consensus GGGGTCC-GCTGGGCCCCCACTTCGGTACTCCCCCTCCTTCC 126
Exon7templ ---------------------------------------- 0
        7F GGGGTCCNGCTGGGCCCCCACTTCGGTACTCCCCCTCCTTCC 71
        7R GGGGTCCAGCTGGGCCCCCACTTCGGTACTCCCCCTCCTTCC 126

 Consensus CAGGTCCTCCAGCCCTTCTGCGACTTTCCAGAAATCGTAGAC 168
Exon7templ ---------------------------------------- 0
        7F CAGGTCCTCCAGCCCTTCTGCGACTTTCCAGAAATCGTAGAC 113
        7R CAGGTCCTCCAGCCCTTCTGCGACTTTCCAGAAATCGTAGAC 168

 Consensus ATTAGCATCAAGCAGGCCCCGCGCGTTGGCCCGGCCGGAGAG 210
Exon7templ ---------------------------------------- 0
        7F ATTAGCATCAAGCAGGCCCCGCGCGTTGGCCCGGCCGGAGAG 155
        7R ATTAGCATCAAGCAGGCCCCGCGCGTTGGCCCGGCCGGAGAG 210

 Consensus CACCGCCTGGTCACTGTTACCAGGACAGACAACCAGATTTTA 252
Exon7templ ---------------------------------------- 0
        7F CACCGCCTGGTCACTGTTACCAGGACAGACAACCAGATTTTA 197
        7R CACCGCCTGGTCACTGTTACCAGGACAGACAACCAGATTTTA 252

 Consensus GTGGGTGCAGGATTCCCCTCCCCTTCAGCCTTACCCCGAGGG 294
Exon7templ ---------------------------------------- 0
        7F GTGGGTGCAGGATTCCCCTCCCCTTCAGCCTTACCCCGAGGG 239
        7R GTGGGTGCAGGATTCCCCTCCCCTTCAGCCTTACCCCGAGGG 294

 Consensus CGGGACCGGCACCCTCGGGTTTCACTGGGCTCTGACGCTTGT 336
Exon7templ ---------------------------------------- 0
        7F CGGGACCGGCACCCTCGGGTTTCACTGGGCTCTGACGCTTGT 281
        7R CGGGACCGGCACCCTCGGGTTTCACTGGGCTCTGACGCTTGT 336
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```
 Consensus CCCTCGCAGGAGGCCGAGTTCCCAGGGCTGCCCGAGGCTCTG 378
Exon7templ ---------GAGGCCGAGTTCCCAGGGCTGCCCGAGGCTCTG 33
        7F CCCTCGCAGGAGGCCGAGTTCCCAGGGCTGCCCGAGGCTCTG 323
        7R CCCTCGCAGGAGGCCGAGTTCCCAGGGCTGCCCGAGGCTCTG 378

 Consensus TCGTTCGTGGCGCTCGTGGACGGCTACTTCCGGCTGACCACG 420
Exon7templ TCGTTCGTGGCGCTCGTGGACGGCTACTTCCGGCTGACCACG 75
        7F TCGTTCGTGGCGCTCGTGGACGGCTACTTCCGGCTGACCACG 365
        7R TCGTTCGTGGCGCTCGTGGACGGCTACTTCCGGCTGACCACG 420

 Consensus GACTCCCAGCACTTCTTCTGCAAGGAGGTGGCACCGCCGAGG 462
Exon7templ GACTCCCAGCACTTCTTCTGCAAGGAGGTGGCACCGCCGAGG 117
        7F GACTCCCAGCACTTCTTCTGCAAGGAGGTGGCACCGCCGAGG 407
        7R GACTCCCAGCACTTCTTCTGCAAGGAGGTGGCACCGCCGAGG 462

 Consensus CTGCTGGAGGAAGTGGCCGAGCAGTGCCACGGCCCCATCACG 504
Exon7templ CTGCTGGAGGAAGTGGCCGAGCAGTGCCACGGCCCCATCAC- 158
        7F CTGCTGGAGGAAGTGGCCGAGCAGTGCCACGGCCCCATCACG 449
        7R CTGCTGGAGGAAGTGGCCGAGCAGTGCCACGGCCCCATCACG 504

 Consensus TAAGGACCTG-CCCCCATTCCCGGCCTCTGTGGCCACTCAGG 546
Exon7templ ---------------------------------------- 158
        7F TAAGGACCTGTCCCCCATTCCCGGCCTCTGTGGCCACTCAGG 491
        7R TAAGGACCTGNCCCCCATTCCCGGCCTCTGTGGCCACTCAGG 546

 Consensus GCCCCTCCCCTTCTCTA-GC---------------------- 588
Exon7templ ---------------------------------------- 158
        7F GCCCCTCCCCTTCTCTATGCCTCAGTGTCCTCACCTTCCAGG 533
        7R GCCCCTCCCCTTCTCTANGCNNNNNNNNNNNNNNNNNNNNN 588

 Consensus -GCCCTGGACAGGGGTCAAGTTTTCAGGTCATAGCTGNNNNN 630
Exon7templ ---------------------------------------- 158
        7F AGCCCTGGACAGGGGTCAAGTTTTCAGGTCATAGCTGNNNNN 575
        7R N------------------------------------- 589

 Consensus NNNNN                                    635
Exon7templ -----                                    158
        7F NNNNN                                    580
        7R -----                                    589
```

```
 Consensus NNNTGTAAAACGACGGCCAGTAAGGATCCCAGGGCTACAGAG 42
Exon8templ ----------------------------------------- 0
        8F ----------------------------------------- 0
        8R NNNTGTAAAACGACGGCCAGTAAGGATCCCAGGGCTACAGAG 42

 Consensus GT-------------------CTCTCTGTCTTCTTCTATCT 84
Exon8templ ----------------------------------------- 0
        8F --NNNNNNNNNNNNNNNNNNNCTCTCTGTCTTCTTCTATCT 40
        8R GTACCTGAATTTGAGCCCAGGTCTCTCTGTCTTCTTCTATCT 84

 Consensus CTGACTCCTCCCCATTCCCTCTCACCTTCCCCCACAGTCTGG 126
Exon8templ ----------------------------------TCTGG 5
        8F CTGACTCCTCCCCATTCCCTCTCACCTTCCCCCACAGTCTGG 82
        8R CTGACTCCTCCCCATTCCCTCTCACCTTCCCCCACAGTCTGG 126

 Consensus ACTTTGCCATCAACAAGCTCAAGACTGGGGGCTCACGTCCTG 168
Exon8templ ACTTTGCCATCAACAAGCTCAAGACTGGGGGCTCACGTCCTG 47
        8F ACTTTGCCATCAACAAGCTCAAGACTGGGGGCTCACGTCCTG 124
        8R ACTTTGCCATCAACAAGCTCAAGACTGGGGGCTCACGTCCTG 168

 Consensus GCTCCTATGTTCTCCGCCGCAGCCCCCAGGACTTTGACAGCT 210
Exon8templ GCTCCTATGTTCTCCGCCGCAGCCCCCAGGACTTTGACAGCT 89
        8F GCTCCTATGTTCTCCGCCGCAGCCCCCAGGACTTTGACAGCT 166
        8R GCTCCTATGTTCTCCGCCGCAGCCCCCAGGACTTTGACAGCT 210

 Consensus TCCTCCTCACTGTCTGTGTCCAGGTCGGTCTACTGCTAGGGT 252
Exon8templ TCCTCCTCACTGTCTGTGTCCAG------------------- 112
        8F TCCTCCTCACTGTCTGTGTCCAGGTCGGTCTACTGCTAGGGT 208
        8R TCCTCCTCACTGTCTGTGTCCAGGTCGGTCTACTGCTAGGGT 252

 Consensus GGGTAGTGGAGGGCTGCCTGGAGGAGGTGACGTTTGAATTGA 294
Exon8templ ----------------------------------------- 112
        8F GGGTAGTGGAGGGCTGCCTGGAGGAGGTGACGTTTGAATTGA 250
        8R GGGTAGTGGAGGGCTGCCTGGAGGAGGTGACGTTTGAATTGA 294

 Consensus GATTTAAAAGATCAGTCAGCATTTGGTTCCTGAAGAATAGGA 336
Exon8templ ----------------------------------------- 112
        8F GATTTAAAAGATCAGTCAGCATTTGGTTCCTGAAGAATAGGA 292
        8R GATTTAAAAGATCAGTCAGCATTTGGTTCCTGAAGAATAGGA 336
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```
 Consensus GGGAAAAGACACCCCCGGTGAACAGAACAGCATATTCAAAGG 378
Exon8templ ---------------------------------------- 112
        8F GGGAAAAGACACCCCCGGTGAACAGAACAGCATATTCAAAGG 334
        8R GGGAAAAGACACCCCCGGTGAACAGAACAGCATATTCAAAGG 378

 Consensus TCTAAAGACTGGAATGAGTTCATGGTGCTTTAGGAGAAAGGA 420
Exon8templ ---------------------------------------- 112
        8F TCTAAAGACTGGAATGAGTTCATGGTGCTTTAGGAGAAAGGA 376
        8R TCTAAAGACTGGAATGAGTTCATGGTGCTTTAGGAGAAAGGA 420

 Consensus C-GAG-C----------------CCTGTAATCCCAGCACT 462
Exon8templ ---------------------------------------- 112
        8F CTGAGGCTGGGCACAGTGGCTTACGCCTGTAATCCCAGCACT 418
        8R CNGAGNCNNNNNNNNNNNNNNNNN---------------- 445

 Consensus TTGGGAGGGTCATAGCTGTTTCCTG                 487
Exon8templ ------------------------                 112
        8F TTGGGAGGGTCATAGCTGTTTCCTG                 443
        8R ------------------------                 445
```

```
 Consensus NNTTTNTNNANNNNNNNNCCGGGGGGNNNAAACCCNNNNCCN 42
Exon9templ ----------------------------------------- 0
9Fnewprime ----------------------------------------- 0
        9R NNTTTNTNNANNNNNNNNCCGGGGGGNNNAAACCCNNNNCCN 42

 Consensus NTNNNCNAAAAAAAAAAAAAAAAAGAAAAAAAAGGAAGAAGG 84
Exon9templ ----------------------------------------- 0
9Fnewprime ----------------------------------------- 0
        9R NTNNNCNAAAAAAAAAAAAAAAAAGAAAAAAAAGGAAGAAGG 84

 Consensus ACTGAGAAGGAGAGTGTCTGTCG---------------G--C 126
Exon9templ ----------------------------------------- 0
9Fnewprime ----------------------NNNNNNNNNNNNNNNGNNC 19
        9R ACTGAGAAGGAGAGTGTCTGTCGCTCAGTCCCACTCAGGGGC 126

 Consensus ---TCTTCTTTGC--AACCCCCTTGGTCCTGATTATAAGGGC 168
Exon9templ --------------AACCCCCTTGGTCCTGATTATAAGGGC 27
9Fnewprime NNNTCTTCTTTGCNNAACCCCCTTGGTCCTGATTATAAGGGC 61
        9R CACTCTTCTTTGCAGAACCCCCTTGGTCCTGATTATAAGGGC 168

 Consensus TGCCTCATCCGGCGCAGCCCCACAGGAACCTTCCTTCTGGTT 210
Exon9templ TGCCTCATCCGGCGCAGCCCCACAGGAACCTTCCTTCTGGTT 69
9Fnewprime TGCCTCATCCGGCGCAGCCCCACAGGAACCTTCCTTCTGGTT 103
        9R TGCCTCATCCGGCGCAGCCCCACAGGAACCTTCCTTCTGGTT 210

 Consensus GGCCTCAGCCGACCCCACAGCAGTCTTCGAGAGCTCCTGGCA 252
Exon9templ GGCCTCAGCCGACCCCACAGCAGTCTTCGAGAGCTCCTGGCA 111
9Fnewprime GGCCTCAGCCGACCCCACAGCAGTCTTCGAGAGCTCCTGGCA 145
        9R GGCCTCAGCCGACCCCACAGCAGTCTTCGAGAGCTCCTGGCA 252

