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A Requirement for Y841 in Jak3 Enzymatic Activity and Hematopoietic Cancers

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A REQUIREMENT FOR Y841 IN JAK3 ENZYMATIC ACTIVITY AND HEMATOPOIETIC CANCERS

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Dedication

For my mother. Without your love and support I would not have made it this far.

A REQUIREMENT FOR Y841 IN JAK3 ENZYMATIC ACTIVITY AND

HEMATOPOIETIC CANCERS

by

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DISSERTATION

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Abstract

A medical need exists for successfully treating people afflicted with leukemia, especially those who develop drug resistant forms. Relapse leukemia cases are particularly high within Hispanic populations where this disease is among the most frequently occurring cancer. Fourteen somatic mutations have been reported in Janus tyrosine kinase 3 (Jak3), including M511I and A573V, from patients with various forms of leukemia. To monitor drug sensitivity, a model system was developed. Indeed, many of these mutations have been shown to possess transforming ability in cell lines such as the IL-3 dependent pro-B cell line Ba/F3. As such, Ba/F3 cells were transformed to cytokine independent growth using a retrovirus system expressing either the Jak3 mutation M511I or A573V. Here we demonstrate that Jak3 M511I and A573V possess this ability to induce cytokine independent Ba/F3 cell growth. Next, cells were used to test the sensitivity to pre-clinical and clinical Jak3 selective inhibitors on cellular viability. The level of sensitivity of transformed Ba/F3 cells varied with treatment to Jak3 inhibitors NC1153 and CP-690,550, while neither responded to the pre-clinical Jak3 inhibitor, EP-009. Screening of one hundred and three patient samples with various forms of leukemia and lymphoma for mutations M511I and A573V in exons 11 and 13 of Jak3 failed to demonstrate the presence of either mutation, respectively, confirming that these mutations are rare. However, this study provides a rational for the use of Jak3 inhibitors with distinct chemical composition for the treatment of Jak3 driven cancers, including patients with the transforming mutations M511I and A573V. This work has also identified a novel Jak3 tyrosine phosphorylation site, Y841, through the use of kinase assays coupled to mass spectrometry. Y841 is evolutionarily conserved across multiple species and Jak family

members, and is positionally conserved in several tyrosine and serine/threonine kinases. Through the use of phospho-deletion mutants, it was determined that Y841 is necessary for full activation of Stat5B, most likely due to its position in the glycine lid of the catalytic domain of Jak3, adjacent to the ATP binding pocket. Phospho-specific antibodies were generated against this site and showed specificity for phospho-peptide over non-phospho peptide, and recognized phosphorylated Jak3 in T-cell cell lines. Interestingly, this work shows that Jak3 mutation A573V has higher phosphorylation at Y841 than Jak3 WT. Furthermore, Jak1, Jak2 and Tyk2 are recognized by the anti-Jak3 Y841 antibody, potentially broadening the scope of this tool to explore hematopoietic cancers driven by other Jak family members.

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Chapter 1

Introduction

1.1 Jak/Stat function in lymphocytes

There are four members of the Janus Kinase (Jak) family, Jak1, Jak2, Jak3 and tyrosine kinase 2 (Tyk2). While Jak1, Jak2 and Tyk2 are ubiquitously expressed in different cell lineages, Jak3 is found primarily in cells of the immune system such as T-, B- and Natural Killer (NK) cells [1]. Jaks share 7 homologous domains, JH1-JH7 (Figure 1). The JH1 kinase domain harbors the kinase function while the JH2 is a pseudokinase domain and negative regulator of kinase activity. JH3 and JH4 make up the SH2-like domain which does not play the role of a typical SH2 domain but may have a role in receptor association [2]. The region within JH5-JH7 contains a 4.1 ezrin radixin, moesin (FERM) domain, necessary for binding to cytokine receptors [3]. Much has been established about Jak function through knockout studies in mice. Jak1 knockout mice die perinatally and have defective lymphoid development [4]. Jak2 deficient mice die at the embryonic stage due to disruption of erythropoiesis [5]. Interestingly, Jak3 knockout mice survive and are fertile though they exhibit a severe combined immunodeficiency (SCID) phenotype [6]. Tyk2 knockout mice also survive and are fertile but have diminished natural killer cell development [7]. Within the last decade the crystal structure of the Jak family kinase domain and the structure of the Jak2 pseudokinase domain were determined [8-12].

Signal Transducers and Activators of Transcription (Stats), are transcription factors that regulate cell growth, survival and differentiation in immune cells [13]. There are seven Stat proteins, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6 containing five shared domains (Figure 2) [14, 15]. An N-terminal domain, a coiled-coil domain for protein-protein interactions, a DNA binding domain, an SH2 domain for docking to the

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receptor and dimer binding, and a transactivation domain [16]. Similar to Jak knockouts, much has been learned about Stats from knockouts in mice. As reviewed in *Regulation of T cell homeostasis by JAKs and STATs* , Stat1 knockout mice show increased death in response to typically "harmless" pathogens [17]. Stat2 knockout mice develop normally but have higher susceptibility to viral infection as well as poor T-cell response to Type1 interferons. Stat3 knockout mice die in the embryonic development stage. Naive T-cells from Stat4 knockout mice are more likely to differentiate into Th2 cells rather than Th1 cells. Stat5a/b double knockout mice have crippled T-cell immune functions though knocking out either 5a or 5b alone shows no significant difference in phenotype. Stat6 knockout mice have reduced ability to differentiate naive T-cells into Th2 cells.

1.2   Signaling through the Interleukin-2 Receptor

The interleukin-2 receptor (IL-2R) initiates three intracellular pathways: the Jak/Stat pathway, the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway.

Signaling through the Jak/Stat pathway is critical in many cell types. Jak3 signals though the common γ chain (γ_c)-cytokines which includes IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21and promote cell survival [18, 19]. The IL-2 receptor complex is composed of the IL-2Rα, IL-2Rβ and γ_c and is formed when binding of the IL-2 ligand occurs. The IL-2Rα subunit is necessary for formation of a high affinity receptor though it does not have signal transduction properties [20]. The IL-2Rβ and γ_c subunits are both necessary for signal transduction [21] and associate with Jak1 and Jak3 respectively [22]. The IL-2Rβ chain contains a Box 1 and Box 2 element that is necessary for Jak1 binding and activation [23].
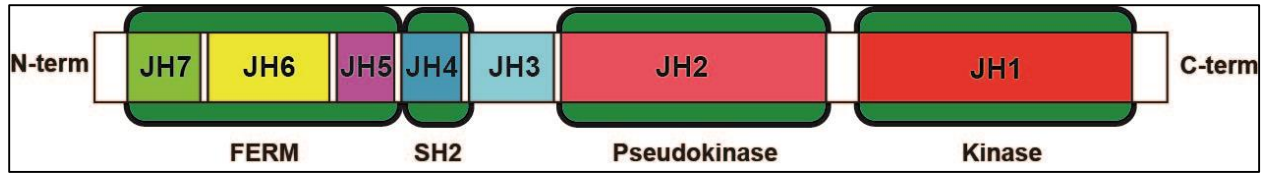
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Figure 1. Schematic model of Jak proteins. JH1 contains the kinase domain, JH2 houses the pseudokinase domain, JH4 holds the SH2-like domain and the JH5-JH7 harbor the FERM domain.

Figure 2. Schematic model of Stat family member domains. Stats harbor 5 domains. An N-terminal domain, a coiled-coil domain, a DNA-binding domain, an SH2 domain, and a transactivation domain.

The γc chain contains a Box 1 and a "Box 2 like" domain which are necessary for binding and activation of Jak3 [24]. Upon binding of cytokine to the receptor Jaks undergo autophosphorylation and then are capable of phosphorylating tyrosine residues within IL-2Rβ creating docking sites for Stat5a and Stat5b [24]. The Stats are then tyrosine phosphorylated which causes them to disengage from the receptor and form dimers (hetero and homo) before ultimately translocating to the nucleus where they drive gene transcription (Figure 3) [17].

The MAPK pathway is mediated by the adapter protein Shc which binds to IL-2Rβ via a JAK phosphorylated tyrosine at position 338 [25]. The MAPK pathway is activated by a Shc/Grb2/Sos complex. Sos is a guanine nucleotide exchange factor that activates the GTPase Ras [26]. Activated Ras then binds to Raf, a serine/threonine kinase, which activates MEK, a tyrosine/threonine kinase, which activates the serine/threonine kinase MAPK. In lymphocytes MAPK is important for T-cell activation, proliferation and differentiation [27].

The PI3K pathway also shares the Shc adaptor molecule but is activated by a Shc/Grb2/Gab2 complex. PI3K is activated by Gab2, an adapter protein and Jak3 substrate that is phosphorylated in response to IL-2 [28]. PI3K then converts phosphotidyl-inositol-4,5-bisphosphate (PIP2) to phosphotidyl-inositol-3,4,5-triphosphate (PIP3) which provides docking sites for Akt, a serine/threonine kinase that acts on many proteins that control apoptosis, cell cycle regulation and growth [29]. One of these proteins is the mammalian target of rapamycin (mTOR), a serine/threonine kinase which controls a number of functions including cell growth, proliferation, survival and gene transcription [30] and is regularly found hyper-activated in cancer [31].

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1.3 Regulation of Jak3 by tyrosine phosphorylation

Several tyrosine phosphorylation sites have been identified in Jak3 that can either negatively, or positively, regulate Jak3. The first tyrosine site to be recognized was Y785 which is located in the linker region between the JH1 and JH2 domains and acts as binding site for SH2B-β. Site directed mutagenesis was used to create the phospho-deletion mutant Jak3 Y785F which was no longer able to be phosphorylated at tyrosine 785 and led to a decrease of 50% total tyrosine phosphorylation of Jak3, although did not have any effect on the catalytic activity of Jak3. It was also found that Y785 is IL-2 inducible in NK 3.3 cells, as the site becomes tyrosine phosphorylated within 5 minutes of IL-2 stimulation [32].

Two additional sites in Jak3, Y980 and Y981, located in the kinase activation loop were identified by *in vitro* kinase assays. Phospho-deletion mutations of Y980F and Y981F determined that Y980 acts as a positive regulator of Jak3, while Y981 acts as a negative regulator of Jak3. Y980 is necessary for Stat5 activation while Y980F mutant results in decreased Stat5 DNA-binding activity. The Y981F mutant increased Stat5 DNA-binding activity [33].

The final two tyrosine phosphorylation sites to be identified are the positive regulators Jak3 Y904 and Y939. These were identified by expressing the triple mutant Jak3 Y785F/Y980F/Y981F in HEK293 cells, immunoprecipitating Jak3 and Western blotting for total tyrosine phosphorylation (pY), which revealed no significant change to total tyrosine phosphorylation [34]. Jak3 was then immunoprecipitated from transfected HEK293 cells and sent for liquid chromatography-tandem mass spectrometry analysis which identified the two sites. These sites are responsible for optimal Jak3

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autophosphorylation, Stat5 tyrosine phosphorylation and transcriptional activity, with both inducible by IL-2.

1.4   Negative regulators of the Jak/Stat pathway

Multiple regulators of the Jak/Stat pathway have been identified and grouped as follows: protein tyrosine phosphatases (PTPs) which can be either cytoplasmic or membrane associated, suppressors of cytokine signaling (SOCS) and protein inhibitors of activated Stats (PIAS).

Protein tyrosine phosphatases include Sh2 domain containing phosphatase 1 and 2 (SHP-1 and 2) and CD45. SHP-1 is a cytoplasmic phosphatase found mostly in hematopoietic cells [35]. Dysregulated SHP-1 has been shown to have a direct effect on Jak3 and Stat3 activation. Loss of SHP-1 causes aberrant JAK3 and Stat3 phosphorylation [36]. SHP-2 shares 55% homology with SHP-1 and is ubiquitously expressed. SHP-2 has been shown to be constitutively associated with Jak1 and Jak3 regardless of cytokine stimulation [37]. CD45 is a transmembrane protein tyrosine phosphatase which can be expressed in hematopoietic cells [38] and is a well-known negative regulator of the entire Jak family [39]. Most recent discoveries include Phospho-Tyrosine Phosphatase 1B (PTP1B) and T Cell Protein Tyrosine Phosphatase (TC-PTP) which share similar catalytic domains which negatively regulate Jak family members. PTP1B is ubiquitously expressed while TC-PTP is primarily expressed in hematopoietic cell lineages [40]. PTP1B recognizes a D/E-pY-pY-K/R motif found in the activation loop of Jak2 and Tyk2 [41] while TC-PTP is specific for a D/E-pY-pY-T/V motif found in the activation loop of Jak1 and Jak3 [42].
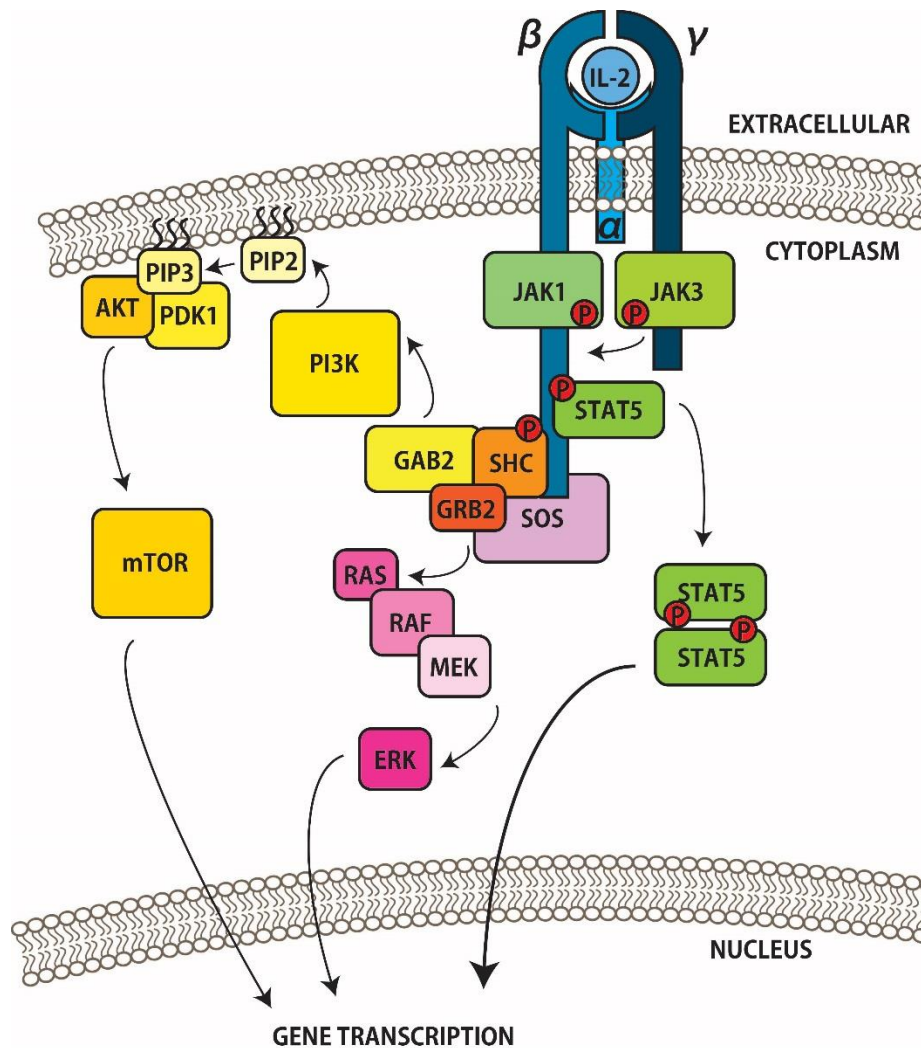
Figure 3. IL-2 Receptor Signaling Network. IL-2 induces trimerization of IL-2R α, β and γ subunits causing Jak molecules to phosphorylate each other and the receptor. Stat5 and other adapter molecules are then recruited to pY sites on the β chain where they become tyrosine and/or serine phosphorylated. Stat5 then disengages from the receptor and dimerizes with another Stat5 molecule before translocating into the nucleus. Simultaneously, the MAPK/Erk and the PI3K pathways become activated. Together these pathways drive gene transcription necessary for cell growth, proliferation and differentiation.

There are 8 known SOCS family members that are inducible negative regulators of cytokine signaling. They act by binding to phosphotyrosine residues on either cytokine receptors or Jaks through centrally located SH2 domains [43]. SOCS1 and SOCS3 both act on Jak family members but in different ways. SOCS1 acts by binding to the tyrosine phosphorylated residues [44] while SOCS3 binds to the phosphorylated cytokine receptor [45] and JAK proteins [46]. SOCS3 is known to inhibit Jak1, Jak2 and Tyk2 [47] but not Jak3 [46]. SOCS1 is induced by IL-2 and inhibits both Jak1 and Jak3 though effects on Jak1 activation are more pronounced. SOCS1 can also interact with IL-2Rβ but this interaction is not necessary to inhibit the Jak proteins [48].

There are four members of the PIAS family, PIAS1, PIAS3, PIASx and PIASy which regulate the Jak/Stat pathway through alterations of the transcriptional profile, in this case Stats [49]. Stats are inhibited in different ways according to their PIAS. PIAS1 and PIAS3 act by blocking the DNA binding activity of Stat1 and Stat3 respectively [50, 51]. PIASy and PIASx take a different route by enlisting the aid of corepressors to block transcriptional activity of Stat1 and Stat4 respectively [52, 53]. PIAS3 can also act as the sole repressor to block transcriptional activity, as is the case with Stat5 [54].

1.5   Tyrosine kinases and cancer

Recently, receptor tyrosine kinases have been implicated in human leukemia. The discovery of the fusion protein BCR-Abl, which causes CML, was the first report that an aberrantly activated tyrosine kinase was shown to cause cancer [55, 56]. The drug Imatinib, a tyrosine kinase inhibitor, was the first "targeted therapy" whose method of action targeted protein tyrosine kinases directly, rather than non-selectively killing rapidly proliferating cells. Imatinib acts by binding to the active site of tyrosine kinases, thus

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eliminating its kinase functionality [57, 58]. Other kinases such as KIT [59] and Flt3 [60] have also been linked to cancer but until recently have proven more difficult to target. Imatinib is undergoing clinical trials for treatment of KIT related cancers and has shown promise in patients with the mutated or amplified protein [61]. Likewise, two drugs Sorafenib and Sunitinib have been used in clinical trials to combat FLT3 driven AMLs with some success [62].

1.6   Jaks and cancer

Jaks have also been shown to be associated with myeloproliferative disorders. The well documented mutation from valine to phenylalanine in amino acid position 617 of Jak2 has been shown to cause diseases such as polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF) [63-66]. PV is an abnormal expansion of red blood cells in the bone marrow while ET is an accumulation of platelets in the bone marrow [67]. MF is another bone marrow disorder where the normal marrow is replaced with fibrous tissue [68]. Ruxolitinib is a Jak inhibitor with specificity for Jak1 and Jak2 [69]. On November 16, 2011 Ruxolitinib became the first Jak inhibitor approved by the FDA for treatment of post-PV and post-ET myelofibrosis [70].

Jak3 has been implicated in leukemias and mutated forms of this protein have been found in patients with AML, AMKL, CML, ALL and recently natural killer/T-cell lymphoma [71-76]. Several point mutations in the DNA sequence of Jak3 have been identified which cause a single or in some cases a double amino acid change and may cause Jak3 to act abnormally. It has also been shown that in some instances these mutated Jak3 proteins are able to transform cytokine dependent lymphocyte cell lines to cytokine independency [71, 72]. There are currently 14 known mutations first identified

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by the following groups. From Yamashita et al, 2010: G62S, Q501H, M511I, R657Q, R918C and L1017M. From Mullighan et al, 2009: S789P. From Walters et al, 2006: P132T, A572V and V722I. From Kiyoi et al, 2007: I87T, M576L and the double mutant A573VA593T. All of the above listed Jak3 mutations are derived from leukemia patients with the exception of L1017M which was discovered in the KCL22 cell line derived from a CML patient [77]. Of note is the fact that these mutations are found throughout 3 of the 4 domains of Jak3. Mutations G62S, I87T, and P132T are located in the FERM domain [71-73]. The Q501H and M511I mutations are located in the linker region between the SH2 domain and the pseudokinase domain [72]. Mutations A572V, A573V, A593T, M576L, R657Q and V722I are found in the pseudokinase domain [71-73]. The S789P mutation is located in the linker region between the kinase and pseudokinase domains [74]. The R918C and L1017M mutations are located in the kinase domain [72, 77]. Interestingly, none of the mutations can be found in the highly conserved SH2 region (Figure 4). A BLAST search also reveals that most of the amino acid positions are conserved across many species including chimps, mice and rats but less than half are conserved in other Jak family members.
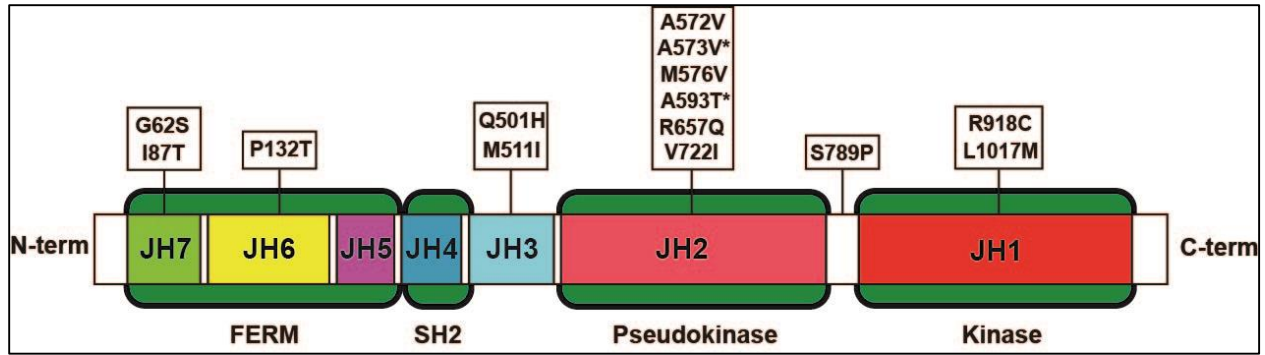
Figure 4. Schematic of locations of Jak3 leukemic mutations. Schematic diagram showing location of mutated amino acids found in leukemic patients in relation to structural domains. *Denotes mutations found in tandem.

1.7   Significance and Hypothesis

Nearly 156,420 new cases of blood cancers are expected to be reported for 2015 [78]. Leukemia, a form of cancer that affects white blood cells found in the bone marrow and blood, will account for approximately 54,270 of these cases and will be responsible for 24,450 deaths. Of these, 14,620 will be Chronic Lymphocytic Leukemia (CLL), 6,660 Chronic Myelogenous Leukemia (CML), 20,830 will be Acute Myelogenous Leukemia (AML) and 6,250 will be Acute Lymphocytic Leukemia (ALL). Chronic leukemias typically have a phenotype of relatively mature cells which accumulate over time. Acute leukemias are characterized by a rapid onset of immature cells. Myelogenous leukemia is a cancer of the myelogenous white blood cells (such as megakaryoblasts and granulocytes) which accumulate in bone marrow and can disrupt normal production of red blood cells. Lymphocytic leukemias on the other hand are cancers of the lymphocytic white blood cells (T-cell, B-cells and Natural Killer cells) which can also disrupt normal production of red blood cells and can spread to other organs [79]. Acute Lymphoblastic Leukemia typically affects children from ages 2-5 years old while myelogenous leukemias most commonly affect adults [80, 81].

Leukemia causes more deaths than any other cancer among children and young adults under the age of 20 and Hispanic children and adolescents under this age have the highest incidence rates of this cancer [82]. This is highlighted by the fact that cancer is now the number one killer of Hispanics in the United States, higher than heart disease which is the has the highest death rate for all other ethnicities [83]. This is of special importance because in El Paso County, Hispanics make up 82.2% of the population [84]. Indeed, for the state of Texas, the highest incidence rates for ALL line up demographically
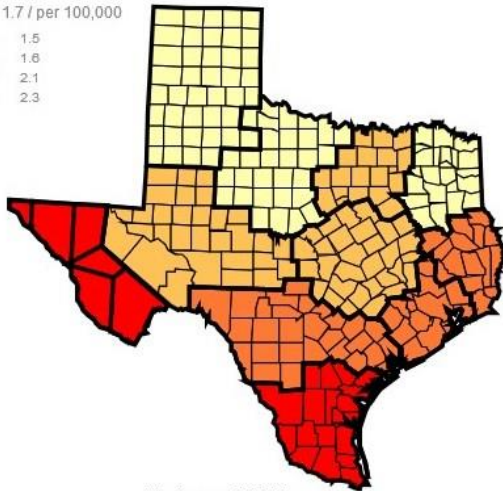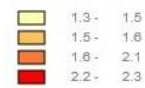
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with the highest concentration of Hispanic people (Figure 5). Therefore there is a critical need to understand the mechanisms involved in mutant Jak3 regulation, to identify inhibitors capable of targeting mutant Jak3 and to determine whether any Jak3 mutations can be found in human cancer patient samples from a biorepository of mostly Hispanic people. It is also of importance to identify novel tyrosine phosphorylation sites and determine whether these sites are differentially regulated in mutant Jak3. We **hypothesize** that **dysregulation of Jak3 mutants leads to an oncogenic phenotype and the novel tyrosine 841 represents a marker of this transformation**.

To identify potential tyrosine phosphorylation sites, a kinase assay was performed using Jak3 immunoprecipitated from transfected HEK293 cells harboring the Y785F/Y980F/Y981F and SDS-PAGE was performed on the samples using a 7.5% gel which was then Coomassie stained and Jak3 bands were excised and sent for mass-spec analysis. This study revealed 11 novel tyrosine phosphorylation sites ranging from the JH1 domain to the JH6 domain. Phospho-specific antibodies were then generated against 7 of sites and were characterized using Western blot and dot blot analysis which revealed anti-pY841 Jak3 as highly selective antibody and a useful tool that may further our understanding of leukemic Jak3 signaling.

