CONTROLLING NUCLEAR JAKS AND STATS FOR SPECIFIC GENE ACTIVATION BY IFN GAMMA

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To all those who believed in me

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LIST OF ABBREVIATIONS

AcH3	Acetylated histone H3
APC	Antigen presenting cell
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPO	Erythropoietin
EPOR	Erythropoietin receptor
FERM	Four point, Ezrin, Radixin, moesin domain; N-terminus of JAK used for receptor association
GAS	Gamma activated sequence
G-CSF	Granulocyte colony stimulating factor
G-CSFR	Granulocyte colony stimulating factor receptor
GH	Growth Hormone
GHR	Growth hormone receptor
GM-CSF	Granulocyte/macrophage colony stimulating factor
H3pY41	Histone H3 tyrosine phosphorylated on residue 41
HP1	heterochromatin protein 1
HSC	Hematopoietic stem cell
IFN	Interferon
IFNAR	Type I IFN receptor
IFNGR	IFN gamma receptor
IL	Interleukin
IP	Immunoprecipitate
IRF	IFN regulatory factor

JAK	Janus kinase
JH	JAK homology
LD	STAT linker domain
LIF	Leukocyte inhibitory factor
ND	STAT N domain
NLS	Nuclear Localization Sequence
OPN	Osteopontin
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
pJAK	Activated JAK
PRL	Prolactin
pSTAT	Tyrosine phosphorylated STAT
PV	Polycythemia Vera
SCID	Severe combined immunodeficiency
SH2	Src homology 2
STAT	Signal transducer and activator of transcription
TAD	Transcriptional activation domain
T_H1	T helper 1
T_H2	T helper 2
TPO	Thrombopoietin
TPOR	Thrombopoietin receptor
WL	Whole cell lysate
WSXWS	Tryptophan, serine, any amino acid, tryptophan, serine
Y	Tyrosine

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IFNγ is a pleotropic cytokine that ligates to specific cell surface receptors to mediate specific gene induction. Moreover, the formation of the ligated receptor complex activates the Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) pathway. It has been previously demonstrated that IFNγ, its receptor subunit IFNGR1 and STAT1 form a macromolecular complex that translocates to the nucleus to accomplish gene activation. In this study, we report on the epigenetics associated with IFNγ signaling. Furthermore we characterize the receptor complex by assessing the association, sub-cellular localization and function of activated JAK1 and JAK2 with the IFNγ-IFNGR1-STAT1 complex. We found that activated JAK1 and JAK2 associate with IFNGR1 throughout signaling and specifically within the nucleus. Furthermore, this JAK-receptor association correlated with an increase in the tyrosine phosphorylation of histone H3 on residue 41 (H3pY41) globally and specifically at the IRF1 promoter. Cumulatively, we propose that the activated IFNγ receptor complex includes JAK1 and JAK2, and that these kinases are directed specifically to relative promoters via their specific interaction with the activated receptor. Finally, we demonstrate that TYK2 is constitutively present in the nucleus of WISH cells and that

treatment with either type I IFN IFNα2 or IFNτ causes the appearance of activated TYK2 and IFNAR1 within the nuclear compartment. Our results suggest that ligands which engage the JAK/STAT pathway in general also use activated JAKs within the nuclear compartment to induce specific epigenetic events.

CHAPTER 1
INTRODUCTION

Extracellular Polypeptide Messages

We are multi-cellular organisms consisting of a vast array of highly differentiated cells. To ensure the success and homeostasis of our overall being, these various cells must be able to respond to their microenvironment and communicate with each other. Moreover, in order to properly orchestrate the simultaneous growth, division, death, mobilization, activation, tissue differentiation, and other innumerable measures, our cells must be able to interpret outside signals and coordinate their actions.

A major way that communication is achieved is via secretion of extracellular polypeptides. These protein messages are made and released from the cell in response to particular stimuli. These messages are often classified as cytokines, growth factors or hormones. Environmental factors and the nature of the protein limit the half-life, distance and direction the message can travel. Nevertheless, these protein messages can then bind to specific cell surface receptors on any cell which has them. The binding of these extracellular protein messages to their cognate receptors is a ligation event which induces a conformational change in the ligand/receptor complex leading to concomitant receptor internalization and activation. Ultimately this activation event is a stimulus which results in immediate physiological changes in the cell and/or chromatin remodeling allowing for specific gene regulation. Thus, the binding of these extracellular protein messages (ligands) to their cognate receptors induces activation, internalization and a signaling event that allows for the cell to respond to particular stimuli. The predominant way that most of these ligands signal once they activate their receptors is via the JAK/STAT pathway Figure 1-1, Table 1-1.

The STATs

The STATs are known to be transcription factors which exist in the cytoplasm and can undergo JAK assisted tyrosine phosphorylation. This phosphorylation is an activation event which allows STATs to dimerize via reciprocal SH2 domain interactions and subsequently enter the nucleus where they bind to palindromic like elements located within the promoters of specific genes (Horvath, 2000). In mammals there are seven STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. The domain architecture of these proteins is shown in Figure 1-2. Conserved among the STATs are five distinct regions: a four-helix bundle transactivation domain, a central β-barrel DNA binding domain, a helical linker domain, an SH2 domain, and an effector domain. The C-terminal transactivation domain is involved in communication with transcription complexes. The effector domain is believed to regulate function specifically through contribution to its nuclear export. The N-terminus of the STATs is the most conserved element and plays a role in stabilizing STAT binding to DNA. Finally, the helical linker domain is simply believed to connect the DNA binding and SH2 domains.

Once a receptor is ligated with commensurate JAK activation and receptor phosphorylation, it is believed that specific STATs are recruited to phosphorylated receptor with docking achieved via SH2 binding contribution from the STAT. Next, the STAT is phosphorylated on a single tyrosine located around position 700. This phosphorylation can be performed by the JAKs or by the receptor itself if it has intrinsic tyrosine kinase activity. Nevertheless, phosphorylation allows the STATs to form homodimers and enter the nucleus. While most ligands predominantly induce STAT homodimers, a few exceptions have been noted; STAT1 is also known to form

heterodimers with STAT2 and STAT3. Regardless, dimerization is believed to be a requirement for DNA binding as STAT monomers cannot bind DNA.

The physiological roles of the STATs have best been characterized using STAT knockout mice (Khwaja, 2006). See Table 1-2 for a list of their phenotypes. Deficiency in STAT2 and STAT3 are embryonically lethal. STAT1 knockout mice quickly succumb to viral and bacterial pathogens due to lack of an innate immune response, however, if the mice are kept in pathogen free conditions they are phenotypically normal and reproductive. Knockout of STAT4 results in mice lacking a T helper 1 (T_H1) cell response. Similarly, STAT6 knockout mice have T helper 2 (T_H2) deficient cellular responses. These STATs have been associated with specific cytokine signaling systems that promote these adaptive immune responses. In mice STAT5 knockout exhibits a sexual dimorphism believed to be caused through growth hormone signaling (Udy et al., 1997). Moreover, male mice deficient in STAT5a are normal phenotypically, while their female counterparts do not develop normal breast tissue and cannot lactate. Males deficient in STAT5b grow more slowly than normal and have liver-produced serum proteins which are more characteristic of female mice. Besides growth hormone signaling regulation, STAT5 also plays a role in the generation of CD4+CD25+Foxp3+ T cells in the thymus and periphery (Burchill et al., 2007). These cells play a role in immune regulation in humans and mutations in either STAT5 gene display immune pathologies associated with decreased CD25 and Foxp3 expression (Cohen et al., 2006).

The DNA element that activated homodimeric STATs bind to was discovered via studying JAK/STAT signaling induced by a cytokine known as IFN gamma (IFNγ). In

this case, dimerized STAT1 was found to enter the nucleus and bind to a palindromic like element, TTC$(N)_{2-4}$GAA, where N is any nucleotide, and was so named the gamma activated sequence (GAS). Since then, the STAT dimers induced by other ligands have also been shown to bind to this same or similar sequence (Horvath, 2000).

The JAKs

In mammals, the JAKs are a family of 4 tyrosine kinases known as TYK2, JAK1, JAK2, and JAK3 (Haan et al., 2006). They are tyrosine kinases ranging from 120-140 kDa and are ubiquitously expressed with the exception of JAK3, which is restricted to hematopoietic cells. The conserved JAK homology (JH) domains are depicted in Figure 1-3. The N-terminus consisting of JH3-JH7 is involved in receptor subunit binding. An SH2 domain overlaps the JH3-JH4 domains and further contributes to receptor binding. The JH2 domain is a pseudokinase domain thought to play a role in autoregulation of JAK activity when associated with non-ligated receptor. The JH1 domain is a classical tyrosine kinase domain which becomes activated via auto tyrosine phosphorylation of the residue located around position 1000 of the JAKs. This phosphorylation is critical to the activity of the kinase and the transmission of signal by the ligated receptor.

The same JAKs are used by different receptor subunits to which they pre-associate. Besides receptor activation and subsequent signal transduction, the JAKs known physiological roles include regulation of cell surface expression via association of receptor subunits, and have also been shown to play roles in the degradation and surface recycling of their associated receptors (Haan et al., 2006). Furthermore, the JAK-receptor association has been referred to as “tight”, an ambiguous term deduced through correlation of degradation rates of receptor subunits and JAKs (Haan et al., 2006) and the lack of gp130-JAK1 disassociation before and after IL-6 treatment

(Behrmann et al., 2004). However, association constants for JAKs and receptors have yet to be determined.