 Consensus ACCTGCTGGGATGGGGGGCTGCACGTAGATGGGGTGGCAGTG 294
Exon9templ ACCTGCTGGGATGGGGGGCTGCACGTAGATGGGGTGGCAGTG 153
9Fnewprime ACCTGCTGGGATGGGGGGCTGCACGTAGATGGGGTGGCAGTG 187
        9R ACCTGCTGGGATGGGGGGCTGCACGTAGATGGGGTGGCAGTG 294

 Consensus ACCCTCACTTCCTGCTGTATCCCCAGACCCAAAGGTGAGCCC 336
Exon9templ ACCCTCACTTCCTGCTGTATCCCCAGACCCAAAG-------- 187
9Fnewprime ACCCTCACTTCCTGCTGTATCCCCAGACCCAAAGGTGAGCCC 229
        9R ACCCTCACTTCCTGCTGTATCCCCAGACCCAAAGGTGAGCCC 336
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```
 Consensus CTTCCTCCCCTGGAATGAGTGGCTGATCTGGGACCCTGGCTT 378
Exon9templ ----------------------------------------- 187
9Fnewprime CTTCCTCCCCTGGAATGAGTGGCTGATCTGGGACCCTGGCTT 271
        9R CTTCCTCCCCTGGAATGAGTGGCTGATCTGGGACCCTGGCTT 378

 Consensus TCTATGTCTGTGACAGCTCCTGTGTGGGTGGCAAGTGGCAGA 420
Exon9templ ----------------------------------------- 187
9Fnewprime TCTATGTCTGTGACAGCTCCTGTGTGGGTGGCAAGTGGCAGA 313
        9R TCTATGTCTGTGACAGCTCCTGTGTGGGTGGCAAGTGGCAGA 420

 Consensus AACTGCAGGTCAAGGTGGGTTAGGGAAGAAAAGGTGATTTGT 462
Exon9templ ----------------------------------------- 187
9Fnewprime AACTGCAGGTCAAGGTGGGTTAGGGAAGAAAAGGTGATTTGT 355
        9R AACTGCAGGTCAAGGTGGGTTAGGGAAGAAAAGGTGATTTGT 462

 Consensus TGGCTCAGGAAGTTAGAGATATATAACCTTTAGGTCTGGCTT 504
Exon9templ ----------------------------------------- 187
9Fnewprime TGGCTCAGGAAGTTAGAGATATATAACCTTTAGGTCTGGCTT 397
        9R TGGCTCAGGAAGTTAGAGATATATAACCTTTAGGTCTGGCTT 504

 Consensus GATCTAGGCACAGCTAGAT--GAGCCA--TC-T--------- 546
Exon9templ ----------------------------------------- 187
9Fnewprime GATCTAGGCACAGCTAGATGTGAGCCATGTCATCTGCACCTA 439
        9R GATCTAGGCACAGCTAGATNNGAGCCANNTCNTNNNNNNNNN 546

 Consensus ---------CAGCTCTCAGCTCTTCCTCTGGGGTCATAGCTG 588
Exon9templ ----------------------------------------- 187
9Fnewprime GTCTCTCTCCAGCTCTCAGCTCTTCCTCTGGGGTCATAGCTG 481
        9R NNNNNNNNN------------------------------- 555

 Consensus TTTCNNN                                   595
Exon9templ -------                                   187
9Fnewprime TTTCNNN                                   488
        9R -------                                   555
```

```
 Consensus NNTTTGTNNNNNNACGGCCAGTGTTGCAGTGAGCTGAGATCG 42
Exon10temp ---------------------------------------- 0
       10F ---------------------------------------- 0
       10R NNTTTGTNNNNNNACGGCCAGTGTTGCAGTGAGCTGAGATCG 42

 Consensus CAC-----------------GG--GA--GA-TGAGACTCCGT 84
Exon10temp ---------------------------------------- 0
       10F ---NNNNNNNNNNNNNNNNGGNNGANNGANTGAGACTCCGT 39
       10R CACCACTGCCCACCCAGCCTGGATGACAGAGTGAGACTCCGT 84

 Consensus CTCAACAGCAGCAGCAACAACAAAACAAAAACAACAACAAAA 126
Exon10temp ---------------------------------------- 0
       10F CTCAACAGCAGCAGCAACAACAAAACAAAAACAACAACAAAA 81
       10R CTCAACAGCAGCAGCAACAACAAAACAAAAACAACAACAAAA 126

 Consensus AGCCATGTGCCCTGAAGTCTTCATCTCAGGGTCGGCTTCTAG 168
Exon10temp ---------------------------------------- 0
       10F AGCCATGTGCCCTGAAGTCTTCATCTCAGGGTCGGCTTCTAG 123
       10R AGCCATGTGCCCTGAAGTCTTCATCTCAGGGTCGGCTTCTAG 168

 Consensus AGGGTACCTCAAACTAAGGCATGAGTTAGCTAACCCTTGGGG 210
Exon10temp ---------------------------------------- 0
       10F AGGGTACCTCAAACTAAGGCATGAGTTAGCTAACCCTTGGGG 165
       10R AGGGTACCTCAAACTAAGGCATGAGTTAGCTAACCCTTGGGG 210

 Consensus ACTTTTCACCTCTGATTTCTGGTTTTTCTCCCTCATCCTCTC 252
Exon10temp ---------------------------------------- 0
       10F ACTTTTCACCTCTGATTTCTGGTTTTTCTCCCTCATCCTCTC 207
       10R ACTTTTCACCTCTGATTTCTGGTTTTTCTCCCTCATCCTCTC 252

 Consensus CCCATAGAAAAGTCCAACCTGATCGTGGTCCAGAGAGGTCAC 294
Exon10temp -------AAAAGTCCAACCTGATCGTGGTCCAGAGAGGTCAC 35
       10F CCCATAGAAAAGTCCAACCTGATCGTGGTCCAGAGAGGTCAC 249
       10R CCCATAGAAAAGTCCAACCTGATCGTGGTCCAGAGAGGTCAC 294

 Consensus AGCCCACCCACATCATCCTTGGTTCAGCCCCAATCCCAATAC 336
Exon10temp AGCCCACCCACATCATCCTTGGTTCAGCCCCAATCCCAATAC 77
       10F AGCCCACCCACATCATCCTTGGTTCAGCCCCAATCCCAATAC 291
       10R AGCCCACCCACATCATCCTTGGTTCAGCCCCAATCCCAATAC 336
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```
 Consensus CAGCTGAGTCAGATGACATTTCACAAGATCCCTGCTGACAGC 378
Exon10temp CAGCTGAGTCAGATGACATTTCACAAGATCCCTGCTGACAGC 119
       10F CAGCTGAGTCAGATGACATTTCACAAGATCCCTGCTGACAGC 333
       10R CAGCTGAGTCAGATGACATTTCACAAGATCCCTGCTGACAGC 378

 Consensus CTGGAGTGGGTAAGAGGCCCTGGGAAATGAGGCGATACCTCA 420
Exon10temp CTGGAGTGG-------------------------------- 128
       10F CTGGAGTGGGTAAGAGGCCCTGGGAAATGAGGCGATACCTCA 375
       10R CTGGAGTGGGTAAGAGGCCCTGGGAAATGAGGCGATACCTCA 420

 Consensus GTCTGGGGTCCAGAGACTCAGATGCGTGGCCTCAGGCATATG 462
Exon10temp ---------------------------------------- 128
       10F GTCTGGGGTCCAGAGACTCAGATGCGTGGCCTCAGGCATATG 417
       10R GTCTGGGGTCCAGAGACTCAGATGCGTGGCCTCAGGCATATG 462

 Consensus CTATAATTTTACCTTGCCTCGGTTTTCCCATCTGTAAAATGG 504
Exon10temp ---------------------------------------- 128
       10F CTATAATTTTACCTTGCCTCGGTTTTCCCATCTGTAAAATGG 459
       10R CTATAATTTTACCTTGCCTCGGTTTTCCCATCTGTAAAATGG 504

 Consensus GGCCAGCAGCTATGTCTCGCTTGGGCTGGG-TCCTGC-G-A- 546
Exon10temp ---------------------------------------- 128
       10F GGCCAGCAGCTATGTCTCGCTTGGGCTGGGATCCTGCAGGAA 501
       10R GGCCAGCAGCTATGTCTCGCTTGGGCTGGGNTCCTGCNGNAC 546

 Consensus CCC------------------TGTCCCCTCACCATTCAGCA 588
Exon10temp ---------------------------------------- 128
       10F CCCCCTCACTGGCCTCTTCTGCTGTCCCCTCACCATTCAGCA 543
       10R CCCNNNNNNNNNNNNNNNNNN------------------- 568

 Consensus TGAGAGGTCATANNTGNNNNNNGNNNN               615
Exon10temp ---------------------------               128
       10F TGAGAGGTCATANNTGNNNNNNGNNNN               570
       10R ---------------------------               568
```

```
 Consensus NTGTNNNNCGACGGCCAGTGAGGCGATACCTCAGTCTGGGGT 42
Exon11temp ---------------------------------------- 0
       11F ---------------------------------------- 0
       11R NTGTNNNNCGACGGCCAGTGAGGCGATACCTCAGTCTGGGGT 42

 Consensus CC----------------G---C-GGCATATGCTATAATTT 84
Exon11temp ---------------------------------------- 0
       11F --NNNNNNNNNNNNNNCNNGNNNCNGGCATATGCTATAATTT 40
       11R CCAGAGACTCAGATGCGTGGCCTCAGGCATATGCTATAATTT 84

 Consensus TACCTTGCCTCGGTTTTCCCATCTGTAAAATGGGGCCAGCAG 126
Exon11temp ---------------------------------------- 0
       11F TACCTTGCCTCGGTTTTCCCATCTGTAAAATGGGGCCAGCAG 82
       11R TACCTTGCCTCGGTTTTCCCATCTGTAAAATGGGGCCAGCAG 126

 Consensus CTATGTCTCGCTTGGGCTGGGATCCTGCAGGAACCCCCTCAC 168
Exon11temp ---------------------------------------- 0
       11F CTATGTCTCGCTTGGGCTGGGATCCTGCAGGAACCCCCTCAC 124
       11R CTATGTCTCGCTTGGGCTGGGATCCTGCAGGAACCCCCTCAC 168

 Consensus TGGCCTCTTCTGCTGTCCCCTCACCATTCAGCATGAGAACCT 210
Exon11temp ------------------------------CATGAGAACCT 11
       11F TGGCCTCTTCTGCTGTCCCCTCACCATTCAGCATGAGAACCT 166
       11R TGGCCTCTTCTGCTGTCCCCTCACCATTCAGCATGAGAACCT 210

 Consensus GGGCCATGGGTCCTTCACCAAGATTTACCGGGGCTGTCGCCA 252
Exon11temp GGGCCATGGGTCCTTCACCAAGATTTACCGGGGCTGTCGCCA 53
       11F GGGCCATGGGTCCTTCACCAAGATTTACCGGGGCTGTCGCCA 208
       11R GGGCCATGGGTCCTTCACCAAGATTTACCGGGGCTGTCGCCA 252

 Consensus TGAGGTGGTGGATGGGGAGGCCCGAAAGACAGAGGTGCTGCT 294
Exon11temp TGAGGTGGTGGATGGGGAGGCCCGAAAGACAGAGGTGCTGCT 95
       11F TGAGGTGGTGGATGGGGAGGCCCGAAAGACAGAGGTGCTGCT 250
       11R TGAGGTGGTGGATGGGGAGGCCCGAAAGACAGAGGTGCTGCT 294

