

2020-01-01

Identification Of A Novel Single Amino Acid Substitution (v666g) Of JAK1 From A Patient With Acute Lymphoblastic Leukemia Impairs JAK3 Mediated Il-2 Signaling

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IDENTIFICATION OF A NOVEL SINGLE AMINO ACID SUBSTITUTION
(V666G) OF JAK1 FROM A PATIENT WITH ACUTE LYMPHOBLASTIC
LEUKEMIA IMPAIRS JAK3 MEDIATED IL-2 SIGNALING

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By
Alice Hernandez Grant
2020

Dedication

To my amazing family. To my husband, the love of my life, Jesse Grant thank you for letting me have it all. You have given me an incredible life, a loving family and many adventures while pursuing my PhD! Your support and parenting has allowed me to pursue my passion for science; while I am in the laboratory, or writing at a coffee shop, I know our children are receiving your unconditional love. To my June. When you came into my life you inspired me to be the best version of myself and gave me strength to pursuing my PhD. Knowing you are at home has kept me organized and on point in the lab so that I can rush home to you at the end of the day. To CJ, my little baby. You are too perfect for the world. I want to use my PhD in a way that will make it better when you are all grown up!

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(V666G) OF JAK1 FROM A PATIENT WITH ACUTE LYMPHOBLASTIC
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by

ALICE HERNANDEZ GRANT B.S. M.A.

DISSERTATION

Presented to the Faculty of the Graduate School of
The University of Texas at El Paso
in Partial Fulfilment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Department of Biological Sciences
THE UNIVERSITY OF TEXAS AT EL PASO

May 2020

Acknowledgements

Dr. Robert A. Kirken, your laboratory sparked my passion in all things JAK! I am very proud of the research we do and how it can potentially translate to ending health disparities within our community. Thank you for mentoring me in this field and in many other ways. You have taught me that believing in people is what makes a great leader. I would also like to thank members of the Kirken Lab. Georgialina, thank you for keeping everything running smoothly in the lab and entertaining. I truly value you as a mentor and friend. I would also like to thank past and present post-docs in our lab Drs. Blanca Ruiz, Elisa Robles, and Armando Estrada. Your advice and expertise has been invaluable to my success. Yes, even if your answer is always 40 μ M, Mando. Dr. Karla Moran, thank you for bringing me luck, being positive and always talking science with me. I had so much fun sharing a bench with you! Yoshira, thank you for going through the PhD experience with me and being a peer mentor. Alex, I am very impressed with you as a scientist and appreciate the quality of work you generated for this project. Rosabril Acuna, thank you for your support and the coffee breaks! Lastly I would like to thank both past and present undergraduate students that kept the lab moving, Denisse Cadena, Karina Damian, Daniela Diaz and Conrad Bencomo.

I would also like to express my sincere gratitude to my committee members: Drs. Cox, Garza, Almeida, and Leung. Thank you for your invaluable feedback and expertise. All of you have influenced me as a scientist, both during lectures, at individual meetings or through collaborations. Dr. Garza, thank you for being my advocate, you always go above and beyond. I would also like to acknowledge the funding that supported my dissertation. I hope the enclosed work helps to fulfil the mission of the BBRC: 5G12MD007592, Coldwell Foundation, Marsh Foundation and RISE: R25 GM 069621.

Abstract

The Janus kinase (JAK) family, notably JAK1, JAK2 and JAK3 are recognized as oncogenic drivers in high risk Acute Lymphoblastic Leukemia (ALL). The bulk of activating JAK mutations are thought to occur within functional hot-spots across Janus Homology (JH) domains. The most frequently mutated regions is the JH2 pseudo-kinase, which provides a negative regulatory role to the adjacent catalytically active JH1 kinase domain. Despite the prevalence of JAK activating mutations and a need for new therapeutic inhibitors, there is a lack of understanding in the allosteric regulation of JAK kinases. Here we sought to identify mutations involved in driving ALL in the El Paso del Norte population, comprised of an 80% Hispanic demographic; an ethnic group associated with high-risk ALL. Using Whole Exome Sequencing, we detected a novel mutation, V666G, localized within the JH2 domain of JAK1. This mutant is proximal to well-known and characterized JAK1 activating mutations. Unexpectedly, the V666G mutation resulted in a JAK1 kinase-dead like phenotype as indicated by a lack of autophosphorylation and cross-phosphorylation by JAK family members. Furthermore, a dominant negative effect of kinase dead JAK1 (K908A) and the JAK1 V666G mutant occurred across JAK family auto and cross activation. JAK3 was autoactivated in the absence of JAK1, however, the presence of inactive JAK1, either K908A or V666G mutations, reduced JAK3 Tyr phosphorylation. In the context of Interleukin-2 (IL-2) signaling, a dominant negative effect by JAK1 V666G was observed on downstream effectors. The presence of JAK1 V666G decreased Tyr phosphorylation on ALL associated JAK3 activating mutants M511I and A573V. These findings reveal a dominant negative role of JAK1 on JAK3 and support a mechanism by which JAK1 may regulate JAK3 autoactivation and IL-2 signaling.

Elucidation of the trans-inhibitory interactions between JAK1 and JAK3 may propagate new ways to allosterically inhibit overactive JAK kinases in cancer.

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Chapter 1: Introduction

1.1 Significance

Cancer exerts the most profound effect on the Hispanic-Mexican-American population health, as the leading cause of death in the U.S. (1). It is estimated that Hispanic children are twice as likely to be diagnosed with cancer than their non-Hispanic counterparts. Moreover, disproportionate health outcomes persist post therapy (2) indicating a role for molecular etiology of cancer health disparities. One of the most prominent Hispanic health disparities in cancer occurs in pediatric leukemia incidence, and mortality (3).

In the U.S, Hispanic children incur the highest rates of leukemia compared to all other races and ethnicities (4). These rates occur most frequently for Acute Lymphoblastic Leukemia (ALL) which accounts for 78% of Hispanic childhood leukemia cases (4) and is second in cancers causing the most deaths in children (5). With current treatment regimens, 80% of childhood ALL is cured (6). However, a subgroup exhibit refractory or relapse ALL where the 5-year free survival rate falls to 15-50%. Hispanics show a 5-year free survival rate for ALL to be 4% lower compared to other children (4).

Current treatment regimens for ALL now include tailoring therapies to fit a patient's needs based on molecular genomic signatures. This has enabled patients to be screened for common activating mutations to predict whether they can benefit from targeted therapies. For example, ALL patients carrying the fusion gene, Breakpoint Cluster Region and Abelson (BCR-ABL) are selected for treatment with imatinib, a tyrosine kinase inhibitor. Currently, Food and Drug Administration (FDA) approved

Janus Kinase (JAK) inhibitors are being tested for their clinical efficacy to treat pediatric B-ALL with JAK activating mutations (7). It is unknown whether Hispanic patients will benefit equally from targeted therapies against known mutations as there is a lack of inclusion or participation of minorities in cancer research and clinical trials. Moreover, less than 2% of genome-wide studies on biospecimens, are from Hispanics (8) and therefore it is unknown whether actionable mutations are responsible for driving certain cancer in this population.

El Paso Del Norte embodies a population of 82% Hispanics, primarily of Mexican origin. According to the Texas Cancer Registry, this demographic is also burdened by the high childhood leukemia incidence rates as previously discussed (Figure 1). Therefore, the unique population demographic of the El Paso-Juarez border provides an opportunity to investigate the complexity of cancer health disparities in Hispanics. Here, we sought to identify and characterize JAK mutational profiles that emerge in the Hispanic population to determine novel targets promoting ALL within such high-risk groups.

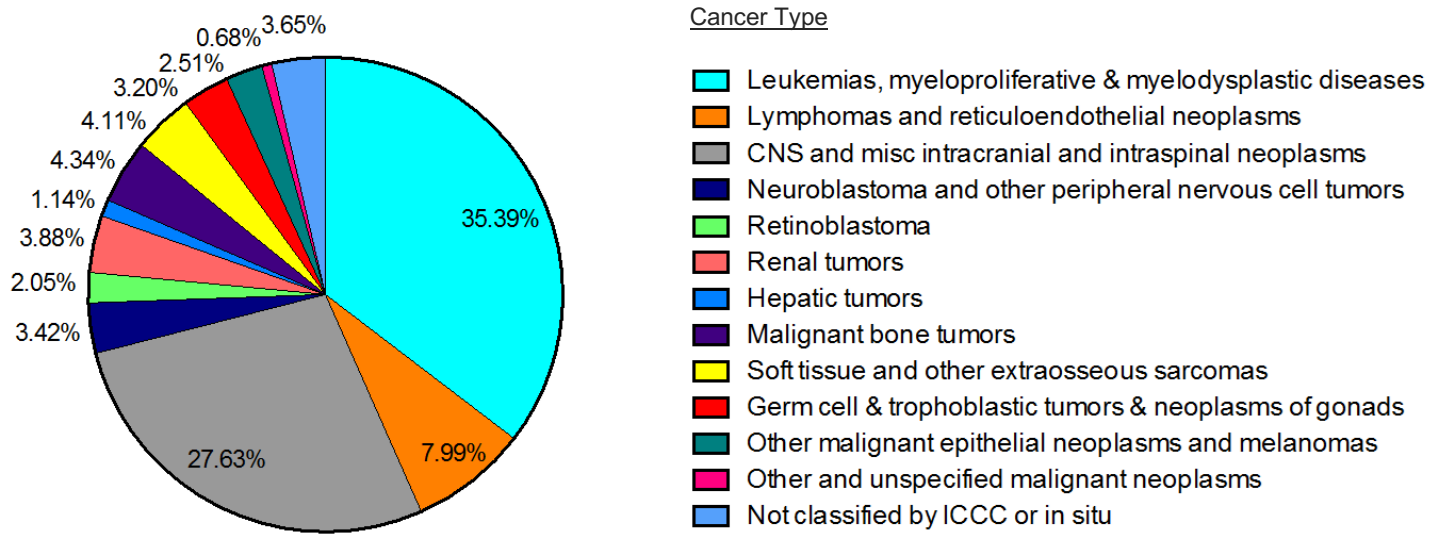


Figure 1. El Paso County Childhood Average Cancer Incidence. Distribution of El Paso childhood incidence by cancer classification. Each section of the diagram represents the percent of childhood cancer incidence within El Paso county.