The cytokines of the hematopoietic system provide a common link connecting the JAK kinases to signaling. They include: interleukins (ILs), colony-stimulating factors, interferons (IFNs), erythropoietin (EPO), and thrombopoietin (TPO). These cytokines all use JAK kinases for their signaling (Khwaja, 2006). Moreover, these cytokines predominantly bind to a family of transmembrane receptors, either monomeric or heterodimeric, that share structural features. The heterodimeric receptors share a common signaling subunit and a unique ligand-binding chain (Rane and Reddy, 2002). These can further be grouped into receptors which share the common β-chain (granulocyte macrophage colony-stimulating factor, IL-3, IL-5), the gp130 subunit (IL-6, leukemia inhibitory factor (LIF), oncostatin M, IL-11), or the common γ-chain (IL-2, IL-4, IL-7, IL-9, IL-13, IL-15). The single chain and heterodimeric group together make up the type I cytokine receptors, which are characterized by the presence of a WSXWS motif, fibronectin type III domains in the extracellular part of the receptor, and by conserved Box1/Box2 regions in the membrane proximal cytoplasmic domain (Khwaja, 2006). The type II cytokine receptors include the IFN and IL-10 receptors, and they lack the WSXWS motif but do have the Box1/Box2 region (Khwaja, 2006). Signaling via these cytokine receptors is initiated by ligand binding, which induces the dimerization or a conformational change of receptor subunits. The JAKs are constitutively associated with the receptor subunits via their FERM domain and the receptor Box1 domain, thereby conferring the functional equivalent of a receptor tyrosine kinase, as these receptors

lack this quality (Behrmann et al., 2004). Receptor oligomerization brings the associated JAKs to close proximity, allowing their auto- or trans-phosphorylation and activation.

The JAKs have a multitude of physiological roles, including regulating the cell surface expression of their associated receptors (Ragimbeau et al., 2003), and the recycling and degradation of their associated receptors (Gauzzi et al., 1997). Gene targeting studies of the JAK kinases in mice show distinct phenotypes and are summarized in Table 1-3 (Igaz et al., 2001). JAK1 knockout mice die perinatally due to profound defects in lymphoid development (Rodig et al., 1998), while JAK2 knockout mice die in the embryonic period due to a lack of erythropoiesis (Parganas et al., 1998). This correlates with the role of JAK2 in erythropoietin (EPO) signaling, a cytokine which plays a vital role in erythropoiesis. Mice deficient in JAK3 are viable, owing to the limited expression of JAK3. They exhibit the murine severe combined immunodeficiency (SCID) phenotype that affects B- and T-cell populations. TYK2 knockout mice are phenotypically normal but have been shown to have exercise intolerance compared to their littermates, suggesting a mitochondrial respiration dis-regulation (Potla et al., 2006).

The JAK/STAT Pathway

Figure 1-1 presents a schematic representation of this commonly used pathway engaged by over 60 ligands in mammals (see Table 1-1). Ligand binds to an extracellular transmembrane receptor inducing receptor oligomerization. Receptors are most often dimeric consisting of either two identical or different receptor subunits. Receptor activation is induced via tyrosine phosphorylation of the JAK protein, which are pre-associated with specific membrane proximal binding sites on the receptor. The JAKs subsequently tyrosine phosphorylate the receptor subunit creating docking sites

for the Src homology 2 (SH2) domains of STATs to bind. Once recruited, the STATs are then also tyrosine phosphorylated by the JAKs. This allows them to form STAT dimers which translocate to the nucleus and modulate specific gene expression (Horvath, 2000). This is basically the classical model of JAK/STAT signaling.

Interferons

Interferons (IFNs) were discovered in the late 1950s as agents which interfered with viral infection. It was later determined that IFNs are secreted polypeptides that exert a wide range of biological activities including: anti-viral, anti-tumoricidal properties, apoptotic potentiation, growth inhibition, and lymphocyte activation to name a few.

IFNs are categorized into three branches according to the specific receptor system engaged (Borden et al., 2007). Type I IFN consists of at least twenty members including: IFNα, β, ω, ε, κ, δ, and τ. In humans IFNα can be further subdivided into 13 members. Besides their strong antiviral properties, they are also structurally homologous, and with the expression of IFNκ, lack introns. Signaling by type I IFN is achieved via engagement of the type I receptor subunits known as IFNAR1 and IFNAR2. Type III IFN includes the members IL-28A, IL-28B, and IL-29. These ligands all contain introns and signal through the IL-10β-receptor and IL-28α-receptor. While type III IFN engage unique receptors, they are believed to induce a similar intracellular signaling event as the type I IFN signaling system (Borden et al., 2007). Type II IFN has only one member, IFNγ. The antiviral properties of IFNγ are ten fold weaker than that of type I IFN members, but it also has potent immunomodulatory function and signals through its own distinct receptor subunits IFNGR1 and IFNGR2.

IFNs are currently portrayed as cytokines which stimulate both intracellular and extracellular networks to regulate and enhance innate and adaptive immune responses,

as well as provide tumor surveillance. Because of these properties, much interest and research has been invested into the mechanism of their signaling and it was the studying of IFN signaling which led to the discovery and characterization of the JAK/STAT pathway, its components, and the GAS element.

IFNγ Signaling. IFNγ is a pleiotropic cytokine produced by activated immune cells including: NKT, NK, T cells, B-cells and professional APCs (Gough et al., 2008; Schroder et al., 2004). The signaling mechanism has been well studied and has become a paradigm for JAK/STAT signaling in general, and is shown in Figure 1-4. IFNγ binds to the extracellular domains of the IFNGR receptor subunits inducing a ligand/receptor complex between the IFNγ, IFNGR1 and IFNGR2. This receptor complex is quickly internalized via receptor-mediated endocytosis. Concurrently, JAK1 and JAK2, which are pre-associated with the intracellular domains of IFNGR1 and IFNGR2 respectively, become activated and phosphorylate tyrosine residue 440 of IFNGR1. STAT1 can then bind to this phosphotyrosine via its SH2 domain and is subsequently tyrosine phosphorylated on position 701 by either JAK. Tyrosine phosphorylated STAT1 dimerizes and is imported into the nucleus via the Ran/importin pathway (Sekimoto et al., 1997). Subsequently, dimerized STAT1 binds to GAS elements located within the promoters of specific genes thereby activating them. Thus, the key role sole ascribed to the receptors and JAKs in IFNγ signaling is simply to create activated STAT1 dimers. Additionally, IFNγ signaling can also activate the commonly used auxiliary pathways: Ras/MAPK, PI3K/Akt, and CamKII potentially aiding in signal amplification and general growth/survival signals. (Gough et al., 2008; Schroder et al., 2004)

The JAK/STAT Dilemma

The current model for JAK/STAT signaling is oversimplified in many regards. First, STATs do not contain a proven nuclear localization sequence (NLS) which permits association with the Ran/importin pathway for nuclear entry. Second, how over 60 ligands redundantly use homodimeric STATs to accomplish specific gene induction profiles remains unexplained. Finally, the translocation and gene regulation by nuclear JAKs has not been addressed with respect to JAK/STAT signaling, nor has its gene targeting mechanism.

STATs Use the Ran/importin Pathway, but Lack an NLS

The nuclear translocation of proteins greater than 40 kDa generally requires the engagement of the energy dependent Ran/importin pathway. This pathway uses the Ran and importin proteins to chaperone cargo containing a NLS through the nuclear pore complex and into the nucleus (Johnson et al., 2004; Lyman et al., 2002; Sekimoto et al., 1997). While it is known that dimerized STATs use this pathway to gain nuclear entry, no such sequence has been empirically proven to exist in the STATs (Johnson et al., 2004). Rather, the association of STATs with other specifically activated signaling components containing the NLS has been shown to chaperone their nuclear entry (Ahmed and Johnson, 2006; Johnson et al., 2004; Kawashima et al., 2006; Lee et al., 2009; Williams et al., 2004).

It has been reported that STAT1 acquires an NLS once it is activated and dimerizes with another activated STAT1 molecule (Nardozzi et al., 2010). Further, it was shown that two juxtaposed STAT1 molecules form a spatially contiguous cluster of polycationic amino acids where each molecule contributes a few cationic residues to form a conformational targeting sequence. As such, these authors contended that this

di-molecular NLS could only be formed by STAT1 dimerization. Additionally, they delineate the half NLS region of each STAT1 molecule to be located within the DNA binding domain formed by homodimeric STAT1 (McBride et al., 2002). Another group went on to similarly show that the STAT1 and STAT2 heterodimer induced by type I IFN, also forms an analogous conformational NLS (Melen et al., 2001). These results fail to consider the association of this STAT heterodimer with IRF9, a known nucleocytoplasmic shuttling protein (Tang et al., 2007). Nevertheless, these dimers containing STAT1 are the only known examples of a di-molecular/conformational NLS nuclear import known to bind to and use importin for nuclear entry (Lange et al., 2007). The group pioneering the conformational STAT1 NLS also showed that STAT1 binds specifically to the importin alpha 5 subunit's C-terminus, specifically Armadillo repeats 8-10 with a K_d of 10^{-6} M or more (Johnson et al., 2004; McBride et al., 2002). As all known K_d s of conventional NLSs bind with a K_d of 10^{-8} M or lower (Johnson et al., 2004), these findings beg the question of physiological relevance. Finally, the demonstration that the STAT1 binding site on importin alpha 5 exists in Armadillo repeats 8-10 (Nardozzi et al., 2010; McBride et al., 2002) is bewildering as binding to Armadillo repeats 2-4 is required for importin alpha activation leading to nuclear pore association and translocation (Lange et al., 2007; Johnson et al., 2004).