 Consensus GAAGGTCATGGATGCCAAGCACAAGAACTGCATGGAGGTGAG 336
Exon11temp GAAGGTCATGGATGCCAAGCACAAGAACTGCATGGAG----- 132
       11F GAAGGTCATGGATGCCAAGCACAAGAACTGCATGGAGGTGAG 292
       11R GAAGGTCATGGATGCCAAGCACAAGAACTGCATGGAGGTGAG 336
```

```
 Consensus AGCAATGTGGACCAGACTTTTGGAGTCGGGGCTGGCTGGAGA 378
Exon11temp ---------------------------------------- 132
       11F AGCAATGTGGACCAGACTTTTGGAGTCGGGGCTGGCTGGAGA 334
       11R AGCAATGTGGACCAGACTTTTGGAGTCGGGGCTGGCTGGAGA 378

 Consensus GGGGGTCGTGGATGCAGAGAAATTTAAAAACACACAGGGACC 420
Exon11temp ---------------------------------------- 132
       11F GGGGGTCGTGGATGCAGAGAAATTTAAAAACACACAGGGACC 376
       11R GGGGGTCGTGGATGCAGAGAAATTTAAAAACACACAGGGACC 420

 Consensus TGGGCGTGGTGGCTCATGCCTGTCATCCCAGCACTTTGGGAG 462
Exon11temp ---------------------------------------- 132
       11F TGGGCGTGGTGGCTCATGCCTGTCATCCCAGCACTTTGGGAG 418
       11R TGGGCGTGGTGGCTCATGCCTGTCATCCCAGCACTTTGGGAG 462

 Consensus GC-GAGGCAGGAGGA-GGT---AAGC--------------- 504
Exon11temp ---------------------------------------- 132
       11F GCTGAGGCAGGAGGATGGTTTGAAGCCAGGAGTTCAAGAACA 460
       11R GCNGAGGCAGGAGGANGGTNN-AAGCNNNNNNNNNNNNNNN 503

 Consensus -CCTAGGCAACATAGCGAGACCTCGTGGTCATAGCTNNNNNN 546
Exon11temp ---------------------------------------- 132
       11F GCCTAGGCAACATAGCGAGACCTCGTGGTCATAGCTNNNNNN 502
       11R N------------------------------------- 504

 Consensus NNNNNN                                  552
Exon11temp ------                                  132
       11F NNNNNN                                  508
       11R ------                                  504
```

```
 Consensus NNNNNNTGTAANNNGACGGCCAGTTCCCGTATCAGAAAATCA 42
Exon12temp ---------------------------------------- 0
       12F ---------------------------------------- 0
       12R NNNNNNTGTAANNNGACGGCCAGTTCCCGTATCAGAAAATCA 42

 Consensus TGGTA----------------G---GA-TC--GGGC--AAG 84
Exon12temp ---------------------------------------- 0
       12F -----NNNNNNNNNNNNNNNNGNNNGANTCNNGGGCNNAAG 37
       12R TGGTAGTGCTGTGTGCACTAATGGCAGACTCCAGGGCCAAAG 84

 Consensus GTGACCTGTGGCC-GGTGTTCCCCTAAGGCAGGTCTGTGAGC 126
Exon12temp ---------------------------------------- 0
       12F GTGACCTGTGGCCNGGTGTTCCCCTAAGGCAGGTCTGTGAGC 79
       12R GTGACCTGTGGCCAGGTGTTCCCCTAAGGCAGGTCTGTGAGC 126

 Consensus ACAAAATTTGGGATTATTGGAGTGGAAGAAACCCACGCATCT 168
Exon12temp ---------------------------------------- 0
       12F ACAAAATTTGGGATTATTGGAGTGGAAGAAACCCACGCATCT 121
       12R ACAAAATTTGGGATTATTGGAGTGGAAGAAACCCACGCATCT 168

 Consensus TCTCTCCCTTCCCACCTTCCCCAGTCATTCCTGGAAGCAGCG 210
Exon12temp ----------------------TCATTCCTGGAAGCAGCG 18
       12F TCTCTCCCTTCCCACCTTCCCCAGTCATTCCTGGAAGCAGCG 163
       12R TCTCTCCCTTCCCACCTTCCCCAGTCATTCCTGGAAGCAGCG 210

 Consensus AGCTTGATGAGCCAAGTGTCGTACCGGCATCTCGTGCTGCTC 252
Exon12temp AGCTTGATGAGCCAAGTGTCGTACCGGCATCTCGTGCTGCTC 60
       12F AGCTTGATGAGCCAAGTGTCGTACCGGCATCTCGTGCTGCTC 205
       12R AGCTTGATGAGCCAAGTGTCGTACCGGCATCTCGTGCTGCTC 252

 Consensus CACGGCGTGTGCATGGCTGGAGACAGTGAGAGCCCCCCACCC 294
Exon12temp CACGGCGTGTGCATGGCTGGAGACA---------------- 85
       12F CACGGCGTGTGCATGGCTGGAGACAGTGAGAGCCCCCCACCC 247
       12R CACGGCGTGTGCATGGCTGGAGACAGTGAGAGCCCCCCACCC 294

 Consensus ACCCACCCCACCCCTGCCTCACCCAAGTCTAGGCTGTTCTTC 336
Exon12temp ---------------------------------------- 85
       12F ACCCACCCCACCCCTGCCTCACCCAAGTCTAGGCTGTTCTTC 289
       12R ACCCACCCCACCCCTGCCTCACCCAAGTCTAGGCTGTTCTTC 336
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```
 Consensus CCACCTCTGTTCTGAGCCGCTATATGACAGCCCCAGCAACAC 378
Exon12temp ------------------------------------------ 85
       12F CCACCTCTGTTCTGAGCCGCTATATGACAGCCCCAGCAACAC 331
       12R CCACCTCTGTTCTGAGCCGCTATATGACAGCCCCAGCAACAC 378

 Consensus ACTGGGCCACCCTGGATGGGAGCCGTGTTCATTACCCTTTAT 420
Exon12temp ------------------------------------------ 85
       12F ACTGGGCCACCCTGGATGGGAGCCGTGTTCATTACCCTTTAT 373
       12R ACTGGGCCACCCTGGATGGGAGCCGTGTTCATTACCCTTTAT 420

 Consensus TTA---CT-TCC-TC--C-----------------TCCAGGT 462
Exon12temp ------------------------------------------ 85
       12F TTATGTCTCTCCATCATCACTCCTTGGAAAGCGGCTCCAGGT 415
       12R TTANNNCTNTCCNTCNNCNNNNNNNNNNNNNNNN------- 455

 Consensus TCTCACCCATATCCAGCGGTCATAGCTGTTTCNNGANNN    501
Exon12temp ----------------------------------------    85
       12F TCTCACCCATATCCAGCGGTCATAGCTGTTTCNNGANNN    454
       12R ----------------------------------------    455
```

```
 Consensus NNTTNNNAAACGACGGCCAGTACAGGGCTCAACACCTTCCAG 42
Exon13temp ----------------------------------------- 0
       13F ----------------------------------------- 0
       13R NNTTNNNAAACGACGGCCAGTACAGGGCTCAACACCTTCCAG 42

 Consensus GCAT-------------TT---A---GGAGGTGGGAGGAGAG 84
Exon13temp ----------------------------------------- 0
       13F ----NNNNNNNNNNNNNTTNNNANNNGGAGGTGGGAGGAGAG 38
       13R GCATTCCAGGCAAATCATTCAGAGATGGAGGTGGGAGGAGAG 84

 Consensus GTGAGTACTGTATGAACAGAGGCAGCAGGGGAGGGAACAGAC 126
Exon13temp ----------------------------------------- 0
       13F GTGAGTACTGTATGAACAGAGGCAGCAGGGGAGGGAACAGAC 80
       13R GTGAGTACTGTATGAACAGAGGCAGCAGGGGAGGGAACAGAC 126

 Consensus AGAGATGAGAGTTTGAGAGACCCTGAGAGCCAGGGTGTTGGC 168
Exon13temp ----------------------------------------- 0
       13F AGAGATGAGAGTTTGAGAGACCCTGAGAGCCAGGGTGTTGGC 122
       13R AGAGATGAGAGTTTGAGAGACCCTGAGAGCCAGGGTGTTGGC 168

 Consensus AGAACCTCCTCAACACAAGTGCAGTTCAGTCTCCCAACCCCG 210
Exon13temp ----------------------------------------- 0
       13F AGAACCTCCTCAACACAAGTGCAGTTCAGTCTCCCAACCCCG 164
       13R AGAACCTCCTCAACACAAGTGCAGTTCAGTCTCCCAACCCCG 210

 Consensus CCTCTCCCTGCTGCCAACCAGGCACCATGGTGCAGGAATTTG 252
Exon13temp --------------------GCACCATGGTGCAGGAATTTG 21
       13F CCTCTCCCTGCTGCCAACCAGGCACCATGGTGCAGGAATTTG 206
       13R CCTCTCCCTGCTGCCAACCAGGCACCATGGTGCAGGAATTTG 252

 Consensus TACACCTGGGGGCCATAGACATGTATCTGCGAAAACGTGGCC 294
Exon13temp TACACCTGGGGGCCATAGACATGTATCTGCGAAAACGTGGCC 63
       13F TACACCTGGGGGCCATAGACATGTATCTGCGAAAACGTGGCC 248
       13R TACACCTGGGGGCCATAGACATGTATCTGCGAAAACGTGGCC 294

 Consensus ACCTGGTGCCAGCCAGCTGGAAGCTGCAGGTGGTCAAACAGC 336
Exon13temp ACCTGGTGCCAGCCAGCTGGAAGCTGCAGGTGGTCAAACAGC 105
       13F ACCTGGTGCCAGCCAGCTGGAAGCTGCAGGTGGTCAAACAGC 290
       13R ACCTGGTGCCAGCCAGCTGGAAGCTGCAGGTGGTCAAACAGC 336
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 Consensus TGGCCTACGCCCTCAACTATCTGGTGAGTGCTCCTCTGCCTG 378
Exon13temp TGGCCTACGCCCTCAACTATCTG------------------ 128
       13F TGGCCTACGCCCTCAACTATCTGGTGAGTGCTCCTCTGCCTG 332
       13R TGGCCTACGCCCTCAACTATCTGGTGAGTGCTCCTCTGCCTG 378

 Consensus CTCCACCCTCCATTCCCAGGGAAGGCTTTCTC-GGG--GAAG 420
Exon13temp ---------------------------------------- 128
       13F CTCCACCCTCCATTCCCAGGGAAGGCTTTCTCTGGGTGGAAG 374
       13R CTCCACCCTCCATTCCCAGGGAAGGCTTTCTCNGGGNNGAAG 420

 Consensus AGGA--------------------ATGCATAGGAGTTTGGT 462
Exon13temp ---------------------------------------- 128
       13F AGGAATTGGGAGTGGGCTCTGTAGTATGCATAGGAGTTTGGT 416
       13R AGGANNNNNNNNNNNNNNNNNNN----------------- 445

 Consensus AAGGGTTCGAGGTCATAGCTGTTTCNNNNN             492
Exon13temp ----------------------------             128
       13F AAGGGTTCGAGGTCATAGCTGTTTCNNNNN             446
       13R ----------------------------              445
```

```
 Consensus NGTNNNNNNNNGGCCAGTGGAGCATGTCTGAGCAGTACCAA- 42
Exon14temp ----------------------------------------- 0
      14F ----------------------------------------N 1
      14R NGTNNNNNNNNGGCCAGTGGAGCATGTCTGAGCAGTACCAAG 42

 Consensus ------------------T-GGAGTTTGCC-AAC-GACTCTT 84
Exon14temp ----------------------------------------- 0
      14F NNNNNNNNNNNNNNNNNNTNGGAGTTTGCCNAACNGACTCTT 43
      14R TGGGTTTTGAAGGATGTATAGGAGTTTGCCAAACAGACTCTT 84