1.2 Background

1.2.1 Interleukin 2 Signaling

JAK kinases take the forefront working immediately downstream of cytokine receptors lacking intrinsic catalytic activity. The JAK family consists of four tyrosine kinases (JAK1, JAK2, JAK3, and Tyrosine-protein kinase 2 (TYK2)) and through paired combinations with seven Signal Transducers and Activators of Transcription (STAT) orchestrate cell specific cytokine signaling (9). Unique to lymphocytes, the common gamma chain (γ_c) receptor recruits JAK3 partnered with JAK1 and together transduce signals from cytokines, Interleukin 2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21 (10).

IL-2 is vital in controlling the life of lymphocytes. The IL-2 ligand is a four-alpha helical structure that signals by engagement with the IL-2 heterotrimeric receptor (11). IL-2 receptor consists of IL-2 receptor β -chain (IL-2R β) (CD122), IL-2R α (CD25) and the γ_c (CD132) (12). The high-affinity IL-2R α / β / γ_c receptor recruits JAK1 to IL-2R β and JAK3 to γ_c via box1 and box2 motifs. IL-2 binding induces trans-phosphorylation between the JAK kinases leading to their activation. Once active, JAK kinases phosphorylate Tyr residues of the γ_c and IL-2R β chain, thereby creating docking sites for Src Homology 2 (SH2) containing proteins. These docking sites allow protein protein interactions triggering the Mitogen activated protein Kinase (MAPK), Phosphatidylinositol 3 Kinase (PI3K) and the JAK/STAT pathways. These ladder pathways directly regulate growth, survival and proliferation respectively.

MAPK Pathway

SH2 containing protein (SHC) binds to phosphorylated Tyr 338 of IL-2R β chain

initiating the MAPK pathway (13). SHC becomes Tyr phosphorylated and recruits Growth factor receptor-bound protein 2 (GRB2). GRB2 works as an adapter protein for Son of seven less (SOS). SOS facilitates activation of RAS by the exchange of GDP to GTP. Now in its active form RAS enables the Ser/Thr kinase RAF to activate Ser/Thr kinase MEK. MEK phosphorylates MAPK and Extracellular signal regulated protein kinase (ERK). Next, ERK phosphorylates and activates cytoplasmic and nuclear proteins, notably transcription factors involved in proliferation. This includes among others C-JUN and ETS domain containing protein (ELK1), that leads to the expression of Fos. Jun and Fos then form the AP-1 heterodimer that function as a transcription factor for key cell-cycle proteins, including D-type cyclins (14).

PI3K Pathway

Similarly, SHC binds to phosphorylated Tyr 338 of IL-2R β chain initiating the Phosphatidylinositol 3 Kinase (PI3K) pathway (15). RAS interacts with the PI3K to induce its activation. Once active, PI3K phosphorylates lipid Phosphatidylinositol-4,5-diphosphate (PIP₂) thereby converting it to Phosphatidylinositol-3,4,5-triphosphate (PIP₃). Next, PIP₃ recruits phosphoinositide dependent protein kinase 1 (PDK1) that in turn phosphorylates protein kinase B (AKT). Finally, AKT phosphorylates and activates proteins involved in survival, and proliferation. Among others, this includes Murine double minute (MDM2) that regulates p53 tumor suppressor; Forkhead box O involved in proliferation and mammalian Target of Rapamycin (mTOR), a kinase implicated in translation and cell growth (16).

JAK/STAT Pathway

In response to IL-2, STAT5A and STAT5B bind to phosphorylated Tyr (pY) residues pY338, pY392 and pY510 of IL-2R β and become Tyr phosphorylated themselves at Y694 and Y699, respectively (17). STAT5A/B then dissociate from their signaling receptor to form dimers, translocate to the nucleus, and then bind to promoter sites on multiple genes that control proliferation and survival. Among others this includes, cyclin D2 involved in proliferation, B-cell lymphoma-extra large (BCL-XL) involved in cell survival and c-MYC involved in the escape from differentiation (18). Taken together IL-2 induces activation of the most recognized oncogenic signaling pathways, MAPK, PI3K and JAK/STAT. Expectedly, Tyr phosphorylation within IL-2 signaling is under tight control by both extrinsic and intrinsic regulation of the JAK kinases (19)

1.2.2 JAK Regulation

The JAK structure consists of seven Janus Homology (JH) domains including JH1-JH7 shared across the JAK kinase family JAK1, JAK2, JAK3 and TYK2 (20). JH1 harbors the kinase domain involved in catalytic activity. JH2 consists of a pseudo-kinase domain that is structurally similar to JH1 but lacks key residues involved in catalytic activity. Importantly, JH2 is thought to directly interact with JH1 domain to inhibit overall JAK activity. JH3-JH7 consist of the 4.1R, Ezrin, Radixin, Moesin (FERM) and Src Homology 2 (SH2) domain and are important for JAKs interacting with their receptor.

JAK Intrinsic regulation

Major progress has been made in understanding the intrinsic regulation of the JAK kinase family by structural studies on JAK1 JH2 domain. This structure lead to the identification of the F-F-V triad within JAK1 JH2 that can act as a conformation switch for

activation (21). Furthermore, the cis interaction between the pseudokinase and kinase of TYK2 (22) has provided a model of inhibitory contact sites for understanding intrinsic negative regulation in cis between the JH1 and JH2 domains. This template of TYK2 inhibitory interactions has lead others to model and predict additional inhibitory interaction sites within JAK1 (23). Others have proposed models of JH2 transinhibition, where partner JAKs interact between JH2 and JH1 domains where the JH2 of one JAK inhibits the JH1 of the other JAK (19). Support for both modes of action are seen throughout the literature and perhaps these two mechanisms are utilized in context specific JAK/STAT signaling.

JAK Extrinsic regulation

As discussed previously the JAK kinases are essential in performing phosphorylation for the IL-2 receptor controlling the activity, localization, and interactions of proteins along the cascade. The JAK kinases also undergo phosphorylation either by a partner JAK or themselves to effect their signaling abilities. Therefore, phosphorylation of key residues along the JAK kinases act as regulators for their catalytic function. Notably, a Tyr in the activation loop of the JAKs and across the general kinase family must undergo phosphorylation for full activation. Thus, one way of regulating a tyrosine kinase extrinsically is via Tyr dephosphorylation. The SH2 Containing Phosphatases (SHP1) and (SHP2) catalyze the dephosphorylation of TYK2, JAK1 (24) and JAK1, JAK2 (25) respectively. Protein Tyrosine Phosphatases 1B (PTP1B) interacts with substrates TYK2 and JAK2 via their SH3 domains where they subsequently undergo dephosphorylation (26). In lymphocytes, T-Cell Protein Tyrosine Phosphatase (TCPTP)

dephosphorylates JAK1 and JAK3. CD45, a receptor tyrosine phosphatase, is capable of dephosphorylating all four JAKs (27).

Other mechanisms for JAK regulation include substrate mimicry by Suppressor of Cytokines Signaling (SOCS1) and (SOCS2). The SOCS proteins contain a Kinase Inhibitory Region (KIR), a short motif, that directly interacts with the JAKs as a false substrate (28) SOCS1 and SOCS3 can also interact with JAKs and several receptors via their SH2 domains creating steric hindrance. Additionally, SOCS initiate the degradation of JAKs and linked receptors via ubiquitination events.

1.2.3 Tyrosine Kinases and Leukemia

Negative regularity mechanisms are in place for tight control of Tyr phosphorylation and or activation of JAK kinases and IL-2 signaling. However, mutagenesis can override these normal cellular controls and promote constitutively active signals and cancer. Select leukemias harbor recurrent chromosomal translocations or somatic point mutations that activate oncogenic tyrosine kinases, leading to constitutive cell signaling. Notably, constitutive phosphorylation of STAT5A/B is a common outcome from mutated tyrosine kinases including: BCR-ABL, mutated forms of Fms-Like Tyrosine Kinase Receptor-3 (FLT3), c-Kit (tyrosine kinase), Tel (ETS transcription factor)-JAK2 and the JAK1 V568F and JAK2 V617F mutants (Figure 2) (29, 30, 31, 32, 33, 34). This is expected because STAT5A/B are critical for lymphoid survival and are normally activated by common γ c cytokines including the aforementioned IL-2 (35).

JAKs and Acute Lymphoblastic Leukemia

The JAK kinase family, notably JAK1, JAK2 and JAK3 are becoming well

recognized as oncogenic drivers in high risk Acute Lymphoblastic Leukemia (ALL), contributing to a conserved 10.7% of cases (36). ALL is a rapid disease progressing cancer of immature lymphocytes. One of the most aggressive leukemias of the B-ALL subtype, is PH-Like ALL associated with high relapse and low survival (37). Although PH-Like ALL is defined by a variety of genetic signatures, most are driven by activating the JAK/STAT pathway (37). Similarly, relapsed T-ALL has been linked to oncogenic JAKs (38, 39). Regardless of the ALL subtype, aberrant activation of the JAK/STAT pathway by driver mutations found within the JAKs are likely to become actionable mutations.

JAK Activating Mutations

The bulk of activating mutations in JAK family members occur within functional hot-spots typically conserved across JH domains. The most frequently mutated region amongst the JAKs is the JH2 pseudo-kinase domain, that provides negative regulation of the active JH1 kinase domain. For example, JAK1 V658F mutation is found in the JH2 domain and occurs in high-risk PH-like B-ALL (40). This Single Nucleotide Polymorphism (SNP) is analogous to the well-known V617F of JAK2 that drives over 90% of Polycythemia Vera cases (41). JAK3 also contains high frequency activating mutations in T-ALL including M511I (42) occurring in the SH2-JH2 linker, and A573V in the JH2 domain (43). Taken together, these highly conserved domains when mutated similarly affect JAK kinase activity due to their structural homology. As mentioned previously it is unknown whether high risk Hispanic ALL patients carry similar or novel JAK activating mutations that could potentially benefit from pipeline targeted therapies.