Further investigation was performed to analyze potential NES and NLS sequences on STAT 3 (Liu et al., 2005), 5 (Iyer and Reich, 2008) and 6 (Chen and Reich, 2010), which have also been shown to use the Ran/importin system for nuclear entry once activated by a ligand (Meyer and Vinkemeier, 2004). While clear and definitively proven nuclear export sequences (NES) for all these STATs were observed,

no NLS sequences were uncovered. Rather, specific sequences required for nuclear entry were pointed out, but not shown to be NLSs. The authors describe new nomenclature for STAT nuclear entry as “nonconventional” NLS. Interestingly, these sequences were found in the coiled-coil region (Meyer and Vinkemeier, 2004), rather than the DNA binding domain as proposed for STAT1. Further, these sequences allowed for constitutive nucleo-cytoplasmic shuttling of these STATs regardless of their activation status. Altogether, it appears that STAT3, 5 and 6 do not use the same mechanism of nuclear entry as STAT1. The inability of these groups to specifically point out and prove NLSs on these STATs makes it highly unlikely that the STATs have NLSs.

STATs Redundantly Target the GAS Element

Signaling specificity also cannot be explained by the targeted DNA sequence bound by all homodimeric STATs. Moreover, all STATs capable of homodimerization: 1, 3, 4, 5, and 6, bind to a GAS or GAS-like element (Ehret et al., 2001; Horvath, 2000). As such, many genes containing GAS elements are redundantly targeted by different STATs. For example, the induction of interferon regulatory factor 1 (IRF1) is achieved by IFNγ (Ahmed and Johnson, 2006; Goenka et al., 1999), IL-6 (Harroch et al., 1994), IL-12 (Galon et al., 1999), and IL-2 (Schwarz et al., 1992), which use homodimeric STAT1, STAT3 STAT4, and STAT5, respectively. Thus, different ligands can redundantly use different STATs to induce the same gene by acting on this same GAS sequence. Interestingly, Prolactin (PRL) and IL-4 have been shown to inhibit IRF1 induction through STAT5 (Luo and Yu-Lee, 1997) and STAT6 (Goenka et al., 1999), respectively. Besides highlighting the lack of targeting specificity provided between the

STATs, this promiscuous binding begs the question of how the STATs uniquely target and regulate GAS containing genes.

There is evidence that homodimeric STATs not only target the same GAS elements, but that they directly compete for them. As mentioned above, the reciprocal regulation of IRF1 by IL-4 and IFNγ is mediated by their activation of STAT6 and STAT1 respectively. Still, the regulation of IRF1 induction depends on the relative amounts of STAT1 and STAT6 which bind to it and simultaneous treatment with both cytokines on HepG2 cells causes both STATs to bind to the IRF1 promoter (Goenka et al., 1999). It has been further shown that treating cells with constant concentrations of IL-4 and IFNγ while increasing the intracellular levels of STAT6 suppresses IRF1 induction by causing increased recruitment of STAT6 to the IRF1 GAS element.

Similarly, it has been demonstrated that STAT5 activated by IL-2, and STAT3 activated by IL-6 compete for the same binding sites in the IL-17 locus of naïve CD4+T cells (Yang et al., 2011). Moreover, increasing the concentrations of IL-6 while holding IL-2 levels constant caused the increase in T_H17 phenotype. The inverse relationship also held true; increasing the concentration of IL-2 respective to IL-6 suppressed this phenotype and expression of IL-17. Again, this was attributed to direct competition for gene occupancy by the STATs activated by their respective ILs. While this group presented only data focusing on the IL-17 locus, they also looked at global chromatin occupancy of STAT4 and STAT6 by polarizing CD4+ naïve T cells towards a T_H1 or T_H2 phenotype (Wei et al., 2010). In this case they found that STAT4 under T_H1 conditions and STAT6 under T_H2 conditions bound to many of the same genes. Moreover, the GAS motif was found to be redundantly targeted. Thus, STAT4 and STAT6, STAT1 and

STAT6, and STAT3 and STAT5 have all been shown to directly target and compete for regulation of target genes containing the GAS element. Finally, it should also be noted that the study comparing STAT4 and STAT6 demonstrated that these STATs also bound to a unique subset of genes. Thus, while STATs can and do compete for occupancy of the same GAS elements, they also bind to specific genes distinctly (reviewed Ehret et al., 2001).

All the above studies failed to take into account the activation status of these STATSs and used diagnostic tools that could not discriminate between phosphorylated and non-phosphorylated STAT. This is an important issue to address as STAT1, 3, 5 (Meyer and Vinkemeier, 2004) and 6 (Chen and Reich, 2010) have all been shown to exist and function in the nucleus when not tyrosine phosphorylated.

JAK2/STAT5 Signaling is Redundantly Used by Several Ligands

The activation of STATs by different ligands in the same cell has raised important questions on the specificity of biological actions. This has been especially noted for the cytokines: IL-3, Erythropoietin (EPO), Thrombopoietin (TPO), Prolactin (PRL), Growth hormone (GH) and Granulocyte-Macrophage colony stimulating factor (GM-CSF) all of which specifically require JAK2 and STAT5 activation to mediate unique gene responses. The mechanism for how these six different ligands specifically activate signaling systems using activated STAT5 has not been established. A study comparing PRL, GH, GM-CSF, and EPO signaling has shown that these ligands all induce STAT5 homodimerization (Gouilleux et al., 1995). This study used COS cells transfected with STAT5 and the appropriate receptor followed by ligand treatment. They found that in all instances the activated STAT5 bound to the same GAS elements found in several genes including the β-casein promoter. However, only PRL and not EPO nor GH, were

able to induce the β-casein promoter *in vivo*. This suggests that additional specifications are required to direct activated STATs to target genomic elements.

Another study using BaF3 cells transfected with receptors for EPO and then stimulated with either IL-3 or EPO, demonstrated exclusive STAT5 signaling and GAS binding (Pallard et al., 1995). Moreover, they showed that treatment with either cytokine permitted cell growth in agreement with the fact that these cells depend on activated STAT5 for their survival. However, EPO but not IL-3 treatment led to the induction of beta-globin (DeMartino et al., 1994) attributing to the specific gene differentiation profile induced by EPO. Thus, the specificity of JAK/STAT signaling could not be solely attributed to activation of redundantly used JAK2 and STAT5.

JAK2V617F Disorders Use Specific Receptors for Signaling

Recently, major attention has been paid toward an oncogenic and constitutively active form of JAK2 in which valine at position 617 is changed to phenylalanine (JAK2V617F). This mutation is believed to abolish the function of the auto-inhibitory region of JAK2 and has been observed in myeloproliferative diseases in which JAK2 and STAT5 are constitutively activated (Funakoshi-Tago et al., 2010; Lu et al., 2005; Reuther, 2008). This mutated form of JAK2 is predominantly found in patients diagnosed with polycythemia vera (PV), thrombocythemia, and primary myelofibrosis which are clinically observed as excessive red blood cells, overproduction of platelet cells and fibrosis of the bone marrow, respectively. Furthermore, transgenic mice containing this JAK2 mutation showed granulocytosis, leukocytosis, and thrombocytosis (Shide et al., 2008). Finally, when bone marrow cells expressing the JAK2 mutant were injected into normal mice, they developed erythrocytosis and subsequent PV-like symptoms (James et al., 2005). Thus, JAK2V617F contributes to oncogenesis via

promoting the aberrant differentiation of hematopoietic stem cells (HSC) and its derivatives into granulocytes, thrombocytes, leukocytes, and erythrocytes. This differentiation suggests aberrant signaling by GM-CSF, TPO, and EPO receptors. Interestingly, JAK2 and STAT5 are redundantly used by these signaling systems to induce HSC differentiation. How JAK2 and STAT5 are uniquely activated by these particular ligands to induce differentiation has not been addressed, nor has it been discerned how only myeloid clonal disorders develop with respect to the JAK2V617F background.

In vitro experiments using BaF3 cells have detailed much of the mechanism of JAK2V617F mediated transformation. BaF3 is a murine bone-marrow derived pro-B-cell line that depends on IL-3 signaling and subsequent JAK2 and STAT5 activation for their survival (Funakoshi-Tago et al., 2010). It was found that when these cells were transfected with JAK2V617F they could transform and become growth factor independent in line with the oncogenic properties of JAK2V617F and its activation of STAT5 (Funakoshi-Tago et al., 2010). Importantly, this transformation was dependent on the co-expression of EPOR, TPOR, and G-CSFR. Moreover, when coexpressed with the mutant JAK2, the respective receptor signaling systems were found to be constitutively active (Funakoshi-Tago et al., 2010; Lu et al., 2005). Thus the JAK2V617F mutation is only oncogenic when coupled to a particular receptor signaling platform. This JAK2V617F-receptor interaction is important in developing the specific phenotypes associated with the respective neoplasias.