 Consensus C-TTC-TCAAACCCTCCNGGGCATTTTCCTGTGTCTGGCCCC 126
Exon14temp ----------------------------------------- 0
      14F CNTTCNTCAAACCCTCCNGGGCATTTTCCTGTGTCTGGCCCC 85
      14R CATTCATCAAACCCTCCNGGGCATTTTCCTGTGTCTGGCCCC 126

 Consensus CTTAGGAGGACAAAGGCCTGCCCCATGGCAATGTCTCTGCCC 168
Exon14temp -----GAGGACAAAGGCCTGCCCCATGGCAATGTCTCTGCCC 37
      14F CTTAGGAGGACAAAGGCCTGCCCCATGGCAATGTCTCTGCCC 127
      14R CTTAGGAGGACAAAGGCCTGCCCCATGGCAATGTCTCTGCCC 168

 Consensus GGAAGGTGCTCCTGGCTCGGGAGGGGGCTGATGGGAGCCCGC 210
Exon14temp GGAAGGTGCTCCTGGCTCGGGAGGGGGCTGATGGGAGCCCGC 79
      14F GGAAGGTGCTCCTGGCTCGGGAGGGGGCTGATGGGAGCCCGC 169
      14R GGAAGGTGCTCCTGGCTCGGGAGGGGGCTGATGGGAGCCCGC 210

 Consensus CCTTCATCAAGCTGAGTGACCCTGGGGTCAGCCCCGCTGTGT 252
Exon14temp CCTTCATCAAGCTGAGTGACCCTGGGGTCAGCCCCGCTGTGT 121
      14F CCTTCATCAAGCTGAGTGACCCTGGGGTCAGCCCCGCTGTGT 211
      14R CCTTCATCAAGCTGAGTGACCCTGGGGTCAGCCCCGCTGTGT 252

 Consensus TAAGCCTGGAGAGTAAGTTCCTGGAGGTGGAGGAGGGAGGGG 294
Exon14temp TAAGCCTGGAGA---------------------------- 133
      14F TAAGCCTGGAGAGTAAGTTCCTGGAGGTGGAGGAGGGAGGGG 253
      14R TAAGCCTGGAGAGTAAGTTCCTGGAGGTGGAGGAGGGAGGGG 294

 Consensus CTGAGCAGGGCAAGGAAGTGGATCCCTGATCCCACTTTCATT 336
Exon14temp ----------------------------------- 133
      14F CTGAGCAGGGCAAGGAAGTGGATCCCTGATCCCACTTTCATT 295
      14R CTGAGCAGGGCAAGGAAGTGGATCCCTGATCCCACTTTCATT 336
```

```
 Consensus CCCTCAGTGCTCACCGACAGGATCCCCTGGGTGGCCCCCGAG 378
Exon14temp ---------------------------------------- 133
       14F CCCTCAGTGCTCACCGACAGGATCCCCTGGGTGGCCCCCGAG 337
       14R CCCTCAGTGCTCACCGACAGGATCCCCTGGGTGGCCCCCGAG 378

 Consensus TGTCTCCGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAG 420
Exon14temp ---------------------------------------- 133
       14F TGTCTCCGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAG 379
       14R TGTCTCCGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAG 420

 Consensus TGGGGCTTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTC 462
Exon14temp ---------------------------------------- 133
       14F TGGGGCTTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTC 421
       14R TGGGGCTTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTC 462

 Consensus ACCATGCCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCC 504
Exon14temp ---------------------------------------- 133
       14F ACCATGCCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCC 463
       14R ACCATGCCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCC 504

 Consensus CCTCACCCGGCATCGGTCTCCGAACCCCCACTT-GACAGAAG 546
Exon14temp ---------------------------------------- 133
       14F CCTCACCCGGCATCGGTCTCCGAACCCCCACTTTGACAGAAG 505
       14R CCTCACCCGGCATCGGTCTCCGAACCCCCACTTNGACAGAAG 546

 Consensus GGCAGAC-GAC--C-------G--------GGGTGGGTCTAT 588
Exon14temp ---------------------------------------- 133
       14F GGCAGACTGACATCCAGTCTGGGGAGATTGGGGTGGGTCTAT 547
       14R GGCAGACNGACNNCNNNNNNNGNNNNNNNN---------- 576

 Consensus TGGGTTGGGGATTGGTCATANNNNNNNNNNGNN         622
Exon14temp ---------------------------------      133
       14F TGGGTTGGGGATTGGTCATANNNNNNNNNNGNN         581
       14R ---------------------------------         576
```

```
 Consensus NGTNNNNNNNNGGCCAGTGGAGCATGTCTGAGCAGTACCAA- 42
Exon15temp ----------------------------------------- 0
       15F ----------------------------------------N 1
       15R NGTNNNNNNNNGGCCAGTGGAGCATGTCTGAGCAGTACCAAG 42

 Consensus ------------------T-GGAGTTTGCC-AAC-GACTCTT 84
Exon15temp ----------------------------------------- 0
       15F NNNNNNNNNNNNNNNNNNTNGGAGTTTGCCNAACNGACTCTT 43
       15R TGGGTTTTGAAGGATGTATAGGAGTTTGCCAAACAGACTCTT 84

 Consensus C-TTC-TCAAACCCTCCNGGGCATTTTCCTGTGTCTGGCCCC 126
Exon15temp ----------------------------------------- 0
       15F CNTTCNTCAAACCCTCCNGGGCATTTTCCTGTGTCTGGCCCC 85
       15R CATTCATCAAACCCTCCNGGGCATTTTCCTGTGTCTGGCCCC 126

 Consensus CTTAGGAGGACAAAGGCCTGCCCCATGGCAATGTCTCTGCCC 168
Exon15temp ----------------------------------------- 0
       15F CTTAGGAGGACAAAGGCCTGCCCCATGGCAATGTCTCTGCCC 127
       15R CTTAGGAGGACAAAGGCCTGCCCCATGGCAATGTCTCTGCCC 168

 Consensus GGAAGGTGCTCCTGGCTCGGGAGGGGGCTGATGGGAGCCCGC 210
Exon15temp ----------------------------------------- 0
       15F GGAAGGTGCTCCTGGCTCGGGAGGGGGCTGATGGGAGCCCGC 169
       15R GGAAGGTGCTCCTGGCTCGGGAGGGGGCTGATGGGAGCCCGC 210

 Consensus CCTTCATCAAGCTGAGTGACCCTGGGGTCAGCCCCGCTGTGT 252
Exon15temp ----------------------------------------- 0
       15F CCTTCATCAAGCTGAGTGACCCTGGGGTCAGCCCCGCTGTGT 211
       15R CCTTCATCAAGCTGAGTGACCCTGGGGTCAGCCCCGCTGTGT 252

 Consensus TAAGCCTGGAGAGTAAGTTCCTGGAGGTGGAGGAGGGAGGGG 294
Exon15temp ----------------------------------------- 0
       15F TAAGCCTGGAGAGTAAGTTCCTGGAGGTGGAGGAGGGAGGGG 253
       15R TAAGCCTGGAGAGTAAGTTCCTGGAGGTGGAGGAGGGAGGGG 294

 Consensus CTGAGCAGGGCAAGGAAGTGGATCCCTGATCCCACTTTCATT 336
Exon15temp ----------------------------------------- 0
       15F CTGAGCAGGGCAAGGAAGTGGATCCCTGATCCCACTTTCATT 295
       15R CTGAGCAGGGCAAGGAAGTGGATCCCTGATCCCACTTTCATT 336
```

```
 Consensus CCCTCAGTGCTCACCGACAGGATCCCCTGGGTGGCCCCCGAG 378
Exon15temp -------TGCTCACCGACAGGATCCCCTGGGTGGCCCCCGAG 35
       15F CCCTCAGTGCTCACCGACAGGATCCCCTGGGTGGCCCCCGAG 337
       15R CCCTCAGTGCTCACCGACAGGATCCCCTGGGTGGCCCCCGAG 378

 Consensus TGTCTCCGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAG 420
Exon15temp TGTCTCCGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAG 77
       15F TGTCTCCGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAG 379
       15R TGTCTCCGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAG 420

 Consensus TGGGGCTTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTC 462
Exon15temp TGGGGCTTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTC 119
       15F TGGGGCTTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTC 421
       15R TGGGGCTTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTC 462

 Consensus ACCATGCCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCC 504
Exon15temp ACCATGCCCATCAGTGCCCTGGATCCTGCTAAG--------- 152
       15F ACCATGCCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCC 463
       15R ACCATGCCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCC 504

 Consensus CCTCACCCGGCATCGGTCTCCGAACCCCCACTT-GACAGAAG 546
Exon15temp ---------------------------------------- 152
       15F CCTCACCCGGCATCGGTCTCCGAACCCCCACTTTGACAGAAG 505
       15R CCTCACCCGGCATCGGTCTCCGAACCCCCACTTNGACAGAAG 546

 Consensus GGCAGAC-GAC--C-------G--------GGGTGGGTCTAT 588
Exon15temp ---------------------------------------- 152
       15F GGCAGACTGACATCCAGTCTGGGGAGATTGGGGTGGGTCTAT 547
       15R GGCAGACNGACNNCNNNNNNNGNNNNNNNN---------- 576

 Consensus TGGGTTGGGGATTGGTCATANNNNNNNNNNGNN         622
Exon15temp ---------------------------------      152
       15F TGGGTTGGGGATTGGTCATANNNNNNNNNNGNN         581
       15R ---------------------------------         576
```

```
 Consensus NGNANANNGNCGGCCAGTCCTGATCCCACTTTCATTCCCTCA 42
Exon16temp ---------------------------------------- 0
       16F ---------------------------------------- 0
       16R NGNANANNGNCGGCCAGTCCTGATCCCACTTTCATTCCCTCA 42

 Consensus GT--------------C--C-GGGTGG-CCCCGAGTGTCTC 84
Exon16temp ---------------------------------------- 0
       16F --NNNNNNNNNNNNNNCNNCNGGGTGGNCCCCGAGTGTCTC 40
       16R GTGCTCACCGACAGGATCCCCTGGGTGGCCCCCGAGTGTCTC 84

 Consensus CGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAGTGGGGC 126
Exon16temp ---------------------------------------- 0
       16F CGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAGTGGGGC 82
       16R CGGGAGGCGCAGACACTTAGCTTGGAAGCTGACAAGTGGGGC 126

 Consensus TTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTCACCATG 168
Exon16temp ---------------------------------------- 0
       16F TTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTCACCATG 124
       16R TTCGGCGCCACGGTCTGGGAAGTGTTTAGTGGCGTCACCATG 168

 Consensus CCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCCCCTCAC 210
Exon16temp ---------------------------------------- 0
       16F CCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCCCCTCAC 166
       16R CCCATCAGTGCCCTGGATCCTGCTAAGGTCAGAGCCCCTCAC 210

 Consensus CCGGCATCGGTCTCCGAACCCCCACTTTGACAGAAGGGCAGA 252
Exon16temp ---------------------------------------- 0
       16F CCGGCATCGGTCTCCGAACCCCCACTTTGACAGAAGGGCAGA 208
       16R CCGGCATCGGTCTCCGAACCCCCACTTTGACAGAAGGGCAGA 252

 Consensus CTGACATCCAGTCTGGGGAGATTGGGGTGGGTCTATTGGGTT 294
Exon16temp ---------------------------------------- 0
       16F CTGACATCCAGTCTGGGGAGATTGGGGTGGGTCTATTGGGTT 250
       16R CTGACATCCAGTCTGGGGAGATTGGGGTGGGTCTATTGGGTT 294

 Consensus GGGGATTACCGACTGCTCCTCTCACCCTCAGAAACTCCAATT 336
Exon16temp ----------------------------AAACTCCAATT 11
       16F GGGGATTACCGACTGCTCCTCTCACCCTCAGAAACTCCAATT 292
       16R GGGGATTACCGACTGCTCCTCTCACCCTCAGAAACTCCAATT 336
```