Chapter 2: Identification of the JAK1 V666G mutation in ALL patient samples

2.1 Introduction

Cancer is the number one cause of death for Hispanics (1). Within these cancers, an unequal burden of leukemia incidence and mortality exists amongst Hispanic children (3). ALL patient outcomes have improved with targeted therapies, yet it represents the second cause of cancer deaths in children (44). Hispanic children have the highest ALL incidence rates and fair far worse than their non-Hispanic counterparts post therapy (45). This is in part due to the lack of inclusion of minorities in cancer research and fully understanding this disease (2). Until we understand how these molecular pathways are “hijacked” in high-risk ALL, it will be difficult to develop new therapies to end cancer health disparities.

Recently, newly developed molecular and genetic technologies have become available to determine driver mutations that result in cancer. Indeed, mutations in kinases have been known to drive certain leukemias; thus, there has been substantial enthusiasm for therapies that target oncogenic tyrosine kinases. The JAK tyrosine kinases are becoming recognized as oncogenic drivers in high risk ALL. However it is unknown whether Hispanic ALL patients display genomic signatures associated with overactivation of the JAK/STAT cascade.

The overall objective of this study was to identify SNPs involved in driving ALL in the El Paso Del Norte population. The goal of this aim was identify novel SNPs in JAK STAT and regulatory effectors from local ALL patient samples. It was **hypothesized** that El Paso Del Norte ALL patient samples contain frequent mutations in the JAK/STAT

signaling cascade that results in dysregulation to promote leukemogenesis. The rationale for this hypothesis is based on studies showing that overactivation of the JAK/STAT cascade is linked with high rates of relapse in ALL.

2.2 Materials and Methods

2.2.1 Patient Samples WES

Data on the genomic DNA from a small cohort of 7 healthy controls and 9 patients diagnosed with ALL from the cancer biorepository at UTEP was used to identify the V666G JAK1 mutant. These data had been previously obtained along with other types of cancer samples and used to establish OncoMiner, a bioinformatics pipeline for the analysis of exonic sequence variants in cancer at UTEPs BBRC bioinformatics core (46). Briefly, DNA was isolated using Purgene Kit A (Qiagen) according to manufacturer's instructions and sent to Otogenetics for whole exome sequencing (WES) (46).

2.2.3 OncoMiner

Subsequently, OncoMiner was used to identify and sort through deleterious genetic variations, including non-synonymous mutations, insertions and deletions. OncoMiner information was gathered for each SNP regarding gene classification, genomic location, available publications on the gene/SNP and a Protein Variation Effect Analyzer (PROVEAN) Score. The PROVEAN score was used to predict deleterious effects of mutations on protein function with a recommended cut off value of -2.5 (47).

2.3 Results

2.3.1 Identification of the JAK1 V666G mutation in ALL patient samples

ALL patient samples were collected from El Paso Del Norte, stored at the UTEP

Tissue Biorepository and sent for WES. SNPs that occurred at least once were 9,967 in controls and 40,561 in ALL patient samples (Figure 2A). SNPs that were unique to ALL were sorted by protein classes (48) using gene ontology (GO) terms (49) and the frequency of each class is represented in the pie with sub levels extending outward (Figure 2B). Complementary to these data, the 9 ALL Patient (P1-P9) SNPs occurring within JAK STAT associated genes that were not present in controls are shown in a heatmap. Patient 4 (P4) shows high frequency mutations in genes implicated in the JAK STAT pathway. One of these SNPs being the JAK1 V666G mutation that is shown with its PROVEAN score of -6.8 analyzed by OncoMiner (Figure 3A). The JAK1 V666G SNP is unreported to our knowledge given its absence in the literature and in our brief search of cancer databases. This search included the Catalogue Of Somatic Mutations In Cancer (COSMIC) database that compiles mutations from cancer patients across multiple databanks including The Cancer Genome Atlas (TCGA) and Genomic Data Commons (GDC) Portal. Lastly, the JAK1 V666 residue within the JH2 is highly conserved amongst the JAK family and across species (Figure 3B) signifying its likely relevance on JAK protein function.

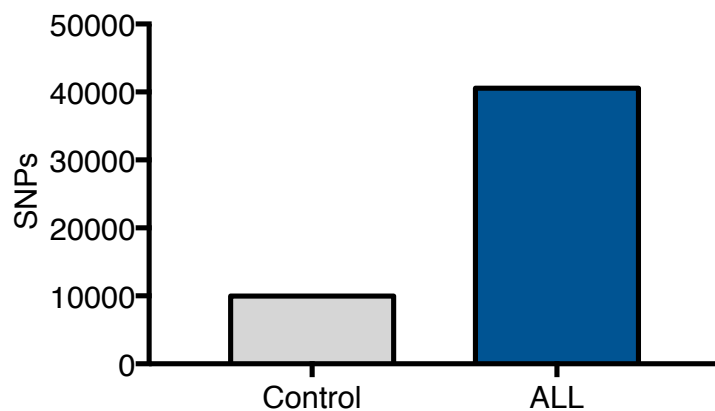
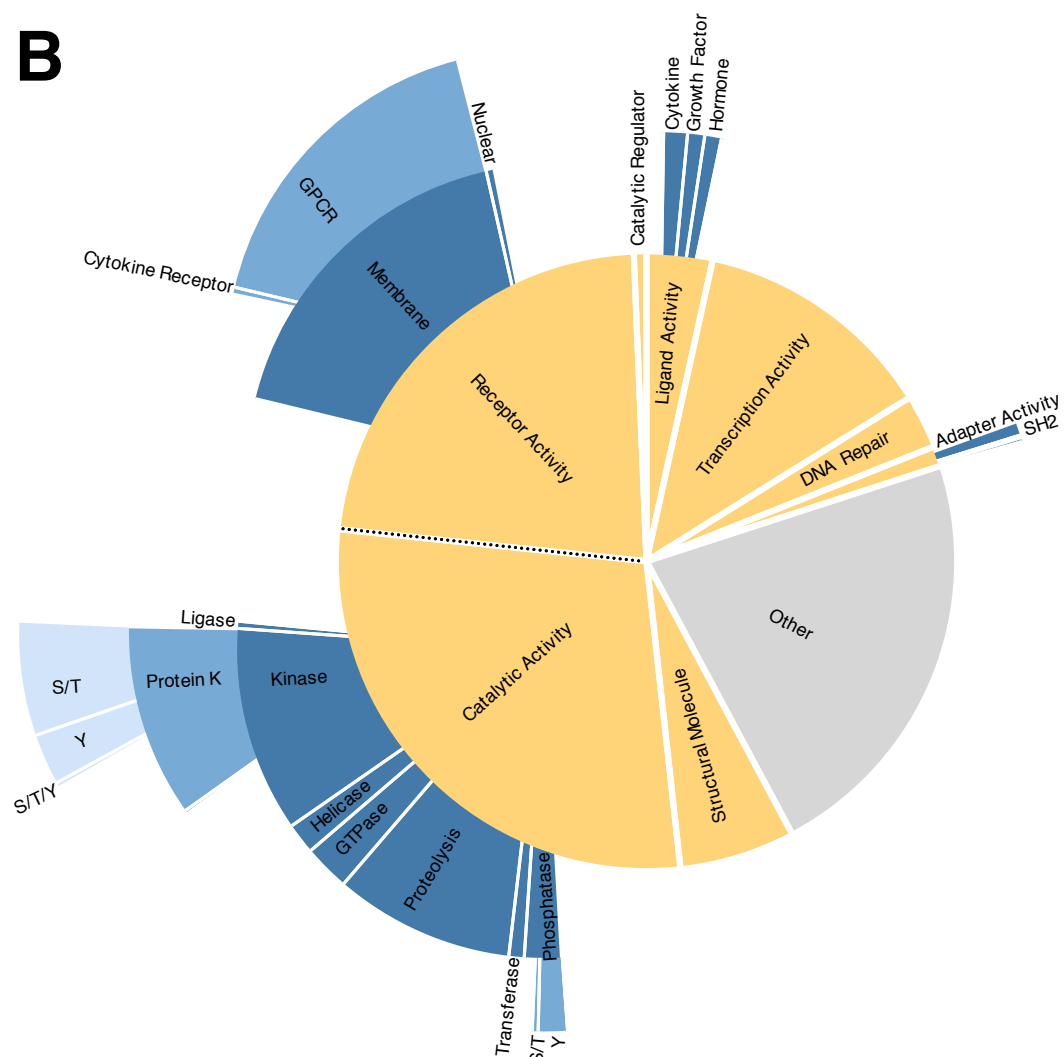
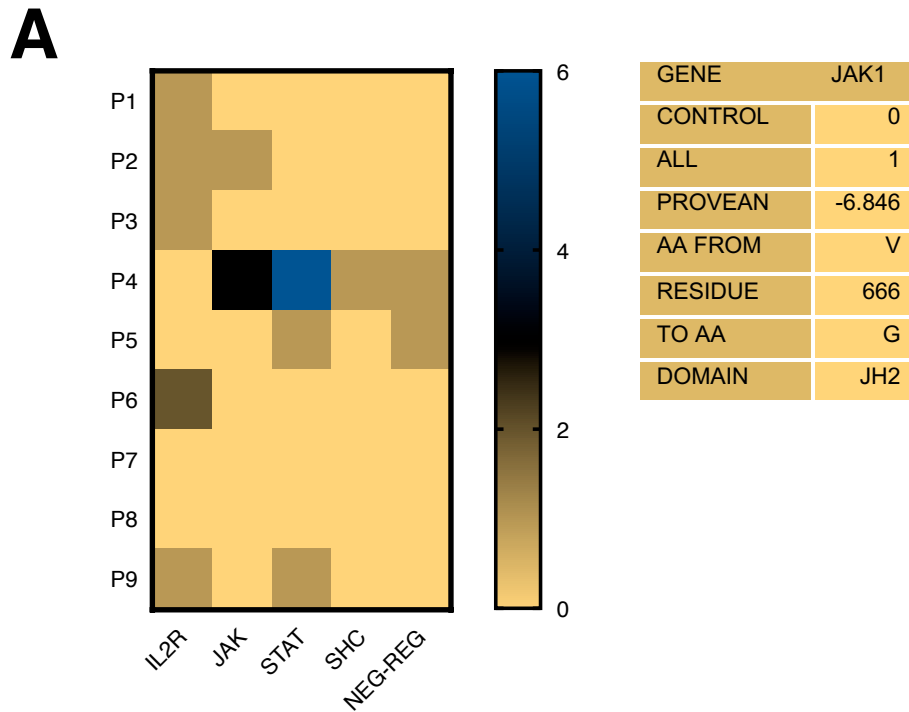
A**B**

Figure 2. Distribution of ALL SNPs from El Paso Del Norte patient samples. A) Comparison of total number of SNPs found by WES of 7 healthy control donors (grey) and 9 ALL patient samples (blue). **B)** Distribution of uniquely occurring ALL SNPs by functional protein classes $n = 9$. Each layer of the diagram represents sublevels of the functional class hierarchy. Catalytic Activity and Receptor Activity overlap (dotted line) and are predominantly kinases and receptors.