Our work focused on 2 Jak3 mutations, M511I and A573V, which have very strong transforming potential and showed differing sensitivities to Jak3 specific inhibition. Jak3 A573V is of special interest as it was sensitive to both inhibitors CP-690,550 (Pfizer) and NC1153 and showed an increase in both total tyrosine phosphorylation and phospho-tyrosine 841, showing that this site acts as a marker for leukemic Jak3 activation.
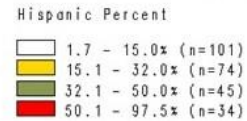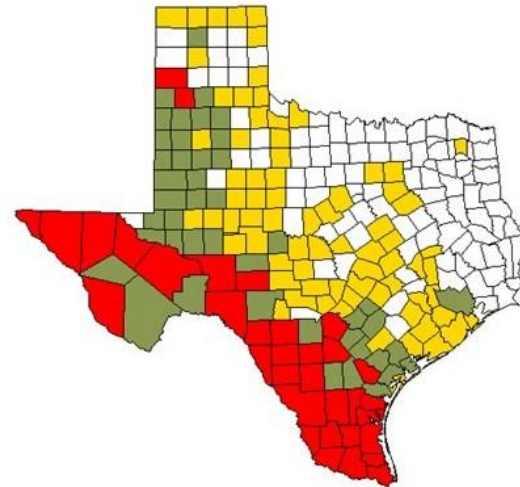
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Figure 5. Incidence of ALL in Texas. On the left incidence rates in Texas of ALL with highest rates in red compared with highest concentration of Hispanic people on the right, also in red.

Chapter 2

Leukemic Jak3 mutations M511I and A573V are transforming and are

sensitive to clinical and pre-clinical Jak3 inhibitors

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2.1   Introduction

Loss of Jak3 regulation within immune cells leads to diseases including Severe Combined Immunodeficiency (SCID) [85, 86], and cancers of the blood, such as leukemia and lymphoma [72, 87].Hematological cancers have been previously reported to be associated with Jak3 gain of function mutations [71, 72, 88, 89]. Indeed, several activating point mutations of Jak3 have been identified from screening patients with acute myelogenous leukemia (AML), acute megakaryoblastic leukemia (AMKL), chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL) and natural killer/T-cell lymphoma [71, 72, 87-90]. The following fourteen somatic mutations spanning Jak3 have been reported in human patients: G62S, I87T, P132T, Q501H, M511I, A572V, A573V, M576L, A593T, R657Q, V722I, S789P, R918C and L1017M. Many of these mutations have been shown to be transforming in Ba/F3 cells, a murine pro B-cell cell line that is normally dependent on IL-3 for growth [71, 72, 88, 89].

To further elucidate the role of mutant Jak3 in leukemia, the present study investigated the kinase activity of the above listed fourteen point mutations as well as their transformational ability, and sensitivity to clinical and pre-clinical inhibitors. Here we provide evidence and that Jak3 A573V and M511I mutations confer transformative properties and are sensitive to the FDA approved Jak3 inhibitor CP-690,550 (Tofacitinib) as well as NC1153. In addition, we assessed the auto-activation ability of the above listed fourteen Jak3 mutations and their ability to activate downstream effectors, Stat5B, in comparison to wild-type (WT) Jak3.

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2.2   Materials and Methods

2.2.1   Cell culture, drug treatment and viability assays:

The human HEK293T cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; Atlanta Biologicals), 2 mM L-glutamine (Corning), and 1 % penicillin/streptomycin (Corning). Murine pro-B-cells, Ba/F3, and human embryonic kidney cells, Hek293, were cultured in RPMI 1640 medium containing 10 % FBS, 2 mM l-glutamine, and 1 % penicillin/streptomycin. Ba/F3 cell culture media was supplemented with 10 IU/ml mouse interleukin-3 (IL-3) (Peprotech). At 48 hours post-transduction cells were washed 3 times with, and resuspended in media devoid of IL-3. Cells were then analyzed by FACS using a Beckman Coulter Gallios™ Flow Cytometer for GFP and propidium iodide (MP Biomedicals). CP-690,550 (Selleck Chemicals), NC1153 [91] or EP-009 [92] treatments were performed at 37 °C using the concentrations and time points indicated. Cellular viability was determined by MTS assay (Promega) according to the manufacturer's instructions. Cell counting was performed by using a hemocytometer (Hausser Scientific) and trypan blue (MP Biomedicals).

2.2.2   Plasmids and site-directed mutagenesis:

Full length wild-type (WT) Jak3 was subcloned into the EcoRI and XhoI sites of MSCV-IRES-GFP (MIG) plasmid, a gift from Tannishtha Reya (Addgene plasmid 20672), or into the TOPO cloning site of pLenti7.3/V5-TOPO (pLenti7.3) plasmid (Invitrogen). Mutant forms of Jak3 were prepared using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Primers used for mutagenesis of Jak3 were as follows: M511I (forward primer 5'-CCAATACCAGCTGAGTCAGATCACACACAAGATCCCTG-3' and reverse primer 5'-

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GAGGGATCTTGTGAAATGT-GATCTGACTCAGCTGGTATTGG-3'); A573V (forward primer 5'-GTCATTCCTGGAAGCAGTGAGCTTGATGAGCCAAG-3' and reverse primer 5'-CTTGGCTCATCAAGCTCACTGCTTCCAGGAATGAC-3'). The pcDNA3.1 human Jak3 and Stat5B cDNAs (OriGene) were obtained as described previously [93, 94]. pSin Cherry and pTrip eGFP, gifts from Dr. Manuel Llano, were used to confirm plasmid fidelity using a Zeiss Axiovert 200 Inverted Fluorescence Microscope and the Zeiss Zen software. All subclones and mutations were verified by DNA sequencing at the Genomic Analysis Core Facility of the Border Biomedical Research Center at The University of Texas at El Paso.

2.2.3 Virus production and infection of target cells:

Lentiviruses were generated by co-transfection of pLenti7.3 plasmid vectors with packaging plasmids pMD.G and pCMVΔR8.91, gifts from Dr. Manuel Llano, into HEK293T cells. Retroviruses were generated by calcium-phosphate co-transfection of 15μg MIG retroviral plasmid vectors and 15μg packaging plasmids pCL-Ampho (Addgene) and 5μg pMD.G, into HEK293T cells. Viral supernatants were collected 72 h post-transfection, centrifuged to remove cell debris, filtered through 0.45-μM filters (Thermo Scientific) and concentrated by ultracentrifugation at 124,750 x g for 2 h on a 20% sucrose cushion. Target cells were plated at 1×10^5 cells in 500 μL of RPMI 1640 culture medium in 24-well plates and infected with 50 μL of either pLenti7.3 Jak3 variants or MIG Jak3 variants.

2.2.4 Transfection and Western blot analysis:

HEK293 cells were seeded into 10 cm dishes 16 hours pre-transfection to yield 90-95% confluency at the time of transfection. Cells were transfected with plasmid

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containing mutant Jak3 or WT Jak3 with three controls, Jak3 K855A, Y981F and/or Y980F and Stat5B using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 hours post-transfection cells were pelleted, lysed, clarified and either immunoprecipitated (IP) for Jak3 using anti-Jak3 polyclonal antibody as previously described [93], or whole cell lysate (WCL) saved. Samples were separated by 7.5 % SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) for Western blot analysis as previously described [95]. Briefly, membranes were probed overnight with mouse monoclonal 4G10 anti-phosphotyrosine (pY) antibody (EMD Millipore) or anti-pY Stat5 (Cell Signaling Technology) developed using horseradish peroxidase-conjugated goat anti-mouse IgG and visualized by enhanced chemiluminescence and X-ray film (Phenix). Membranes were stripped as previously described [93] and re-probed with Jak3 (Abcam) or Stat5 (Santa Cruz) antibodies to ensure equal protein loading.

2.3   Results

2.3.1   Leukemia related Jak3 transforming mutants are capable of auto-activation and phosphorylation of downstream effectors.

To assess whether previously identified transforming mutations within Jak3 are tyrosine phosphorylated, HEK293 cells were transfected with plasmids for either Jak3 WT, Jak3 kinase dead mutant, K855A, Jak3 hyperactive active mutant Y981F, or one of fourteen mutant Jak3 plasmids identified in human leukemia patients [72, 87-89]. At 24 hours post-transfection cells were harvested, lysed, immunoprecipitated for Jak3 and Western blot analysis and densitometry was performed to assess total tyrosine phosphorylation (Figure 6A-B). As shown in these figures, all Jak3 variants displayed

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equal or greater tyrosine phosphorylation compared to the WT enzyme. Kinase dead Jak3 (K855A) showed no activity. Similarly, HEK293 cells were transfected as above but with the inclusion of WT Stat5B as a substrate for Jak3 kinase activity (Figure 12C-D). All Jak3 variants were competent to induce Stat5 tyrosine phosphorylation. Together, these results confirm that the tested Jak3 mutations are catalytically active and are able to associate with and activate downstream effector proteins such as Stat5 in the absence of cytokine stimulation.

2.3.2 Generation of viral constructs to determine transformational ability in Ba/F3 cells

To transform Ba/F3 from IL-3 dependent to IL-3 independent growth, the Murine Stem Cell Virus – Internal Ribosomal Entry Site – Green Fluorescent Protein (MIG) viral vector was used to generate viral constructs using pcL-Ampho and pMD.G plasmids for packaging and envelope of the viruses as shown in figure 7A. Jak3 WT was cloned into the EcoR1 and Xho1 restriction sites of the MIG vector and site-directed mutagenesis was used to generate plasmids for the 14 leukemic Jak3 mutations. HEK293 cells were then transfected with 2µg of each plasmid for Jak3 WT and the 14 mutations G62S, I87T, P132T, Q501H, M511I, A572V, A573V, M576L, A593T, R657Q, V722I, S789P, R918C and L1017M, to be used for Western blot analysis to determine whether plasmids were correctly expressing Jak3 and GFP. Figure 7B revealed that Jak3 was not properly cloned into the MIG Jak3 G62S, M576L and R657Q plasmids, while the MIG Jak3 A572V plasmid was not expressing GFP.
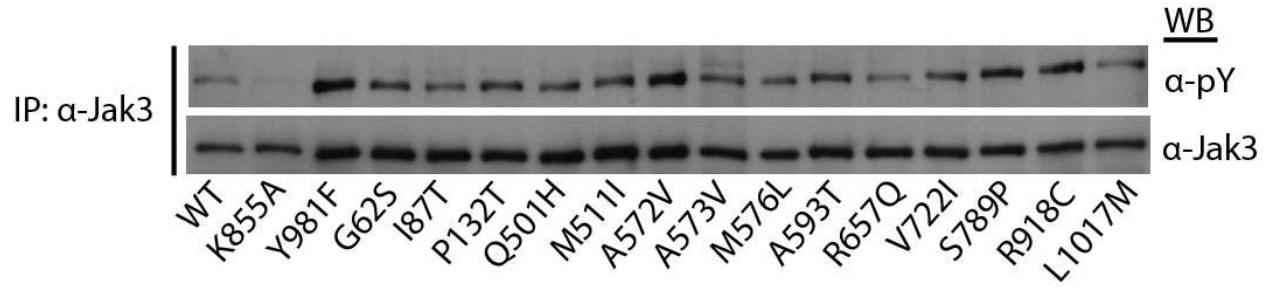
Further experiments to test the fidelity of the plasmids were performed utilizing co-transfection of the plasmids with pSin Cherry which shows red under a fluorescent microscope. A defect in the promotor or other regions of the plasmid can also affect the

22

expression of a second plasmid's genes, therefore pSin Cherry was used as the control for expression defects, while pTrip eGFP was a control for GFP expression. HEK293 cells were co-transfected with 2µg of pTrip eGFP or MIG Jak3 WT or MIG Jak3 mutants and 2µg pSin Cherry plasmid, and 48 hours post-transfection cells were analyzed through fluorescent microscopy for green and red dye expression. None of the transfected plasmids had an effect on expression of the pSin Cherry plasmid (Figure 8A-P), but as shown in the Western blot from Figure 13B, MIG Jak3 A572V did not express GFP (Figure 14H). Expression of GFP was also confirmed by flow cytometry (Figure 9A-O) and once again MIG Jak3 A572V did not express GFP (Figure 9I). Plasmids for MIG Jak3 G62S, A572V, M576L and R657Q were not used to generate virus.

2.3.3 Optimization of viral transduction for Kit225 and Ba/F3 cells

In order to maximize transduction efficiency, 2 viruses were generated to determine whether the addition of the pMD.G plasmid which encodes the VSV-G envelope protein that allows for viral entry provided greater transduction in HEK293 cells. It was determined that transduction was increased by 5.5% when the pMD.G plasmid was used to generate the virus (Figure 10A). Ba/F3 or Kit225 cells were then transduced using either a virus generated using the pTrip eGFP vector or the MIG Jak3 WT vector. Transduction efficiency was compared between the two and while the pTrip eGFP virus had better efficiency, the MIG Jak3 WT virus was still able to transduce both the Ba/F3 (Figure 10B) and Kit225 cells (Figure 10C).
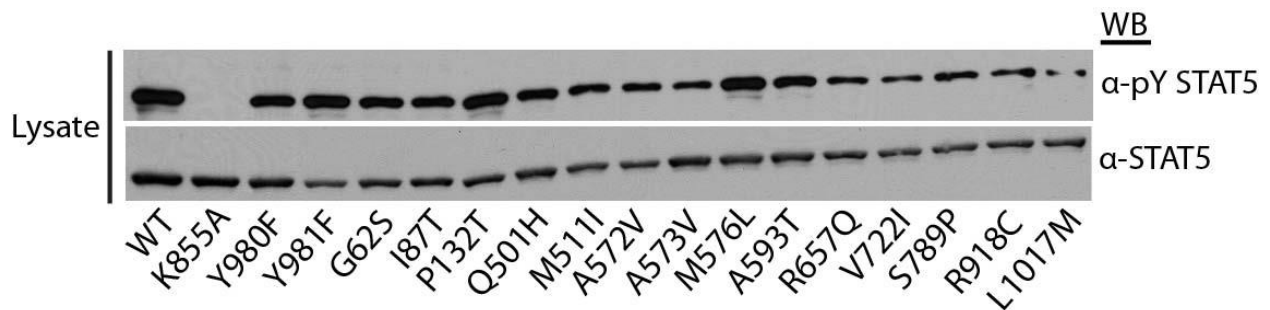
23

A

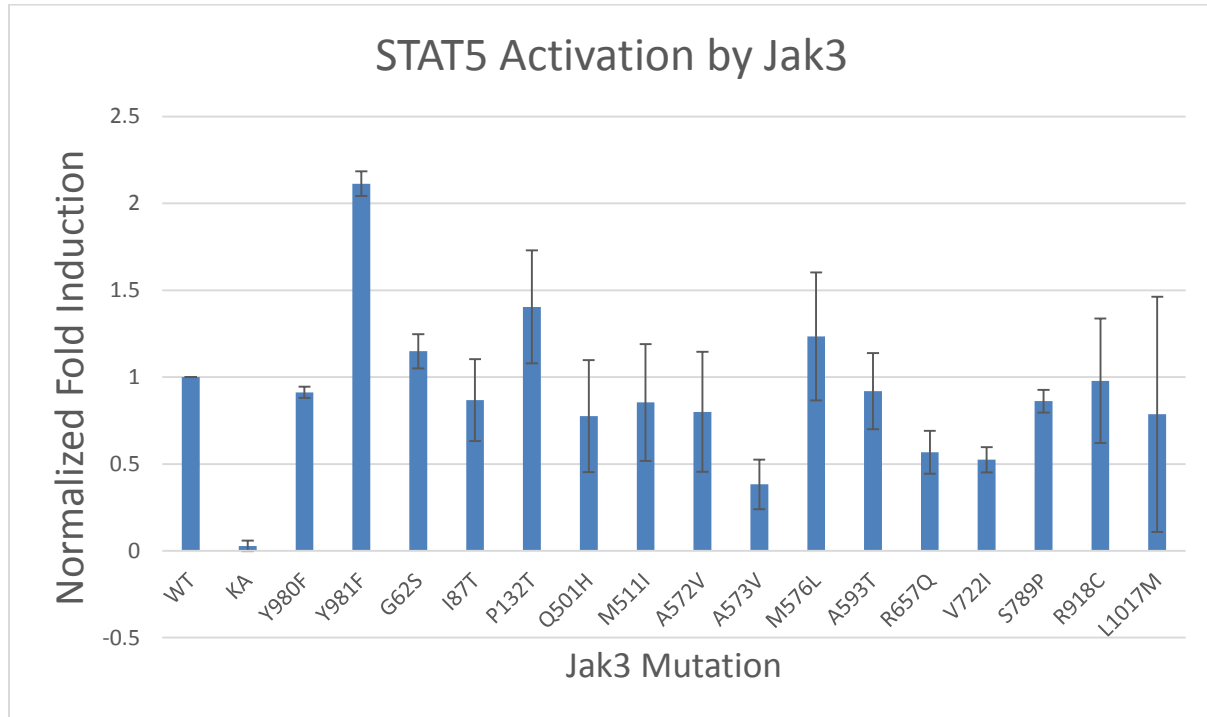

IP: α-Jak3

WB

α-pY

α-Jak3

WT K855A Y981F G62S I87T P132T Q501H M511I A572V A573V M576L A593T R657Q V722I S789P R918C L1017M

B

Jak3 Auto-Kinase activity

Normalized Fold Induction

7
6
5
4
3
2
1
0
-1

WT KA G62S I87T P132T Q501H M511I A572V A573V M576L A593T R657Q V722I S789P R918C L1017M

Jak3 Mutation

C

Lysate

WB

α-pY STAT5

α-STAT5

WT K855A Y980F Y981F G62S I87T P132T Q501H M511I A572V A573V M576L A593T R657Q V722I S789P R918C L1017M

D

STAT5 Activation by Jak3

Figure 6. Hyperactivation of Jak3 somatic mutations and Stat5B activation. A. Hek293 cells were transfected with Jak3 plasmids for WT, catalytically inactive mutant (K855A), hyperactive mutant (Y981F), or one of fourteen Jak3 mutations previously identified in leukemia patients as indicated. At 24 hours post-transfection, cells were lysed, clarified, immunoprecipitated for Jak3 and Western blot analysis was performed for anti-phosphotyrosine (pY) and total Jak3. B. Densitometric analysis of A, n=2. C. Hek293 cells were transfected as above with the inclusion of a Stat5B WT plasmid and Jak3 (Y980F). Cells were lysed 24 hours post-transfection and Western blot analysis was performed for pY-Stat5 and total Stat5. D. Densitometric analysis of C, n=2. Statistical analysis was done using a paired student t-test.
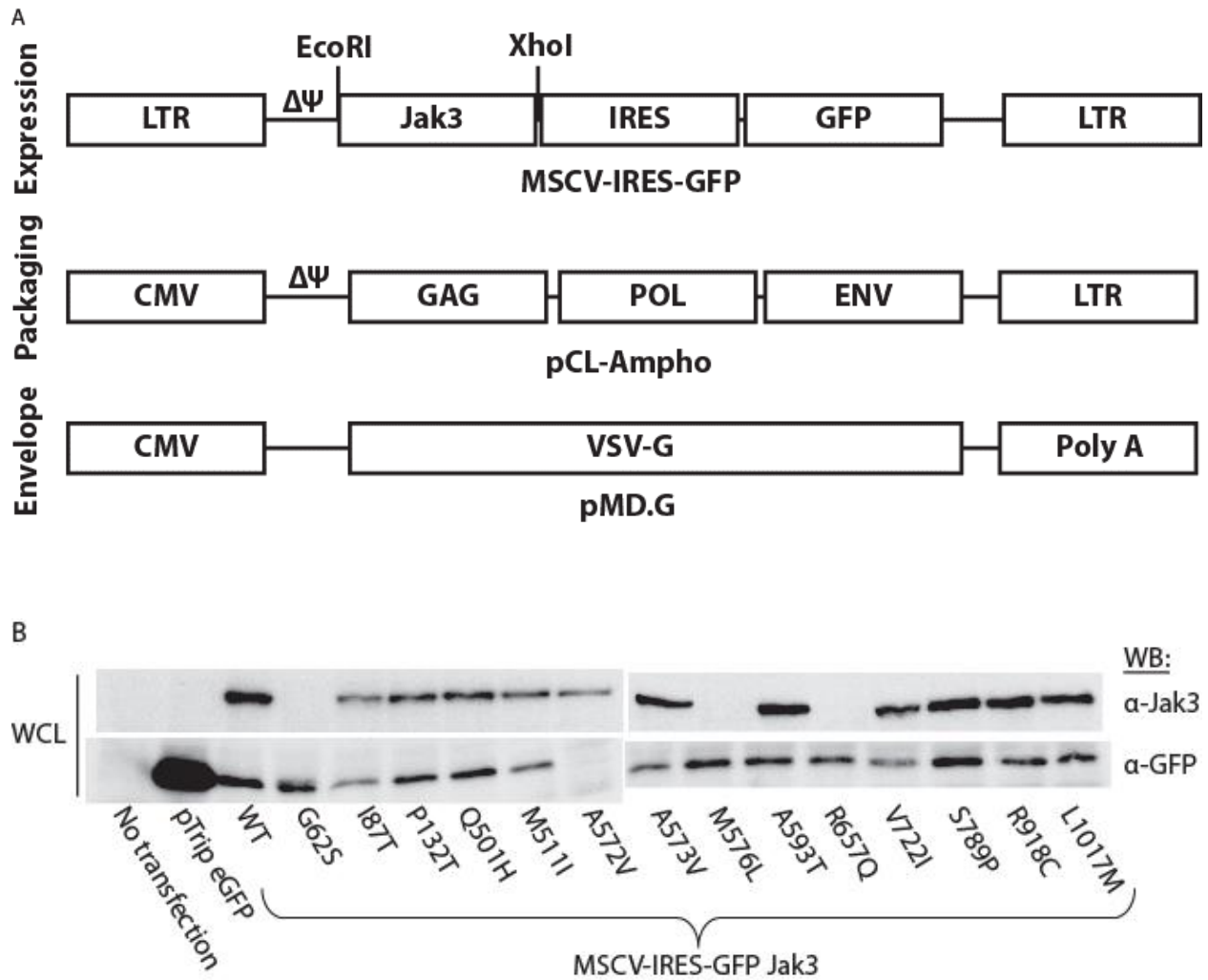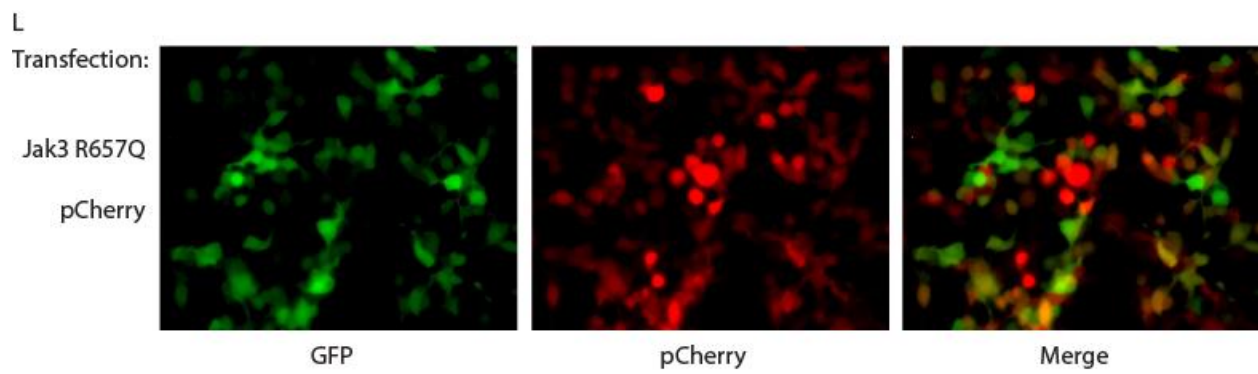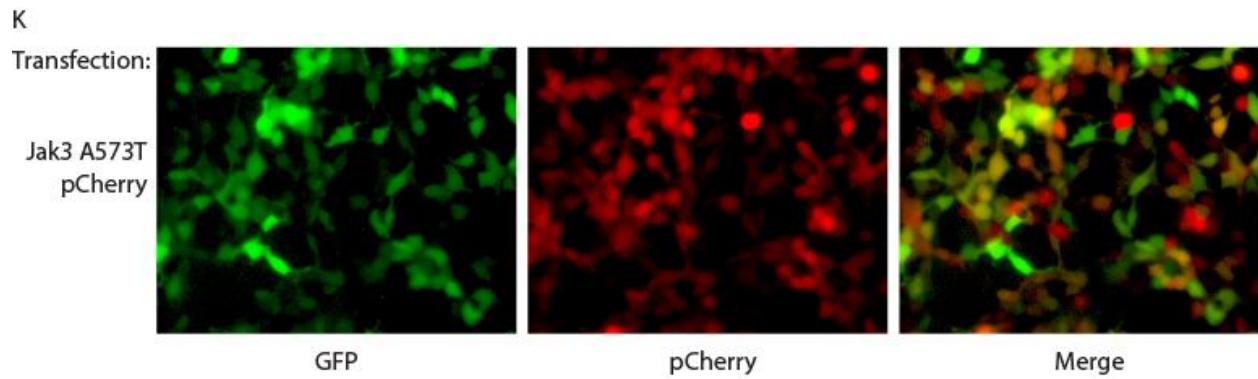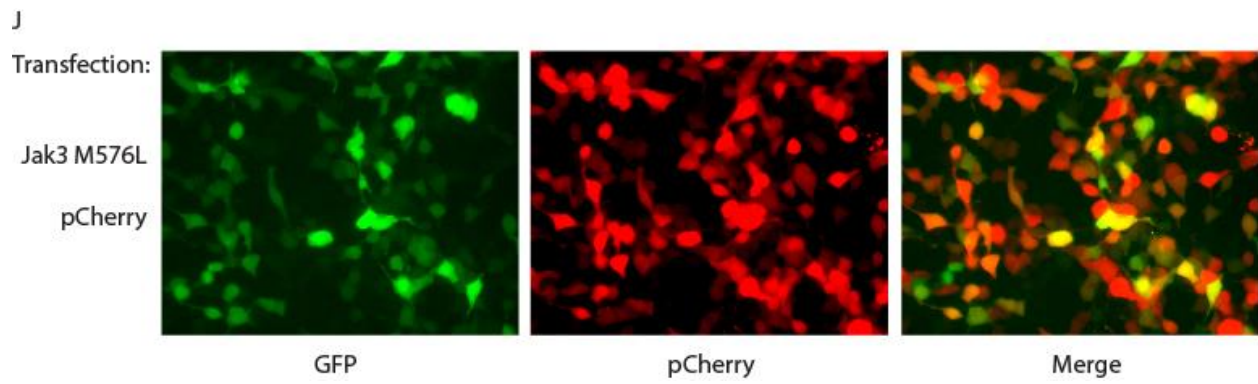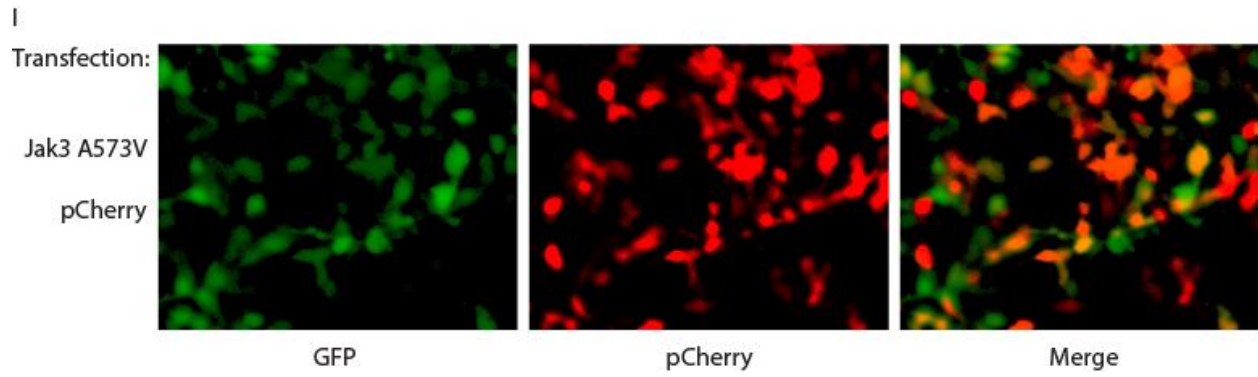
Figure 7. Generation of MIG Jak3 virus. A. Schematic representation of the expression,

packaging and envelope plasmids needed to construct the MSCV-IRES-GFP (MIG) virus.