```
 Consensus TTATGAGGACCGGCAGCAGCTGCCGGCCCCCAAGTGGACAGA 378
Exon16temp TTATGAGGACCGGCAGCAGCTGCCGGCCCCCAAGTGGACAGA 53
       16F TTATGAGGACCGGCAGCAGCTGCCGGCCCCCAAGTGGACAGA 334
       16R TTATGAGGACCGGCAGCAGCTGCCGGCCCCCAAGTGGACAGA 378

 Consensus GCTGGCCCTGCTGATTCAACAGTGCATGGCCTATGAGCCGGT 420
Exon16temp GCTGGCCCTGCTGATTCAACAGTGCATGGCCTATGAGCCGGT 95
       16F GCTGGCCCTGCTGATTCAACAGTGCATGGCCTATGAGCCGGT 376
       16R GCTGGCCCTGCTGATTCAACAGTGCATGGCCTATGAGCCGGT 420

 Consensus CCAGAGGCCCTCCTTCCGAGCCGTCATTCGTGACCTCAATAG 462
Exon16temp CCAGAGGCCCTCCTTCCGAGCCGTCATTCGTGACCTCAATAG 137
       16F CCAGAGGCCCTCCTTCCGAGCCGTCATTCGTGACCTCAATAG 418
       16R CCAGAGGCCCTCCTTCCGAGCCGTCATTCGTGACCTCAATAG 462

 Consensus CCTCATCTCTTCAGGTGCCCGCTGGGACGGGTTGGGTGGGGA 504
Exon16temp CCTCATCTCTTCAG-------------------------- 151
       16F CCTCATCTCTTCAGGTGCCCGCTGGGACGGGTTGGGTGGGGA 460
       16R CCTCATCTCTTCAGGTGCCCGCTGGGACGGGTTGGGTGGGGA 504

 Consensus GGGCTGTGATGTCATAT-GGGCCCAG--GAA--A--G----- 546
Exon16temp ---------------------------------------- 151
       16F GGGCTGTGATGTCATATTGGGCCCAGTGGAAGGAGCGTGGTT 502
       16R GGGCTGTGATGTCATATNGGGCCCAGNNGAANNANNGNNNNN 546

 Consensus --------GCCACGCCCTGTGTGTCTGGTGAGGTTGGTCATN 588
Exon16temp ---------------------------------------- 151
       16F TGCAGCAGGCCACGCCCTGTGTGTCTGGTGAGGTTGGTCATN 544
       16R NNNNNNNN------------------------------- 554

 Consensus NNNNNNNNNNGAN                           601
Exon16temp ------------                            151
       16F NNNNNNNNNNGAN                           557
       16R ------------                            554
```

```
 Consensus GGACGGCCAGTGCACAGCAAGTCAA-T-AG-AGTGGGGC--A 42
Exon17temp ---------------------------------------- 0
  17(18)F+ ----------------------GTYAG-AGTGGGGC--A 14
  17(18)R- GGACGGCCAGTGCACAGCAAGTCAACTCAGGAGTGGGGCCCA 42

 Consensus GGATGAGAGGCGCTGCTTACCACTGCCCATGCCCCCACCCCA 84
Exon17temp ---------------------------------------- 0
  17(18)F+ GGATGAGAGGCGCTGCTTACCACTGCCCATGCCCCCACCCCA 56
  17(18)R- GGATGAGAGGCGCTGCTTACCACTGCCCATGCCCCCACCCCA 84

 Consensus GACTATGAGCTCCTCTCAGACCCCACACCTGGTGCCCTGGCA 126
Exon17temp -ACTATGAGCTCCTCTCAGACCCCACACCTGGTGCCCTGGCA 41
  17(18)F+ GACTATGAGCTCCTCTCAGACCCCACACCTGGTGCCCTGGCA 98
  17(18)R- GACTATGAGCTCCTCTCAGACCCCACACCTGGTGCCCTGGCA 126

 Consensus CCTCGTGATGGGCTGTGGAATGGTGCCCAGCTCTATGCCTGC 168
Exon17temp CCTCGTGATGGGCTGTGGAATGGTGCCCAGCTCTATGCCTGC 83
  17(18)F+ CCTCGTGATGGGCTGTGGAATGGTGCCCAGCTCTATGCCTGC 140
  17(18)R- CCTCGTGATGGGCTGTGGAATGGTGCCCAGCTCTATGCCTGC 168

 Consensus CAAGACCCCACGAT-TTCGAGGAGAGACACCTCAAGTACATC 210
Exon17temp CAAGACCCCACGATCTTCGAGGAGAGACACCTCAAGTACATC 125
  17(18)F+ CAAGACCCCACGATCTTCGAGGAGAGACACCTCAAGTACATC 182
  17(18)R- CAAGACCCCACGATYTTCGAGGAGAGACACCTCAAGTACATC 210

 Consensus TCACAGCTGGGCAAGGTAAGGTGGGCAGGGCCAGGGTGGGTT 252
Exon17temp TCACAGCTGGGCAAG-------------------------- 140
  17(18)F+ TCACAGCTGGGCAAGGTAAGGTGGGCAGGGCCAGGGTGGGTT 224
  17(18)R- TCACAGCTGGGCAAGGTAAGGTGGGCAGGGCCAGGGTGGGTT 252

 Consensus GGAGAGGGCAGGGCAGCATCCAGGTGCCTGGACATCAGTCCC 294
Exon17temp ---------------------------------------- 140
  17(18)F+ GGAGAGGGCAGGGCAGCATCCAGGTGCCTGGACATCAGTCCC 266
  17(18)R- GGAGAGGGCAGGGCAGCATCCAGGTGCCTGGACATCAGTCCC 294

 Consensus GCTATCCCCCAGGGCAACTTTGGCAGCGTGGAGCTGTGCCGC 336
Exon17temp ---------------------------------------- 140
  17(18)F+ GCTATCCCCCAGGGCAACTTTGGCAGCGTGGAGCTGTGCCGC 308
  17(18)R- GCTATCCCCCAGGGCAACTTTGGCAGCGTGGAGCTGTGCCGC 336
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```
 Consensus TATGACCCGCTAGGCGACAATACAGGTGCCCTGGTGGCCGTG 378
Exon17temp ---------------------------------------- 140
  17(18)F+ TATGACCCGCTAGGCGACAATACAGGTGCCCTGGTGGCCGTG 350
  17(18)R- TATGACCCGCTAGGCGACAATACAGGTGCCCTGGTGGCCGTG 378

 Consensus AAACAGCTGCAGCACAGCGGGCCA-ACCAGCAGAGGGACTTT 420
Exon17temp ---------------------------------------- 140
  17(18)F+ AAACAGCTGCAGCACAGCGGGCCARACCAGCAGAGGGACTTT 392
  17(18)R- AAACAGCTGCAGCACAGCGGGCCAGACCAGCAGAGGGACTTT 420

 Consensus CAGCGGGAGATTCA-ATCCTCAAAGCACTGCACAGTGATTTC 462
Exon17temp ---------------------------------------- 140
  17(18)F+ CAGCGGGAGATTCARATCCTCAAAGCACTGCACAGTGATTTC 434
  17(18)R- CAGCGGGAGATTCAGATCCTCAAAGCACTGCACAGTGATTTC 462

 Consensus ATTGTCAAGTATCGTGGTGTCAGCTATGGCCCGGGTGAGCCA 504
Exon17temp ---------------------------------------- 140
  17(18)F+ ATTGTCAAGTATCGTGGTGTCAGCTATGGCCCGGGTGAGCCA 476
  17(18)R- ATTGTCAAGTATCGTGGTGTCAGCTATGGCCCGGGTGAGCCA 504

 Consensus GCTCCCGGATGAGTGAACCAAGACGTATGGGTGCTTTTCAAA 546
Exon17temp ---------------------------------------- 140
  17(18)F+ GCTCCCGGATGAGTGAACCAAGACGTATGGGTGCTTTTCAAA 518
  17(18)R- GCTCCCGGATGAGTGAACCAAGACGTATGGGTGCTTTTCAAA 546

 Consensus GTGCACATTCTTACCCTCCTGCCAGGCCACT--AG--AG--T 588
Exon17temp ---------------------------------------- 140
  17(18)F+ GTGCACATTCTTACCCTCCTGCCAGGCCACTTTAGGTAGGCT 560
  17(18)R- GTGCACATTCTTACCCTCCTGCCAGGCCACT--AG-WAGAYT 585

 Consensus GGGAACGTGATGGTCATAGCTGGTTTCCK             617
Exon17temp ----------------------------             140
  17(18)F+ GGGAACGTGATGGTCATAGCTGGTTTCCK             589
  17(18)R- ----------------------------             585
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```
 Consensus GGACGGCCAGTGCACAGCAAGTCAA-T-AG-AGTGGGGC--A 42
Exon18temp ----------------------------------------- 0
      18F+ ----------------------GTYAG-AGTGGGGC--A 14
      18R- GGACGGCCAGTGCACAGCAAGTCAACTCAGGAGTGGGGCCCA 42

 Consensus GGATGAGAGGCGCTGCTTACCACTGCCCATGCCCCCACCCCA 84
Exon18temp ----------------------------------------- 0
      18F+ GGATGAGAGGCGCTGCTTACCACTGCCCATGCCCCCACCCCA 56
      18R- GGATGAGAGGCGCTGCTTACCACTGCCCATGCCCCCACCCCA 84

 Consensus GACTATGAGCTCCTCTCAGACCCCACACCTGGTGCCCTGGCA 126
Exon18temp ----------------------------------------- 0
      18F+ GACTATGAGCTCCTCTCAGACCCCACACCTGGTGCCCTGGCA 98
      18R- GACTATGAGCTCCTCTCAGACCCCACACCTGGTGCCCTGGCA 126

 Consensus CCTCGTGATGGGCTGTGGAATGGTGCCCAGCTCTATGCCTGC 168
Exon18temp ----------------------------------------- 0
      18F+ CCTCGTGATGGGCTGTGGAATGGTGCCCAGCTCTATGCCTGC 140
      18R- CCTCGTGATGGGCTGTGGAATGGTGCCCAGCTCTATGCCTGC 168

 Consensus CAAGACCCCACGAT-TTCGAGGAGAGACACCTCAAGTACATC 210
Exon18temp ----------------------------------------- 0
      18F+ CAAGACCCCACGATCTTCGAGGAGAGACACCTCAAGTACATC 182
      18R- CAAGACCCCACGATYTTCGAGGAGAGACACCTCAAGTACATC 210

 Consensus TCACAGCTGGGCAAGGTAAGGTGGGCAGGGCCAGGGTGGGTT 252
Exon18temp ----------------------------------------- 0
      18F+ TCACAGCTGGGCAAGGTAAGGTGGGCAGGGCCAGGGTGGGTT 224
      18R- TCACAGCTGGGCAAGGTAAGGTGGGCAGGGCCAGGGTGGGTT 252

 Consensus GGAGAGGGCAGGGCAGCATCCAGGTGCCTGGACATCAGTCCC 294
Exon18temp ----------------------------------------- 0
      18F+ GGAGAGGGCAGGGCAGCATCCAGGTGCCTGGACATCAGTCCC 266
      18R- GGAGAGGGCAGGGCAGCATCCAGGTGCCTGGACATCAGTCCC 294

 Consensus GCTATCCCCCAGGGCAACTTTGGCAGCGTGGAGCTGTGCCGC 336
Exon18temp ----------GGCAACTTTGGCAGCGTGGAGCTGTGCCGC 30
      18F+ GCTATCCCCCAGGGCAACTTTGGCAGCGTGGAGCTGTGCCGC 308
      18R- GCTATCCCCCAGGGCAACTTTGGCAGCGTGGAGCTGTGCCGC 336
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```
 Consensus TATGACCCGCTAGGCGACAATACAGGTGCCCTGGTGGCCGTG 378
Exon18temp TATGACCCGCTAGGCGACAATACAGGTGCCCTGGTGGCCGTG 72
      18F+ TATGACCCGCTAGGCGACAATACAGGTGCCCTGGTGGCCGTG 350
      18R- TATGACCCGCTAGGCGACAATACAGGTGCCCTGGTGGCCGTG 378