B

JAK FAMILY *

JAK1	D	V	E	N	I	M	V	E	E	F	V	E	G
JAK2	G	D	E	N	I	L	V	Q	E	F	V	K	F
JAK3	G	D	S	T	-	M	V	Q	E	F	V	H	L
TYK2	G	P	E	N	I	M	V	T	E	Y	V	E	H

SPECIES *

HUMAN	D	V	E	N	I	M	V	E	E	F	V	E	G
MOUSE	D	V	E	N	I	M	V	E	E	F	V	E	G
ZEBRAFISH	H	Q	E	N	I	M	V	E	E	F	V	Q	Y
RAT	D	V	E	N	I	M	V	E	E	F	V	E	G
CHIMP	H	Q	E	N	I	M	V	E	E	F	V	Q	Y
CARP	D	V	E	N	I	M	V	E	E	F	V	E	G
COW	D	V	E	N	I	M	V	E	E	F	V	E	G
DOG	D	V	E	N	I	M	V	E	E	F	V	E	G
CHICKEN	D	L	E	N	I	M	V	E	E	F	V	E	F
CHAMELEON	D	V	E	N	I	M	V	E	E	Y	V	E	F
FROG	D	V	E	N	I	M	V	E	E	F	V	D	F
PUFFER-FISH	H	Q	E	N	I	M	V	E	E	F	V	Q	L

Figure 3. Identification of the Jak1 V666G mutation in ALL patient samples. A) Patients (P1-P9) are shown with uniquely occurring ALL SNPs within genes involved in the JAK STAT pathway and represented in a heatmap. Patient 4 contains JAK1 V666G shown with relevant SNP information including Provean score. **B)** Val 666 of JAK1 is highly conserved across JAK family and species.

2.4 Discussion

Although these data are from a small cohort we detected a high frequency of SNPs in JAK/STAT associated genes to occur in primarily one ALL patient. This patient contained both novel and reported SNPs within JAK/STAT genes. As mentioned previously, Hispanic samples are insufficiently available or absent in Biobanks and thus consequently underrepresented in clinical research. Here we detected a novel SNP within JAK1 that was not previously reported in any established cancer databases.

Our group identified V666G in human JAK1, occurring at a highly conserved residue within the JH2 domain. The potential significance of JAK1 V666G mutation prompted our interest due its location within the JH2/pseudokinase domain, a known hot spot for activating mutations. Specifically, JAK1 V666G is located within the JH2 hinge in close proximity to well-known JAK1 activating mutant V658F. Thus, JAK1 V666G, was chosen as a candidate for subsequent investigations for its regulation of JAK/STAT proteins and cell activity.

Chapter 3: Auto and Cross-Phosphorylation of JAK1 is lost by V666G

3.1 Introduction

Autophosphorylation is a shared mechanism of tyrosine kinases. It is not surprising that overexpression of JAK1, JAK2, JAK3 and TYK2 can lead to cytokine independent growth and constitutive STAT activation (50). This is because many kinases when expressed at high protein volume become phosphorylated and consequently activated (51). Accordingly, to detect the activating potential of the JAK1 V666G mutant we assessed tyrosine phosphorylation in response to conventional overexpression and kinase assays.

Many JAK kinases rely on partner JAKs to undergo phosphorylation and it is therefore useful to assess cross-phosphorylation between JAK1 V666G with partner JAK kinases. The dominant role between JAK kinases in transactivation is an ongoing pursuit in pathway specific contexts. However, the cross activation between JAK kinases solely by overexpression is largely unexplored. Therefore, we assessed the potential of the JAK1 V666G mutant in its ability to phosphorylate partner JAKs and vice versa.

The goal of this aim was to biophysically characterize the impact of JAK mutant proteins on auto and cross-phosphorylation. It was **hypothesized** that the newly identified V666G mutation would enhance activation of JAK1. The rationale for this hypothesis is based on studies showing that a disruption in the JH2, negative and regulatory, pseudokinase domain can lead to an oncogenic JAK kinase.

3.2 Materials and Methods

3.2.1 Cell Culture

The JAK1 deficient, human U4C cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum ((FBS); Atlanta Biologicals), 2mM L- glutamine (Corning), and 1% penicillin/streptomycin (Corning).

3.2.3 Plasmids and Site-directed Mutagenesis

The human JAK1 MYC tag (RC213878), JAK1 GFP tag (RG213878) JAK2 (RC220503) and JAK3 (pcDNA3.1) plasmids were purchased from Origene. STAT5B cDNA, was obtained from the home laboratory originally purchased from OriGene as described previously (52). Mutant forms of JAK family were prepared using the QuikChange XL site-directed mutagenesis kit (Agilent) according to the instructions of the manufacturer. The primers used for the following JAK family mutants are indicated in the table immediately below (Table 1). All subclones and mutations were verified by DNA sequencing at the Genomic Analysis Core Facility of the BBRC, at UTEP.

Table 1. JAK1 Mutational Primers

GENE		FORWARD	REVERSE
JAK1	V666G	5'-CGTGGAGAATATCATGGGGGAAGAGTTTGTGGAAG-3'	5'-CTTCCACAAACTCTTCCCCCATGATATTCTCCACG-3'
	K908A	5'AGGGGAGCAGGTGGCTGTTGCATCTCTGAAGCCTG3'	5'CAGGGCTTCAGAGATGCAACAGCCACCTGCTCCCCT3'

3.2.4 Transfections

Approximately 16 hours pre-transfection U4C cells were seeded into 10 cm dishes to yield 90–95% confluency at the time of transfection. For all following experiments, cells were transfected with approximately 15µg of indicated JAK plasmid along with 10µg of STAT5B. MYC tagged JAK1 WT and JAK1 constructs, including kinase dead (K908A) and V666G along with STAT5B were transfected in U4C cells and used for overexpression of JAK1 and kinase assays. Similarly, for cross activation of JAK1 with JAK family members, cells were transfected with JAK1 WT GFP tag, JAK2

WT, or JAK3 WT with STAT5B either alone or with JAK1 WT MYC tagged or JAK1 MYC tagged constructs K908A or V666G. All transient transfections of U4C cells were performed according to manufactures instructions, (Lipofectamine 2000, Invitrogen). Cells were harvested 24 hours post-transfection and subsequently pelleted, lysed, clarified and immunoprecipitated (IP) with either Origene antibody against GFP tag (TA150041), or Santa Cruz antibodies against c-MYC (sc40), JAK1 (sc376996) JAK2 (sc390539) or using a polyclonal antibody generated against the COOH terminus of JAK3 described previously (53).

3.2.5 Kinase Assay

For kinase assays, JAK1 was IP using indicated antibody as described above. IP protein was washed with kinase buffer (10mM Mops, 3mM EDTA, 001% Triton, 5% Glycerol, 01% Bme, 1mg/ml BSA, and 10mM MgCl₂, at 7 PH) and either incubated in kinase buffer or kinase buffer supplemented with 100 μM ATP. Kinase reactions were carried out at 32 °C for 20 min followed by vigorous washing of the beads with cold kinase buffer followed by a lysis buffer wash and the reaction stopped immediately by adding 4X Sample Buffer.

3.2.6 Western Blots

Samples were separated by 7.5% or 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) for Western Blot (WB) analysis as previously described (53). Briefly, membranes were probed overnight with mouse monoclonal anti-phosphoTyr (pTyr) antibody (4G10, EMD Millipore) in immunoprecipitation experiments and developed using horseradish peroxidase-

conjugated goat anti-mouse and visualized by chemiluminescence using LICOR and Image Studio Lite software. Membranes were stripped and re-probed for JAK1 (sc295, Santa Cruz) JAK2 (06-1310, Millipore) or JAK3 (ab78116-100, Abcam). Similarly, membranes were probed against pTyr STAT5 (05-495, Millipore) for corresponding lysate when indicated and re-probed for total STAT5 (610191, BD-Transduction).

3.3 Results

3.3.1 Autoactivation and STAT5 Phosphorylation is lost in JAK1 V666G

To investigate the impact of V666G on JAK1 kinase activity we examined autoactivation by overexpression and measured Tyr phosphorylation of JAK1 and STAT5 substrate. Compared to JAK1 WT, autoactivation of JAK1 V666G was similar to that of kinase dead control K908A, with significant decreases in Tyr phosphorylation (Figure 4A). Compared to JAK1 WT, JAK1 V666G Tyr phosphorylation of STAT5 was significantly decreased, similar to kinase dead (Figure 4B). JAK1 V666G was then challenged by kinase assay and even in the presence of ATP, Tyr phosphorylation was reduced similar to kinase dead when compared to WT (Figure 4C).