B. Whole cell lysate from HEK293 cells transfected with MIG plasmids as indicated.

Plasmids with G62S, M576L and R657Q did not properly express Jak3, while MIG Jak3

A572V did not properly express GFP.

A
Transfection:

pTrip eGFP
pCherry

| GFP | pCherry | Merge |

B
Transfection:

Jak3 WT
pCherry

| GFP | pCherry | Merge |

C
Transfection:

Jak3 G62S
pCherry

| GFP | pCherry | Merge |

D
Transfection:

Jak3 I87T
pCherry

| GFP | pCherry | Merge |

27

E
Transfection:

Jak3 P132T

pCherry

GFP   pCherry   Merge

F
Transfection:

Jak3 Q501H

pCherry

GFP   pCherry   Merge

G
Transfection:

Jak3 M511I

pCherry

GFP   pCherry   Merge

H
Transfection:

Jak3 A572V

pCherry

GFP   pCherry   Merge

28

I

Transfection:

Jak3 A573V

pCherry

| GFP | pCherry | Merge |

J

Transfection:

Jak3 M576L

pCherry

| GFP | pCherry | Merge |

K

Transfection:

Jak3 A573T
pCherry

| GFP | pCherry | Merge |

L

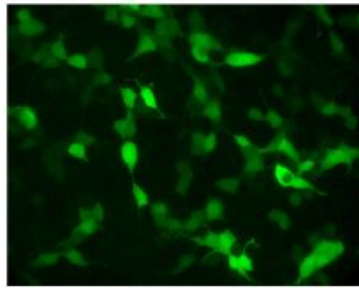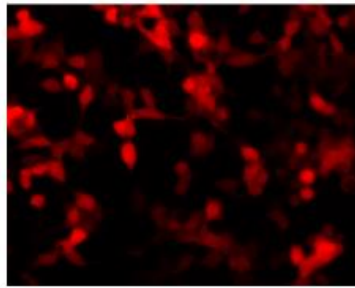Transfection:

Jak3 R657Q

pCherry

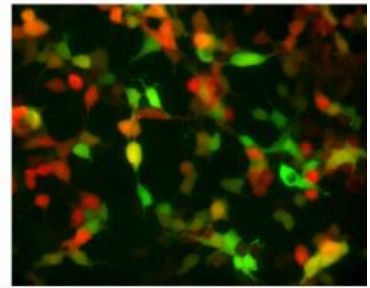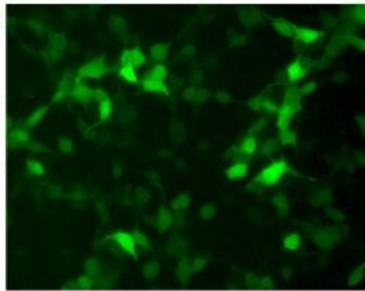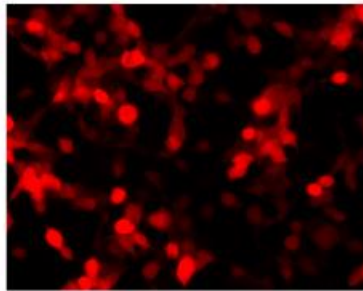| GFP | pCherry | Merge |

M
Transfection:

Jak3 V722I

pCherry

GFP   pCherry   Merge

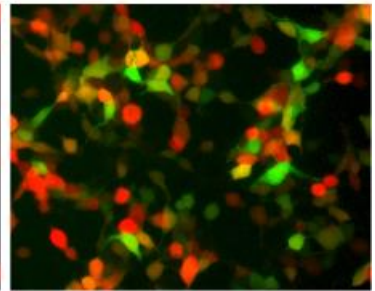**N**
Transfection:
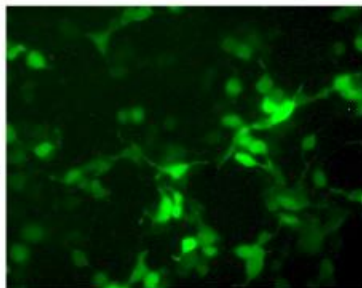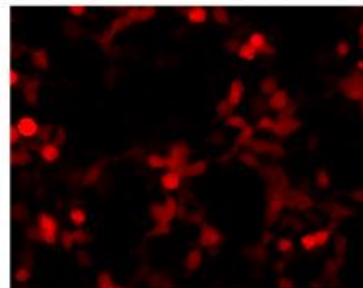
Jak3 S789P

pCherry

GFP   pCherry   Merge

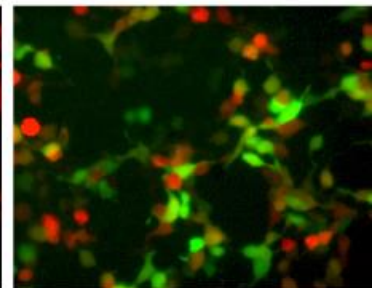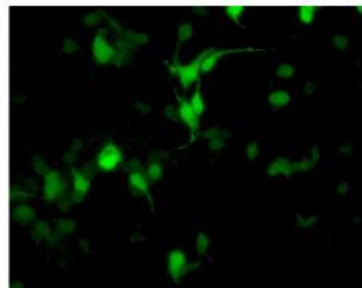**O**
Transfection:

Jak3 R918C

pCherry

GFP   pCherry   Merge

P
Transfection:

Jak3 L1017M

pCherry
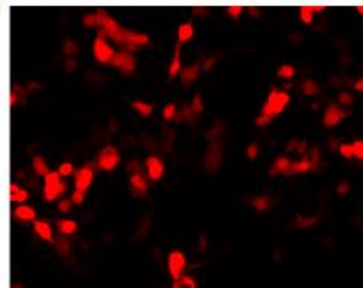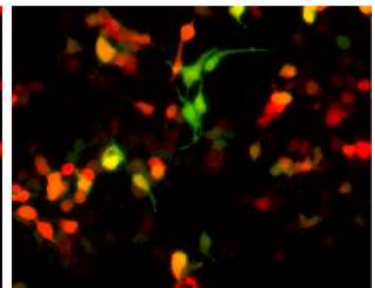
GFP   pCherry   Merge

Figure 8. Confirmation of MIG Jak3 plasmid fidelity. Hek293T cells were transfected with 2 µg each of the MIG Jak3 plasmids or pTrip eGFP and pCherry plasmids and incubated for 48 h at 37 °C. Cells were then visualized using fluorescent microscopy for GFP and Cherry expression. All cells were transfected with pCherry and pTrip eGFP or MIG Jak3 plasmids as follows: A. pTrip eGFP. B. MIG Jak3 WT. C. MIG Jak3 G62S. D. MIG Jak3 I87T. E. MIG Jak3 P132T. F. MIG Jak3 Q501H. G. MIG Jak3 M511I. H. MIG Jak3 A572V. I. MIG Jak3 A573V. J. MIG Jak3 M576L. K. MIG Jak3 A593T. L. MIG Jak3 R657Q. M. MIG Jak3 V722I. N. MIG Jak3 S789P. O. MIG Jak3 R918C. P. MIG Jak3 L1017M.