 Consensus AAACAGCTGCAGCACAGCGGGCCA-ACCAGCAGAGGGACTTT 420
Exon18temp AAACAGCTGCAGCACAGCGGGCCAGACCAGCAGAGGGACTTT 114
      18F+ AAACAGCTGCAGCACAGCGGGCCARACCAGCAGAGGGACTTT 392
      18R- AAACAGCTGCAGCACAGCGGGCCAGACCAGCAGAGGGACTTT 420

 Consensus CAGCGGGAGATTCA-ATCCTCAAAGCACTGCACAGTGATTTC 462
Exon18temp CAGCGGGAGATTCAGATCCTCAAAGCACTGCACAGTGATTTC 156
      18F+ CAGCGGGAGATTCARATCCTCAAAGCACTGCACAGTGATTTC 434
      18R- CAGCGGGAGATTCAGATCCTCAAAGCACTGCACAGTGATTTC 462

 Consensus ATTGTCAAGTATCGTGGTGTCAGCTATGGCCCGGGTGAGCCA 504
Exon18temp ATTGTCAAGTATCGTGGTGTCAGCTATGGCCCGG-------- 190
      18F+ ATTGTCAAGTATCGTGGTGTCAGCTATGGCCCGGGTGAGCCA 476
      18R- ATTGTCAAGTATCGTGGTGTCAGCTATGGCCCGGGTGAGCCA 504

 Consensus GCTCCCGGATGAGTGAACCAAGACGTATGGGTGCTTTTCAAA 546
Exon18temp ---------------------------------------- 190
      18F+ GCTCCCGGATGAGTGAACCAAGACGTATGGGTGCTTTTCAAA 518
      18R- GCTCCCGGATGAGTGAACCAAGACGTATGGGTGCTTTTCAAA 546

 Consensus GTGCACATTCTTACCCTCCTGCCAGGCCACT--AG--AG--T 588
Exon18temp ---------------------------------------- 190
      18F+ GTGCACATTCTTACCCTCCTGCCAGGCCACTTTAGGTAGGCT 560
      18R- GTGCACATTCTTACCCTCCTGCCAGGCCACT--AG-WAGAYT 585

 Consensus GGGAACGTGATGGTCATAGCTGGTTTCCK             617
Exon18temp ---------------------------             190
      18F+ GGGAACGTGATGGTCATAGCTGGTTTCCK             589
      18R- ---------------------------             585
```

```
 Consensus ATTWYRGGAGTGGGGCCAGGATGAGAGGCGCTGCTTACCACT 42
Exon 18 te ----------------------------------------- 0
    18 F + ATTWYRGGAGTGGGGCCAGGATGAGAGGCGCTGCTTACCACT 42

 Consensus GCCCATGCCCCCACCCCAGACTATGAGCTCCTCTCAGACCCC 84
Exon 18 te ----------------------------------------- 0
    18 F + GCCCATGCCCCCACCCCAGACTATGAGCTCCTCTCAGACCCC 84

 Consensus ACACCTGGTGCCCTGGCACCTCGTGATGGGCTGTGGAATGGT 126
Exon 18 te ----------------------------------------- 0
    18 F + ACACCTGGTGCCCTGGCACCTCGTGATGGGCTGTGGAATGGT 126

 Consensus GCCCAGCTCTATGCCTGCCAAGACCCCACGATCTTCGAGGAG 168
Exon 18 te ----------------------------------------- 0
    18 F + GCCCAGCTCTATGCCTGCCAAGACCCCACGATCTTCGAGGAG 168

 Consensus AGACACCTCAAGTACATCTCACAGCTGGGCAAGGTAAGGTGG 210
Exon 18 te ----------------------------------------- 0
    18 F + AGACACCTCAAGTACATCTCACAGCTGGGCAAGGTAAGGTGG 210

 Consensus GCAGGGCCAGGGTGGGTTGGAGAGGGCAGGGCAGCATCCAGG 252
Exon 18 te ----------------------------------------- 0
    18 F + GCAGGGCCAGGGTGGGTTGGAGAGGGCAGGGCAGCATCCAGG 252

 Consensus TGCCTGGACATCAGTCCCGCTATCCCCCAGGGCAACTTTGGC 294
Exon 18 te ----------------------------GGCAACTTTGGC 12
    18 F + TGCCTGGACATCAGTCCCGCTATCCCCCAGGGCAACTTTGGC 294

 Consensus AGCGTGGAGCTGTGCCGCTATGACCCGCTAGGCGACAATACA 336
Exon 18 te AGCGTGGAGCTGTGCCGCTATGACCCGCTAGGCGACAATACA 54
    18 F + AGCGTGGAGCTGTGCCGCTATGACCCGCTAGGCGACAATACA 336

 Consensus GGTGCCCTGGTGGCCGTGAAACAGCTGCAGCACAGCGGGCCA 378
Exon 18 te GGTGCCCTGGTGGCCGTGAAACAGCTGCAGCACAGCGGGCCA 96
    18 F + GGTGCCCTGGTGGCCGTGAAACAGCTGCAGCACAGCGGGCCA 378

 Consensus GACCAGCA-AGGGACTTTCAGCGGGAGATTCA-ATCCTCAAA 420
Exon 18 te GACCAGCAGAGGGACTTTCAGCGGGAGATTCAGATCCTCAAA 138
    18 F + GACCAGCARAGGGACTTTCAGCGGGAGATTCARATCCTCAAA 420
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 Consensus GCACTGCACAGTGATTTCATTGTCAAGTATCGTGGTGTCAGC 462
Exon 18 te GCACTGCACAGTGATTTCATTGTCAAGTATCGTGGTGTCAGC 180
   18 F + GCACTGCACAGTGATTTCATTGTCAAGTATCGTGGTGTCAGC 462

 Consensus TATGGCCCGGGTGAGCCAGCTCCCGGATGAGTGAACCAAGAC 504
Exon 18 te TATGGCCCGG------------------------------ 190
   18 F + TATGGCCCGGGTGAGCCAGCTCCCGGATGAGTGAACCAAGAC 504

 Consensus GTATGGGTGCTTTTCAAAGTGCACATTCTTACCCTCCTGCCA 546
Exon 18 te ----------------------------------------- 190
   18 F + GTATGGGTGCTTTTCAAAGTGCACATTCTTACCCTCCTGCCA 546

 Consensus GGCCACTTTAGGTAGGCTGGGAACGTGATGGTCATAGCKGTT 588
Exon 18 te ----------------------------------------- 190
   18 F + GGCCACTTTAGGTAGGCTGGGAACGTGATGGTCATAGCKGTT 588

 Consensus TCSK                                       592
Exon 18 te ----                                       190
   18 F + TCSK                                       592
```

```
 Consensus NGTAAAACGACGGCCAGTGCAAAACTGAGGTCGAGAGGGACA 42
Exon19temp ---------------------------------------- 0
       19F ---------------------------------------- 0
       19R NGTAAAACGACGGCCAGTGCAAAACTGAGGTCGAGAGGGACA 42

 Consensus CAA---------------G-GGGA---A-GGGGGGA-GAGC 84
Exon19temp ---------------------------------------- 0
       19F ---NNNNNNNNNNNNNNNGNGGGAN--ANGGGGGGANGAGC 37
       19R CAAGGTCCCACTGTGAAAGGGGGGAAGAATGGGGGGACGAGC 84

 Consensus AGGGCTGGGCCCTGCTGTGACAGATCCTGCCTTCTCCAGGCC 126
Exon19temp ----------------------------------GCC 3
       19F AGGGCTGGGCCCTGCTGTGACAGATCCTGCCTTCTCCAGGCC 79
       19R AGGGCTGGGCCCTGCTGTGACAGATCCTGCCTTCTCCAGGCC 126

 Consensus GCCAGAGCCTGCGGCTGGTCATGGAGTACCTGCCCAGCGGCT 168
Exon19temp GCCAGAGCCTGCGGCTGGTCATGGAGTACCTGCCCAGCGGCT 45
       19F GCCAGAGCCTGCGGCTGGTCATGGAGTACCTGCCCAGCGGCT 121
       19R GCCAGAGCCTGCGGCTGGTCATGGAGTACCTGCCCAGCGGCT 168

 Consensus GCTTGCGCGACTTCCTGCAGCGGCACCGCGCGCGCCTCGATG 210
Exon19temp GCTTGCGCGACTTCCTGCAGCGGCACCGCGCGCGCCTCGATG 87
       19F GCTTGCGCGACTTCCTGCAGCGGCACCGCGCGCGCCTCGATG 163
       19R GCTTGCGCGACTTCCTGCAGCGGCACCGCGCGCGCCTCGATG 210

 Consensus CCAGCCGCCTCCTTCTCTATTCCTCGCAGATCTGCAAGGTGC 252
Exon19temp CCAGCCGCCTCCTTCTCTATTCCTCGCAGATCTGCAAG---- 125
       19F CCAGCCGCCTCCTTCTCTATTCCTCGCAGATCTGCAAGGTGC 205
       19R CCAGCCGCCTCCTTCTCTATTCCTCGCAGATCTGCAAGGTGC 252

 Consensus GAGGGGGCGCCCCGGGACTTGTGGGGATTCAGCTGGCACGGC 294
Exon19temp ---------------------------------------- 125
       19F GAGGGGGCGCCCCGGGACTTGTGGGGATTCAGCTGGCACGGC 247
       19R GAGGGGGCGCCCCGGGACTTGTGGGGATTCAGCTGGCACGGC 294

 Consensus CTGGGCAGGGGTCTGCTTGGAGGTCGCGGTGAAGGCTGAGGA 336
Exon19temp ---------------------------------------- 125
       19F CTGGGCAGGGGTCTGCTTGGAGGTCGCGGTGAAGGCTGAGGA 289
       19R CTGGGCAGGGGTCTGCTTGGAGGTCGCGGTGAAGGCTGAGGA 336
```

```
 Consensus G--GTT--GG--CC-------GG-N----TGGGGTTGGCTTA 378
Exon19temp ---------------------------------------- 125
      19F GTGGTTTGGGGTCCAGGTCTCGGGNGTGGTGGGGTTGGCTTA 331
      19R GNNGTTNNGGNNCCNNNNNNNGGNNNNNN------------ 365

 Consensus GGGCTCAGGATCAGAGGTCATAGCTGTTTCNNNANN       414
Exon19temp ----------------------------------       125
      19F GGGCTCAGGATCAGAGGTCATAGCTGTTTCNNNANN       367
      19R ----------------------------------       365
```

```
 Consensus NNTAAAACGACGGCCAGTCAGAACTTCAGTGGAGGATGGCT- 42
Exon20temp ----------------------------------------- 0
       20F ----------------------------------------N 1
       20R NNTAAAACGACGGCCAGTCAGAACTTCAGTGGAGGATGGCTC 42

 Consensus --------------GTTGGGGTCTGGGTTGGGGTGCCAGGT 84
Exon20temp ----------------------------------------- 0
       20F NNNNNNNNNNNNNNNGTTGGGGTCTGGGTTGGGGTGCCAGGT 43
       20R GGGGGTAGGGTTATAGTTGGGGTCTGGGTTGGGGTGCCAGGT 84

 Consensus CACGCTTGGGGTACCTGCCGGATTATCCTGGGATCCTCTCTG 126
Exon20temp ----------------------------------------- 0
       20F CACGCTTGGGGTACCTGCCGGATTATCCTGGGATCCTCTCTG 85
       20R CACGCTTGGGGTACCTGCCGGATTATCCTGGGATCCTCTCTG 126