3.3.2 Cross-activation of JAK1 is lost by V666G mutation and impairs Tyr

Phosphorylation of WT JAK1 JAK2 and JAK3

To investigate whether JAK1 V666G could be activated by WT JAK family members we examined cross-activation by overexpression of JAK1-MYC constructs along with either WT JAK1-GFP, JAK2 or JAK3 and measured Tyr phosphorylation of both JAKs. Similar to kinase dead JAK1, JAK1-MYC V666G could not be phosphorylated by JAK-GFP WT. However, in the presence of MYC tagged kinase dead JAK1 and, JAK1

V666G, Tyr phosphorylation of JAK1-GFP WT was significantly decreased compared to samples with either no MYC tagged JAK1 or JAK1-MYC WT. There was no significant difference in Tyr phosphorylation of JAK1-GFP between that with no JAK1-MYC and JAK1-MYC WT (Figure 5A). JAK1-MYC V666G could not be phosphorylated by JAK2 WT. In the presence kinase dead JAK1 and, JAK1 V666G, Tyr phosphorylation of JAK2 WT was significantly decreased compared to samples with either no JAK1 or JAK1 WT. Tyr phosphorylation of JAK2 was significantly greater in the presence of JAK1 WT compared to no JAK1 (Figure 5B). JAK1-MYC V666G could not be phosphorylated by JAK3 WT. Again, in the presence of kinase dead JAK1 and, JAK1 V666G, Tyr phosphorylation of JAK3 WT was significantly decreased compared to samples with either no JAK1 or JAK1 WT. Furthermore JAK3 with no JAK1 was Tyr phosphorylated to the same extent as JAK3 in the presence JAK1 WT (Figure 5C). Taken together, neither WT JAK1, JAK2 or JAK3 were capable of phosphorylating JAK1 V666G. And in the presence of kinase dead JAK1 K908A and JAK1 V666G autophosphorylation and or cross-phosphorylation of WT JAK1, JAK2 and JAK3 was significantly reduced.

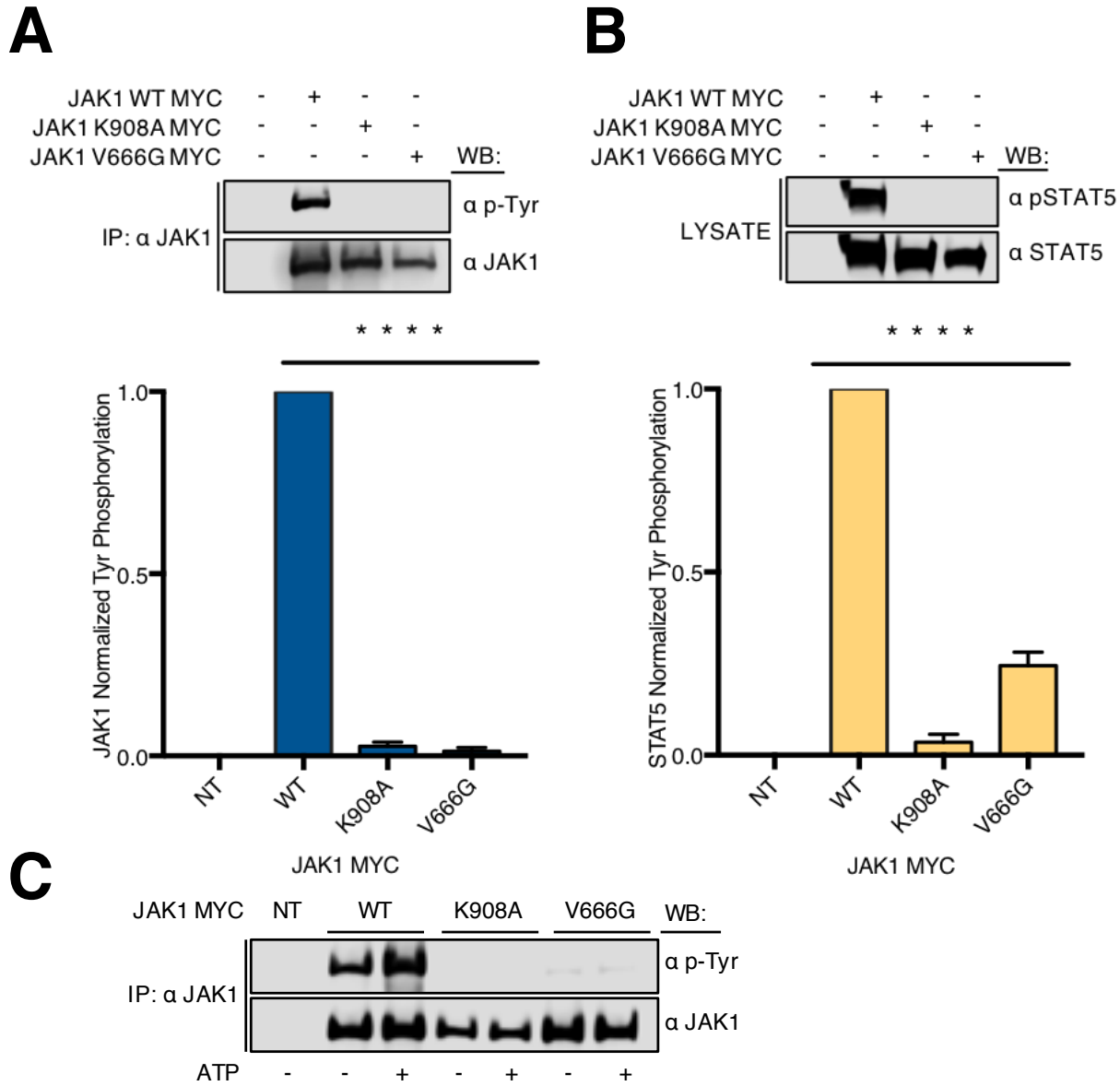


Figure 4. Autoactivation and STAT5 Phosphorylation is lost in JAK1 V666G. Immunoblotting in U4C cells, non-transfected (NT) or transiently transfected to overexpress either JAK1 Wild Type (WT), or JAK1 MYC constructs, kinase dead (K908A) or JH2 mutant (V666G) along with STAT5. Representative blots are shown with quantified phosphorylation normalized to total protein using densitometry were N=3. **A)** Overexpressed JAK1 was immunoprecipitated (IP) to assess autoactivation indicated by JAK1 Tyr phosphorylation. Data represent means \pm SEM; n = 3 independent experiments. ***P < 0.0001 using a one-way ANOVA with post-hoc Tukey's test for multiple comparisons. **B)** Corresponding lysate was used to assess JAK1 substrate phosphorylation indicated by STAT5 Tyr phosphorylation. Data were analyzed same as A); ***P < 0.0001. **C)** IP JAK1 was challenged by kinase assay treated with 100 μ M ATP for 20 min and blotted for Tyr phosphorylation.

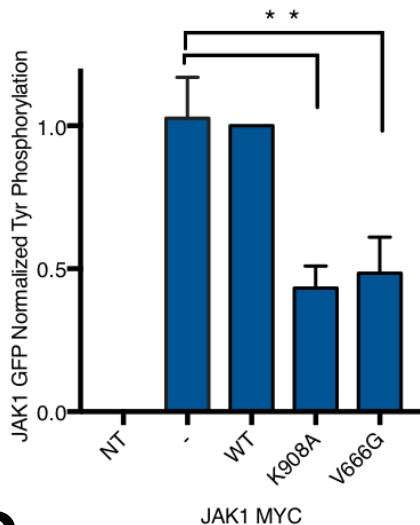
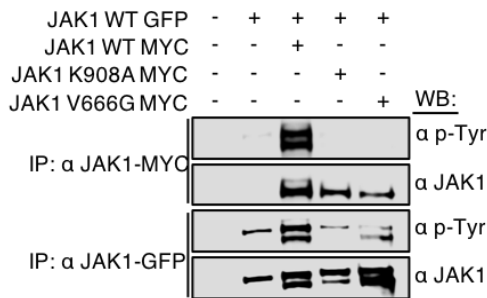
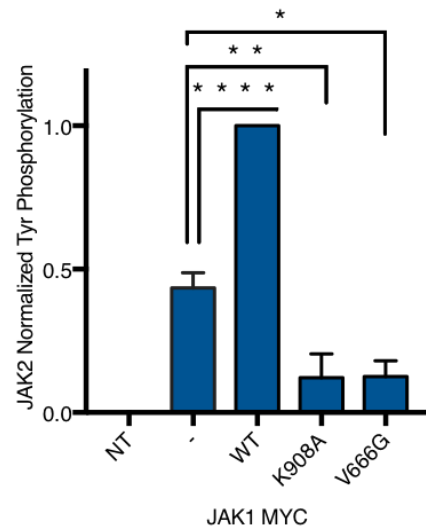
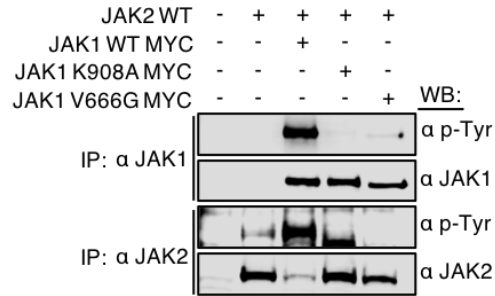
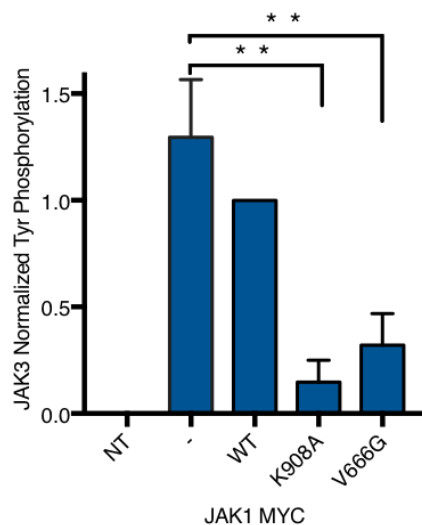
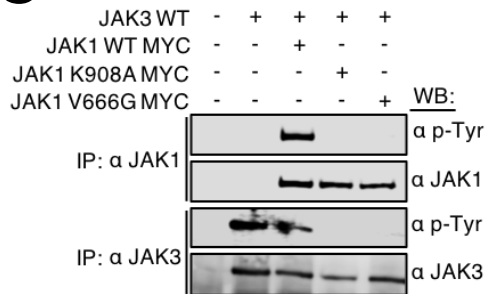
A**B****Dis****C**

Figure 5. JAK1 V666G inhibits Tyr phosphorylation of WT JAK1, JAK2 and JAK3. Immunoblotting in U4C cells, NT or transiently transfected to overexpress either no JAK1 (-), JAK1 WT-MYC, or JAK1 MYC constructs, K908A or V666G along with either WT JAK1-GFP, JAK2 or JAK3. Representative blots are shown with quantified Tyr phosphorylation normalized to total protein using densitometry and plotted with mean and \pm SEM from at least 3 independent experiments **A)** JAK1-GFP WT was overexpressed with either no JAK1(-), JAK1-MYC WT, K908A, or V666G and IP for MYC and GFP tags subsequently assessed for cross activation indicated by Tyr phosphorylation. Graphed data represent JAK1 WT GFP normalized Tyr phosphorylation $n = 4$ $**P < 0.0062$ using a one-way ANOVA with post-hoc Tukey's test for multiple comparisons. **B)** JAK2 WT was overexpressed with either -, JAK1-MYC WT, K908A, or V666G and IP for JAK1 and JAK2 subsequently assessed for cross activation indicated by Tyr phosphorylation. Graphed data represent JAK2 normalized Tyr phosphorylation $n = 3$ $*P < 0.0101$, $**P < 0.0092$ $P^{****} < 0.0001$ analyzed as A). **C)** JAK3 WT was overexpressed with either -, JAK1-MYC WT, K908A, or V666G and IP for JAK1 and JAK3 subsequently assessed for cross activation indicated by Tyr phosphorylation. Graphed data represent JAK3 normalized Tyr phosphorylation $n = 3$ $**P < 0.0055$ analyzed as A).