A   Non-transfected

B   pTrip eGFP

C   MIG Jak3 WT

D

MIG Jak3 G62S

E

MIG Jak3 I87T

F

MIG Jak3 P132T

G

MIG Jak3 Q501H

H

MIG Jak3 M511I

I

MIG Jak3 A572V

34

J

N1 12.1%   N2 6.8%

N3 74.8%   N4 6.3%

B 9.7%

MIG Jak3 A573V

K

N1 9.6%   N2 5.3%

N3 79.6%   N4 5.6%

B 8.2%

MIG Jak3 M576L

L

N1 10.7%   N2 6.3%

N3 76.7%   N4 6.3%

B 9.2%

MIG Jak3 A593T

M MIG Jak3 R657Q

N MIG Jak3 V722I

O MIG Jak3 S789P

P

MIG Jak3 R918C

Q

MIG Jak3 L1017M
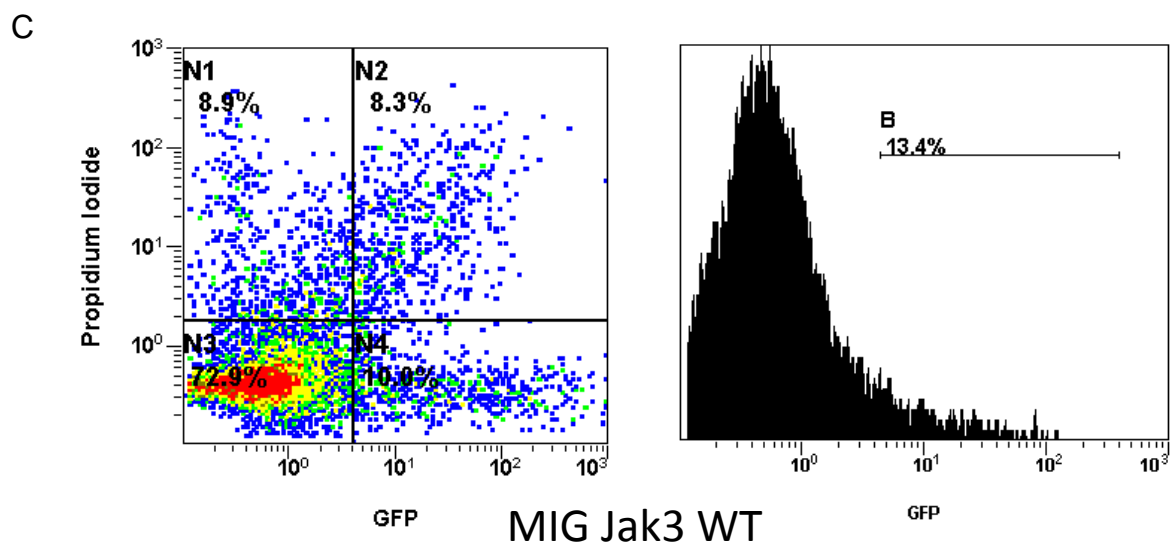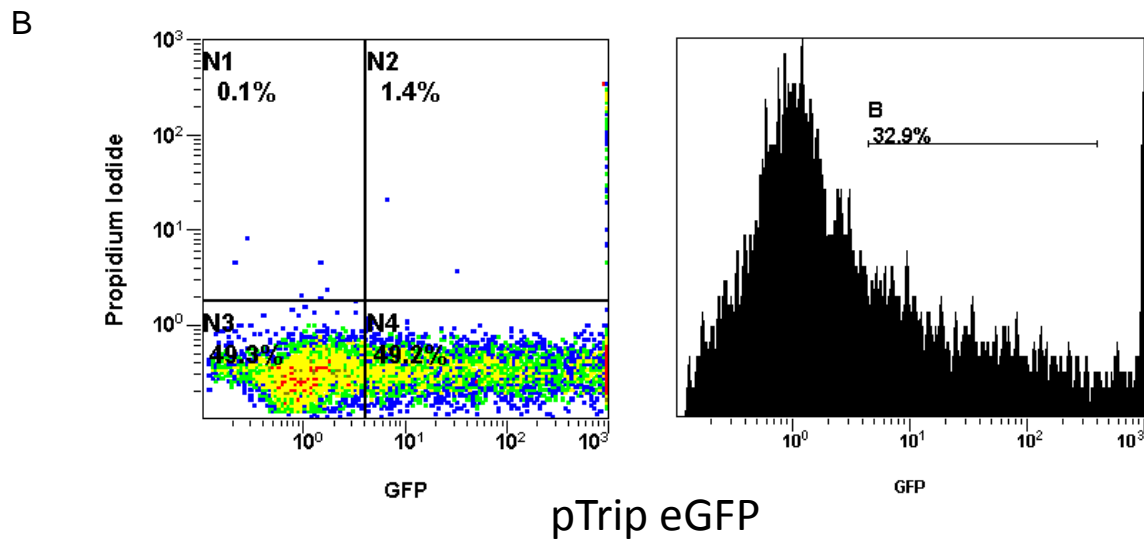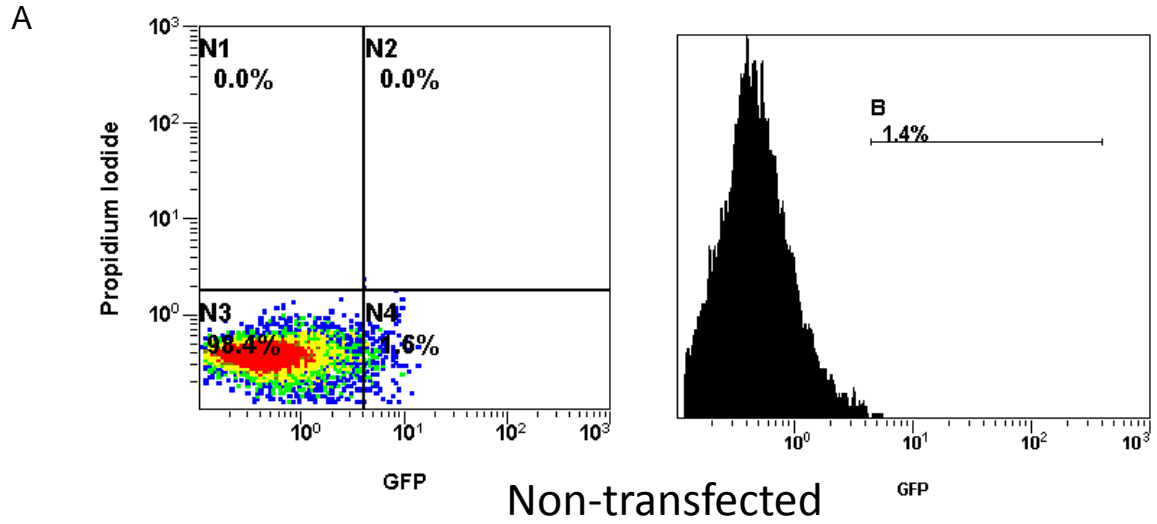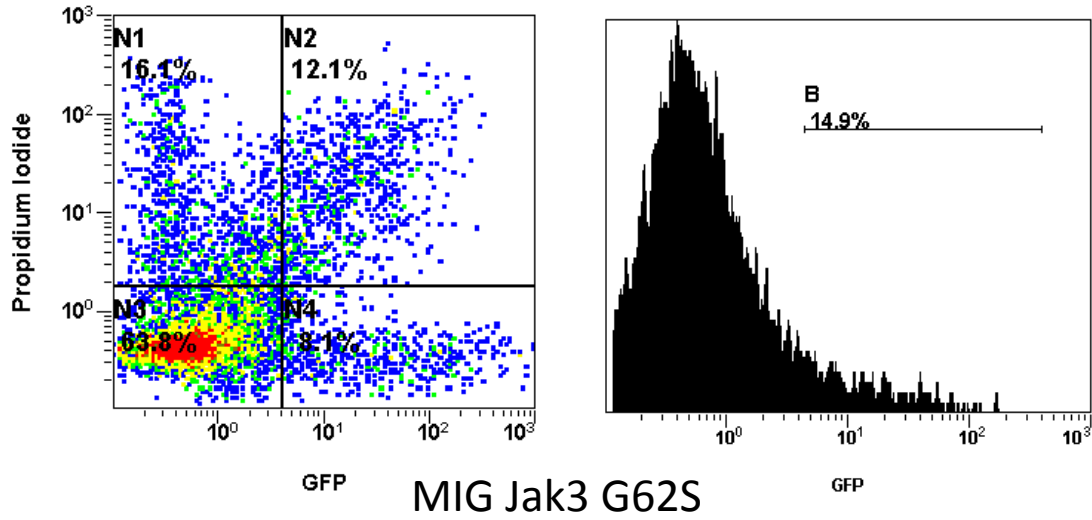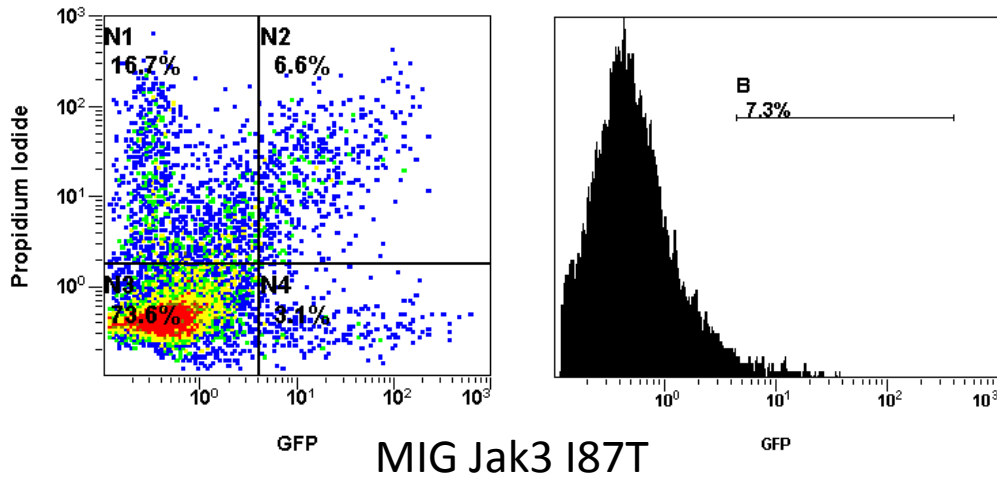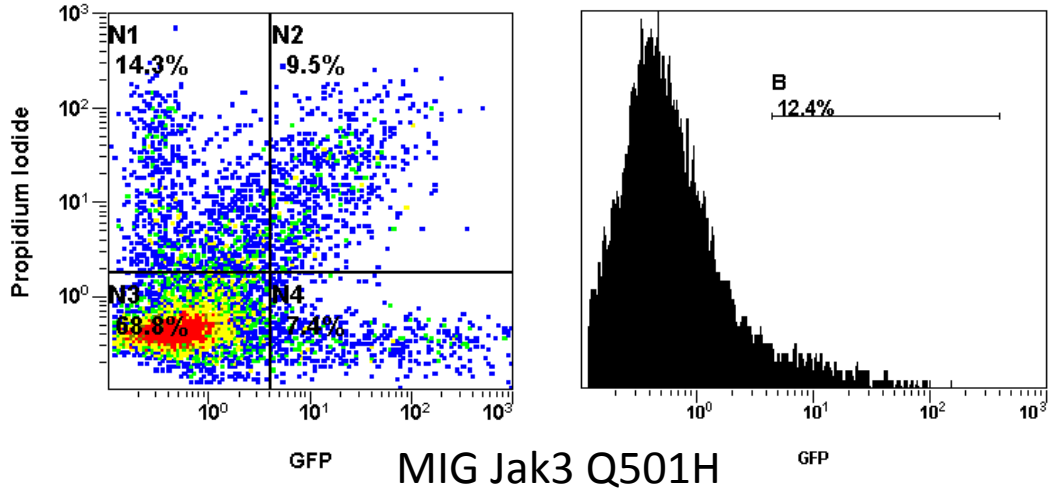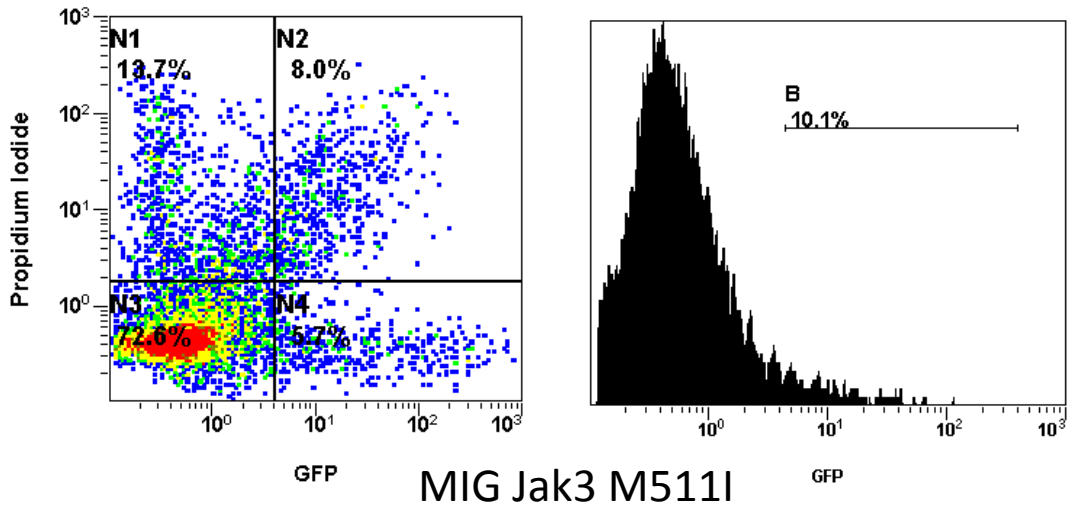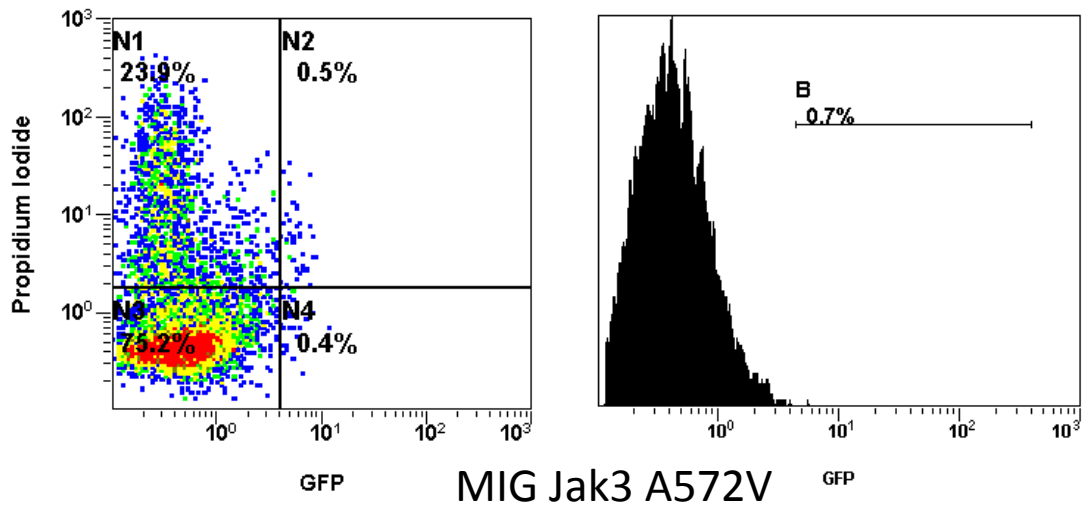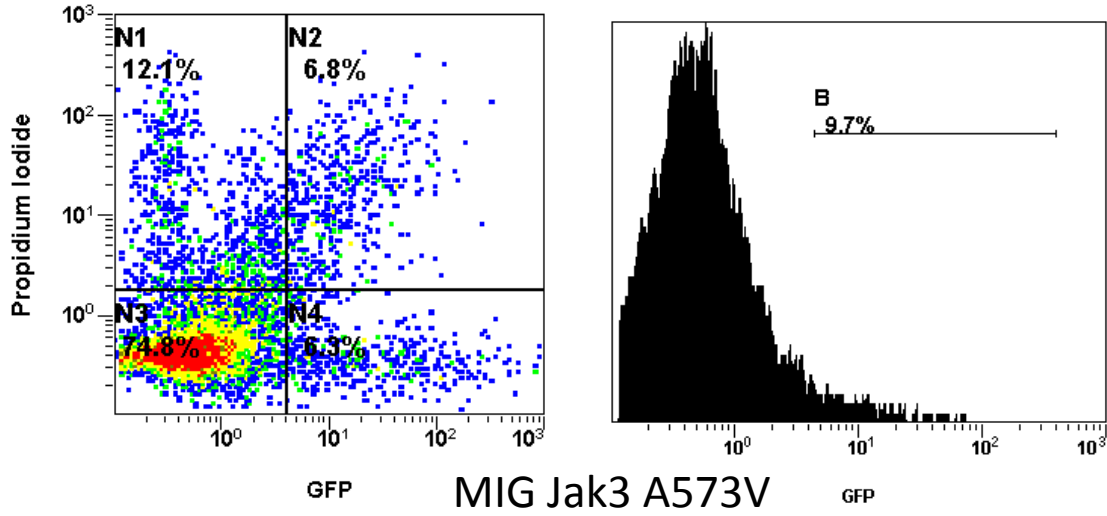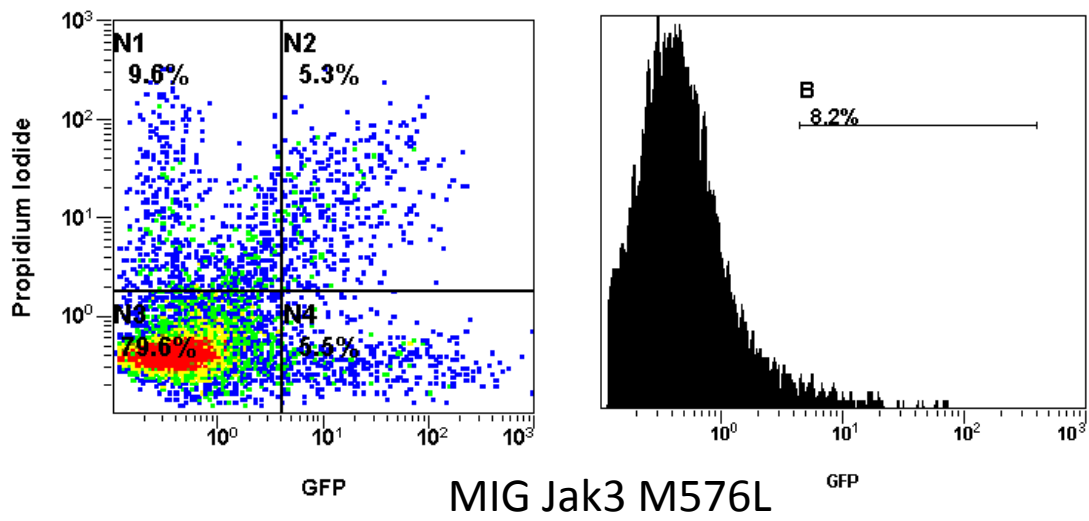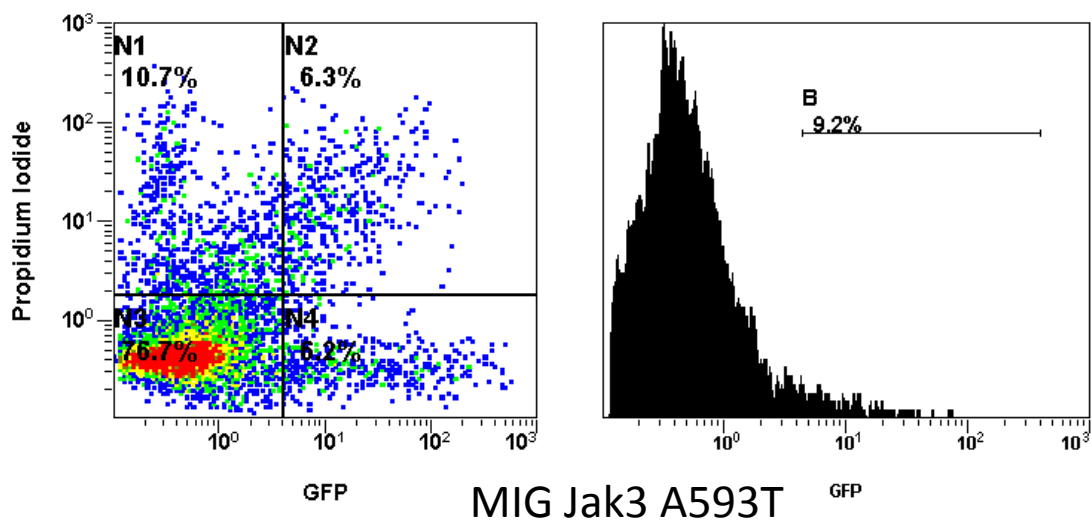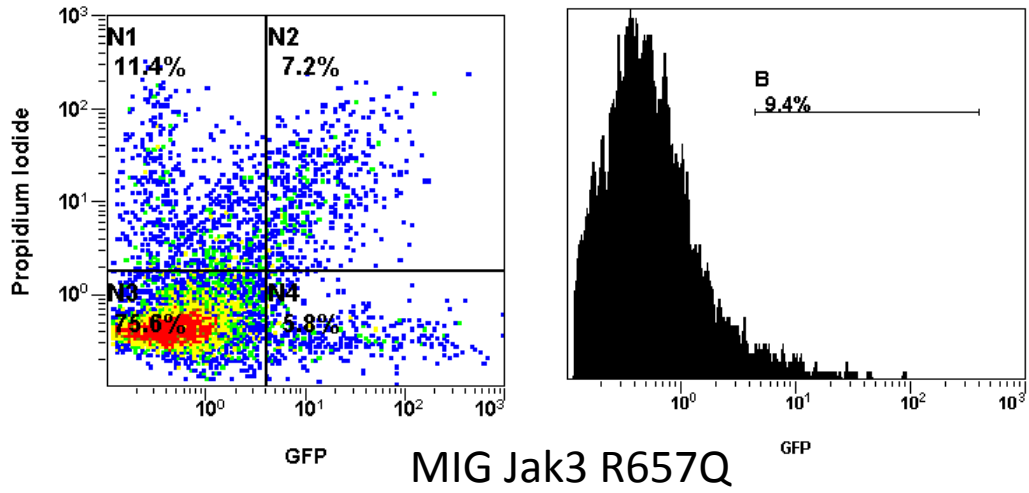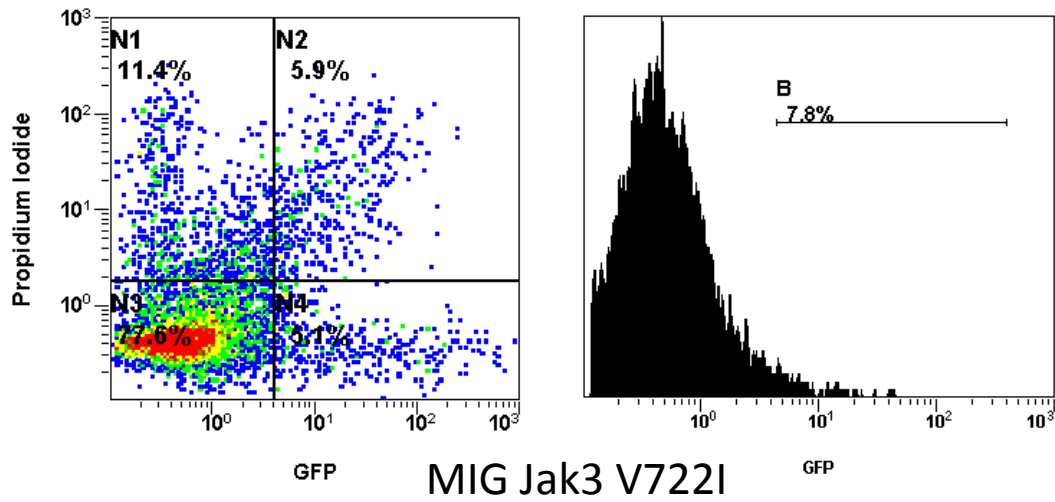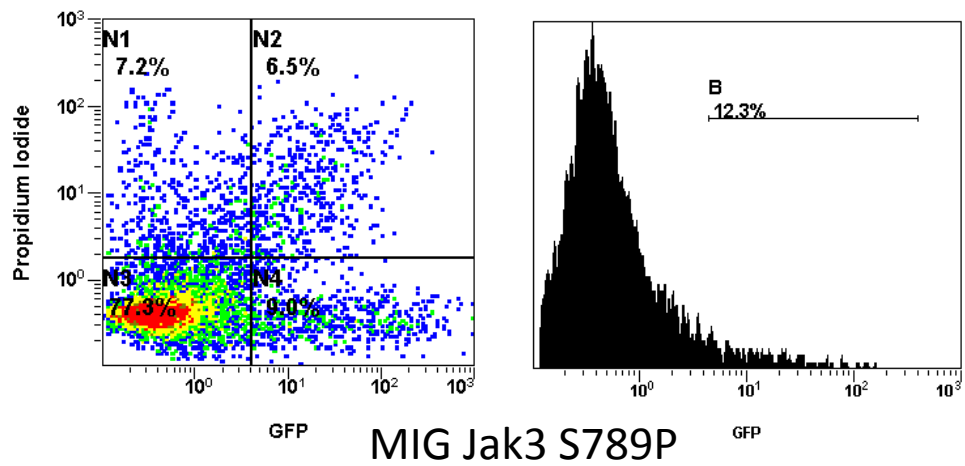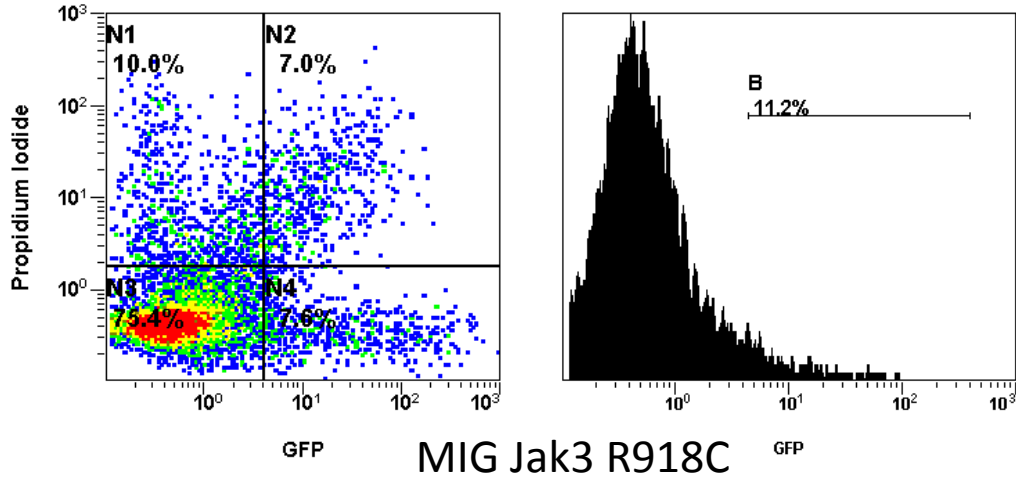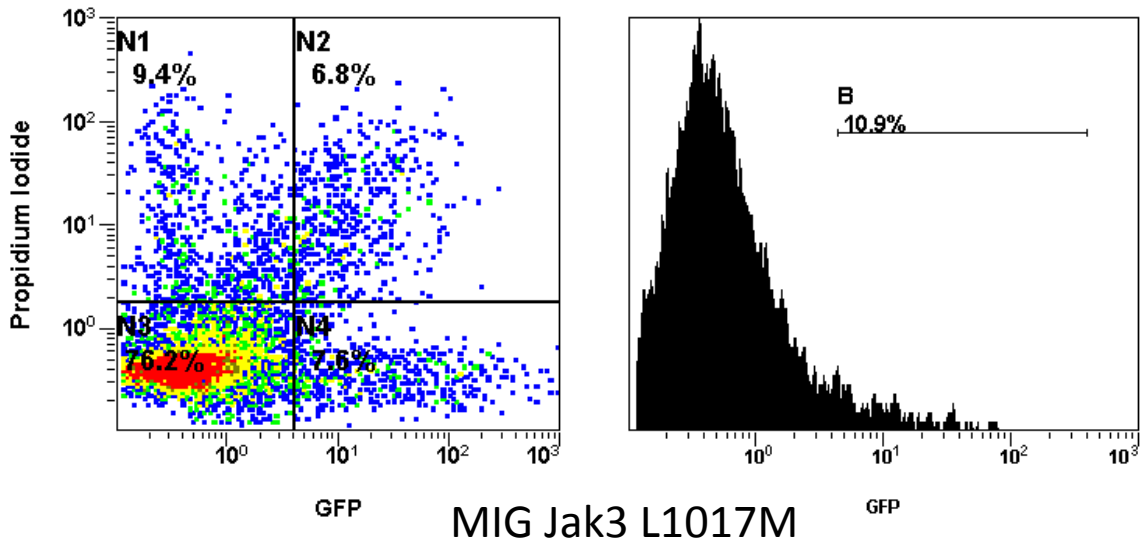
Figure 9. MIG Jak3 plasmid fidelity analyzed by FACS. Confirmation of GFP expression by the indicated MIG Jak3 mutants. Cells were analyzed by flow cytometry for GFP expression and cell viability by propidium iodide staining at 48 hours post-transfection. MIG Jak3 A572V (Panel I) failed to express GFP. A. Non-transfected. B. pTrip eGFP. C. MIG Jak3 WT. D. MIG Jak3 G62S. E. MIG Jak3 I87T. F. MIG Jak3 P132T. G. MIG Jak3 Q501H. H. MIG Jak3 M511I. I. MIG Jak3 A572V. J. MIG Jak3 A573V. K. MIG Jak3 M576L. L. MIG Jak3 A593T. M. MIG Jak3 R657Q. N. MIG Jak3 V722I. O. MIG Jak3 S789P. P. MIG Jak3 R918C. Q. MIG Jak3 L1017M.
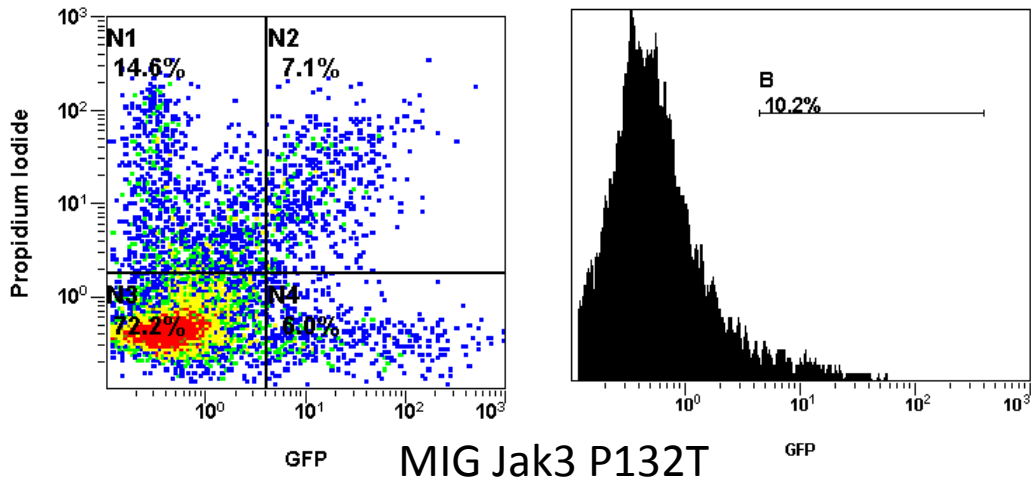
A

Non-transduced

MIG Jak3 WT
pcL Ampho

MIG Jak3 WT
pcL Ampho
pMD.G

B

Non-transduced

pTrip eGFP
50 µL

MIG Jak3 WT
100 µL

C

Non-transduced

pTrip eGFP
50 µL
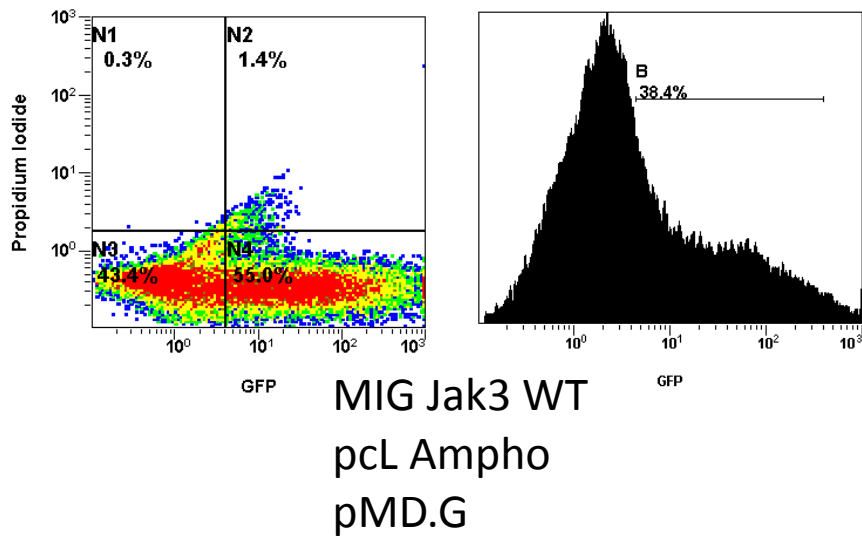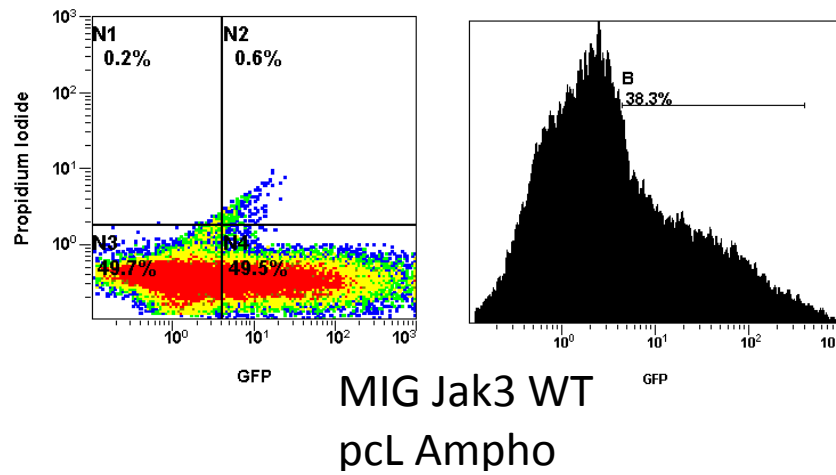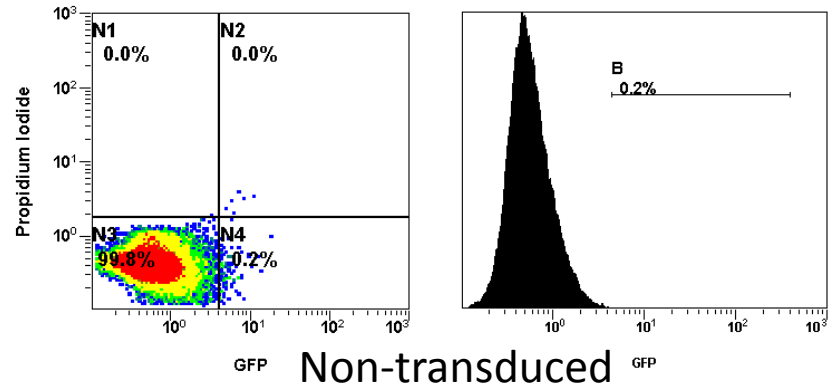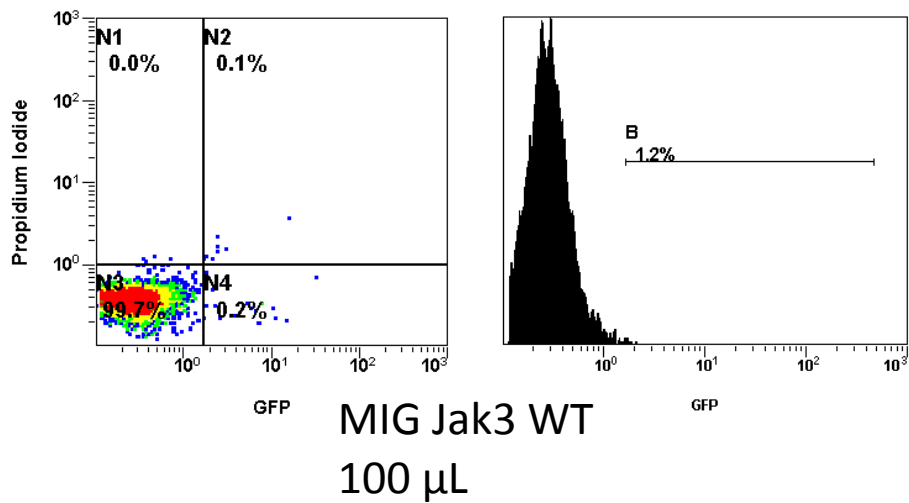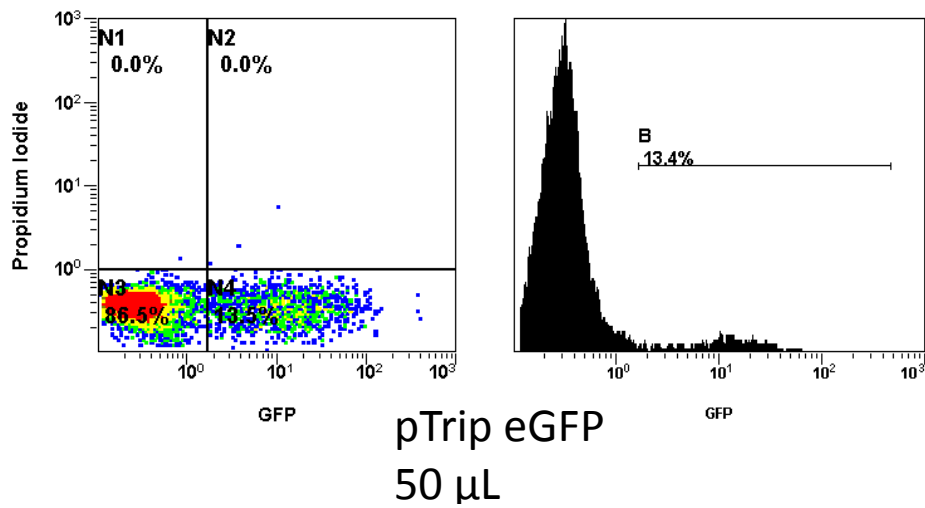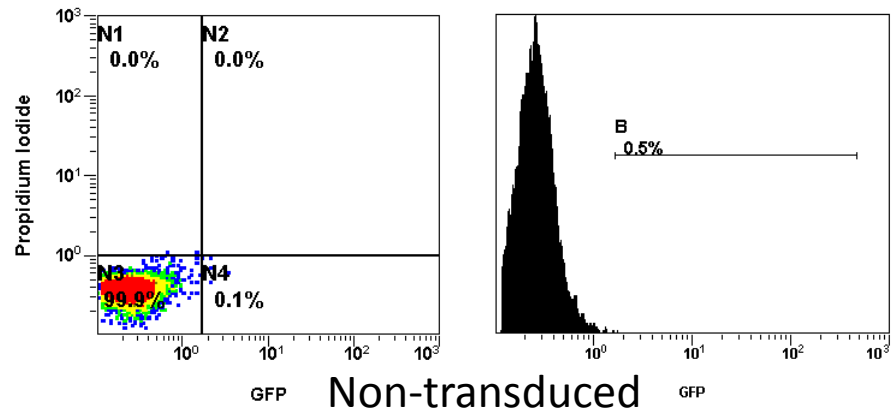
MIG Jak3 WT
100 µL

Figure 10. Optimization of MIG Jak3 transduction. At 24 hours post-transduction cells were harvested and analyzed by flow cytometry for GFP expression A. HEK293 cells transduced with MIG virus made with or without the pMD.G plasmid encoding VSV-G reveal enhanced transduction with VSV-G envelope protein. B. MIG Jak3 WT transduced Ba/F3 cells have low efficiency compared to cells transduced with pTrip eGFP virus. C. MIG Jak3 WT transduced Kit225 cells have low efficiency compared to cells transduced with pTrip eGFP virus.

2.3.4 Jak3 mutants M511I and A573V are able to generate stable transduced IL-3 independent Ba/F3 cells

To determine whether any of these Jak3 mutations have transforming potential. Ba/F3 and Kit225 cells were transduced with MIG Jak3 variants and 48 hours post-transduction underwent IL-3 starvation. At 48 hours post IL-3 starvation, the remaining viable cell population of transduced cells was analyzed by FACS with gating on the FS/SS histogram. MIG Jak3 transformed cells were evaluated for GFP expression, as an indicator of transduction efficiency, and for propidium iodide staining as an indicator of cellular viability 96 hours post-transduction and 48 hours post-IL-3 withdrawal in Ba/F3 cells (Figure 11A-K) or IL-2 withdrawal in Kit225 cells (Figure 12A-K). At 9 days post-cytokine withdrawal all transducted Kit225 cells had died and only the Ba/F3 MIG Jak3 M511I and A573V transduced cells were still living, though they had a low viability GFP expression was greater than 90% (Figure 12A-B). At 30 days of IL-3 withdrawal both MIG Jak3 M511I and A573V transduced Ba/F3 cells were almost 100 % GFP positive and were greater than 75 % viable (Figure 12C-D); This indicated that both cell lines had been successfully transformed into IL-3 independent cell growth. Ba/F3 transformed cells were then lysed and Western blotted for Jak3 to confirm protein expression (Figure 12E).