 Consensus CACGCTCACACCGCCCGCCCGCAGGGCATGGAGTACCTGGGC 168
Exon20temp ----------------------GGCATGGAGTACCTGGGC 18
       20F CACGCTCACACCGCCCGCCCGCAGGGCATGGAGTACCTGGGC 127
       20R CACGCTCACACCGCCCGCCCGCAGGGCATGGAGTACCTGGGC 168

 Consensus TCCCGCCGCTGCGTGCACCGCGACCTGGCCGCCCGAAACATC 210
Exon20temp TCCCGCCGCTGCGTGCACCGCGACCTGGCCGCCCGAAACATC 60
       20F TCCCGCCGCTGCGTGCACCGCGACCTGGCCGCCCGAAACATC 169
       20R TCCCGCCGCTGCGTGCACCGCGACCTGGCCGCCCGAAACATC 210

 Consensus CTCGTGGAGAGCGAGGCACACGTCAAGATCGCTGACTTCGGC 252
Exon20temp CTCGTGGAGAGCGAGGCACACGTCAAGATCGCTGACTTCGGC 102
       20F CTCGTGGAGAGCGAGGCACACGTCAAGATCGCTGACTTCGGC 211
       20R CTCGTGGAGAGCGAGGCACACGTCAAGATCGCTGACTTCGGC 252

 Consensus CTAGCTAAGCTGCTGCCGCTTGACAAAGACTACTACGTGGTC 294
Exon20temp CTAGCTAAGCTGCTGCCGCTTGACAAAGACTACTACGTGGTC 144
       20F CTAGCTAAGCTGCTGCCGCTTGACAAAGACTACTACGTGGTC 253
       20R CTAGCTAAGCTGCTGCCGCTTGACAAAGACTACTACGTGGTC 294

 Consensus CGCGAGCCAGGCCAGAGCCCCATTTTCTGGTGGGGAACCCGC 336
Exon20temp CGCGAGCCAGGCCAGAGCCCCATTTTCTG------------ 173
       20F CGCGAGCCAGGCCAGAGCCCCATTTTCTGGTGGGGAACCCGC 295
       20R CGCGAGCCAGGCCAGAGCCCCATTTTCTGGTGGGGAACCCGC 336
```

```
 Consensus GCCTAGGCTCCGCCCCTAN-CCCCACGGCTCTGGCTCCGCCC 378
Exon20temp ---------------------------------------- 173
       20F GCCTAGGCTCCGCCCCTANTCCCCACGGCTCTGGCTCCGCCC 337
       20R GCCTAGGCTCCGCCCCTANNCCCCACGGCTCTGGCTCCGCCC 378

 Consensus CCAGCCATGCCCCCGCCCCCTCCCGCTGCTTTGCTCCCCAGC 420
Exon20temp ---------------------------------------- 173
       20F CCAGCCATGCCCCCGCCCCCTCCCGCTGCTTTGCTCCCCAGC 379
       20R CCAGCCATGCCCCCGCCCCCTCCCGCTGCTTTGCTCCCCAGC 420

 Consensus CTTAGCCCCGCCCTTCCTCCGCTGCAGCTTTGGCCCCTCCCA 462
Exon20temp ---------------------------------------- 173
       20F CTTAGCCCCGCCCTTCCTCCGCTGCAGCTTTGGCCCCTCCCA 421
       20R CTTAGCCCCGCCCTTCCTCCGCTGCAGCTTTGGCCCCTCCCA 462

 Consensus CTCCCCAGAGCCCCGCCCCCTCAACAGCACTGGCTCCTCT-T 504
Exon20temp ---------------------------------------- 173
       20F CTCCCCAGAGCCCCGCCCCCTCAACAGCACTGGCTCCTCTGT 463
       20R CTCCCCAGAGCCCCGCCCCCTCAACAGCACTGGCTCCTCTNT 504

 Consensus CTCCCGCTGCCCTGC-----G-------------------G 546
Exon20temp ---------------------------------------- 173
       20F CTCCCGCTGCCCTGCTGTCAGCGGCCCCCAGCCTTAGCCCCG 505
       20R CTCCCGCTGCCCTGCNNNNNGNNNNNNNNNNNNNNCNNNN- 545

 Consensus CCCTTCTCTCAGCTCTCGCCGGTCATAGCTGTNNCNNG     584
Exon20temp ------------------------------------     173
       20F CCCTTCTCTCAGCTCTCGCCGGTCATAGCTGTNNCNNG    543
       20R ------------------------------------     545
```

```
 Consensus NTTNTNNNAAACGACGGCCNGTGAATCCACCTATCCCACAGC 42
Exon21temp ----------------------------------------- 0
       21F ----------------------------------------- 0
       21R NTTNTNNNAAACGACGGCCNGTGAATCCACCTATCCCACAGC 42

 Consensus CAGGGAA----------------GGG--TGACCTGCTC--- 84
Exon21temp ----------------------------------------- 0
       21F -------NNNNNNNNNNNNNNNNNGGGNNTGACCTGCTCNNN 35
       21R CAGGGAAACCGAGACCCTGGAGACGGGACTGACCTGCTCACA 84

 Consensus GTCCCCACCTACCCTGACCAGTTCCCCATTCCAAGGCTGCCC 126
Exon21temp ----------------------------------------- 0
       21F GTCCCCACCTACCCTGACCAGTTCCCCATTCCAAGGCTGCCC 77
       21R GTCCCCACCTACCCTGACCAGTTCCCCATTCCAAGGCTGCCC 126

 Consensus CCCTCTTCCTGTCCTTTCTACACCCTCGCATCTCAAGACCTT 168
Exon21temp ----------------------------------------- 0
       21F CCCTCTTCCTGTCCTTTCTACACCCTCGCATCTCAAGACCTT 119
       21R CCCTCTTCCTGTCCTTTCTACACCCTCGCATCTCAAGACCTT 168

 Consensus GTCCCCTCTCCAGGTATGCCCCCGAATCCCTCTCGGACAACA 210
Exon21temp ------------GTATGCCCCCGAATCCCTCTCGGACAACA 29
       21F GTCCCCTCTCCAGGTATGCCCCCGAATCCCTCTCGGACAACA 161
       21R GTCCCCTCTCCAGGTATGCCCCCGAATCCCTCTCGGACAACA 210

 Consensus TCTTCTCTCGCCAGTCAGACGTCTGGAGCTTCGGGGTCGTCC 252
Exon21temp TCTTCTCTCGCCAGTCAGACGTCTGGAGCTTCGGGGTCGTCC 71
       21F TCTTCTCTCGCCAGTCAGACGTCTGGAGCTTCGGGGTCGTCC 203
       21R TCTTCTCTCGCCAGTCAGACGTCTGGAGCTTCGGGGTCGTCC 252

 Consensus TGTACGAGCTCTTCACCTACTGCGACAAAAGCTGCAGCCCCT 294
Exon21temp TGTACGAGCTCTTCACCTACTGCGACAAAAGCTGCAGCCCCT 113
       21F TGTACGAGCTCTTCACCTACTGCGACAAAAGCTGCAGCCCCT 245
       21R TGTACGAGCTCTTCACCTACTGCGACAAAAGCTGCAGCCCCT 294

 Consensus CGGCCGTGAGTCGGCTTCCCA-N-CCCCCAGCCTTCTTCTCC 336
Exon21temp CGGCC-------------------------------------- 118
       21F CGGCCGTGAGTCGGCTTCCCANNNCCCCCAGCCTTCTTCTCC 287
       21R CGGCCGTGAGTCGGCTTCCCAGNGCCCCCAGCCTTCTTCTCC 336
```

```
 Consensus CTCCACGCCCCTCG--GCCA----C----------------- 378
Exon21temp ---------------------------------------- 118
      21F CTCCACGCCCCTCGTGGCCAATCTCCAACCTGTCTGCGCCTG 329
      21R CTCCACGCCCCTCGNNGCCANNNNCNNNNNNNNNNNNNNNN 378

 Consensus CGTCCCTCTTTAGCATGGGGTCACGGTCATAGCTGTTTCNNN 420
Exon21temp ---------------------------------------- 118
      21F CGTCCCTCTTTAGCATGGGGTCACGGTCATAGCTGTTTCNNN 371
      21R ---------------------------------------- 378

 Consensus AAAA                                     424
Exon21temp ----                                     118
      21F AAAA                                     375
      21R ----                                     378
```

```
 Consensus NNNTNNNAAACGACGGCCAGTACCTTTCTGACCCCTTCACGG 42
Exon22temp ----------------------------------------- 0
       22F ----------------------------------------- 0
       22R NNNTNNNAAACGACGGCCAGTACCTTTCTGACCCCTTCACGG 42

 Consensus TNCAG------------C------TC---GATGGCCCCTACC 84
Exon22temp ----------------------------------------- 0
       22F -----NNNNNNNNNNNNCNNNNNNTCNNNGATGGCCCCTACC 37
       22R TNCAGGCAGCCCCTCCCCGCTCCATCACAGATGGCCCCTACC 84

 Consensus CCCACCACGGGTGGCCCCTCCCCCTCCACCCACGGAGGCTCC 126
Exon22temp ----------------------------------------- 0
       22F CCCACCACGGGTGGCCCCTCCCCCTCCACCCACGGAGGCTCC 79
       22R CCCACCACGGGTGGCCCCTCCCCCTCCACCCACGGAGGCTCC 126

 Consensus TCCCCCACCACATGCGCTCCTCCTTGGCTCCAGGAGTTCCTG 168
Exon22temp -------------------------------GAGTTCCTG 9
       22F TCCCCCACCACATGCGCTCCTCCTTGGCTCCAGGAGTTCCTG 121
       22R TCCCCCACCACATGCGCTCCTCCTTGGCTCCAGGAGTTCCTG 168

 Consensus CGGATGATGGGATGTGAGCGGGATGTCCCCGCCCTCTGCCGC 210
Exon22temp CGGATGATGGGATGTGAGCGGGATGTCCCCGCCCTCTGCCGC 51
       22F CGGATGATGGGATGTGAGCGGGATGTCCCCGCCCTCTGCCGC 163
       22R CGGATGATGGGATGTGAGCGGGATGTCCCCGCCCTCTGCCGC 210

 Consensus CTCTTGGAACTGCTGGAGGAGGGCCAGAGGCTGCCGGCGCCT 252
Exon22temp CTCTTGGAACTGCTGGAGGAGGGCCAGAGGCTGCCGGCGCCT 93
       22F CTCTTGGAACTGCTGGAGGAGGGCCAGAGGCTGCCGGCGCCT 205
       22R CTCTTGGAACTGCTGGAGGAGGGCCAGAGGCTGCCGGCGCCT 252

 Consensus CCTGCCTGCCCTGCTGAGGTGAGCGCCGCAGGGCTAGCCTCA 294
Exon22temp CCTGCCTGCCCTGCTGAG---------------------- 111
       22F CCTGCCTGCCCTGCTGAGGTGAGCGCCGCAGGGCTAGCCTCA 247
       22R CCTGCCTGCCCTGCTGAGGTGAGCGCCGCAGGGCTAGCCTCA 294

 Consensus GTTTCCCAGTCTGTAGATTGGGCCGGGGTCTCGGGCAAGCCA 336
Exon22temp ------------------------------------ 111
       22F GTTTCCCAGTCTGTAGATTGGGCCGGGGTCTCGGGCAAGCCA 289
       22R GTTTCCCAGTCTGTAGATTGGGCCGGGGTCTCGGGCAAGCCA 336
```

```
 Consensus GCTGGCGCCTGAGTCTCTGTACTGAGAAGAAAGGCTAGAGTG 378
Exon22temp ------------------------------------------ 111
       22F GCTGGCGCCTGAGTCTCTGTACTGAGAAGAAAGGCTAGAGTG 331
       22R GCTGGCGCCTGAGTCTCTGTACTGAGAAGAAAGGCTAGAGTG 378