3.4 Discussion

JAK1 V666G lead to a kinase dead-like phenotype in contrast to our predictions of overactivation. These observations identified the V666 residue in human JAK1 conserved across the JAK family as a site necessary for kinase autoactivation.

Additionally, Tyr phosphorylation of JAK1, JAK2 and JAK3, was disrupted reciprocally by the presence of either the JH1 kinase dead JAK1 mutant or newly identified JH2 JAK1 V666G mutant. Specifically, the absence of JAK1 allowed minimal yet sustained autoactivation of JAK2 and moderate activation of JAK1 and JAK3 that was hindered in the presence of an inactive JAK1. It is likely that JAK2 and JAK3 rather than autophosphorylate, preferentially cross-phosphorylate with JAK1. Furthermore, inactive JAK1 inhibited autophosphorylation of JAK1 WT. These data indicate a dominant negative effect by JAK1 on autophosphorylation and cross-phosphorylation between JAK family members.

Chapter 4: JAK1 V666G inhibits JAK3 mediated IL-2 signaling

4.1 Introduction

Understanding the transactivation between partner JAK kinases in common γ chain signaling is a constant pursuit. One aspect of transactivation includes defining each role of JAK1 and JAK3 in IL-2 signaling. JAK1 has been recognized for initiating Tyr phosphorylation of JAK3 in response to IL-2 where JAK3 then reciprocates phosphorylation (54). Once active, JAK3 takes the bulk action of phosphorylating key Tyr residues along IL-2R β chain (55) creating docking sites for SH2 containing proteins. As discussed previously (see introduction) these SH2 containing proteins will initiate the MAPK, PI3K, and JAK/STAT pathways. Pertaining to the JAK/STAT pathway, the SH2 domain of STAT5 binds phosphorylated Tyr along IL-2R β chain. This recruitment allows STAT5 to become phosphorylated mostly by JAK1. For example, containing an active JAK3 and a kinase-dead JAK1 results in loss of STAT5 phosphorylation and activation (56).

Similar to a kinase dead JAK1 by JH1 mutation the inactivation of JAK1 via JH2 mutations have been shown to reduce STAT5 basal activity even in the presence of JH1 R657Q JAK3 activating mutant (57). Furthermore JH2 activating JAK3 mutants including M511I and A573V have been shown to require IL-2 receptor, and JAK1 for their transforming capabilities (58). It seems that the presence of JAK1 is necessary for JAK3 activation and downstream IL-2 signaling. However, it is unclear if the absence of JAK1 and the presence of inactive JAK1 by JH1 (K908A) and JH2 (V666G) mutations effect JAK3 activation and IL-2 signaling differently. Deciphering these outcomes could provide

support for trans-inhibition between JAK kinases and henceforth new ways to extrinsically target overactive JAKs. Insights into the mechanism of action behind inactive JH2 mutants can also propagate new ways to inhibit JAKs allosterically.

The goal of this aim was to determine the functional impact of novel JAK mutation V666G on IL-2 signaling with JAK3 WT or activating JAK3 M511I and A573V involved in ALL. It was **hypothesized** that the newly identified V666G JAK1 mutant would reduce JAK3 Tyr phosphorylation and IL-2 downstream signaling. The rationale for this hypothesis is based on studies showing that IL-2 requires a functional JAK1 kinase for both WT and oncogenic JAK3 signaling.

4.2 Materials and Methods

4.2.1 Plasmids and Site-directed Mutagenesis,

U4C cell lines were maintained as described previously (Chapter 2). Complementary to aforementioned JAK1 and JAK3 plasmids, IL-2R β , γ c, and mutant M511I and A573V JAK3 (see Table 2) were obtained from the home laboratory as described previously (59).

Table 2. JAK3 Mutational Primers

GENE		FORWARD	REVERSE
JAK3	M511I	5'-CCAATACCAGCTGAGTCA GATCACACACAAGATCCCTG-3'	5'-GAGGGATCTTGTGAAATGT-GATCTGACTCAGCTGG TATTGG-3'
	A573V	5'-GTCATTCCCTGG AAGCAGTGAGCTTGATGAGCCAAG-3'	5'-CTTGGCTCATCAAGCTCACTGCTTCCAGG AATGAC-3'

4.2.2 Transfections and IL-2

For all following experiments, U4C cells were transfected with 5 μ g IL-2R β , and 5 μ g γ c to reconstitute IL-2 signaling components. In this reconstituted system, the first set of experiments included 15 μ g JAK3 WT expressed alone or with JAK1 MYC constructs (WT, K908A and V666G) and STAT5 and used for immunoprecipitation.

Complementary to this scheme a separate experiment was conducted using total cell lysate to examine the effects of JAK1 V666G on IL-2 induced activation of downstream STAT5, ERK and AKT. This reconstituted pathway included the same experimental scheme as above with JAK1 MYC constructs and JAK3 adjusted to approximately 500ng to prevent autoactivation and respond to IL-2.

The next set of experiments included 15µg of JAK3 WT or JAK3 constructs M511I or A573V expressed with either JAK1 MYC WT or V666G, or a combination and used for IP. The combination was included to assess whether JAK1 WT could rescue the inhibitory effects of JAK1 V666G. Again, complementary to this scheme, a separate set of experiments was conducted using lysate to examine the effects of JAK1 V666G on JAK3 M511I or A573V IL-2 induced activation of downstream STAT5. This reconstituted pathway included the same experimental scheme with JAK1 MYC and JAK3 constructs adjusted to approximately 500ng to prevent autoactivation and respond to IL-2.

Cells used for immunoprecipitation experiments received complete media while cells used for lysate experiments were made quiescent by receiving 1% FBS media and incubated for twenty-four hours post transfection. All cells were treated with 400 IU/ml IL-2 for 10 minutes prior to immunoprecipitation while cells that were made quiescent were either stimulated with or without IL-2 for 10 minutes and analyzed with lysate. Cells were subsequently harvested, pelleted, lysed, clarified and either used for immunoprecipitation or immediately probed for activation. IP JAK1 or JAK3 were Western blotted for pTyr then stripped and re-probed for total JAK1 or JAK3. Similarly,

lysate experiments were probed for pTyr STAT5, pERK (Cell signaling, 4370S), or pAKT (Cell signaling, D25E6), then stripped and re-probed for total STAT5, ERK (Cell signaling 4695) or AKT (Cell signaling, C67E7) where indicated.

4.3 Results

4.3.1 JAK1 V666G inhibits JAK3 mediated IL-2 signaling

We reasoned that a dominant presence of JAK1 V666G on JAK3 WT as seen in our kinase overexpression studies may translate over in the context of IL-2 signaling. To investigate whether JAK1 V666G could inhibit JAK3 in the presence of IL-2 and signaling components, we examined activation of JAK3 and downstream JAK/STAT, MAPK and PI3K proteins, STAT5, ERK and AKT respectively. Challenged with IL-2, overexpressed JAK1 V666G showed reduced Tyr phosphorylation compared to JAK1 WT even in the presence of JAK3 and IL-2 signaling components. In this same context, JAK3 showed a reduction in Tyr phosphorylation when in the presence of JAK1 V666G compared to that expressed with no JAK1 or JAK1 WT. A Similar trend in decreased Tyr phosphorylation occurred in STAT5 from corresponding lysate (Figure 6A).

To determine whether JAK1 V666G could inhibit downstream IL-2 signaling, U4C cells were transfected with the same scheme as described in Figure 6A with the exception of expressing the minimum amount of JAK needed to respond to IL-2. Challenged with IL-2, STAT5 showed a reduction in Tyr phosphorylation in the presence of JAK1 V666G compared to JAK1 WT. Phosphorylation of ERK and AKT were significantly reduced in the presence of JAK1 V666G compared to samples with no JAK1 or JAK1 WT (Figure 6B).

4.3.2 JAK1 V666G inhibits JAK3 M511I and A573V mediated IL-2 signaling.

To investigate whether JAK1 V666G could inhibit activating JAK3 mutants M511I and A573V, we examined Tyr phosphorylation of JAK3 mutants in response to IL-2. Challenged with IL-2 overexpressed JAK1 V666G showed reduced Tyr phosphorylation compared to JAK1 WT even in the presence of M511I JAK3 activating mutant. JAK3 M511I showed a reduction in Tyr phosphorylation when in the presence of JAK1 V666G. Furthermore, the inhibitory effects of JAK1 V666G could not be rescued by the presence of JAK1 WT, as decreases in Tyr phosphorylation continued in JAK3 M511I. Likewise, cells transfected with the same scheme as described above but instead with the minimum amount of JAK constructs needed to respond to IL-2, JAK1 V666G continued to inhibit downstream STAT5 phosphorylation of JAK3 M511I in response to IL-2. Even in the presence of both JAK1 V666G and JAK1 WT, a reduction in Tyr phosphorylation of STAT5 continued (Figure 7A).