Nuclear JAKs

Further investigation into the mechanism of JAK2V617F signaling has revealed very interesting and unconventional findings, particularly in discerning a nuclear role for

JAK2 (Dawson et al., 2009; Liu et al., 2011). Moreover, JAK2 activated either by the mutation above or by cytokines, cause JAK2 to target and phosphorylate histone H3 located within the promoter of target genes thereby activating them. As such, treatment of cells with the cytokines LIF, IL-3, and PDGF2 all increase global H3Y41 phosphorylation (Dawson et al., 2009). However, the specific gene targeting mechanism of JAK2 has not been elucidated, though unique engagement with specific receptor signaling systems is an attractive notion.

It has also been shown that JAK2 and JAK2V617F also play an indirect role in epigenetics by binding to and phosphorylating the histone methyltransferase PRMT5, both outside and within the nucleus (Liu et al., 2011). This phosphorylation inactivates PRMT5, causes global chromatin removal of symmetrically dimethylated histones H2A and H4 at position R3, and results in specific gene regulation. Thus, JAK2 plays both a direct and indirect role in epigenetics and specific gene expression. Interestingly, histone H3 (Griffiths et al., 2011) and PRMT5 (Liu et al., 2011) have also been shown to be a substrate for JAK1, implying redundancy in JAK usage.

It must also be noted that the nuclear localization of JAKs are not a novel discovery. JAK1 (Griffiths et al., 2011; Hao et al., 2005; Lobie et al., 1996), JAK2 (Lobie et al., 1996; Mertani et al., 2003; Nilsson et al., 2006), and TYK2 (Ragimbeau et al., 2001) have all been shown to be constitutively present or play an active role within the nucleus of the cells studied. Importantly, growth hormone treatment was shown to induce the translocation of activated JAK2 to the nucleus in GHR transfected CHO cells (Lobie et al., 1996) and in CWSV-1 cells (Ram and Waxman, 1997), while IFNα

treatment of TYK2-GFP transfected U1A cells was shown not to alter the nuclear cytoplasmic distribution of total activated TYK2 (Ragimbeau et al., 2001).

Concerted Receptor-STAT Gene Activation

It has been suggested that the association of homodimeric STATs with other proteins is responsible for their specificity of gene induction (Ahmed and Johnson, 2006; Johnson et al., 2004). For example, the acetyltransferase p300 has been shown to associate with STAT1 (Horvath, 2000), 3 (Giraud et al., 2002; Horvath, 2000; Lee et al., 2009), and 5 (Pfitzner et al., 1998), to accomplish promoter activation via histone acetylation. However, the redundant use of p300 and others cofactors which contribute to STAT signaling responses via general gene activating mechanisms still does not explain signaling specificity. It is interesting to note that the association of cotranscription factors such as p300 have been found to interact with many receptor systems where they may interface with activated STATs and JAKs (Chia et al., 2010; Lee et al., 2009; Tang et al., 2007). Thus, the recruitment of STATs to unique receptor subunits may allow them to engage other unique and redundantly used cofactors to mediate their specific gene response.

A multitude of evidence from many different labs has strongly suggested that the ligand, receptor and JAKs play a much grander role in signaling than mere JAK/STAT activation. Where investigated, ligands, receptors, and JAKs have all been observed in the nucleus in addition to STATs following ligand stimulation (reviewed in Subramanium et al., 2001). In many instances, it has been shown that the ligated receptor associates with STATs in the nucleus where they function as a complex to activate specific promoters.

The mechanism of EGF signaling has been extensively detailed and highlights this association. It has been shown that upon ligation by EGF, the receptor EGFR is rapidly internalized via receptor mediated endocytosis, undergoing retrograde transport leading to importin-β mediated nuclear entry. Nuclear EGFR complexes with STAT3 and STAT5 to activate the promoters of cyclin D1, iNOS, cfos (Lo et al., 2005) and aurora B (Hung et al., 2008), respectively. Similar to the EGF, osteopontin (OPN) mediated ligation of CD44 induces cyclin D1 activation via a nuclear complex consisting of CD44, STAT3, and p300 (Lee et al., 2009). In this case it should be noted that STAT3 nuclear accumulation and cyclin D1 expression was inhibited by transfecting cells with CD44 mutants either lacking an intracellular domain or bearing a mutation in its nuclear localization sequence. Thus, a full length receptor capable of nuclear translocation and STAT association was required for gene activation and STAT nuclear import. Finally, GH induction and nuclear translocation of STAT5 (Chia et al., 2010; Ram and Waxman, 1997), JAK2 (Mertani et al., 2003; Ram and Waxman, 1997), GH, and GHR (Mertani et al., 2003), has also been shown, however the association of these components within the nucleus or at specific promoters has not been determined.

The IFNγ Signaling Mechanism. Our lab has focused on the mechanism of IFNγ signaling. We and others (Bader and Weitzerbin, 1994) have shown that IFNγ and its alpha receptor subunit (IFNGR1) are rapidly internalized and translocated to the nucleus (Ahmed and Johnson, 2006; Larkin et al., 2000). IFNγ binds first to the extracellular domains of IFNGR subunits activating the receptor and inducing receptor-mediated endocytosis. Upon internalization, IFNγ binds via its C-terminus to residues 253-287 of IFNGR1 (Szente and Johnson, 1994). JAK1 associates with human IFNGR1

on residues 266-269 throughout activation (Gough et al., 2008; Schroder et al., 2004), however, the binding of IFNγ to the intracellular domain of IFNGR1 potentiates the movement of JAK2 from IFNGR2 to 283-309 and 404-432 on murine IFNGR1 (Szente et al., 1995). Interestingly, there is overlap for the binding of these proteins on IFNGR1. STAT1 is recruited to residue 440 of IFNGR1 and is tyrosine phosphorylated by the JAKs. The polycationic sequence of IFNγ is then recognized by importinα and the entire activated receptor complex consisting of IFNγ, IFNGR1, and STAT1, are imported into the nucleus. There, the receptor complex binds to target genes such as IRF1 and IDO1 to promote their activation (Ahmed and Johnson, 2006). An important question remains as to whether JAK1 and JAK2 are part of this activated nuclear receptor complex.

The finding that JAK1 and JAK2 function in the nucleus to specifically activate target genes by phosphorylating histone H3 within target promoters begs the question of how the JAKs are directed to specific histones and how this role fits into conventional JAK/STAT signaling. The epigenetic gene activation mediated by JAK2 is performed through several cytokines utilizing receptor systems that activate JAK2 (Dawson et al., 2009; Griffiths et al., 2011). This has led us to ask whether IFNγ is one of these cytokines as its signaling depends on both JAK1 and JAK2 (Briscoe et al., 1996). Further, the fact that JAK2 requires a receptor association to accomplish specific gene induction (Gouilleux et al., 1995; Pallard et al., 1995) and that JAK-receptor association is considered “tight” (Behrmann et al., 2004; Haan et al., 2006) strongly suggest that the JAKs complex with the receptor throughout signaling and particularly within the nucleus. Thus, we thought it was logical to assess the associations of JAK1 and JAK2 with the IFNγ-IFNGR1-STAT1 receptor complex as part of IFNγ signaling. Particularly, we

sought to assess histone H3pY41 levels globally and at specific promoters following IFNγ stimulation.

Table 1-1. Ligands that signal using Janus kinases (JAKs) and signal transducers and activators of transcription (STATs).

Ligand	JAK(s)	STAT(s)
EPO	JAK2	STAT5
TPO	JAK2	STAT5
PRL	JAK2	STAT5
GM-CSF	JAK2	STAT5
G-CSF	JAK1, JAK2, TYK2	STAT3
IL-11	JAK1, JAK2, TYK2	STAT3
Serotonin	JAK2	STAT3
IFNγ	JAK1, JAK2	STAT1
IL-4	JAK1, JAK3	STAT6
Insulin	JAK1	STAT1, STAT5
Thrombin	JAK2	STAT1, STAT3
IL-6	JAK1, JAK2, TYK2	STAT1, STAT3
GH	JAK2	STAT3, STAT5
IL-2	JAK1, JAK3	STAT3, STAT5
IL-3	JAK2	STAT3, STAT5
IL-7	JAK1, JAK3	STAT3, STAT5
LIF	JAK1, JAK2, TYK2	STAT1, STAT3, STAT5
IL-10	JAK1, TYK2	STAT1, STAT3, STAT5
IL-5	JAK2	STAT1, STAT3, STAT5
IL-9	JAK1, JAK3	STAT1, STAT3, STAT5
Angiotensin	JAK2, TYK2	STAT1, STAT2, STAT3
Leptin	JAK2	STAT3, STAT5, STAT6
IL-12	JAK2, TYK2	STAT1, STAT3, STAT4, STAT6
IL-23	JAK2, TYK2	STAT1, STAT3, STAT4, STAT6
Type I IFNs	JAK1, TYK2	STAT1, STAT2, STAT3-6

Adapted with modifications from Schindler, 2002 (Schindler, 2002)