 Consensus TGAGGCCGATGAGGATCCTGGCCCCCACTTGGCTACTCTCTC 420
Exon22temp ------------------------------------------ 111
       22F TGAGGCCGATGAGGATCCTGGCCCCCACTTGGCTACTCTCTC 373
       22R TGAGGCCGATGAGGATCCTGGCCCCCACTTGGCTACTCTCTC 420

 Consensus ACTGTGTGGCAAGTCAGAGCACTTTCAGAGCCTCAGTTTACC 462
Exon22temp ------------------------------------------ 111
       22F ACTGTGTGGCAAGTCAGAGCACTTTCAGAGCCTCAGTTTACC 415
       22R ACTGTGTGGCAAGTCAGAGCACTTTCAGAGCCTCAGTTTACC 462

 Consensus CTTTTCCAAAA-GAGAAT---AAT-CCT-------------- 504
Exon22temp ------------------------------------------ 111
       22F CTTTTCCAAAATGAGAATAATAATGCCTTATAGGGTGAGGGA 457
       22R CTTTTCCAAAANGAGAATNNNAATNCCTNNNNNNNNNNNNNN 504

 Consensus ------GACTCCTGAACACCTGTGCCTATGGGTCATAGCTGT 546
Exon22temp ------------------------------------------ 111
       22F AGATTAGACTCCTGAACACCTGTGCCTATGGGTCATAGCTGT 499
       22R NNNNNN----------------------------------- 510

 Consensus TTCNNNANN                                  555
Exon22temp ---------                                  111
       22F TTCNNNANN                                  508
       22R ---------                                  510
```

```
 Consensus NNNNAAACGNCGGCCAGTGATCATGCCATTGCACTCCAGCCT 42
Exon23temp ----------------------------------------- 0
      23aF ----------------------------------------- 0
      23aR NNNNAAACGNCGGCCAGTGATCATGCCATTGCACTCCAGCCT 42
      23bF ----------------------------------------- 0
      23bR ----------------------------------------- 0

 Consensus G---------------T-C--CT--A-AAA-C-AAAACAAA 84
Exon23temp ----------------------------------------- 0
      23aF -NGNNNNNNNNNNNNNTNCNNCTNNANAAA-CNAAAACAAA 40
      23aR GGACAACAGAGCTAGACTCCGTCTCAAAAAAACAAAAACAAA 84
      23bF ----------------------------------------- 0
      23bR ----------------------------------------- 0

 Consensus TACGCTGAATGGGAGT----------GAC-GC-CAG-CACG 126
Exon23temp ----------------------------------------- 0
      23aF TACGCTGAATGGGAGTTGTGTCCTTTGGACTGCTCAGGCACG 82
      23aR TACGCTGAATGGGAGTTGTGTCCTTTGGACTGCTCAGGCACG 126
      23bF ----------------------------------------- 0
      23bR ----------------NNNNNNNAAACGACGGC-CAGTCACG 25

 Consensus ACCCCATTATCTGTCCCCCGCCC-------------CT--T 168
Exon23temp ---------------------------GTTCACGAGCTCAT 14
      23aF ACCCCATTATCTGTCCCCCGCCCCTCAGGTTCACGAGCTCAT 124
      23aR ACCCCATTATCTGTCCCCCGCCCCTCAGGTTCACGAGCTCAT 168
      23bF ----------------------GNNNNNNNNNNNNNCTNNT 19
      23bR ACCCCATTATCTGTCCCCCGCCCCTCAGGTTCACGAGCTCAT 67

 Consensus ---GCTGTGCTGGGCCCCTAGCCCACAGGACCGGCCATCATT 210
Exon23temp GAAGCTGTGCTGGGCCCCTAGCCCACAGGACCGGCCATCATT 56
      23aF GAAGCTGTGCTGGGCCCCTAGCCCACAGGACCGGCCATCATT 166
      23aR GAAGCTGTGCTGGGCCCCTAGCCCACAGGACCGGCCATCATT 210
      23bF NNNGCTGTGCTGGGCCCCTAGCCCACAGGACCGGCCATCATT 61
      23bR GAAGCTGTGCTGGGCCCCTAGCCCACAGGACCGGCCATCATT 109

 Consensus CAGCGCCCTGGGCCCCCAGCTGGACATGCTGTGGAGCGGAAG 252
Exon23temp CAGCGCCCTGGGCCCCCAGCTGGACATGCTGTGGAGCGGAAG 98
      23aF CAGCGCCCTGGGCCCCCAGCTGGACATGCTGTGGAGCGGAAG 208
      23aR CAGCGCCCTGGGCCCCCAGCTGGACATGCTGTGGAGCGGAAG 252
      23bF CAGCGCCCTGGGCCCCCAGCTGGACATGCTGTGGAGCGGAAG 103
      23bR CAGCGCCCTGGGCCCCCAGCTGGACATGCTGTGGAGCGGAAG 151
```

```
 Consensus CCGGGGGTGTGAGACTCATGCCTTCACTGCTCACCCAGAGGG 294
Exon23temp CCGGGGGTGTGAGACTCATGCCTTCACTGCTCACCCAGAGGG 140
      23aF CCGGGGGTGTGAGACTCATGCCTTCACTGCTCACCCAGAGGG 250
      23aR CCGGGGGTGTGAGACTCATGCCTTCACTGCTCACCCAGAGGG 294
      23bF CCGGGGGTGTGAGACTCATGCCTTCACTGCTCACCCAGAGGG 145
      23bR CCGGGGGTGTGAGACTCATGCCTTCACTGCTCACCCAGAGGG 193

 Consensus CAAACACCACTCCCTGTCCTTTTCATAGCTCCTGCCCGCAGA 336
Exon23temp CAAACACCACTCCCTGTCCTTTTCATAG-------------- 168
      23aF CAAACACCACTCCCTGTCCTTTTCATAGCTCCTGCCCGCAGA 292
      23aR CAAACACCACTCCCTGTCCTTTTCATAGCTCCTGCCCGCAGA 336
      23bF CAAACACCACTCCCTGTCCTTTTCATAGCTCCTGCCCGCAGA 187
      23bR CAAACACCACTCCCTGTCCTTTTCATAGCTCCTGCCCGCAGA 235

 Consensus CCTCTGGATTAGGTCTCTGTTGACTGGCTGTGTGACCTTAGG 378
Exon23temp ---------------------------------------- 168
      23aF CCTCTGGATTAGGTCTCTGTTGACTGGCTGTGTGACCTTAGG 334
      23aR CCTCTGGATTAGGTCTCTGTTGACTGGCTGTGTGACCTTAGG 378
      23bF CCTCTGGATTAGGTCTCTGTTGACTGGCTGTGTGACCTTAGG 229
      23bR CCTCTGGATTAGGTCTCTGTTGACTGGCTGTGTGACCTTAGG 277

 Consensus CCCGGAGCTGCCCCTCTCTGGGCCTCAGAGGCCTTATGAGGG 420
Exon23temp ---------------------------------------- 168
      23aF CCCGGAGCTGCCCCTCTCTGGGCCTCAGAGGCCTTATGAGGG 376
      23aR CCCGGAGCTGCCCCTCTCTGGGCCTCAGAGGCCTTATGAGGG 420
      23bF CCCGGAGCTGCCCCTCTCTGGGCCTCAGAGGCCTTATGAGGG 271
      23bR CCCGGAGCTGCCCCTCTCTGGGCCTCAGAGGCCTTATGAGGG 319

 Consensus TCCTCTACTTCAGGAACACCCCC-NGACATTGCATTTGGGGG 462
Exon23temp ---------------------------------------- 168
      23aF TCCTCTACTTCAGGAACACCCCCNNGACATTGCATTTGGGGG 418
      23aR TCCTCTACTTCAGGAACACCCCCANGACATTGCATTTGGGGG 462
      23bF TCCTCTACTTCAGGAACACCCCCNNGACATTGCATTTGGGGG 313
      23bR TCCTCTACTTCAGGAACACCCCCANGACATTGCATTTGGGGG 361

 Consensus GGCTCCCGTGGCCTGTAGAATAGCCTGTGGCCTTTGCAATTT 504
Exon23temp ---------------------------------------- 168
      23aF GGCTCCCGTGGCCTGTAGAATAGCCTGTGGCCTTTGCAATTT 460
      23aR GGCTCCCGTGGCCTGTAGAATAGCCTGTGGCCTTTGCAATTT 504
      23bF GGCTCCCGTGGCCTGTAGAATAGCCTGTGGCCTTTGCAATTT 355
      23bR GGCTCCCGTGGCCTGTAGAATAGCCTGTGGCCTTTGCAATTT 403
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 Consensus GTTAAGGTTCAAGACAGA-GGGCATA---------GGG---- 546
Exon23temp ---------------------------------------- 168
      23aF GTTAAGGTTCAAGACAGATGGGCATATGTGTCAGTGGGGCTC 502
      23aR GTTAAGGTTCAAGACAGANGGGCATANNNNNNNN-GGGNNNN 545
      23bF GTTAAGGTTCAAGACAGATGGGCATATGTGTCAGTGGGGCTC 397
      23bR GTTAAGGTTCAAGACAGATGGGCATATGTGTCAGTGGGGCTC 445

 Consensus ------------CCCAAAGAAGCAAGGAACCAA--T-A-AG- 588
Exon23temp ---------------------------------------- 168
      23aF TCTGAGTCCTGGCCCAAAGAAGCAAGGAACCAAGGTCATAG- 543
      23aR NNNNNNNNNNNN---------------------------- 557
      23bF TCTGAGTCCTGGCCCAAAGAAGCAAGGAACCAAATTTA-AGA 438
      23bR TCTGAGTCCTGGCCCAAAGAAGCAAGGAACCAAATTTA-AGA 486

 Consensus CT-T-------TCCCAACCCCTTAAGCCCTGGCCCCCTGAGT 630
Exon23temp ---------------------------------------- 168
      23aF CTGTTNNNNNN------------------------------ 554
      23aR ---------------------------------------- 557
      23bF CTCTCGCATCTTCCCAACCCCTTAAGCCCTGGCCCCCTGAGT 480
      23bR CTCTCGCATCTTCCCAACCCCTTAAGCCCTGGCCCCCTGAGT 528

 Consensus TTCCTTTTCTGTCTCTCTCTTTTTATTTTTTTTATTTTTA-T 672
Exon23temp ---------------------------------------- 168
      23aF ---------------------------------------- 554
      23aR ---------------------------------------- 557
      23bF TTCCTTTTCTGTCTCTCTCTTTTTATTTTTTTTATTTTTATT 522
      23bR TTCCTTTTCTGTCTCTCTCTTTTTATTTTTTTTATTTTTANT 570

 Consensus T------------AGAGCCTCGCTCTGTTACCCAGGGTGGG 714
Exon23temp ---------------------------------------- 168
      23aF ---------------------------------------- 554
      23aR ---------------------------------------- 557
      23bF TTTATTTTTGAGACAGAGCCTCGCTCTGTTACCCAGGGTGGG 564
      23bR TNNNNNNNNNNNNN---------------------------- 584

 Consensus GTCATAGNTGTTNNNNN                          731
Exon23temp -----------------                         168
      23aF -----------------                         554
      23aR -----------------                         557
      23bF GTCATAGNTGTTNNNNN                         581
      23bR -----------------                         584
```

Vita

Damaris Rosado was born in El Paso, Texas. The only daughter or Maria Trinidad Rosado, she graduated from Irvin High School, El Paso, Texas, in the spring of 2004 and entered the University of Texas at El Paso in the fall. While pursuing a bachelor's degree in biological sciences, she worked for the Research Initiative for Scientific Enhancement (RISE) where she performed HIV and cancer research and as well performed osteogenesis imperfecta through the Howard Hughes Medical Institute (HHMI) at Baylor College of Medicine. In the fall of 2010, she entered the Graduate School at the University of Texas at El Paso.

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