Similarly, JAK3 A573V could not phosphorylate JAK1 V666G and revealed reduced Tyr phosphorylation in its presence. The inhibitory effects of JAK1 V666G could not be rescued by the addition of JAK1 WT, as decreased Tyr phosphorylation continued in JAK3 A573V. Likewise, cells transfected with the same scheme described above but instead with the amount of JAK constructs needed to respond to IL-2, JAK1 V666G continued to inhibit STAT5 phosphorylation of JAK3 A573V in response to IL-2. In the presence of both JAK1 V666G and JAK1 WT, a reduction in Tyr phosphorylation of STAT5 continued (Figure 7B). These data demonstrate that “constitutively active” JAK3 mutants are incapable of activating the JAK1 V666G. Additionally, activating JAK3 mutants cannot

escape the inhibitory effects of JAK1 V666G even when JAK1 WT is available for rescue.

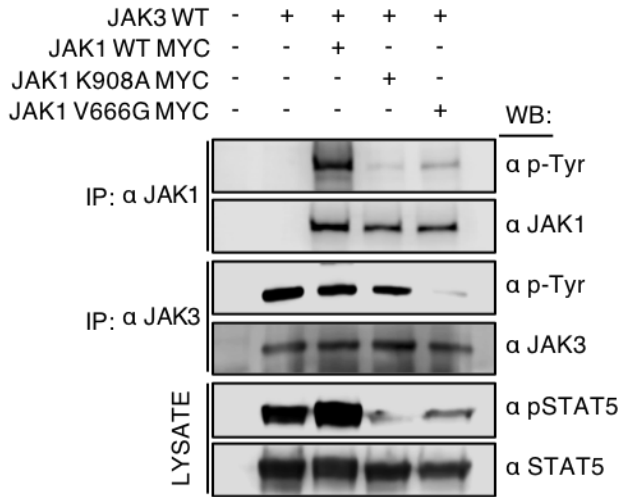
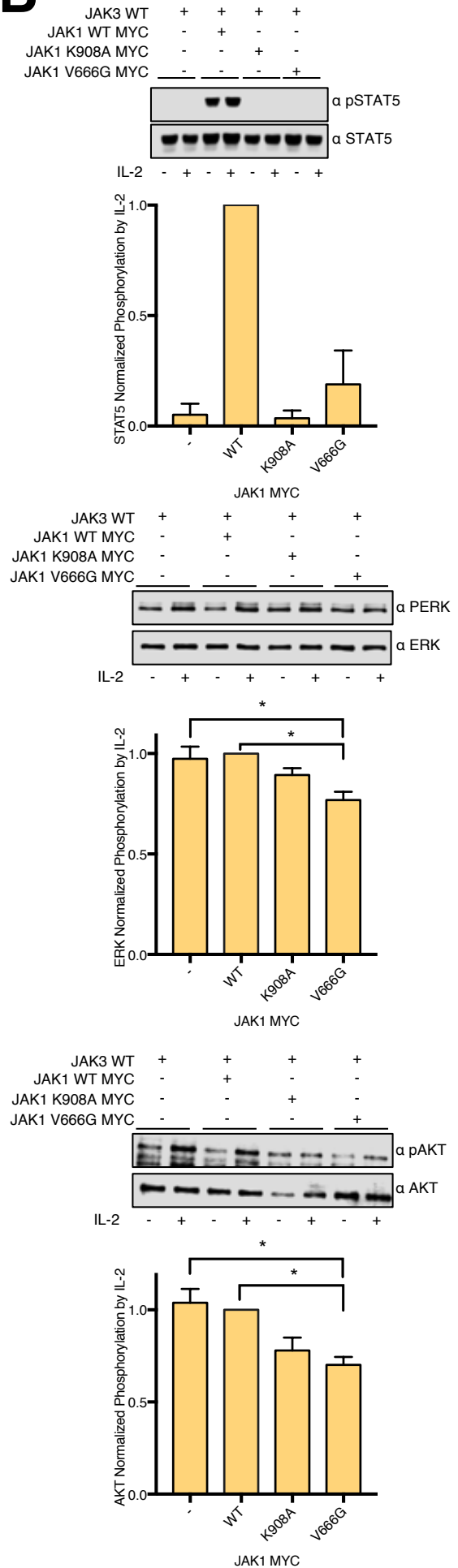
A

Figure 6. JAK1 V666G inhibits JAK3 mediated IL-2 signaling. Immunoblotting in U4C cells, NT or transiently transfected with no JAK1, JAK1 WT-MYC, or JAK1 MYC constructs, K908A or V666G along with JAK3, IL-2R β , γ c and STAT5 challenged with IL-2. **A)** Expressed with IL-2 signaling components, overexpressed JAK1 and JAK3 were IP and examined for Tyr phosphorylation. Corresponding lysate was examined for downstream phosphorylation of STAT5. **B)** Expressed with IL-2 signaling components, and minimum JAK1 and JAK3 needed for IL-2 induction, lysate was examined for STAT5, ERK AND AKT phosphorylation. Graphed data include samples treated with IL-2 represented by normalized phosphorylation. Data were analyzed using a one-way ANOVA with post-hoc Tukey's test for multiple comparisons. ERK phosphorylation n=3 *P<0.0283. AKT phosphorylation n=4 *P<0.0119.

B

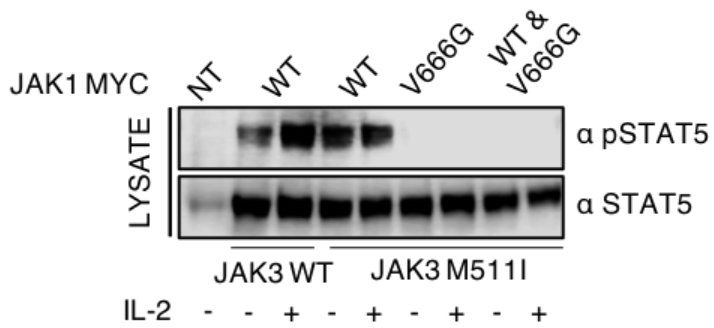
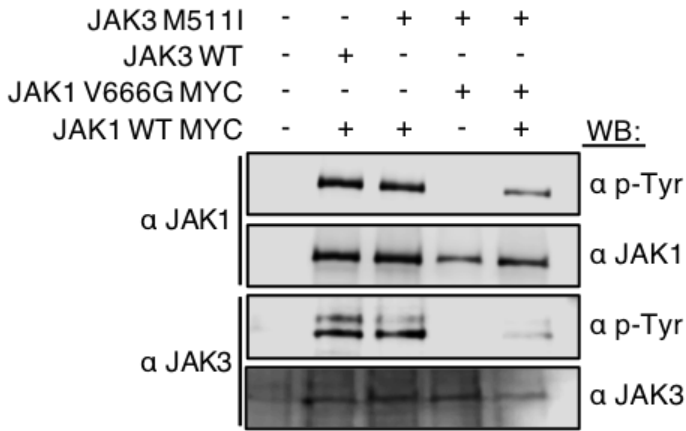
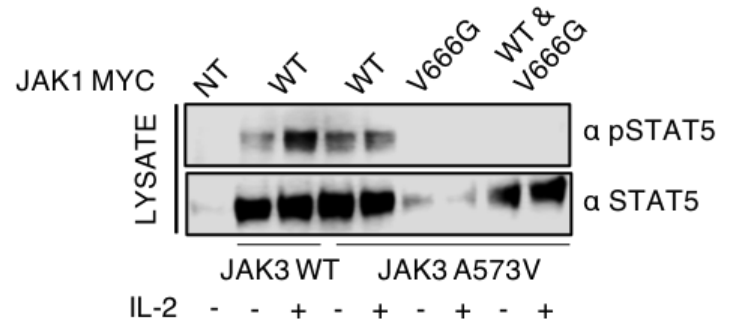
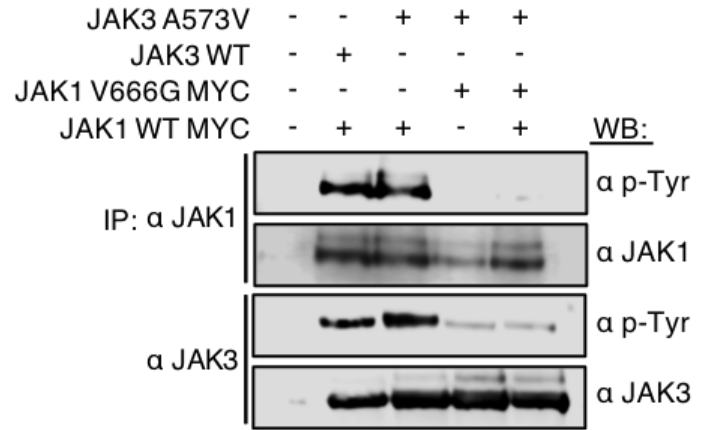
A**B**

Figure 7. JAK1 V666G inhibits JAK3 M511I and A573V IL-2 signaling. Immunoblotting in U4C cells, NT or transiently transfected with JAK1 MYC constructs, and M511I or A573V and IL-2 signaling components **A**) Challenged with IL-2, overexpressed JAK1 and JAK3 M511I were IP and examined for Tyr phosphorylation in the presence of JAK1 V666G or JAK1 V666G with JAK1 WT rescue. Below, similar to the latter transfection scheme using the minimum of JAK1 and JAK3 constructs needed for IL-2 induction, lysate was examined for STAT5 phosphorylation **B**) The same context as (A) but instead expressed with JAK3 A573V were IP JAKs were examined for Tyr phosphorylation and again below a complementary transfection scheme showing STAT5 phosphorylation in response to IL-2.