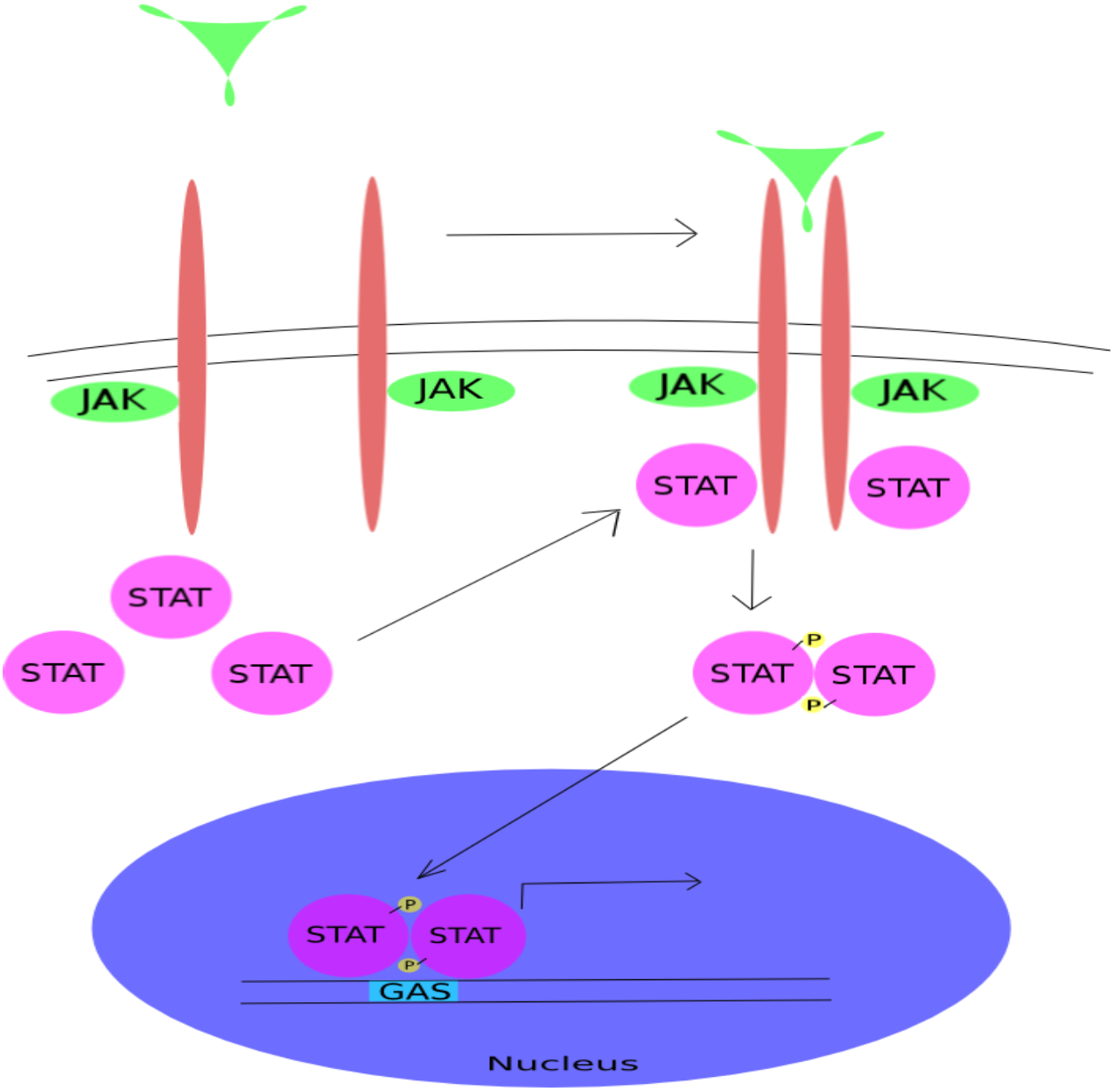

Figure 1-1. Schematic model of the JAK/STAT signaling pathway. A ligand binds to its receptor(s), activating the JAKs that are pre-associated with their receptor subunits. The JAKs then phosphorylate the cytoplasmic domain of their receptors, creating docking sites for the SH2 domain of STATs. Once the STATs are recruited to the receptor, they are phosphorylated on key tyrosine and by JAKs. This activation of STATs allows them to form homodimers that subsequently translocate to the nucleus to bind specific sequences (GAS) on genomic DNA to activate gene expression.

Figure 1-2. Domain architecture of STAT proteins. Defined abbreviations for the structural and functional regions of the STAT proteins: ND, N-domain responsible for STAT-STAT interactions (yellow box); COILED COIL, coiled-coil domain contributes to protein interactions (green box); DNA, DNA-binding domain (red box); LD, linker domain contributes to transcription (orange box); SH2, Src homology 2 domain responsible for receptor binding and STAT dimerization (blue box). All STATs are activated by tyrosine (Y) phosphorylation around position 700. The transcriptional activation domains (TAD, purple box), aids in recruiting transcriptional cofactors.

Table 1-2. Phenotypes of STAT knockout mice.

Targeted Gene	Phenotype
STAT1	No innate response to viral or bacterial infection
STAT2	Viable and fertile, defective type I IFN functions
STAT3	Early embryonic lethal
STAT4	Lack of TH1 function
STAT5A	No breast development or lactation
STAT5B	No breast development or lactation
STAT6	Lack of TH2 function

Adapted with modification from Darnell, 1997.

Table 1-3. Phenotypes of JAK knockout mice.

Targeted Gene	Phenotype
TYK2	Normal, but lack of physical endurance
JAK1	Perinatal lethal, low birth weight, no nursing
JAK2	Embryonic lethal, lack of erythropoiesis
JAK3	SCID

Adapted with modifications from Igaz et al., 2001.

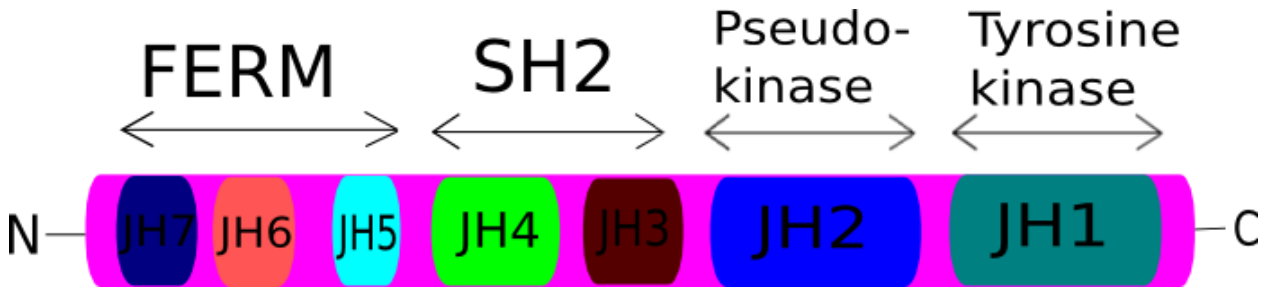

Figure 1-3. Domain architecture of JAK proteins. FERM domain encompasses JH7-JH5 and is required for receptor association. An SH2 domain exists in JH4-JH3 and may contribute to receptor binding. JH2 is the pseudokinase domain and is believed to play an autoinhibitory role of the JH1 domain which is a classical tyrosine kinase domain. Around position 1000 within the JH1 domain lies tyrosine residues which become phosphorylated with the active JAK.