2.3.5 Jak3 Transformed Ba/F3 cell lines are sensitive to Jak3 inhibitors, NC1153 and CP-690,550

MTS assay was used to determine the sensitivity of Ba/F3 M511I and A573V cells to the following Jak3 selective inhibitors: NC1153, EP-009 and and the pan-Jak inhibitor CP-690,550 (Figure 14A). The Mannich base NC1153 and its derivative, EP-009, have been shown to be selective in targeting Jak3 resulting in reduced tyrosine kinase activity
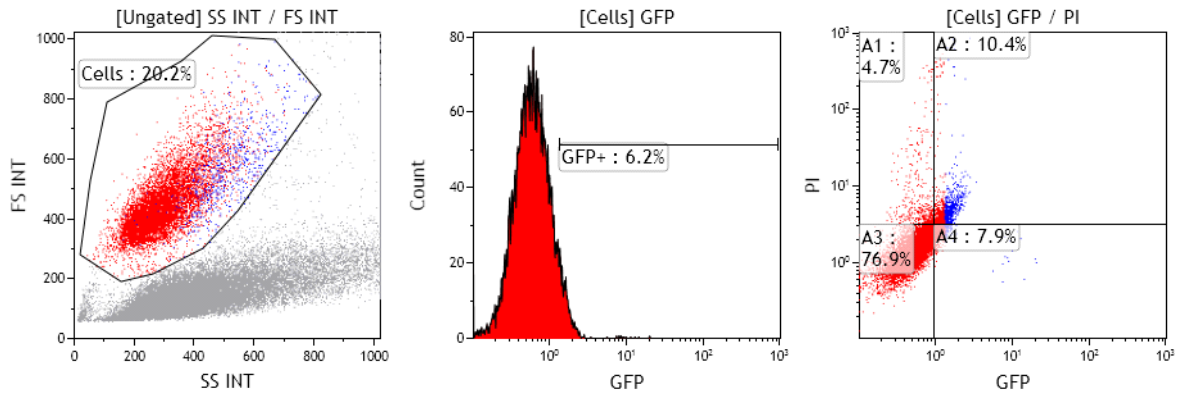
43

Non-transduced

MIG Jak3 I87T

MIG Jak3 P132T

D

[Ungated] SS INT / FS INT

Cells : 26.2%

[Cells] GFP

GFP+ : 2.3%

[Cells] GFP / PI

A1 : 4.6% A2 : 4.6%
A3 : 86.6% A4 : 4.1%

MIG Jak3 Q501H

E

[Ungated] SS INT / FS INT

Cells : 13.2%

[Cells] GFP

GFP+ : 6.3%

[Cells] GFP / PI

A1 : 14.9% A2 : 14.0%
A3 : 67.8% A4 : 3.3%

MIG Jak3 M511I

F

[Ungated] SS INT / FS INT

Cells : 14.7%

[Cells] GFP

GFP+ : 7.3%

[Cells] GFP / PI

A1 : 21.6% A2 : 18.9%
A3 : 57.8% A4 : 1.6%

MIG Jak3 A573V

45

G

MIG Jak3 A593T

H

MIG Jak3 V722I

I

MIG Jak3 S789P

J

[Ungated] SS INT / FS INT   [Cells] GFP   [Cells] GFP / PI

Cells : 19.2%   GFP+ : 10.8%   A1 : 7.8%   A2 : 9.5%

A3 : 74.4%   A4 : 8.3%

MIG Jak3 R918C

K

[Ungated] SS INT / FS INT   [Cells] GFP   [Cells] GFP / PI

Cells : 15.3%   GFP+ : 2.9%   A1 : 6.2%   A2 : 5.2%

A3 : 85.9%   A4 : 2.7%

MIG Jak3 L1017M

Figure 11. Generation of stable IL-3 dependent mutant Jak3 positive Ba/F3 cell line.

Ba/F3 cells were transduced with indicated MIG Jak3 viruses. At 2 days post-transduction cells were put on selection by washing the cells 3 times and resuspending in IL-3 free media. 2 days post-IL-3 withdrawal cells were analyzed by flow cytometry for GFP expression, gated on living cell population. A. Non-transfected. B. MIG Jak3 I87T. C. MIG Jak3 P132T. D. MIG Jak3 Q501H. E. MIG Jak3 M511I. F. MIG Jak3 A573V. G. MIG Jak3 A593T. H. MIG Jak3 V722I. I. MIG Jak3 S789P. J. MIG Jak3 R918C. K. MIG Jak3 L1017M.
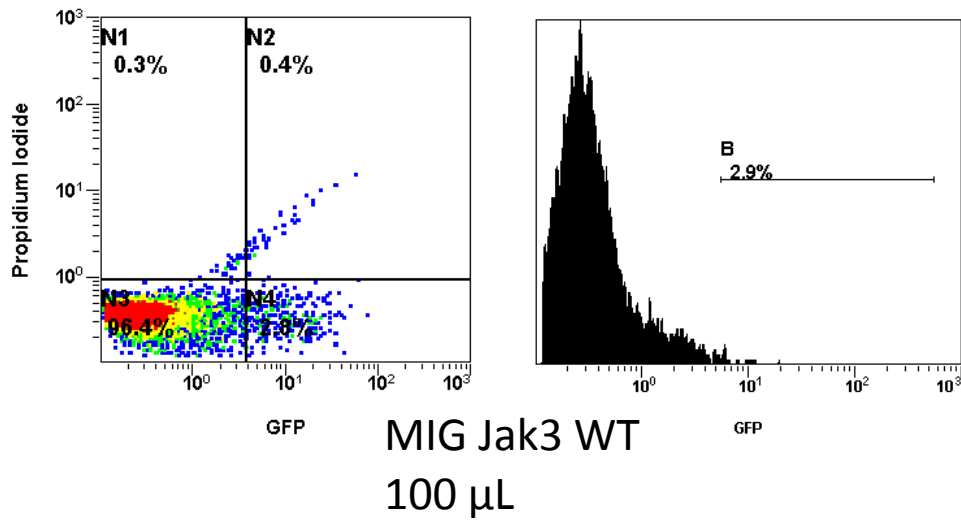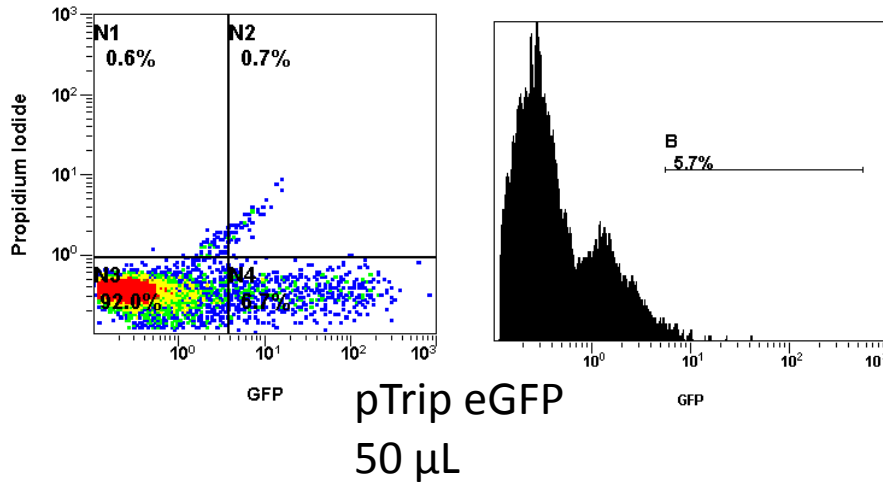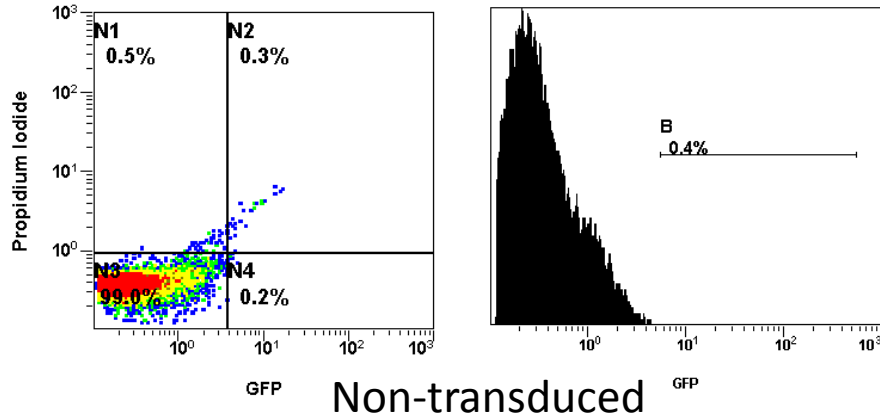
A — Non-transduced

B — MIG Jak3 I87T

C — MIG Jak3 P132T

D

MIG Jak3 Q501H

E

MIG Jak3 M511I

F

MIG Jak3 A573V

G

MIG Jak3 A593T

H

MIG Jak3 V722I

I

MIG Jak3 S789P

J

MIG Jak3 R918C

K

MIG Jak3 L1017M
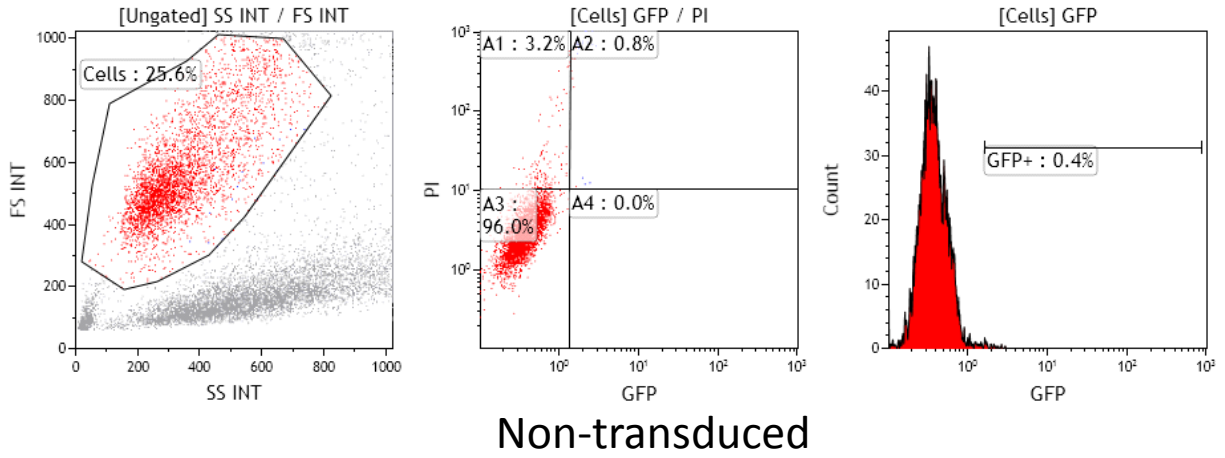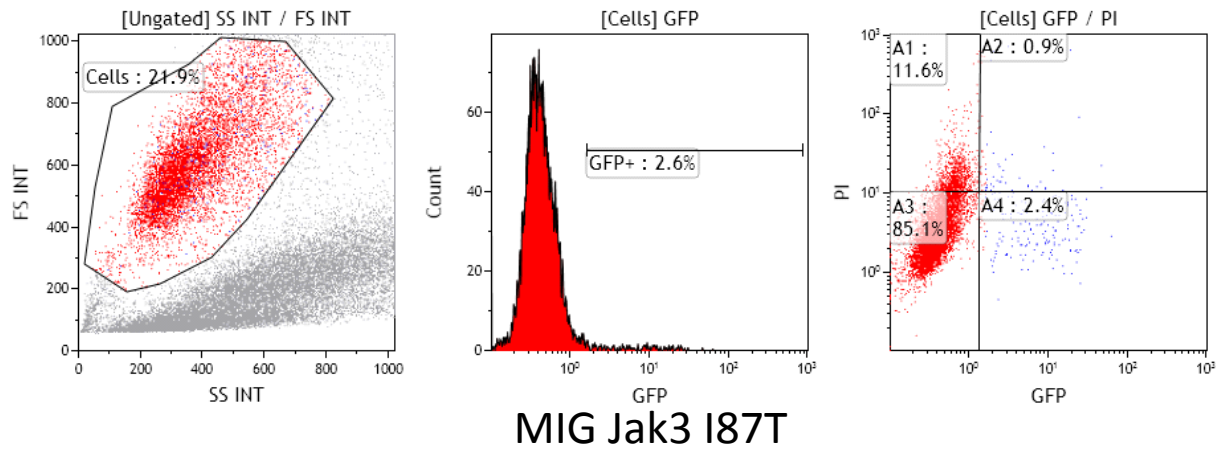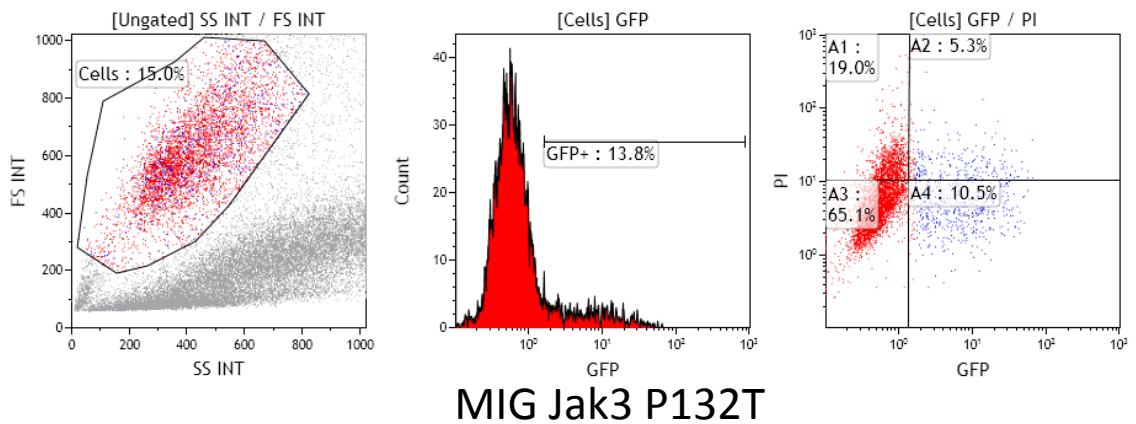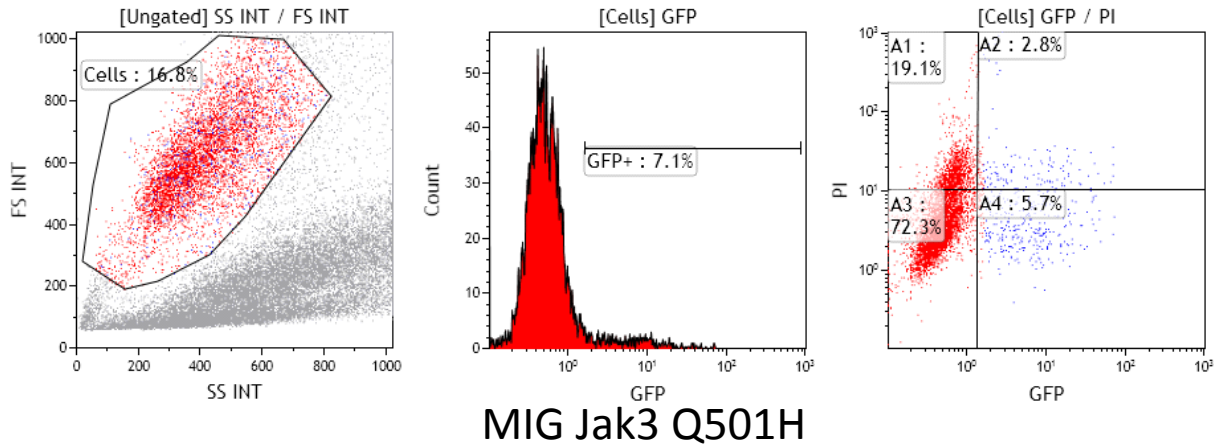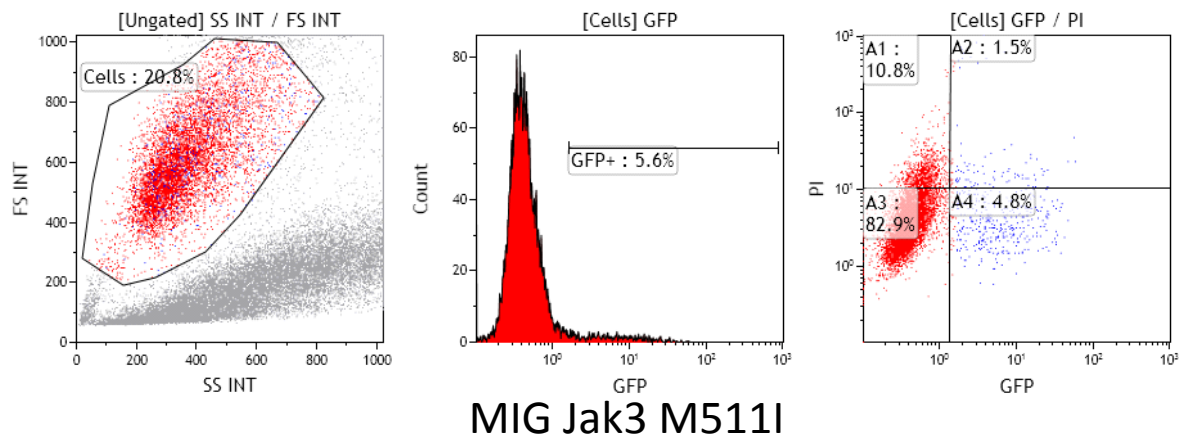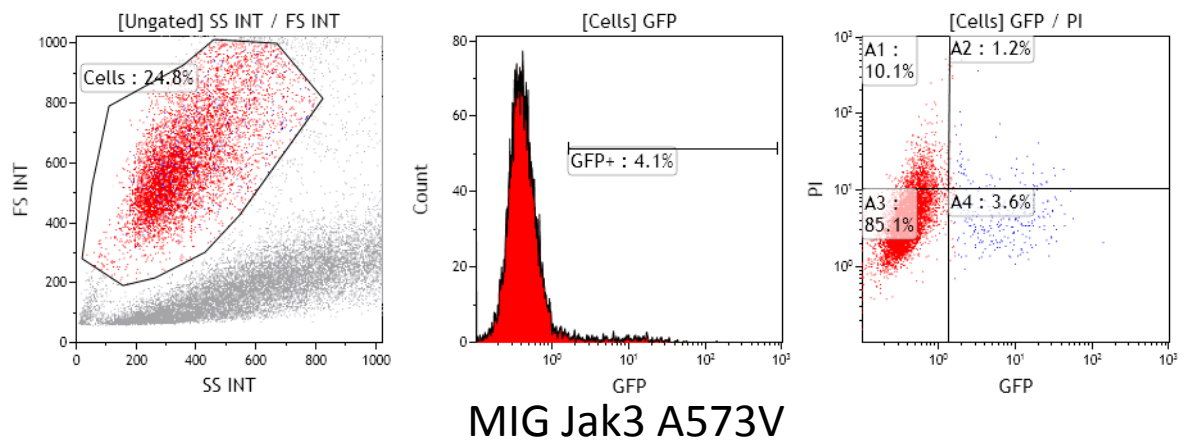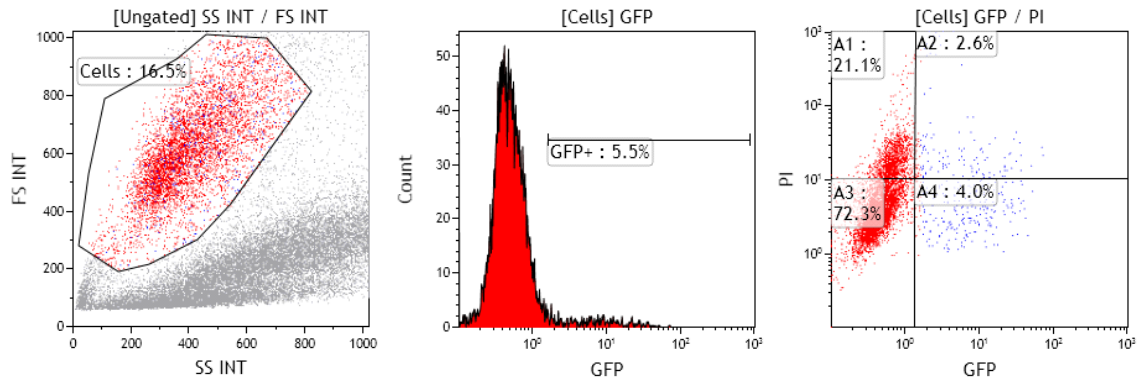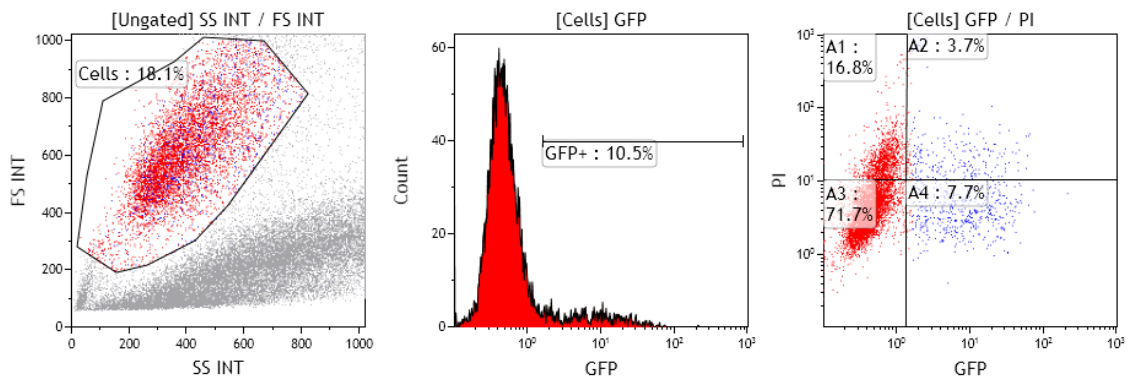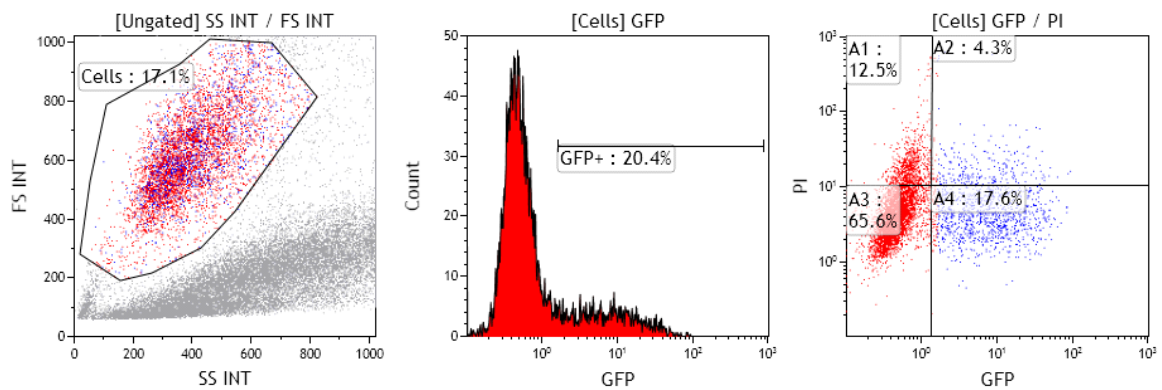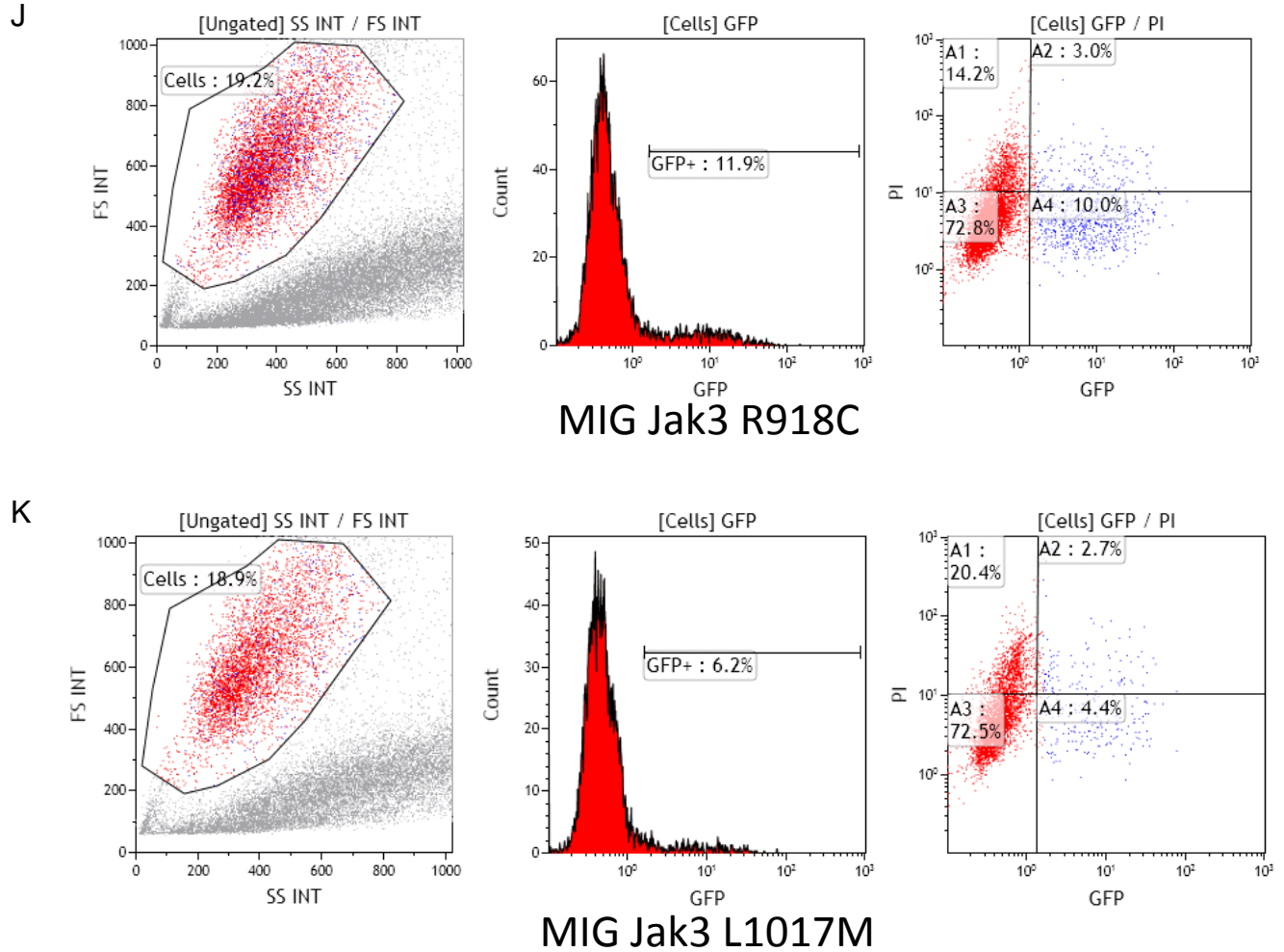
Figure 12. Generation of stable IL-3 dependent mutant Jak3 positive Ba/F3 cell line.