4.4 Discussion

These findings demonstrate that direct Tyr phosphorylation of JAK1, necessary for IL-2 signaling, can be disrupted by the presence of a mutation within the JH2 domain. The exchange of a single amino acid residue Val in the hinge region of JH2 results in a kinase that is functionally inactive. In conjunction with others we show that an inactive JAK1 prevents optimal IL-2 induced activation of STAT5 (56). Additionally, JAK1 V666G abrogates JAK3 Tyr phosphorylation and dominantly impairs IL-2 mediated signaling in STAT5, as well as ERK and AKT. However, we also show that in the absence of JAK1, JAK3 can sustain IL-2 signaling indicated by downstream phosphorylation of ERK and AKT that is reduced in the presence of JAK1 V666G. This finding indicates a direct and dominant negative effect of an inactive JAK1 by JH2 mutation on JAK3 activity.

Lastly, JAK1 V666G dominant negative effect continues in the presence of JAK3 activating mutants M511I and A573V. These JAK3 mutants constitutively phosphorylate key Tyr residues along IL-2 receptor and recruit STAT5 to sustain oncogenesis. Here we show that JAK3 M511I and A573V in the presence of JAK1 V666G decrease their signaling potential. Others have demonstrated this effect on JAK3 M511I with traditional JAK1 JH1 kinase dead mutants (58) but here we show the same effect with a JH2 kinase dead-like mutant V666G. And even with a rescue JAK1 WT present, together expressed with JAK1 V666G, JAK3 M511I and A573V phosphorylation remains decreased.

Chapter 5: Future Directions

5.1 Future Directions

It is surprising that a single point mutation within the JH2 could have such a negative effect on both autoactivation, cross-phosphorylation and IL-2 induced transphosphorylation. Solved structures for JAK1 and family members were used to aid in understanding the mechanism of action responsible for JAK1 V666G. As discussed previously, the cis interaction between the pseudokinase and kinase of TYK2 (22) has provided a model of inhibitory contact sites between JH1 and JH2 domains. This model has led others to predict inhibitory interaction sites within JAK1 including an inhibitory salt bridge located between the pseudokinase hinge 668E and kinase domain loop 893R (23). Given that our Gly mutation is proximal to this hypothetical salt bridge, the structural consequence of JAK1 V666G was modeled by superimposing on the TYK2 structure [Protein Data Bank (PDB) ID code 3NZ0].

The linear structure of JAK1 is shown with JH and established domains where the V666G location is indicated within the JH2 pseudo-kinase domain and its structural consequence modeled immediately below (Figure 8). This model reveals its proximity near the predicted salt bridge at the JH1 and JH2 cis interface.

A couple lines of thought are offered that may explain the inhibitory effect of JAK1 V666G in cis. First, modeled against Canté-Barrett proposed inhibitory salt-bridge the exchange from Val for Gly may allow flexibility and subsequently strengthen electrostatic interactions between the JH1 and JH2 interface. Second, recent evidence suggests that ATP binding within the JH2 of JAK family members provides some form of structural

stability enhancing JH1 activity (60, 61). Thus, along the lines that ATP bound JH2 facilitates activation, perhaps the presence of V666G disrupts ATP binding and compromises JH1 stability resulting in an inactive kinase. Future directions include modeling the binding of ATP to JAK1 JH2 in the presence of V666G. Interestingly, many FDA approved kinase inhibitors target residues flanking the hinge of the canonical kinase domains using non-conserved residues for specificity (62). Given the inhibitory effect of V666G within the JH2 hinge, perhaps this region could similarly be targeted to effectively inhibit oncogenic JAK1 kinases allosterically.

The ladder interpretations alone do not fully account for the inhibitory effects of JAK1 V666G on partner JAKs which supports an inhibitory mechanism in trans. To date there are no crystal structures of partner JAKs interacting in trans so the structural basis by which separate JH1 and JH2 domains interact is limited. Val 666 is found within the hinge of the JH2 near the hub of activating mutants, notably V658F (analogous to JAK2 V617F). Proposed by Brooks, the V617F hotspot along with the activation loop in JAK2 JH2 is predicted to interact in trans with the active site of another JAK2 JH1 (63). This ladder theory is perhaps one of the closest trans-inhibitory interaction one could model against other JAKs. We propose the V666G mutation strengthens a similar interface between the JH2 JAK1 and JH1 JAK3. Val 666 is one residue away from the JH2 gatekeeper E667 (64) and the identity of a kinase's gatekeeper has been shown to influence the conformational positioning of its activation loop (Hari et al., 2013). Thus, we speculate that V666G promotes an activation loop extended away from the JAK1 JH2 enhancing its interaction with the active site of JAK3 JH1. Future directions include

modeling the position of the activation loop within JAK1 JH2 in the presence of V666G. Structural studies on JAK JH1 JH2 domain interactions are needed to explore the mechanistic action of JAK1 V666G. Defining the physical contacts between JAK1 V666G and JAK3 could reveal new targetable regions against oncogenic JAKs. Importantly, the residues within this inhibitory interface could very well be outside the JAK3 ATP pocket that can be exploited by new drugs for allosteric inhibition. It is also speculated that this highly conserved Val residue may be conserved across other Tyr kinases and pseudokinases. Future directions, determining if this Val is conserved across such kinomes could mean that new targetable regions would not be restricted to JAKs.

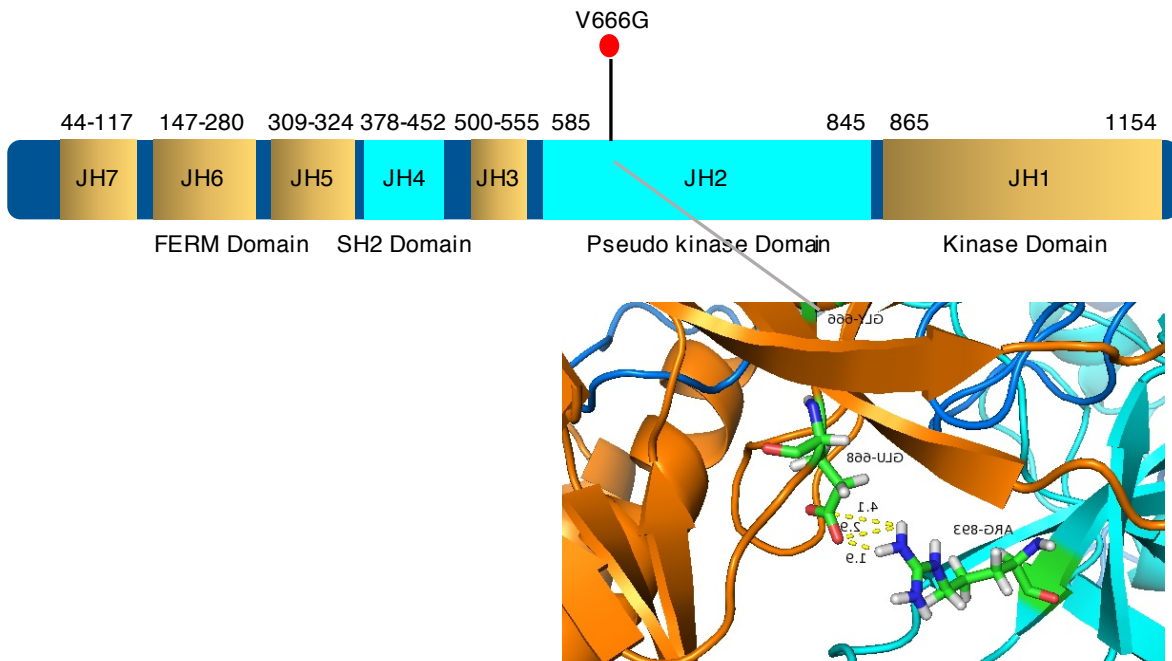


Figure 8. JAK1 V666G is predicted to be within the JH1 JH2 cis interface.

Linear model of JAK1 with V666G shown in the JH2 domain and modeled against TYK2 JH1 JH2 inhibitory interaction. JAK1 V666G within JH2 (orange) at the interface near Glu⁶⁶⁸ and JH1 (Teal) Asp⁸⁹³ hypothetical salt bridge (green).

Chapter 6: Overview

6.1 Overview

To identify how ALL is driving Hispanic health disparities, El Paso Del Norte ALL patient samples were screened for mutations involved in the JAK/STAT pathway. Using WES a novel mutation, V666G, in the JH2 of JAK1 was detected. The potential significance of this JAK1 V666G mutation prompted our interest due its location within the pseudokinase hinge in close proximity to well-known JAK1 activating mutants. Unexpectedly, the V666G mutation lead to a kinase-dead like phenotype on JAK1 as indicated by a lack of autophosphorylation and cross-phosphorylation by JAK family members. Furthermore, a consistent dominant negative effect of kinase dead JAK1 K908A and the newly identified JAK1 V666G JH2 mutant was seen on JAK family member cross phosphorylation. Particularly, the absence of JAK1 allowed sustained autoactivation of JAK3 yet the presence of inactive JAK1 by JH1 and JH2 mutations could reduce this activity indicated by Tyr phosphorylation. In the context of IL-2 signaling, a dominant negative effect of JAK1 V666G continued upon JAK3 transphosphorylation and downstream signaling. This trans-inhibitory effect of JAK1 V666G continued amongst ALL associated JAK3 activating mutants M511I and A573V. These findings demonstrate a dominant negative role of JAK1 on JAK3 and support that JAK1 JH2 domain can directly effect activation of JAK3. As mentioned previously future directions modeling this inhibitory interaction between the poorly understood JAK1 JH2 pseudokinase and JAK3 could yield insights about how to inhibit oncogenic JAKs.

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Vita

Alice Hernandez Grant earned her Bachelor of Science degree in Psychology from The University of Texas at El Paso (UTEP) in 2012. Subsequently, she obtained her Masters of Arts degree in Experimental Psychology in the field of behavioral neuroscience from UTEP in 2015. She went on to pursue her PhD in a new field, cancer immunology, when she joined the doctoral program in 2015 in the Department of Bioscience. In the Biological Science program at UTEP, she has been studying tyrosine kinases and cell signaling in lymphocytes as they relate to Leukemia. The major focus of her research has been the identification of novel mutants and their structural and functional consequence on Janus Kinase (JAK1), a tyrosine kinase frequently dysregulated in Acute Lymphoblastic Leukemia. Work from Dr. Grants dissertation, on a nonconical role for JAK1 pseudokinase, supervised by Dr. Robert A. Kirken, will be submitted for publication in a top tier peer-reviewed scientific journal.