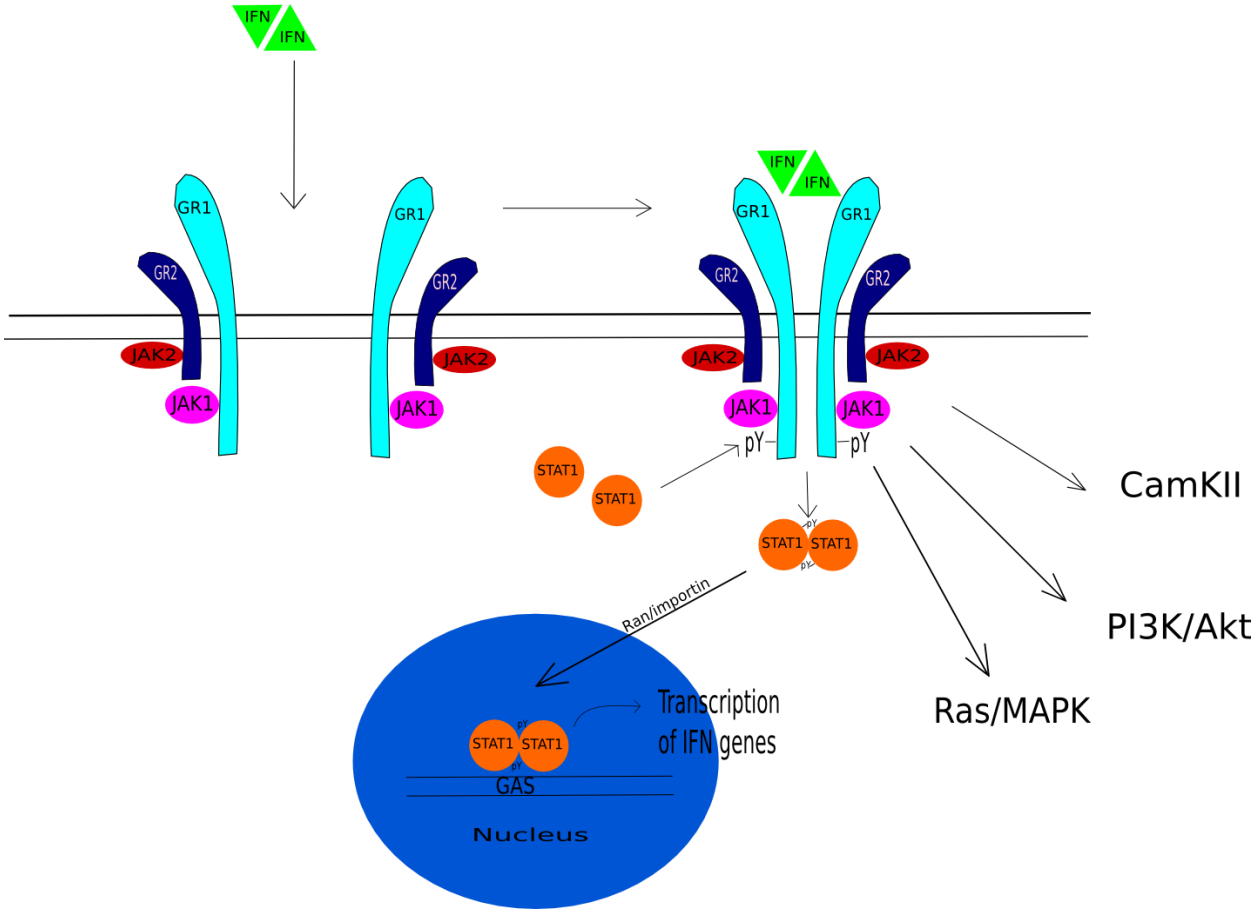

Figure 1-4. Canonical IFNγ signaling. Ligand binding to IFNGR induces a conformational change in IFNGR1 and IFNGR2. This binding is believed to occur with a dimer of IFNγ binding to two IFNGRs, such that the stoichiometry is 2:2. Subsequent to ligation, JAK2 and JAK1 become activated and phosphorylate tyrosine (pY) 440 of IFNGR1. STAT1 is recruited to this pY and is phosphorylated on residue 701. Phosphorylated STAT1 forms a homodimer and is imported into the nucleus via the Ran/importin pathway where it binds to the GAS element thereby inducing target genes. The PI3K/Akt, Ras/MAPK, and CamKII pathways can also be activated.

CHAPTER 2
MATERIALS AND METHODS

Cell Culture and Antibodies

WISH cells were purchased from American Type Culture Collection (ATCC) and were grown in MEME (Sigma-Aldrich) with 10% FBS and antibiotics. For all experiments, cells were serum starved for at least 4 hours, washed twice with PBS and then given serum free media with or without 250 ng/ml IFNγ (Invitrogen), 500 ng/ml IFNτ, or 10 kU/ml IFNα2. The following polyclonal antisera were purchased from Santa Cruz: IFNGR1 (sc-700), STAT1 (sc-346), pSTAT1 (sc-7988-R), pSTAT2 (sc-21689-R), TYK2 (sc-169), pTYK2 (sc-11763), pJAK1 (sc-16773-R), pJAK2 (sc-16566-R), normal rabbit IgG (sc-2027), β -Tubulin (sc-9104), β-Lamin (sc-20682), and Histone H3 (sc-10809). Antibody to acetylhistone was from Active Motif (06-599). Antibody to IFNAR1 was from Epitomics (EP899Y). Antibody to JAK2 was from Millipore (06-1310). Additional antibodies to pJAK2 were also purchased from Cell Signaling: 3771 (polyclonal) and 3776 (monoclonal). Antibody to tyrosine phosphorylated Histone H3 was from Abcam (ab26310).

Chromatin Immunoprecipitation (ChIP) Assay

WISH cells were treated or not with IFNγ for 1 hour. Cells were then washed twice with cold PBS and treated with 1% formaldehyde for 10 min at 37°C. The rest of the procedure was conducted using the ChIP kit from Millipore (#17-295), as per the manufacturer's protocol. Sonication was conducted to get DNA fragments of ~500 bp. Control IgG, or different antibodies, were used for each immunoprecipitation as indicated. DNA fragments eluted were used for PCR with the following primers that spanned the GAS element in their promoters. Human IFN regulatory factor-1 (IRF1)

promoter region was amplified with the primers 5’- CGCCCTGTACTTCCCCTT-3’ (-403 to-386) and 5’-CACCGAGCAATCCAAACACTTA-3’ (-222 to -344). As a control, PCR was conducted with the primers from the human β-actin promoter 5’-CTCGCTCTCGCTCTTTTTTTTTTTC-3’ (-967 to -941) and 5’-CTCGAGCCATAAAAGGCAACT-3’ (-844 to -864). The PCR conditions were: 94°C for 5 min, followed by 35 cycles at 94°C for 15 s, 60°C for 30 s, and 68°C for 20 s. This was followed by annealing at 68°C for 5 min. Following ChIP with different antibodies indicated, the DNA protein complex was used to elute the associated proteins by boiling with the electrophoresis buffer and was analyzed by Western blotting, as mentioned below.

Nuclear Fractionation and Nuclear JAK2 Activation

Following treatment, WISH cells were washed twice in cold PBS, removed by scraping in lysis buffer, and pelleted via low speed centrifugation. The supernatant was removed and saved as cytoplasmic extract while the pellet, containing intact nuclei, was gently resuspended in lysis buffer. The centrifugation, re-suspension, and decanting was then repeated twice more. Isolated nuclei were confirmed by trypan blue staining. Nuclear extracts were prepared by sonicating the final nuclear pellet in lysis buffer, followed by centrifugation to remove insoluble material. Whole cell lysate was independently generated by simply sonicating cells following their scraping in lysis buffer. To determine activation of JAK2 within the nucleus, WISH cells were treated with IFNγ for 10 min and nuclei were collected as described above. Pooled nuclei were resuspended in kinase buffer consisting of 10 mM PBS, 100 μM Na_3VO_4, 5 mM $MgCl_2$, and 300 μM ATP, and equal volumes were aliquoted in separate tubes followed by

incubation at 37^{o} C for the indicated times. Nuclei were then recollected by centrifugation and nuclear lysates obtained as described above.

Indirect Immunofluorescence Assay and Confocal Microscopy

WISH cells on coverslips were fixed with 2% paraformaldehyde and processed as described elsewhere (Kima et al., 2010). In brief, incubation with primary antibodies was done at 1:50 dilution and was performed in binding buffer supplemented with 0.05% saponin. Coverslips were incubated with goat anti-rabbit secondary antibodies conjugated to FITC (Molecular probes) at a 1:200 dilution. Finally, the nucleic acid dye propidium iodide was added to all coverslips and the excess removed by washings with PBS. Coverslips were then mounted on glass slides with ProLong antifade (Molecular Probes), and the images observed were obtained using a Zeiss Axiovert 200 M confocal microscope under 40x water immersion objective and an auxiliary 2x magnification. 3D reconstruction of images from 2D images was done using 17 image sections from the stack, 4 µm above and below the focal plane through the nucleus of the cells (a 0.5 µm displacement each along the Z-axis) and sections were merged to render a 3D reconstruction of the cells. All images were further clarified using LSM 5 Pascal v3.2 SP2 (Zeiss, Heidelberg, Germany).

Analysis of Proteins Bound to Biotinylated GAS Promoter DNA

To identify the proteins associated with the GAS promoter, a nucleotide sequence from human IRF-1 promoter containing the GAS motif, 5'-TGATTTCCCCGAAATG -3', was chosen. An oligonucleotide containing a BamHI site followed by 5 copies of this sequence and another oligonucleotide with complementary sequence were annealed and then inserted into the pGL3-Basic vector using standard cloning techniques. Sequence analysis showed two insertion events, generating a

vector with 10 GAS elements; the first five tandem copies were separated from the second five by six basepairs: 5'-GATCCG-3'. The GAS copies were amplified out of this vector via PCR using primers biotinylated at the 5' ends: biotin-5'-GGTGCCAGAACATTTCT-3' and biotin-5'-TACTGTTGGTAAAGCCACC-3'. The resulting biotinylated x10 GAS PCR product was precipitated out using standard ethanol/sodium acetate protocol, and the final DNA pellet was redissolved in a minimal amount of water and analyzed via a 1.4% agarose gel. Equal amounts of this DNA were incubated with 1 mg of whole cell lysates and left to rock at 4^{o} C overnight followed by incubation with Neutravidin-agarose (Thermo-Scientific) for 2 hours. Precipitated material was sedimented and washed with PBS. The bound proteins were eluted with SDS sample buffer, boiled, electrophoresed, and analyzed by Western blotting.

Western Blot Analysis and Immunoprecipitation

Cells were washed with PBS and harvested in lysis buffer (10 mM HEPES (pH 7.9), 100 mM KCl, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 2 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF. Whole cell lysate was generated via sonication on ice and insoluble material removed via centrifugation at 14k rpm for 10 min at 4^{o} C. Protein concentration was measured using 660 nm protein assay reagent (Pierce). Protein (10 µg each) was electrophoresed on an acrylamide gel, transferred to PVDF, and probed with the antibodies indicated. HRP-conjugated secondary antibodies were used, and detection was conducted by chemiluminescence (Pierce). Immunoprecipitation was conducted by incubating specific antibodies with equal amounts of lysate, followed by incubation with Protein A-Agarose (Santa Cruz), for at least 2 hours. Precipitated material was sedimented and washed thrice with PBS.