A-K. Ba/F3 cells were transduced with the indicated MIG Jak3 virus. At 11 days post-transduction cells were put on selection by washing the cells 3 times and resuspending in IL-3 free media. At 9 days post-IL-3 withdrawal cells were analyzed by flow cytometry for GFP expression, selecting for remaining viable cell population. A. Non-transfected. B. MIG Jak3 I87T. C. MIG Jak3 P132T. D. MIG Jak3 Q501H. E. MIG Jak3 M511I. F. MIG Jak3 A573V. G. MIG Jak3 A593T. H. MIG Jak3 V722I. I. MIG Jak3 S789P. J. MIG Jak3 R918C. K. MIG Jak3 L1017M.
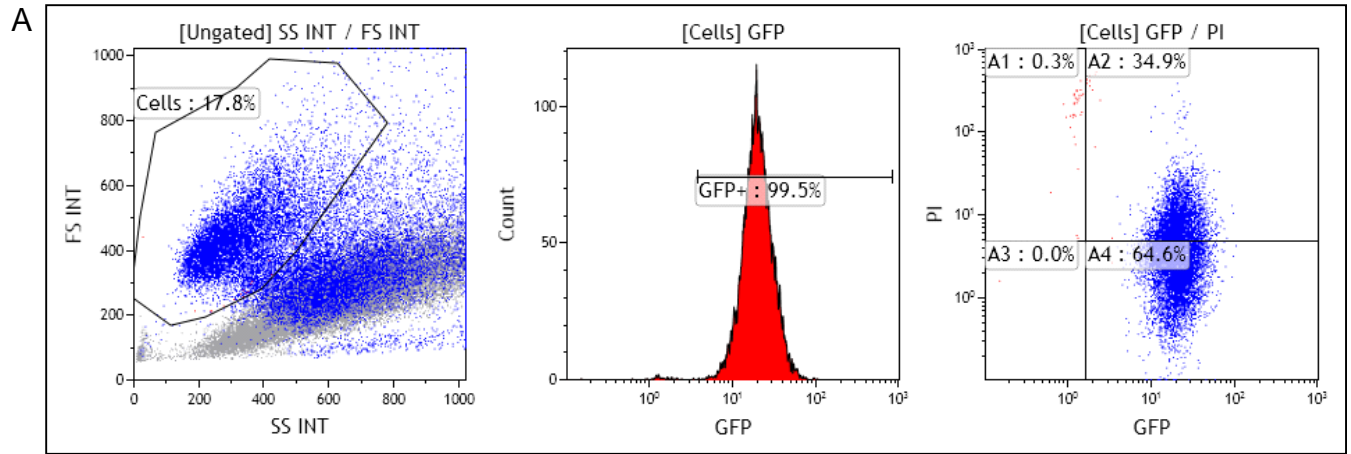
MIG Jak3 M511I
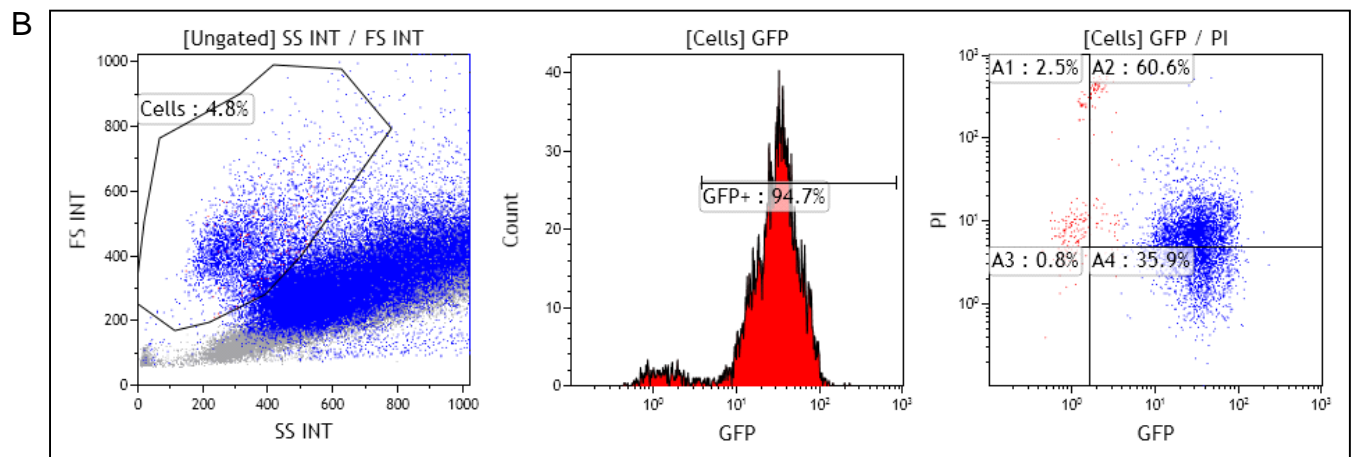
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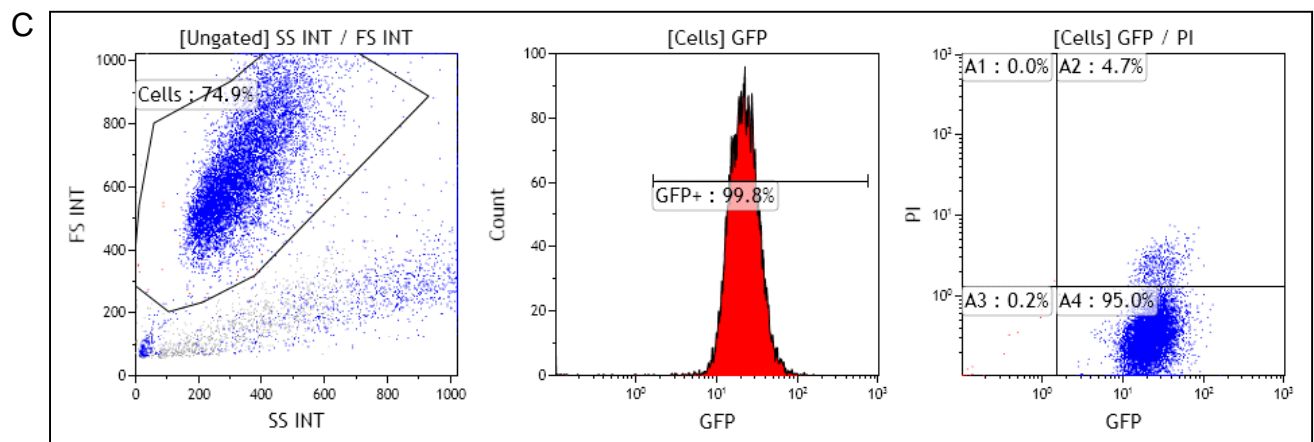
MIG Jak3 M511I
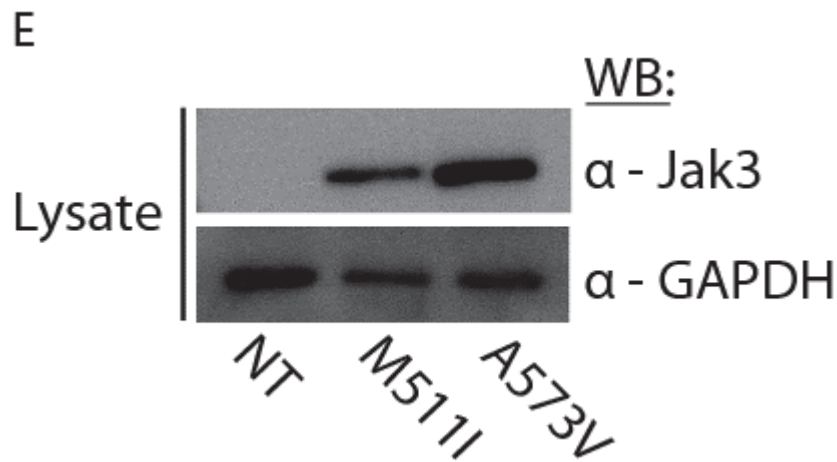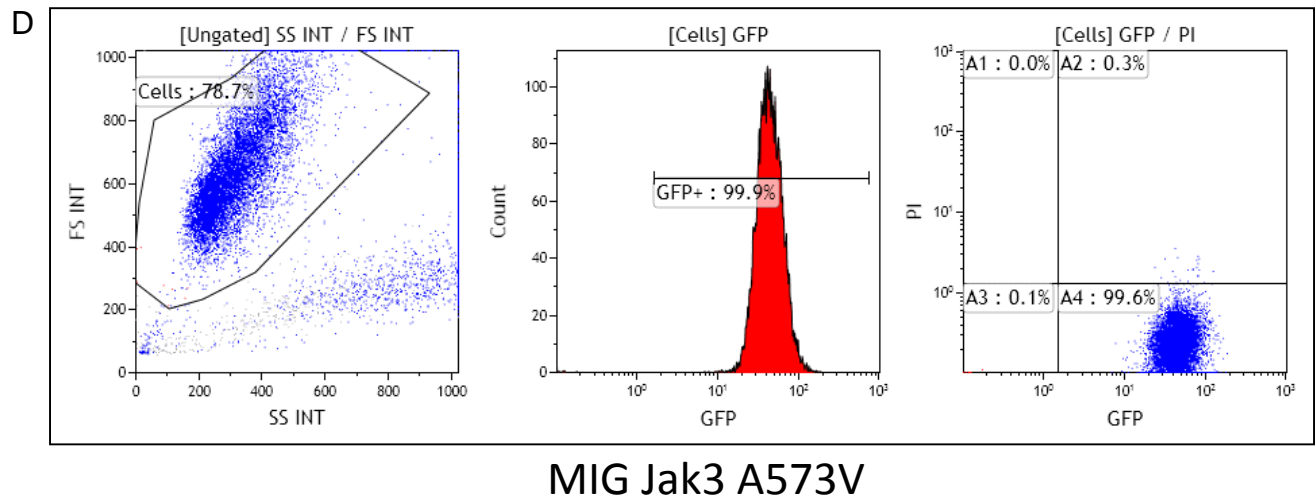
MIG Jak3 A573V

Figure 13. Generation of stable IL-3 independent Jak3 positive, M511I and A573V, Ba/F3 cell lines. Ba/F3 cells were transduced with either MIG Jak3 M511I (A) or MIG Jak3 A573V (B). At 9 days post-IL-3 withdrawal cells were analyzed by flow cytometry for GFP expression. At 30 days post-IL-3 withdrawal MIG Jak3 M511I (C) or A573V (D) transduced cells were analyzed by flow cytometry for GFP expression D. E. Transduced Ba/F3 cells were lysed and Western blot analysis was performed for Jak3 and GAPDH.

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A
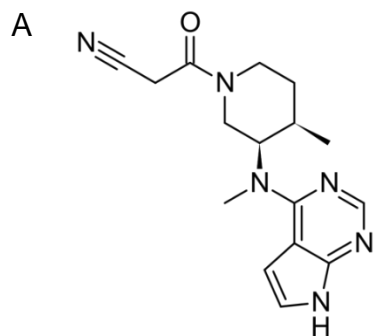
CP-690,550
$C_{16}H_{20}N_6O$
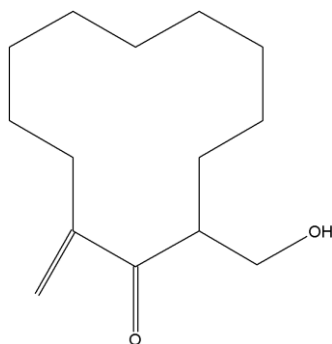
EP009
$C_{14}H_{24}O$

NC1153
$C_{18}H_{36}N_2O$

54

B
Jak3 M511I

CP-690,550

EP009

NC1153

C
Jak3 A573V
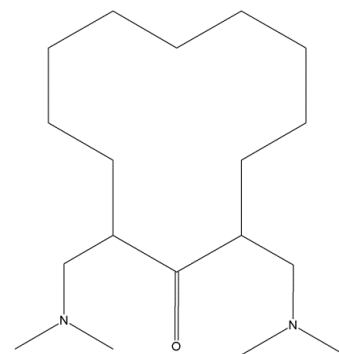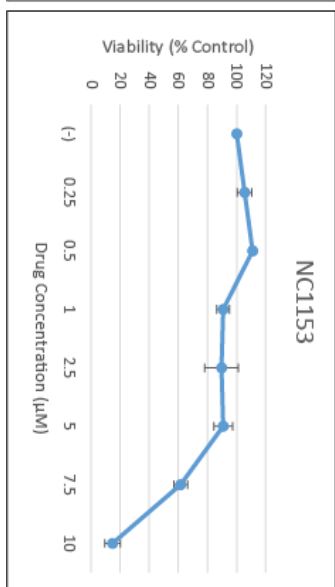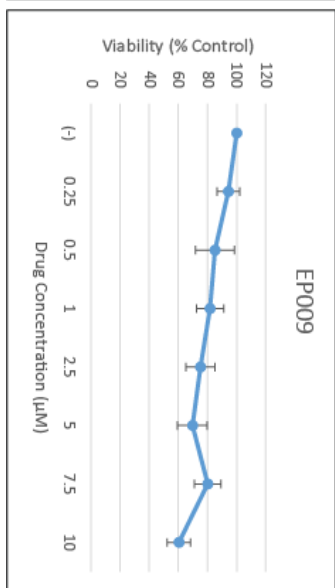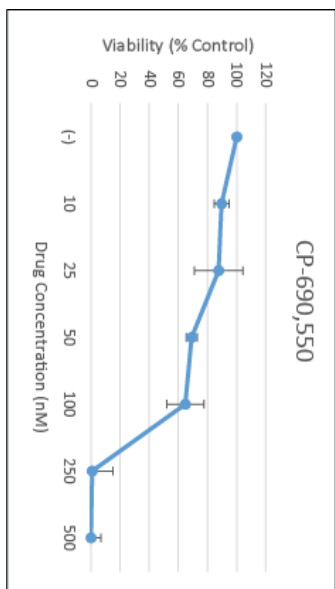
CP-690,550
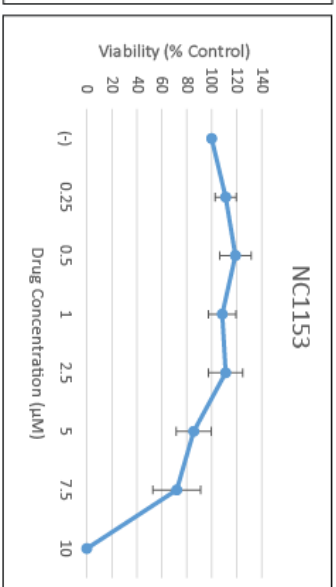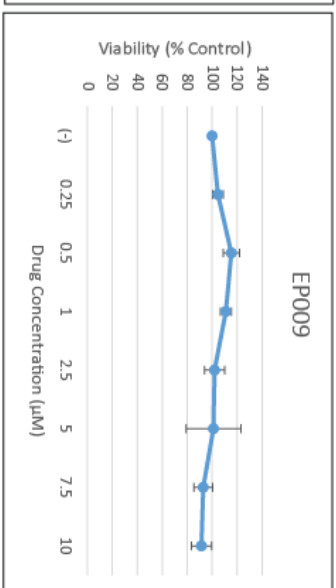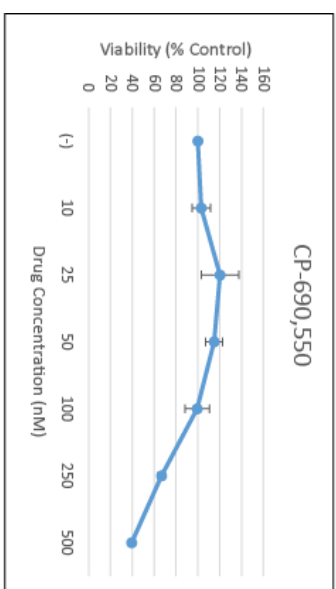
EP009

NC1153

Figure 14. Cytotoxicity of Jak3 inhibitors on M511I and A573V Jak3 transformed Ba/F3 cells. A. Schematic representation of inhibitors CP-690,550, EP-009 and NC1153. B. MIG Jak3 M511I transformed Ba/F3 cells were cultured for 24 h at 37 °C with increasing amounts of either NC1153 (0-10µM), EP-009 (0-10µM) or CP-690,550 (0-500 nM) and cell viability measured by MTS tetrazolium salt assay. Values represent mean absorbance (OD490-OD650 nm) normalized to vehicle (PBS or DMSO) treated control cells. Error bars represent standard deviation of n=3. C. MIG Jak3 A573V transformed Ba/F3 cells were cultured as described above.

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and induction of apoptosis in Jak3 expressing lymphocytes [91, 92, 96]. Similarly, CP-690,550 has been shown to be an effective driver of apoptosis in a Jak3 transformed Ba/F3 model but also has inhibitory effects on Jak1 and Jak2 [97]. Ba/F3 cells transformed with M511I and A573V showed a dose dependent sensitivity to NC1153 and CP-690,550 while neither Jak3 transforming mutant responded to EP-009 treatment (Figure 14B-C).

2.4   Discussion

The 14 Jak3 mutations showed a broad range of autophosphorylation and Stat5 activation profiles which did not provide strong evidence of the mechanisms that led to an oncogenic phenotype. These mutations may act to protect against proteosomal degradation of the protein or disable regulatory mechanisms such as phosphatases to escape the wild-type phenotype.

Our studies involved the creation of a virus that could stably infect Ba/F3 cells in order to determine the transforming ability of fourteen Jak3 mutations identified in leukemia patients [72, 87-89]. In contrast to what others have shown regarding the expression of this group of fourteen mutants, in our hands, only the M511I and A573V mutations were successful at transforming Ba/F3 cells using the MIG vector. However, these transformations were only a transient effect associated with downregulation of Jak3 expression through promotor silencing.

This work, in combination with the work of others' [72, 87, 88], describes the transforming potential of known Jak3 somatic mutations in driving leukemia, including ALL. Here we have shown that the previously identified Jak3 mutations, M511I and A573V, are transforming and sensitive to Jak3 kinase inhibitors. Importantly, the FDA

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approved pan-Jak inhibitor CP-690,550 is approved for the treatment of auto-immune disorders such as rheumatoid arthritis and is undergoing clinical trials for psoriasis and ankylosing spondylitis [98, 99]. CP-690,550 can target all Jaks making it less than ideal in the treatment of disease due to the potential for off-target effects [100]. Therefore, the Jak3 specific inhibitor NC1153 and its derivative EP-009 were chosen for their specificity to Jak3 to limit off-target effects. EP-009 is currently in the pre-clinical stages of development and has shown promise in targeting Jak3-driven tumor T-cells both *in vitro* and *in vivo* [92]. The M511I mutation is located in the JH3 domain, a linker between the SH2 domain and the pseudokinase JH2 domain and shows limited sensitivity to EP-009 (Figure 14B) while the A573V mutation, which is located in the JH2 domain, has almost no sensitivity to EP-009 (Figure 14C). NC1153 on the other hand is effective against both mutations (Figure 14B and C). Together this data implies that the subtle differences between the two inhibitors affect their interactions with Jak3, possibly through the JH2 domain.

This data may be used in a clinical setting when choosing an inhibitor for use against certain hematological cancers. This data specifically points to CP-690,550 as a treatment for Jak3 driven leukemias harboring the M511I and A573V mutations, though it also points to a need for more specific inhibitors such as NC1153 which are also effective and have reduced off-target effects.

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Chapter 3

Characterization of Jak3 Y841 Phosphorylation

3.1 Introduction

Previously, five Jak3 tyrosine phosphorylation sites were mapped in human Jak3 as follows: Y785, Y904, Y939, Y980 and Y981. However, preliminary data generated in our lab identified 11 possible novel tyrosine phosphorylation sites from Jak3 transfected HEK293 cells. For these assays cells were immunoprecipitated for Jak3, subjected to kinase assays coupled to liquid chromatography-tandem mass spectrometry (15A-K). The 11 sites identified are Y105, Y190, Y238, Y399, Y633, Y637, Y738, Y762, Y824, Y841 and Y929 (Figure 16A). Upon identification, phospho-specific antibodies were generated against 7 of these sites, Y105, Y633, Y637, Y738, Y762, Y841 and Y929 using either immunized rabbits or murine hybridoma cell lines (Figure 16B).

We next sought to characterize the phospho-specific antibodies and to determine the functional role of the newly identified sites. Characterization of the antibodies was performed using dot blot and Western blot analysis to determine whether the phospho-specific antibodies could recognize phosphorylated Jak3. For these assays Jak3 transfected HEK293 cells, natural killer like cell line YT, or the IL-2 dependent Kit225 T-cell cell line were employed. To determine the functional role of Jak3 phospho-sites, phospho-deletion tyrosine to phenylalanine (Y-F) mutations were used in transfected Hek293 cells to assess whether total Jak3 tyrosine phosphorylation or Stat5B activation was affected.

60

A pY105

b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11} b_{12}

R / F / Y* / F / P / N / W / F / G / L / E / K / C

y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

3262

Intensity

y_8

y_3
b_3*

y_9

b_4*

b_9*

y_5

b_5* b_6*

b_7* b_8*

y_2 y_4 y_6 y_7

$b_{10}*$ $b_{11}*$

0

0   250   500   750   1000   1250   1500

m/z

B pY190

b_1 b_2 b_3 b_4 b_5 b_6

K / T / V / S / Y* / K / A

y_6 y_5 y_4 y_3 y_2 y_1

1341

Intensity

y_3*

y_2

b_3

b_5* y_4*

0

200   300   400   500   600

m/z

61

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C

pY238

K / Y* / I / M / D / L / E / R / L

b₁ b₂ b₃ b₄ b₅ b₆ b₇ b₈

b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8
y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

D

pY399

K / T / G / G / S / R / P / G / S / Y* / V / L / R / R

b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11} b_{12} b_{13}
y_{13} y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

E

pY633

K Q L A Y* A L N Y L E D K G

b₁ b₂ b₃ b₄ b₅ b₆ b₇ b₈ b₉ b₁₀ b₁₁ b₁₂ b₁₃

y₁₃ y₁₂ y₁₁ y₁₀ y₉ y₈ y₇ y₆ y₅ y₄ y₃ y₂ y₁

F

pY637

K Q L A Y A L N Y* L E D K G

b₁ b₂ b₃ b₄ b₅ b₆ b₇ b₈ b₉ b₁₀ b₁₁ b₁₂ b₁₃

y₁₃ y₁₂ y₁₁ y₁₀ y₉ y₈ y₇ y₆ y₅ y₄ y₃ y₂ y₁

G

pY738

b₁ b₂ b₃ b₄ b₅ b₆ b₇ b₈

K / L / Q / F / Y* / E / D / R / Q

y₈ y₇ y₆ y₅ y₄ y₃ y₂ y₁

49685

Intensity

b_3
y_2
b_4 y_3
b_5*
y_4
b_6*
y_5*
b_7*

0   100   200   300   400   500   600   700   800   900   1000

m/z

H

pY762

b₁ b₂ b₃ b₄ b₅ b₆ b₇ b₈ b₉ b₁₀ b₁₁ b₁₂ b₁₃ b₁₄ b₁₅ b₁₆ b₁₇ b₁₈ b₁₉ b₂₀ b₂₁ b₂₂ b₂₃ b₂₄

K / W / T / E / L / A / L / L / I / Q / Q / C / M / A / Y* / E / P / V / Q / R / P / S / F / R / A

y₂₄ y₂₃ y₂₂ y₂₁ y₂₀ y₁₉ y₁₈ y₁₇ y₁₆ y₁₅ y₁₄ y₁₃ y₁₂ y₁₁ y₁₀ y₉ y₈ y₇ y₆ y₅ y₄ y₃ y₂ y₁

5081

Intensity

$y_{17}*$ $y_{19}*$
$y_{16}*$ $y_{18}*$
$y_{15}*$
$y_{13}*$ $y_{14}*$
b_9
y_8 y_9
$y_{11}*$
b_5
b_7
y_{10} $y_{12}*$
b_8
y_6
y_5 b_4
b_6
b_{10}
$y_{20}*$ $y_{21}*$
$y_{22}*$
b_{12}
b_{13}
b_{14}
$b_{15}*$ $b_{16}*$

0   250   500   750   1000   1250   1500   1750

m/z

64

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I

pY824

$$\begin{array}{c} & b_1 & b_2 & b_3 & b_4 & b_5 & b_6 & b_7 & b_8 \\ K/Y^*/I/S/Q/L/G/K/G \\ & y_8 & y_7 & y_6 & y_5 & y_4 & y_3 & y_2 & y_1 \end{array}$$

J

pY841

$$\begin{array}{c} & b_1 & b_2 & b_3 & b_4 & b_5 & b_6 & b_7 & b_8 & b_9 & b_{10} & b_{11} & b_{12} & b_{13} & b_{14} & b_{15} & b_{16} \\ R/Y^*/D/P/L/G/D/N/T/G/A/L/V/A/V/K/Q \\ & y_{16} & y_{15} & y_{14} & y_{13} & y_{12} & y_{11} & y_{10} & y_9 & y_8 & y_7 & y_6 & y_5 & y_4 & y_3 & y_2 & y_1 \end{array}$$

65

K

pY929

b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11}

R / L / L / L / Y* / S / S / Q / I / C / K / G

y_{11} y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

Figure 15. Mass spectra of newly identified tyrosine phosphorylation sites. Mass spectra are shown for novel tyrosine phosphorylation sites of Jak3 revealed by mass spectrometry analysis from Jak3 autokinase assays. A. Mass spectrum of pY105. B. Mass spectrum of pY190. C. Mass spectrum of pY238. D. Mass spectrum of pY399. E. Mass spectrum of pY633. F. Mass spectrum of pY637. G. Mass spectrum of pY738. H. Mass spectrum of pY762. I. Mass spectrum of pY824. J. Mass spectrum of pY841. K. Mass spectrum of pY929.