CHAPTER 3
RESULTS

pJAK2 and pJAK1 Are Recruited to the GAS Element in the IRF1 Promoter

We previously showed by ChIP analysis that treatment of cells with IFNγ resulted in binding of IFNγ, IFNGR1, STAT1α, but not IFNGR2 to the GAS element in the promoter region of the IRF1 gene (Ahmed and Johnson, 2006). A similar ChIP analysis was performed for activated JAK2 (pJAK2) and JAK1 (pJAK1) using WISH cells treated with IFNγ for 1 hour. Sonicated chromatin containing approximately 500-bp fragments were immunoprecipitated with antibodies to STAT1, IFNGR1, pJAK1, pJAK2, acetylated histone H3 (AcH3), and histone H3 phosphorylated on tyrosine 41 (H3pY41), followed by PCR with primers flanking the GAS element from IRF1. The PCR product selected for amplification extended from nucleotides -403 to -222 in the promoter of the IRF1 gene. As a control, PCR product for the promoter of β-actin gene, -967 to -844, was chosen. As shown in Figure 3-1A, STAT1, IFNGR1, pJAK1, pJAK2, AcH3, and H3pY41 were associated with the GAS element of the IRF1 promoter in IFNγ treated cells. As we previously showed the presence of IFNGR1 and STAT1 at the GAS promoter (Ahmed and Johnson, 2006), their presence here can also be considered as an internal positive control. Phosphorylation of AcH3 on Y41, H3pY41, results in disassociation of the transcription repressor heterochromatin protein 1α (HP1α) from chromatin, resulting in transcription of genes such as IRF1, which are repressed by HP1α (Dawson et al., 2009). Untreated cells showed IRF1 promoter only in input and anti-AcH3 precipitated chromatin. β-actin promoter was precipitated only by anti-AcH3 in IFNγ treated cells. Thus, in addition to the presence of STAT1 and IFNGR1, IFNγ treated cells also showed the presence of pJAK1 and pJAK2 in the promoter of the IRF-1 gene.

Association of pJAK1 and pJAK2 with IFNGR1 in Cells Treated with IFNγ

IFNγ treatment of cells results in the formation of a complex consisting of IFNγ, pSTAT1α, and IFNGR1 where pSTAT1α is activated STAT1α (Ahmed and Johnson, 2006). This complex is actively transported into the nucleus where the nuclear localization sequence (NLS) is provided by the IFNγ. We were interested in determining if pJAK1 and pJAK2 were associated with IFNGR1 in the complex as this would provide a mechanism for the specific presence of the JAKs at the IRF1 promoter. Accordingly, WISH cells were treated with IFNγ and whole-cell lysates were immunoprecipitated (IP) with antibodies to IFNGR1 and analyzed by Western blots. As shown in Figure 3-1B, both pJAK1 and pJAK2 bound to IFNGR1 stably over 15 to 60 minutes with similar levels of pJAK2 over the time period and maximal pJAK1 binding at 60 minutes. pJAK2 has been shown to bind to IFNGR1 in IFNγ treated cells (Szente et al., 1995). These observations provide the mechanism for activation of the JAKs and for their specific presence at the promoter site of an IFNγ activated gene.

Cell lysates were also Western blotted for tyrosine phosphorylation of histone H3 (H3pY41) after IFNγ treatment. As shown in Figure 3-1C, increased H3pY41 was observed at 30 and 60 minutes, with a peak at 30 minutes. These results are consistent with nuclear pJAK2, and perhaps pJAK1, phosphorylation of histone H3 at Y41, which is associated with specific gene activation.

pJAK2 is Present in the Nucleus of Cells only after Treatment with IFNγ

In order to further determine if pJAK2 was present in the nucleus of WISH cells solely as a result of IFNγ treatment, we analyzed whole cell and nuclear lysates for both pJAK2 and JAK2. pJAK2 was present in the nucleus only after treatment of cells with IFNγ, while JAK2 was present constitutively (Figure 3-2A). JAK2 contains a classical

cationic NLS, which may allow movement in an inactive state between the cytoplasm and nucleus (Lobie et al., 1996). To ascertain the purity of nuclear fractionation, β-tubulin and β-lamin were used as markers of nuclear and cytoplasmic fractions, respectively. pJAK2 appeared in the nucleus in association with IFNGR1, which we have previously shown to function as a transcription/cotranscription factor in IFNγ activated genes (Ahmed and Johnson, 2006). This does not preclude activation of JAK2 in the nucleus via nuclear IFNGR1.

In order to determine possible activation of JAK2 in the nucleus in addition to the known activation in the cytoplasm, we treated WISH cells with IFNγ for 10 minutes, isolated the nuclei, and determined if there was an increase in nuclear pJAK2 over time. As shown in Figure 3-2B, pJAK2 was present in the nucleus at 0 minutes of nuclear isolation of IFNγ treated cells by Western blot. The level of nuclear pJAK2 increased at 5 and 15 minutes and decreased at 30 minutes. Thus, there was further activation of JAK2 in the isolated nucleus, which suggests that the nuclear IFNGR1 of IFNγ treated cells was capable of phosphorylation of nuclear JAK2. These activation events are consistent with our demonstration of an IFNγ/IFNGR1 complex in the nucleus of IFNγ treated cells, but are inexplicable in the models where IFNγ treatment of cells results only in the presence of pSTAT1 in the nucleus. It is noteworthy that pSTAT1 levels were maximal at 0 minutes of isolated nuclei and decreased thereafter over time. By comparison, pY41H3 levels increased in nuclei over time. Immunoprecipitation of IFNGR1 from isolated nuclei of IFNγ treated cells showed IFNGR1 and pJAK2 association, which would be expected in nuclear IFNGR1 involvement with nuclear activation of JAK2 (Figure 3-2C). Thus, the increase in pJAK2 appears to be related to

nuclear events other than further nuclear activation of pSTAT1.

pJAK2, IFNGR1, and STAT1 Directly Associate with GAS Promoter Element of Cells Treated with IFNγ

We have previously shown that IFNGR1 and STAT1 directly associate with the GAS promoter element in IFNγ treated cells (Ahmed and Johnson, 2006). In order to verify the association of pJAK2, a biotinylated GAS promoter was generated and incubated with whole cell lysates from WISH cells treated with IFNγ. Following incubation with lysates, the mixture was then incubated with Neutravidin conjugated to agarose. Following washing, the bound proteins were eluted, electrophoresed, and probed with antibodies to pJAK2, IFNGR1, and STAT1. As shown in Figure 3-2D, all these proteins were associated with the GAS promoter of IFNγ treated cells at 30 and 60 minutes. Thus, pJAK2, IFNGR1, and STAT1 are associated with the GAS promoter element only after treatment of cells with IFNγ.

Immunofluorescence of pJAK2 in the Nucleus of Cells Treated with IFNγ

To complement the ChIP analysis and Western blots, we performed immunofluorescence using confocal microscopy on WISH cells treated with IFNγ. Cells were stained for JAK2 and pJAK2. As shown in Figure 3-3A, JAK2 was present throughout the cell, but pJAK2 was absent from untreated cells. By contrast, pJAK2 was observed in the cells after treatment with IFNγ for 30 minutes, with a strong presence in the nucleus (Figure 3-3B). The predominant presence of pJAK2 in the nucleus was also observed at 60 minutes post IFNγ treatment (Figure 3-3C). Thus, similar to ChIP and Western blots, JAK2 was present in both the cytoplasm and nucleus of untreated and IFNγ treated cells. pJAK2 was present in the cytoplasm and nucleus only after treatment of cells with IFNγ.

pTYK2 is Present in the Nucleus of Cells only after Treatment with Type I IFN while total TYK2 is constitutively present

In order to determine if other JAK/STAT utilizing ligands besides IFNγ also induce the nuclear localization of activated JAKs besides JAK2 and JAK1 we characterized the nuclear and cytoplasmic distribution of TYK2 and pTYK2 by sub-cellular fractionation of WISH cells treated with IFNα2 or IFNτ. For both of these ligands we were able to show the constitutive presence of TYK2 in nuclear extracts. However, pTYK2 was present in nuclear lysates only after treatment with either type I IFN (Figure 3-4). Like JAK2, TYK2 also contains a putative classical cationic NLS, which may allow movement in an inactive state between the cytoplasm and nucleus. To ascertain the purity of nuclear fractionation, β-tubulin and β-lamin were again used as markers of nuclear and cytoplasmic fractions, respectively.

To further verify the authenticity of type I IFN signaling, the nucelocytoplasmic profile of pSTAT1, and pSTAT2 were also examined. Figure 3-4 shows that these activated STATs are present in both compartments only after type I IFN treatment in accord with the known signaling mechanism of type I IFN. Because TYK2 is known to associate with the IFNAR1 subunit to activate STAT1 and 2 we also examined its nucleocytoplasmic profile (Figure 3-4). We found that in response to either IFNα2 or IFNτ, IFNAR1 accumulates in the nuclear fraction over a period of one hour, while its cytoplasmic presence decreases.

pJAK2 and IFNGR1 Are Associated with Histone H3 in IFNγ Treated Cells

We showed in Figure 3-1C that treatment of WISH cells with IFNγ resulted in phosphorylation of histone H3 at tyrosine 41. We test here for the association of pJAK2, IFNGR1, and pSTAT1 with histone H3 in IFNγ treated cells. WISH cells were treated

with IFNγ for 30 and 60 minutes and whole cell lysates were immunoprecipitated against histone H3 followed by Western blotting for the indicated proteins. As shown in Figure 3-5, pJAK2, IFNGR1, and pSTAT1 were associated with histone H3 at 30 and 60 minutes of IFNγ treatment, but not to any significant extent in untreated cells. Interestingly, STAT1 protein was associated with histone H3 in untreated cells, decreased at 30 minutes of IFNγ treatment and then increased proportionally to increased pSTAT1. This would suggest that unphosphorylated STAT1 protein is associated with histone H3 in untreated cells, exits after IFNγ treatment, and returns as pSTAT1. This is consistent with studies in *Drosophila* that show that unphosphorylated STAT is present in the nucleus of cells and functions as a heterochromatin stabilizer. Exit from the nucleus or disassociation from histone H3/heterochromatin was associated with heterochromatin destabilization and gene activation (Yan et al., 2011). The association of pJAK2 and IFNGR1 with histone H3 is consistent with pJAK2 phosphorylation of tyrosine 41 on the protein.