66
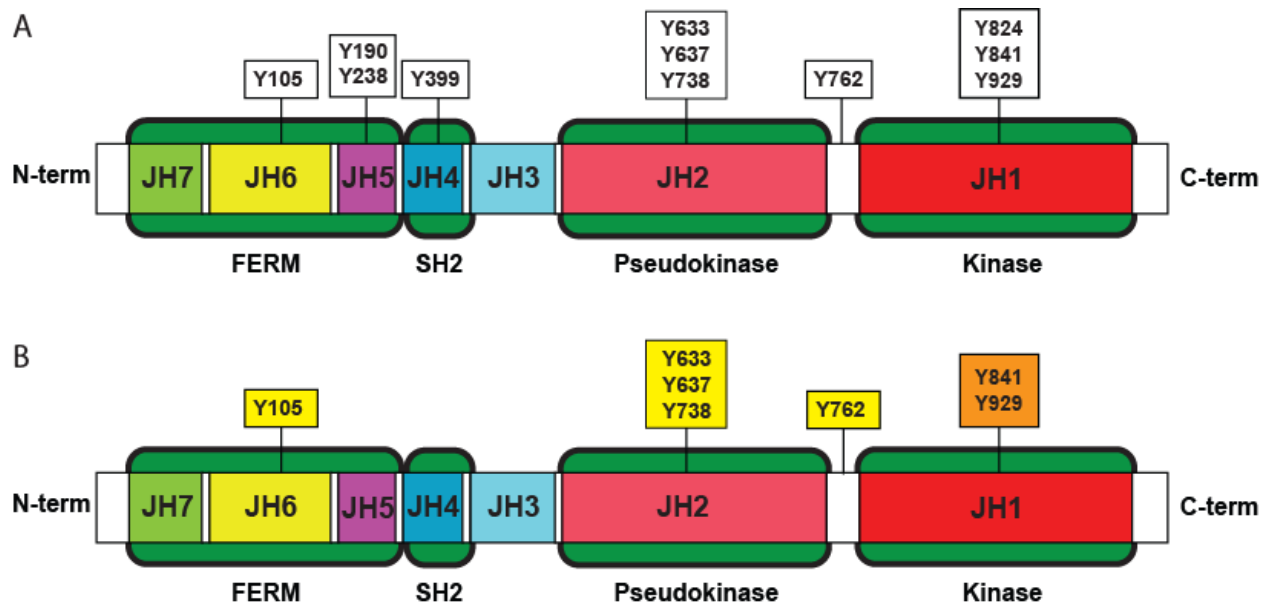
Figure 16. Schematic of novel tyrosine phospho-site positions. A. The 11 novel Jak3 tyrosine phosphorylation sites and the domains in which they can be found. B. Yellow denotes antibody generated using immunized rabbit, while orange denotes antibodies generated using a murine hybridoma cell line.

3.2 Materials and Methods

3.2.1 Cell Culture and Treatments:

The human NK-like cell line YT, T-cell cell line Kit225 and the adherent HEK293 cell line were maintained in RPMI 1640 (Thermo Scientific Inc.) medium with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Cellgro), and penicillin-streptomycin (50 IU/ml and 50 µg/ml, respectively) (Cellgro) (complete media) at 37 °C with 5% CO2. KIT225 cells were supplemented with 100 IU/ml human recombinant IL2 (NCI Preclinical Repository). Kit225 and YT cells were made quiescent by growing to exhaustion (> 5X105 cells/mL) and then stimulated with 10,000 IU of human recombinant IL2 (NCI Preclinical Repository) for the indicated times. Treatments were performed at 37 °C using 10^8 cells per treatment. Unstimulated cells were used as control. For pervanadate treatments, 100 X pervanadate was prepared as previously described for a final concentration of 2.5mM [101], and treatments were incubated with $1x10^8$ cells for 30 minutes at 37 °C.

3.2.2 Transfections:

HEK293 cells were transfected with 10 µg of pcDNA3.1/human Jak3 plasmid (Invitrogen) alone or with 10 µg Stat5B plasmid (OriGene) per confluent 10 cm dish. Cells were harvested 48 h post transfection. Transient transfections of HEK293 cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

3.2.3 Solubilization of Proteins, Immunoprecipitation and Western Blot

Cells were pelleted and solubilized in lysis buffer (10 mM Tris-HCl (pH 7.6), 5 mM EDTA (pH 8.0), 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4 , 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml aprotinin, 2 µg/ml

68

leupeptin, and 1 µg/ml pepstatin A, and rotating at 4 °C for 1 h. Whole cell lysates were clarified by centrifugation (15,000 x g, 15 min, 4 °C). For immunoprecipitation reactions, supernatants were rotated with 3 µL of α-Jak3 rabbit polyclonal antibody for 2 hours at 4 °C. Immune complexes were captured by incubation with Protein A-Sepharose beads (Rockland Immunochemicals) rotating for 1 h at 4 °C. The beads where then washed three times with ice cold lysis buffer and eluted by boiling 5 min in 2 x SDS sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.02% bromophenol blue, 10% glycerol, pH 6.8). Samples were resolved in 7.5% SDS-PAGE and transferred to polyvinyl-difluoride (PVDF) membrane and blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Western blot analysis was performed by incubating the membrane with anti-pY 841 Jak3 monoclonal antibody, anti-pY antibody (Millipore) and anti-pY Stat5 antibody overnight at 4 °C, or anti-Jak3 (Abcam) and anti-Stat5 (Santa Cruz) for 1 hour at room temperature. Assays were developed with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and X-ray film.

3.2.4 Dot Blot Analysis:

Phosphopeptides and non-phosphopeptides were diluted in water and in increasing concentration were absorbed to methanol activated PVDF membranes. The membranes were allowed to dry, re-activated with methanol and then blocked in 1% BSA 1 h at room temperature followed by incubation with phosphospecific antibodies as described previously [95].

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3.2.5 *In vitro* Kinase Assay:

HEK293 cells were transfected with the appropriate expression vectors for Jak3 and then lysed in Triton lysis buffer. Jak3 proteins were immunoprecipitated with anti-Jak3 antibody and captured with Protein A-Sepharose as described above. The beads were washed three times with cold lysis buffer and once with ice cold kinase buffer (25 mM HEPES (pH 7.3), 1% Triton X-100, 100 mM NaCl, 10 mM $MgCl_2$, 3mM $MnCl_2$ and 50 µM sodium orthovanadate). The kinase reactions were initiated by addition of 100 µM ATP and were incubated at 37 °C for 20 minutes. The reactions were quenched by washing the Protein A-Sepharose beads with lysis buffer and eluting the material with 2 X SDS sample buffer. Samples were resolved by 7.5% SDS-PAGE and tyrosine phosphorylation levels of Jak3 were assessed by Western blotting with anti-pY antibodies.

3.3   Results

3.3.1 Phosphospecific antibodies anti-pY633, pY738, pY762, pY841 and pY929 selectively recognize phospho compared to non-phospho peptide

To determine whether the pY Jak3 specific antibodies cross react with non-phosphorylated forms of the peptides, dot blot analysis with the immunizing phospho-peptides and corresponding non-phospho peptides was performed. Increasing amounts of phospho or non-phospho peptide for sites 633, 841 and 929 (1ng – 1000ng) or 738 and 762 (3ng – 3000ng) were spotted on PVDF membranes and immunoblotted with anti-pY 633, 738, 762, 841 or 929 Jak3 antibodies at 1:5000 dilution. The anti-pY 633, 738, 762, 841 or 929 Jak3 antibodies primarily recognized the phosphorylated peptide but not

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its non-phosphorylated counterpart, indicating that these phospho-specific antibodies do not cross-react significantly with the non-phosphorylated peptide (Figure 17A-E).

3.3.2 pY 841 Jak3 recognizes phosphorylated protein in autokinase assay and pervanadate treated cells

To determine whether anti-pY841 Jak3 antibody recognizes phosphorylated protein, HEK293 cells were transfected with WT Jak3 and cells were harvested 48 hours post-transfection, lysed and immunoprecipitated for Jak3. Beads were washed 3 X with triton lysis buffer and once with kinase buffer, then incubated at 37 °C for 20 minutes with 100 µM ATP. Reactions were quenched with cold lysis buffer and beads were eluted using 2X sample buffer. Samples were separated by 7.5% SDS-PAGE, transferred to PVDF and immunoblotted overnight with anti-pY841 Jak3 antibody at 1:1000 dilution. Anti-pY841 Jak3 was able to recognize the phosphorylated protein (Figure 18A).

Further characterization was performed by stimulating Kit225 and YT cells with IL-2 for 15 minutes and treating with pervanadate, a tyrosine phosphatase inhibitor, for 30 minutes. Cells were flash frozen using dry ice post treatment, lysed, immunoprecipitated in the case of YT cells for Jak3, or whole cell lysate used for the Kit225 cells, separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with anti-pY841 Jak3 antibody and 1:1000 dilution overnight at 4°C. In YT cells pY841 was constitutively phosphorylated between non-IL-2 and IL-2 stimulated cells and had higher phosphorylation in the pervanadate treated cells (Figure 18B). Kit225 cells were constitutively phosphorylated at Y841 with IL-2 stimulation and pervanadate treatment (Figure 18C).

71
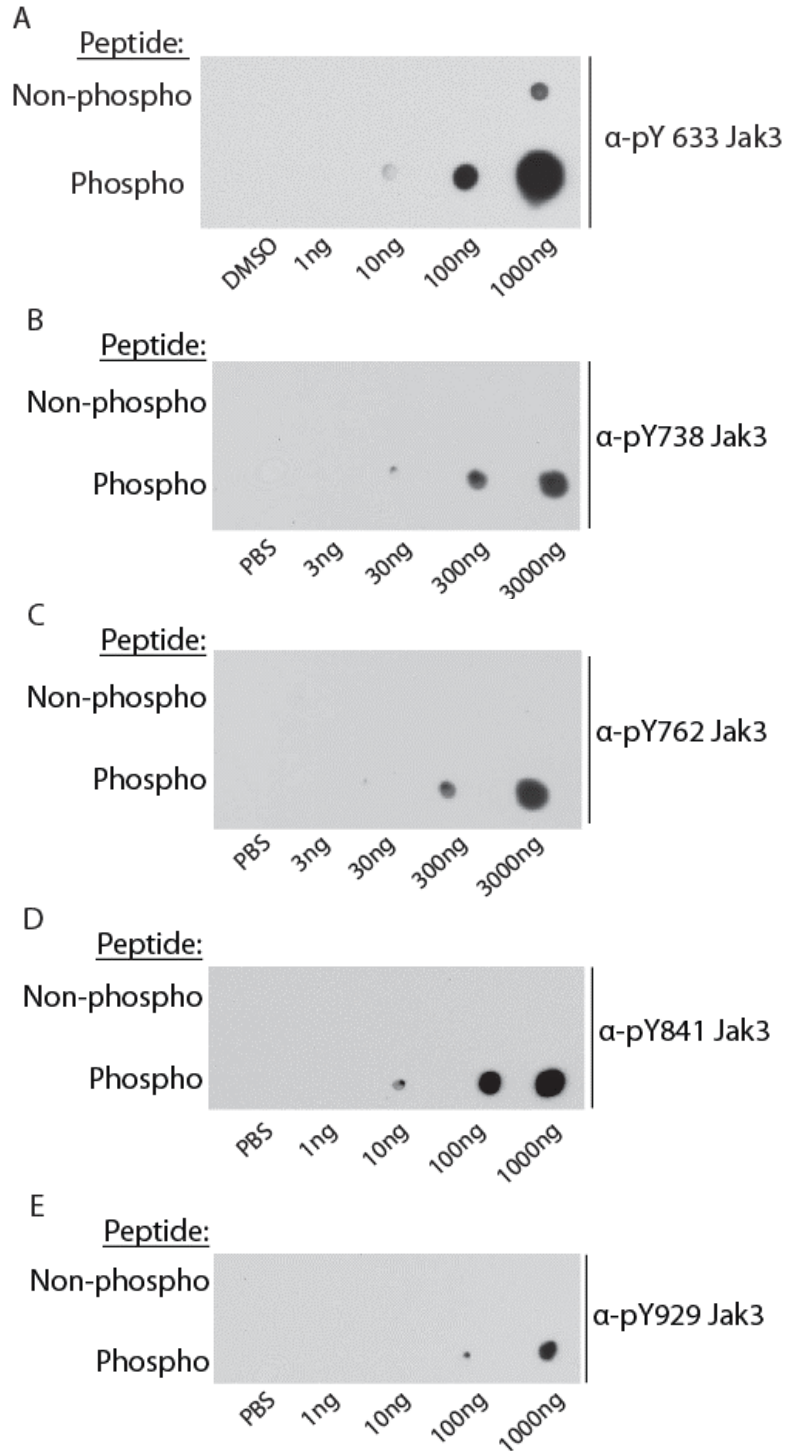
Figure 17. Dot blot analysis for phospho versus non-phosphopeptide specificity with indicated antibodies. A. Rabbit poly clonal anti-pY633 Jak3 antibody was tested against increasing concentrations (1 ng, 10 ng, 100 ng and 1000 ng) of non-phospho peptide (top) vs. phospho peptide (bottom). B. Purified rabbit anti-pY738 Jak3 antibody was tested against increasing concentrations (3 ng, 30 ng, 300 ng and 3000 ng) of non-phospho peptide (top) vs. phospho peptide (bottom). C. Purified rabbit anti-pY762 Jak3 antibody was tested against increasing concentrations (3 ng, 30 ng, 300 ng and 3000 ng) of non-phospho peptide (top) vs. phospho peptide (bottom).. D. Mouse monoclonal anti-pY841 Jak3 antibody was tested against increasing concentrations (1 ng, 10 ng, 100 ng and 1000 ng) of non-phospho peptide (top) vs. phospho peptide (bottom). E. Mouse monoclonal anti-pY929 Jak3 antibody was tested against increasing concentrations (1 ng, 10 ng, 100 ng and 1000 ng) of non-phospho peptide (top) vs. phospho peptide (bottom).

To determine whether Y841 Jak3 phosphorylation was regulated early during IL-2 stimulation, YT cells were stimulated over 8 time points using 10,000 IU of IL-2. Cells were then flash frozen and lysed, immunoprecipitated for Jak3, separated by SDS-PAGE and transferred to PVDF membrane. The membrane was then probed for anti-pY841 Jak3 overnight at 4°C. Y841 of Jak3 was phosphorylated constitutively in YT cells regardless of IL-2 stimulation (Figure 18D).

3.3.3 Jak3 Y841 is critical for full activation of Stat5B

In order to determine the role of novel Jak3 tyrosine phosphorylation sites, Jak3 phospho-deletion mutant plasmids were generated with tyrosine substituted for phenylalanine so that expressed mutant Jak3 protein could not become tyrosine phosphorylated at those sites. Mutants were made for Jak3 Y105, Y633, Y637, Y738, Y762, Y841 and Y929, and 10 µg were transfected into HEK293 cells with WT and a catalytically inactive mutant K855A, harvested at 48 hrs, and immunoprecipitated for Jak3. A 7.5% SDS-PAGE was performed on immunoprecipitated Jak3 and transferred to PVDF and incubated overnight with anti-pY antibody at 4 °C. Western blot analysis of immunoprecipated Jak3 revealed a reduction in total tyrosine phosphorylation compared with WT Jak3 for mutants Y105F, Y841F and Y929 (Figure 19A).

To determine the effect on Stat5 activation, HEK293 cells were co-transfected with Jak3 WT or mutants and Stat5B plasmids. Cells were harvested at 48 hrs and SDS-PAGE was performed for 20µg of whole cell lysate, transferred to PVDF and Western blotted for anti-pY Stat5 overnight as a marker of activated Stat5 and densitometric analysis revealed Jak3 Y841F mutant significantly reduced Stat5B activation by 90% (p=0.003, paired student t-test) (Figure 19B and C).

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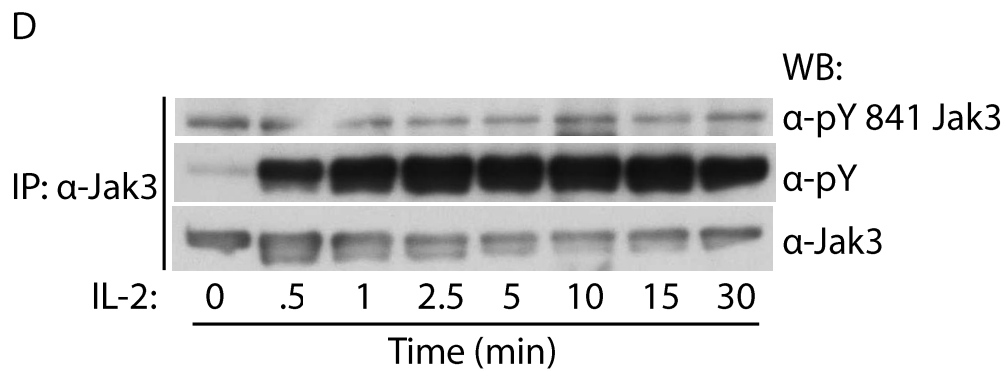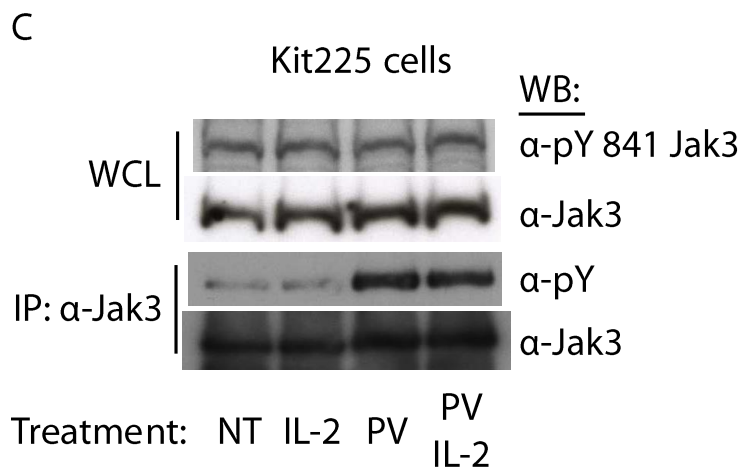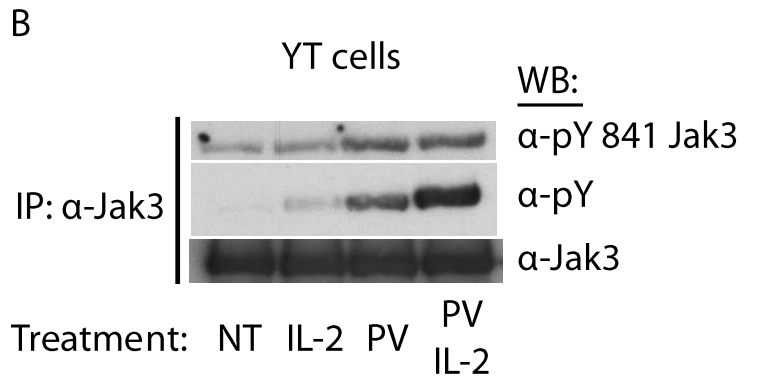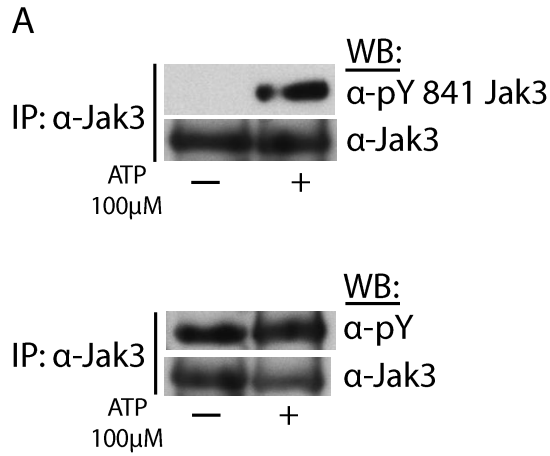
Figure 18. Anti-pY841 Jak3 recognizes phosphorylated protein. A. Kinase assay using 100 μM ATP with immunoprecipitated Jak3 and Western blotted with anti-pY841 Jak3. B. IL-2 and/or pervanadate (PV) treated YT or C. Kit225 cells were immunoprecipitated for Jak3 and probed with anti-pY841 Jak3 antibody. D. IL-2 stimulation time course in YT cells, immunoprecipitated for Jak3 and probed for anti-pY841 Jak3.

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A

IP: α-Jak3

WB: α-pY / α-Jak3

WT K855A Y105F Y633F Y637F Y738F Y762F Y841F Y929F

B

WCL

WB: α-pY STAT5 / α-STAT5

WT K855A Y105F Y633F Y637F Y738F Y762F Y841F Y929F
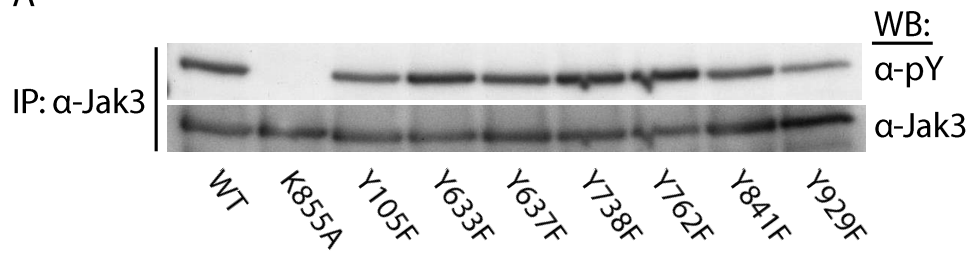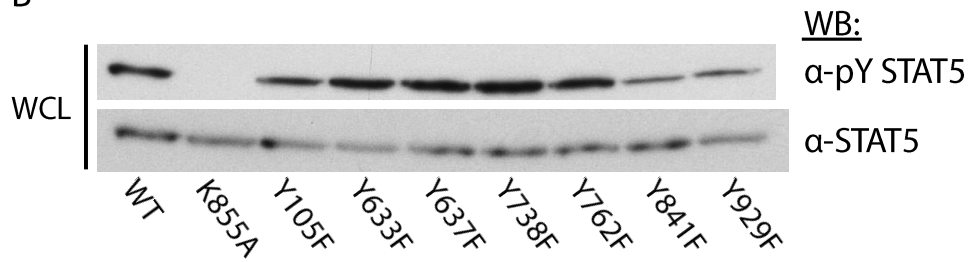
C
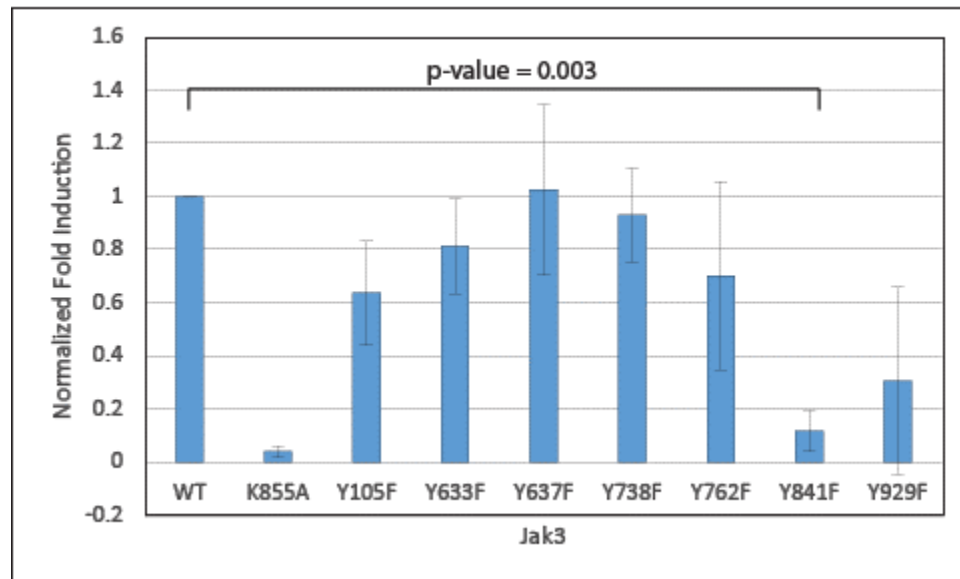
Figure 19. Jak3 Y841F mutation reduces Stat5B activation. A. Hek293 cells were transfected with 10 µg WT or mutant Jak3 plasmids and incubated for 48 h at 37 °C. Cells were harvested at 48 hours to assess auto-kinase activity. B. Hek293 cells were transfected with 2 µg WT or mutant Jak3 plasmids and 2 µg Stat5B plasmids and harvested at 48 h at 37 °C. Western blot was performed for Stat5B activation which showed a decrease in pY Stat5B with Jak3 Y841F mutant. (C) Densitometric analysis of Figure 8B shows significant reduction of pY-Stat5 by Jak3 Y841F.

A

Front View

B

Back View

C

Top View
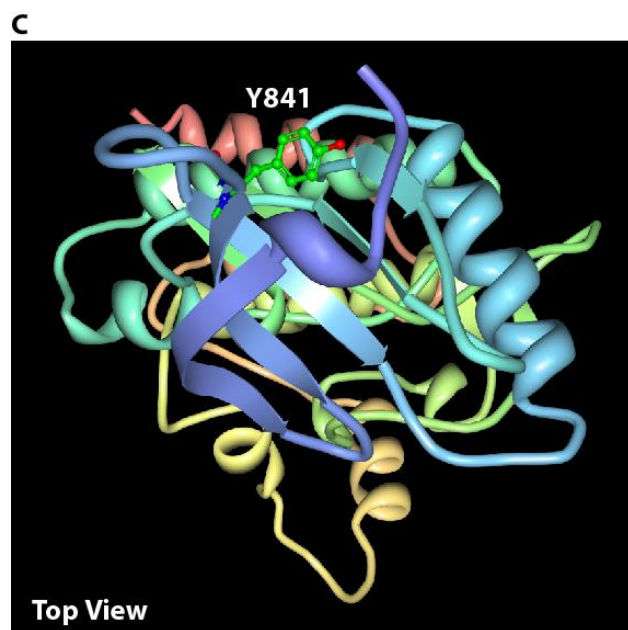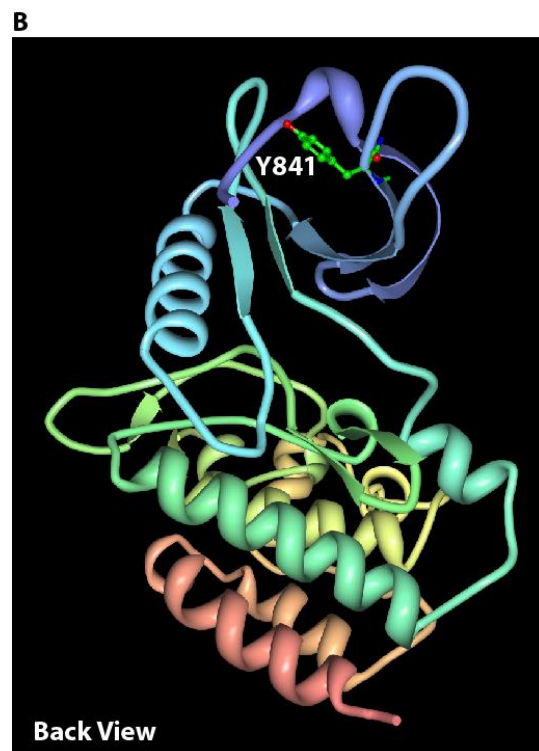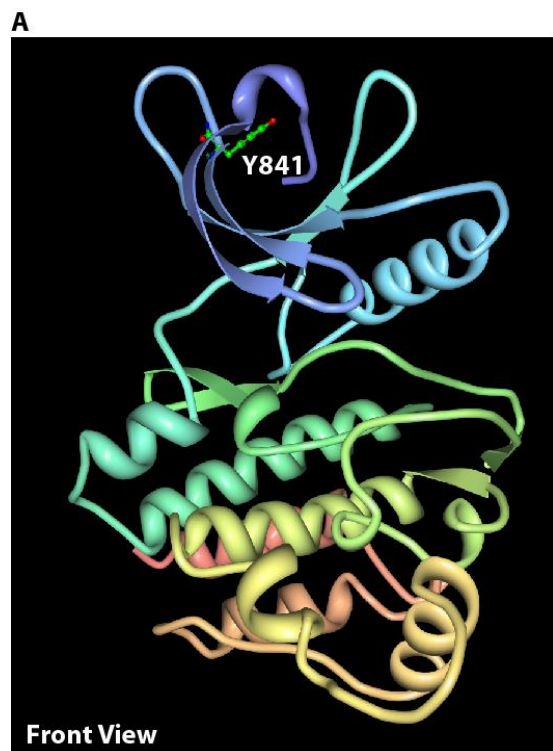
Figure 20. Structural representation of Jak3 JH1 domain with Y841 Crystal structure of Jak3 JH1 domain with Y841 using PDB 1YVJ. A. Front view the ATP binding pocking of Jak3 catalytic domain JH1 with tyrosine residue 841 show. B. Side view showing the glycine lid of the JH1 domain. C. Top view of Jak3 catalytic domain.

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3.4   Discussion

In order to understand the functional role of the novel tyrosine phosphorylation sites of Jak3, a two-step approach was used. First, we determined how using phospho-deletion mutants of each of 7 of those sites, Jak3 Y105F, Y633F, Y637F, Y738F, Y762F, Y841F and Y929F, individually affected total Jak3 phosphorylation or its ability to activate Stat5B. Our results show that while several mutations Y105F, Y841F and Y929F reduce total Jak3 phosphorylation, the Y841F stands out as it significantly reduced the ability to activate Stat5B by 90% (p=0.003) (Figure 19C).

Second, we generated and characterized phospho-specific antibodies against those sites which can serve as a useful tool in understanding how Jak3 functions *in vivo*. Anti- pY633, pY738, pY762, pY841 and pY929 Jak3 showed excellent specificity in recognizing phospho over non-phospho peptides in dot blot assays (Figure 9A-E). The anti-pY841 Jak3 antibody was also able to recognize phosphorylated Jak3 from whole cell lysate and immunoprecipitated cells, making it an excellent tool for studying the regulation of Jak3 phosphorylation (Figure 18A-C). Indeed, this antibody may be used to identify dysregulated Jak3 in human hematopoietic diseases.

Tyrosine 841 is located on the glycine lid located in the catalytic JH1 domain of Jak3, near the ATP binding pocket as shown in figure 20 using the crystal structure PDB 1YVJ. Y841 is adjacent to the outer lip of the pocket as shown in figures 20A and 20B. Mutating Y841 to phenylalanine attenuates both Jak3 autophosphorylation and Stat5B activation, highlighting the importance of this site. It is interesting to speculate that this site may serve a role as providing a stable interaction surface for molecules such as Stats in order for them to achieve maximum phosphorylation.

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Chapter 4

Jak3 phospho-tyrosine 841 is differentially regulated in leukemic Jak3

M511I and A573V

4.1   Introduction

Jak3 leukemic mutants M511I and A573V possess the ability to transform Ba/F3 cells to cytokine independent growth showing that these mutations allow for dysregulation to occur and disrupt normal signaling (Figure 13). In order to examine how these mutations are uncoupling normal Jak3 regulation, the phosphorylation of Y841 was examined using the anti-pY841 Jak3 antibody that was generated, as it is known that this site is important for the regulation of Stat5, a downstream target of Jak3.

Jak3 Y841 is conserved across all Jak family members, Jak1, Jak2 and Tyk2. Jak2 Y868, the corresponding conserved tyrosine residue to Jak3 Y841, is reported to be autophosphorylated and important for signaling and full activation of murine Jak2 [102]. There are no reports of the corresponding residues in Jak1 (Y882) nor Tyk2 (Y916). Our work is the first to show that Y882 of Jak1 is phosphorylated in a kinase assay and the site is recognized by the anti-Jak3 Y841 antibody. This tool may serve as a novel way of detecting other dysregulated Jak molecules.

Hispanics are part of an under-served community and have the highest rates of mortality due to cancer [83]. We utilized a bio-repository of human tumor samples from a predominantly Hispanic population in order to find both novel and reoccurring Jak3 mutations to understand how to more effectively treat patients with leukemia and lymphoma.

4.2   Materials and Methods

4.2.1   Cell Culture and Treatments:

The HEK293, MT2, Hut78 and Hut102 cell lines were maintained in RPMI 1640 (Thermo Scientific Inc.) medium with 10% fetal bovine serum (FBS) (Atlanta Biologicals),

2 mM L-glutamine (Cellgro), and penicillin-streptomycin (50 IU/ml and 50 µg/ml, respectively) (Cellgro) (complete media) at 37 °C with 5% CO2.

4.2.2 Transfections:

HEK293 cells were transfected with 10 µg of plasmid pcDNA3.1/human Jak3 (Invitrogen), Jak1, Jak2 or Tyk2 (Origene) per confluent 10 cm dish. Jak1, Jak2 and Tyk2 contain the MYC and FLAG tags. Cells were harvested 48 h post transfection. Transient transfections of HEK293 cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

4.2.3 Solubilization of Proteins, Immunoprecipitation and Western Blot

Cells were pelleted and solubilized in lysis buffer (10 mM Tris-HCl (pH 7.6), 5 mM EDTA (pH 8.0), 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4 , 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 µg/ml pepstatin A, and rotating at 4 °C for 1 h. Whole cell lysates were clarified by centrifugation (15,000x g, 15 min, 4°C). For immunoprecipitation reactions, supernatants were rotated with 3 µL (HEK293) or 5 µL (MT2, Hut78, Hut102) of anti-Jak3 rabbit polyclonal antibody for 2 hours at 4 °C. Immune complexes were captured by incubation with Protein A-Sepharose beads (Rockland Immunochemicals) rotating for 1 h at 4 °C. The beads where then washed three times with ice cold lysis buffer and eluted by boiling 5 min in 2 x SDS sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.02% bromophenol blue, 10% glycerol, pH 6.8). Samples were resolved in 7.5% SDS-PAGE and transferred to polyvinyl-difluoride (PVDF) membrane and blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Western blot analysis was performed by incubating the membrane with anti-pY 841 Jak3 monoclonal antibody,

anti-pY antibody (Millipore) antibody overnight at 4 °C, or anti-Jak3 (Abcam) for 1 hour at room temperature. Assays were developed with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and x-ray film.

4.2.4 *in vitro* Kinase Assay:

HEK293 cells were transfected with the appropriate expression vectors for Jak1, Jak2, Jak3 and Tyk2, and lysed in Triton lysis buffer. Jak proteins were immunoprecipitated with anti-Jak3 or anti-myc antibody and captured by protein A-Sepharose as described above. The beads were washed three times with cold lysis buffer and once with ice cold kinase buffer, 25 mM HEPES (pH 7.3), 1% Triton X-100, 100 mM NaCl, 10 mM $MgCl_2$, 3mM $MnCl_2$ and 50 µM sodium orthovanadate. The kinase reaction was initiated by addition of 100 µM ATP and were incubated at 37 °C for 20 minutes and quenched the reaction by washing the protein A-Sepharose beads with lysis buffer and eluting the material with 2 X SDS sample buffer. Samples were resolved by SDS-PAGE and tyrosine phosphorylation levels of Jak3 were assessed by Western blotting with anti-pY841 Jak3 monoclonal antibody, anti-pY antibody (Millipore) antibody, or anti-Jak3 (Abcam) and anti-myc (Santa Cruz) for 1 hour at room temperature. Assays were developed with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and x-ray film.

4.2.5 Purification of genomic DNA and sequencing:

Qiagen DNeasy Blood & Tissue Kit was used to purify genomic DNA (gDNA) from leukemia and lymphoma primary cells, obtained from The University of Texas at El Paso

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tumor biorepository comprised primarily of Hispanic donors, according to manufacturer's protocol. DNA concentration was measured using NanoDrop 3000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and was verified using 1 % agarose gel (Ultra-pure Agarose with 1x TBE) containing 0.002% EtBr. Patient gDNA was amplified using the following primers: Exon 11 (forward primer 5'-GTTGCAGTGAGCTGAGATCG-3' and reverse primer 5'-TCTCATGCTGAATGGTGAGG-3'); Exon 13 (forward primer 5'-TCCCGTATCAGAAAATCATGG-3' and reverse primer 5'-GCTGGATATGGGTGAGAACC-3'). Subsequently, PCR product was sequenced using a 3130x/Genetic Analyzer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) in the Border Biomedical Research Center housed at The University of Texas at El Paso.

4.3 Results

4.3.1 Jak3 pY841 is upregulated in leukemic Jak3 mutant A573V

To determine whether leukemic Jak3 mutants M511I and A573V are differentially regulated, plasmids for Jak3 WT and mutants K855A, M511I A573V were transfected into HEK293 cells and harvested 48 hours. Cells were lysed and immunoprecipitated for Jak3, separated by SDS-PAGE, transferred to PVDF membrane and probed overnight with anti-Jak3-pY841 antibody. Jak3 A573 had a 1.7 fold increase in phosphorylation at Y841 while M511I was comparable to wild-type (Figure 21A-B). Interestingly, the catalytically inactive mutant K855A was only reduced by approximately 50% compared to wild-type, indicating phosphorylation by an unknown tyrosine kinase.

The HTLV-1 transformed T-cell leukemia/lymphoma cells lines MT2 and Hut102 were examined for increased Jak3 Y841 phosphorylation. In Hut102 cells, which are derived from a 26 year old black T-cell lymphoma patient [103], Jak3 Y841 was increased,
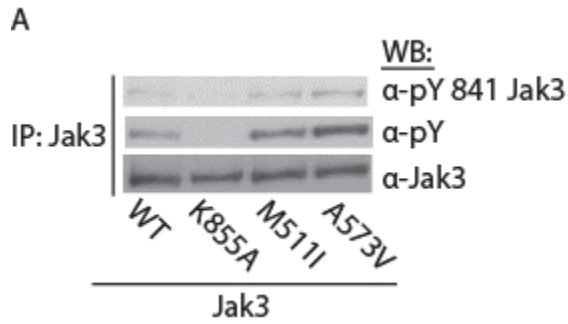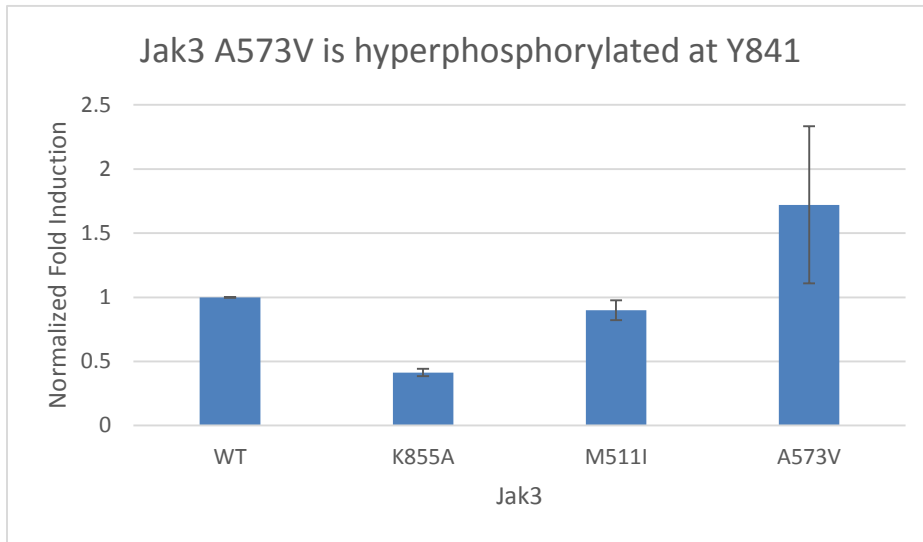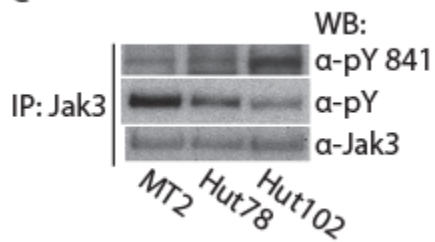
Figure 21. Jak3 Y841 is highly phosphorylated in leukemic Jak3 A573V. A. HEK293 cells transfected with WT, K855A, M511I or A573V Jak3 were immunoprecipitated for Jak3, separated by SDS-PAGE and transferred to PVDF. Membrane was then Western blotted using anti-pY841 Jak3. B. Densitometric analysis of A. C. HTLV-1transformed cell line Hut102 has increased phosphorylation at Jak3 Y841.

despite having less total phosphorylation than MT-2 cells and control cell line Hut78 (Figure 21C).

4.3.2 Anti-pY841 Jak3 antibody recognizes phosphorylated tyrosine residues in Jak1, Jak2 and Tyk2

A kinase assay was performed on immunoprecipitated Jak1, Jak2 and Tyk2 to determine if anti-pY841 Jak3 antibody was able to cross react with Jak1, Jak2 or Tyk2. The kinase assay was performed using 100 µM ATP for 20 minutes at 37 °C. Jak1, Jak2 Tyk2 tyrosine phosphorylation was recognized by the anti-pY841 Jak3 antibody (Figure 22A). Sequence alignment shows that the tyrosine sites are conserved across all Jak family members and in Jak3, the Y841 is conserved across multiple species (Figure 22 B and C).

4.3.3 Common Jak3 mutations are not found in Hispanic leukemia and lymphoma patients

Sequencing primers were generated to identify Jak3 mutations in leukemia and lymphoma patient samples from a mostly Hispanic tumor biorepository. Exons 3, 4, 11, 13, 15, 16, 18 and 19, where 12 of the 14 mutations identified are located, were sequenced using both forward and reverse primers in 103 patient samples but no new nor previously identified Jak3 mutations were found (data not shown).
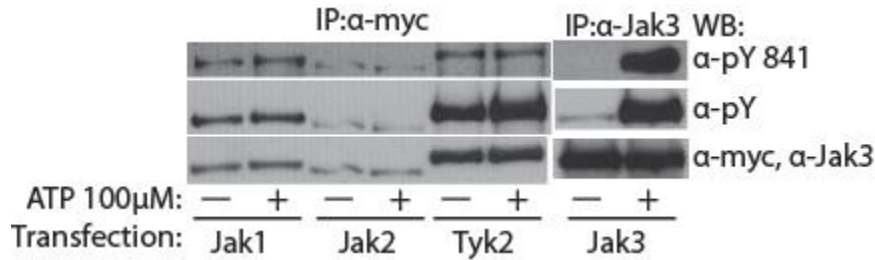
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Figure 22. Anti-pY841 Jak3 antibody recognizes phosphorylated Jak1, Jak2 and

Tyk2 in a kinase assay. A. A kinase assay was performed on Jak1, Jak2, Tyk2 or Jak3

that was immunoprecipitated from transfected HEK293 cells. B. Sequence alignment of

Jak family members coinciding with Jak3 Y841. C. Sequence alignment of Jak3 across

multiple species shows high homology.

```
Human Jak3   ---GKGNFGSVELCRYDPLGDN---
Human Lck    ERLGAGQFGEVWMGYYNGHTKVAVK
             *  *:**.*  :   *:      .

Human Jak3   ---GKGNFGSVELCRYDPLGDNT---
Human Lyn    KRLGAGQFGEVWMGYYNNSTKVAVKT
             *  *:**.*  :   *:     . :

Human Jak3   ---GKGNFGSVELCRYDPLGDNTGALV---
Human Hck    KKLGAGQFGEVWMATYNKHTKVAVKTMKPG
             *  *:**.*  :.  *:     . :    :

Human Jak3   ---GKGNFGSVELCRYDPLGD---
Human Blk    RKLGSGQFGEVWMGYYKNNMKVAII
             *.*:**.*  :   *.     .

Human Jak3    ---SVELCRYDPLGDNTGA---
Human PDGFR   IYIITEYCRYGDLVDYLHRNKH'
                .*  ***    * *

Human Jak3    ---VELCRYDPLGDNTGALVAVK---
Human VEGFR1  MVIVEYCKYGNLSNYLKSKRDLFFLN
               ** *:*   *.:    :    :

Human Jak3    ---SVELCRYDPLGDNTGALVAV---
Human VEGFR3  LMVIVEFCKYGNLSNFLRAKRDAFP(
                **:*:*   *.:    *    .

Human Jak3    ---GKGNFGSVELCRYDPLGDNTGA
Human Zap70   IELGCGNFGSVRQGVYRMRKKQIDV
               *  ******.     *      .:  .

Human Jak3    ---GSVELCRYDPLGDNTGALVAVK---
Human Flt3    IKGFLVKCCAYNSLGTSCETILLNSPGP
               *:  *  *:  **  .   :::   .

Human Jak3    GKGNFGSVEL-CRYDPLGDNTGALVAVK---
Human c-Kit   GAGAFGKVVEATAYGLIKSDAAMTVAVKMLK
              *  *  **.*       *   :  .::.   ****

Human Jak3    ---GKGNFGSVELCRYDPLG---
Human ERK2    SYIGEGAYGMVCS-AYDNVNKVR
               *:*  :*  *       **  :
```

Figure 23. Sequence alignment of Jak3 Y841 against other tyrosine and serine/threonine kinases. Tyrosine 841 is positionally conserved across four Src kinase family members, growth factor receptors PDGFR, VEGFR1 and VEGFR3, known oncogenic tyrosine kinases c-Kit and Flt3, and the serine/threonine kinase ERK2.

a kinase. It is interesting to speculate that this site is necessary not only for full activation of Stat5 by Jak3 (Figure 19B), but for all Jak family members to be able to activate Stats fully, and potentially for a large range of kinases to fully activate their substrates.

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4.4   Discussion

Current studies of Jak3 mutations and cancer have focused on the transforming ability of Jak3 and targeting aberrant Jak3 activation using selective inhibitors such as CP-690,550. Limited studies have made a mechanistic approach to determining how mutant Jak3 is dysregulated in leukemias and lymphomas [97]. This work is the first to look at dysregulation by means of differential tyrosine phosphorylation profiles. Anti-pY841 Jak3 serves as a useful and unique tool to understanding the biochemical changes occurring in leukemic Jak3 and moving forward will help to expand the current knowledge of Jak3 phosphorylation sites and their role in normal regulation of the protein and its substrates, not only in normal cells but also in disease. The value of the anti-pY841 Jak3 antibody is underscored by the identification of dysregulated Jak3 in the leukemic Jak3 mutant A573V (Figure 21A) and in the T-cell lymphoma cell line Hut102 (Figure 21C). It will be interesting to explore a broad range of leukemia/lymphoma derived cell lines.

This can be further expanded to Jak1, Jak2 and Tyk2, which share a highly conserved sequence around what is Jak3 Y841, which can help to better understand the role of tyrosine phosphorylation in Jak driven diseases. Perhaps even more interesting is a limited BLAST search has revealed that the Jak3 tyrosine 841 is positionally conserved in many tyrosine and serine/threonine kinases (Figure 23). The tyrosine is notably conserved in the Src family members Lck, Hck, Blk, and Lyn, the growth factor receptors PDGFR, VEGFR1 and VEGFR3, oncogenes Flt3 and c-Kit, Zap70 and the serine/threonine kinase Erk2. Several studies have identified conserved regions of the catalytic domain shared by hundreds of kinases that are necessary for peptide binding, catalysis, phospho-transfer and ATP binding [104-106]. The newly identified tyrosine

represents a potential novel mechanism that is required for optimal substrate phosphorylation by kinases. It is extremely exciting to speculate that this site is required not just by Jak3 but all Jak family members and potentially many more unrelated kinase families.

This is also the first study that has focused on identifying mutant Jak3 in a Hispanic population and it is interesting that the incidence is zero, in stark contrast to other ethnic populations. The majority of studies performed have focused on identifying Jak3 mutations in Asian communities, predominantly Japanese with limited Singaporean, Thai, and Chinese populations, with mutant Jak3 incidence from as low as 8.7% to as high as 35.4% [72, 87, 88, 107, 108]. This work highlights the difference of leukemia and lymphoma mechanics between ethnic populations and that targeting Jak3 for leukemia therapy in Hispanics may not be as effective as in an Asian population. Using a personalized medicine approach is necessary to determine the best treatment on a per patient basis.

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Chapter 5

Future Directions

5.1 Future Directions

Determine the role of gamma common cytokines in Jak3 Y841 phosphorylation

Tyrosine phosphorylation is induced by many gamma common cytokines, not just IL-2. Therefore it is important to determine whether Y841 is regulated by cytokines such as IL-4, IL-7, IL-9, IL-15 and IL-21. This can shed light on how tyrosine phosphorylation profiles differ under different stimuli. The T-cell cell line Kit225 will serve as an excellent model as it contains many of the gamma common cytokine receptors.

Explore Jak1, Jak2 and Tyk2 tyrosine phosphorylation of Y882, Y868 and Y916 respectively

We have now generated a useful tool that can be used to analyze not only Jak3 specific phosphorylation but also that of Jak1, Jak2 and Tyk2. First, tyrosine to phenylalanine mutants will be generated against Jak1 Y882, Jak2 Y868 and Tyk2 Y916 to ensure specificity of our antibody against these sites. Then, our research will focus on how these proteins are phosphorylated in response to both gamma common cytokines for Jak1, and also stimuli such as leptins, erythropoietin, B-cell receptor activation, GM-CSF, interferons and others for Jak2 and Tyk2.

Role of Jak3 Y841, Jak1 Y882 and Jak2 Y868 in disease

As we have seen that Jak3 A573V has hyper-phosphorylated Y841, it would be interesting to see if this phenomenon occurs in human patient samples. Using the human tumor biorepository at UTEP, it will be possible to examine cases of leukemia and lymphoma for differential regulation of Jak tyrosine phosphorylation. This could provide novel insight into the mechanism of diseases such as these.

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Determine the role of the conserved tyrosine site on activation of Stats by other Jaks and if this role is limited to Jak family members or has a broader role in kinase function

In order to determine whether the conserved tyrosine residue plays a role in other Jak family members, a HEK293 reconstitution system can be used to introduce phospho-deletion mutations into other Jak family members to see if Stat activation is reduced. This methodology can also be applied to other kinases that share the conserved tyrosine residue to examine total phosphorylation and activation of other substrates. This site has the potential to be necessary for hundreds of kinases and may lead to furthering our understanding of kinase functionality at the biochemical level. Initial sequence alignments of 189 of the 511 kinases has revealed 31 with the conserved tyrosine, including many of the calmodulin dependent kinases, FMS-like tyrosine kinases, the Erk family of kinases and the bromodomain containing kinases. This knowledge has the potential to change our understanding of how kinases may function.

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