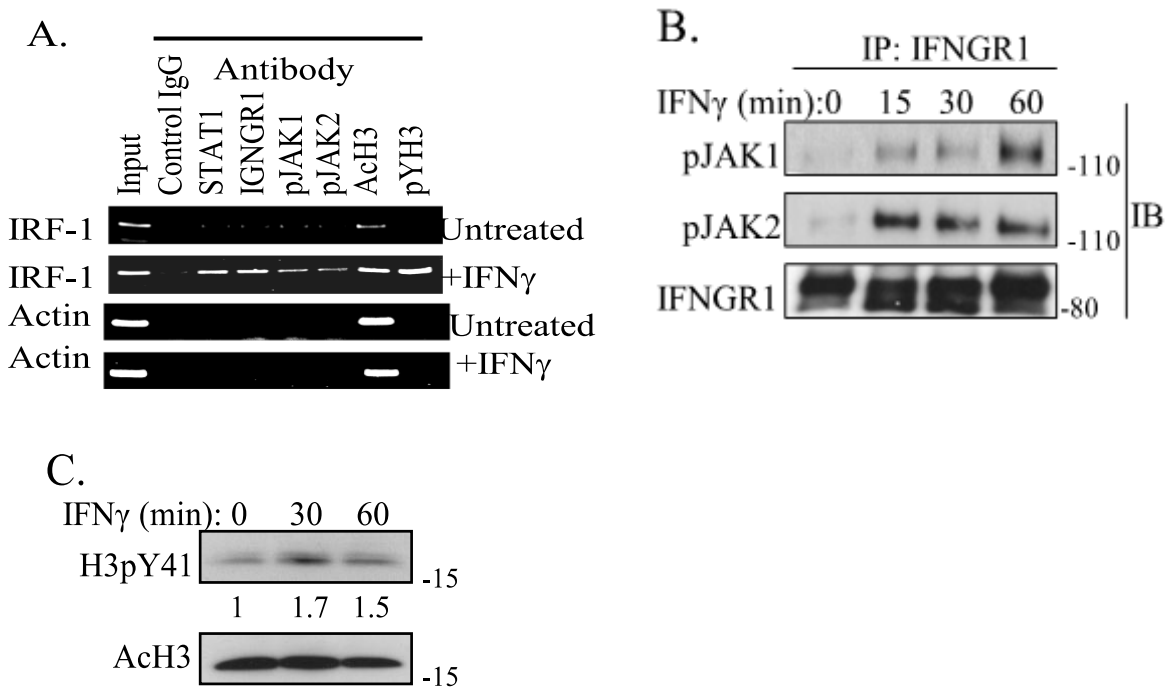

Figure 3-1. IFNγ stimulation induces the association of STAT1, IFNGR1, pJAK1, pJAK2, and H3pY41 with the IRF1 promoter. A) WISH cells were treated with or without IFNγ (250 ng/ml) for 1 hour, then treated with 1% formaldehyde for 10 minutes. Chromatin from cross-linked cells was sheared by sonication and immunoprecipitated with the specific antibodies indicated, followed by incubation with protein A-Sepharose saturated with salmon sperm DNA. The detection of immunoprecipitated IRF-1 or β-actin promoter was conducted by PCR with promoter-specific primers. PCR products were run on a 1.4% agarose gel. B) The IFNγ induced association of pJAK1 and pJAK2 with IFNGR1 correlates with the tyrosine phosphorylation of histone H3. WISH cells were incubated with or without IFNγ (250 ng/ml) for the indicated times, then whole cell lysates were obtained. Equal amounts of protein were immunoprecipitated using IFNGR1 antibody. Immunoprecipitated material was then washed, subject to PAGE, and then Western blotted using the indicated antibodies. C) Phosphorylation of histone H3 on tyrosine 41 (H3pY41) of IFNγ treated cells. Ten μg of whole cell lysates were analyzed by Western blot for H3pY41, then stripped and re-probed for AcH3. Numbers at the bottom of pYH3 blot represent relative intensity of bands as measured by using Image J program downloaded from the National Institute of Health. The results are representative of three experiments.

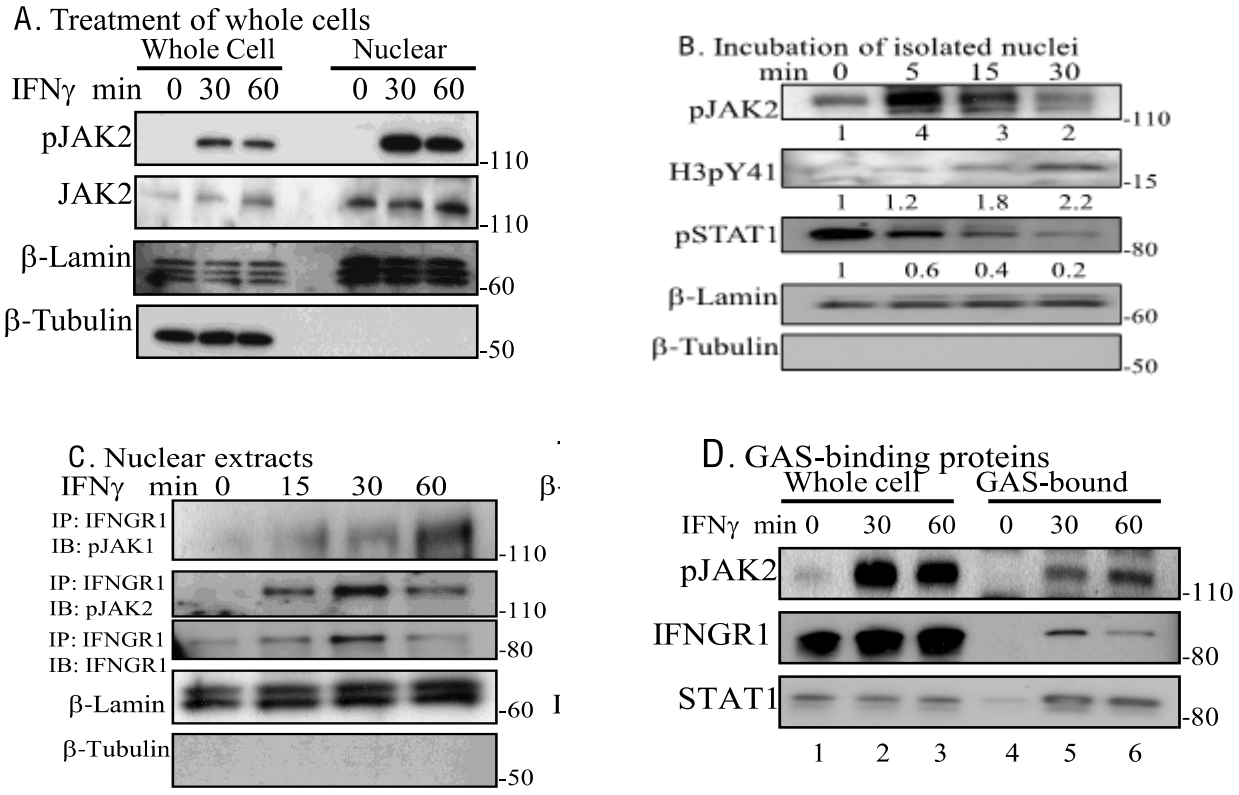

Figure 3-2. JAK2 is constitutively present in the nucleus and is activated upon IFNγ treatment. A) WISH cells were incubated with or without IFNγ (250 ng/ml) for the indicated times, then whole cell (WL) and nuclear lysates were independently obtained (see Materials and Methods), and analyzed by Western blot with the indicated antibodies. B) WISH cells were treated with IFNγ (250 ng/ml) for 10 min and their nuclei were purified (see Materials and Methods). Isolated nuclei were then incubated at 37^{o} C in kinase buffer for the indicated times. Lysates were obtained and subjected to Western blotting for the indicated proteins. Numbers at the bottom represent relative intensity of bands as measured by using Image J program. C) Cells were treated with 250 ng/ml IFNγ for the indicated times and nuclear extracts were immunoprecipitated with antibody to IFNGR1. Eluted proteins were Western blotted with antibodies to pJAK1, pJAK2, and IFNGR1. D) pJAK2, IFNGR1, and STAT1 are associated with the GAS promoter. A biotinylated double-stranded oligomer containing five copies of the GAS element taken from the IRF1 promoter was incubated with equal amounts of whole cell lysates (WL) from WISH cells that were untreated or treated with IFNγ for 30 min or 60 min. This complex was then added to Neutravidin conjugated to agarose (GAS probe). The bound proteins were washed, eluted, electrophoresed, and probed sequentially with antibodies to pJAK2, IFNGR1, and STAT1. The results are representative of at least two experiments.

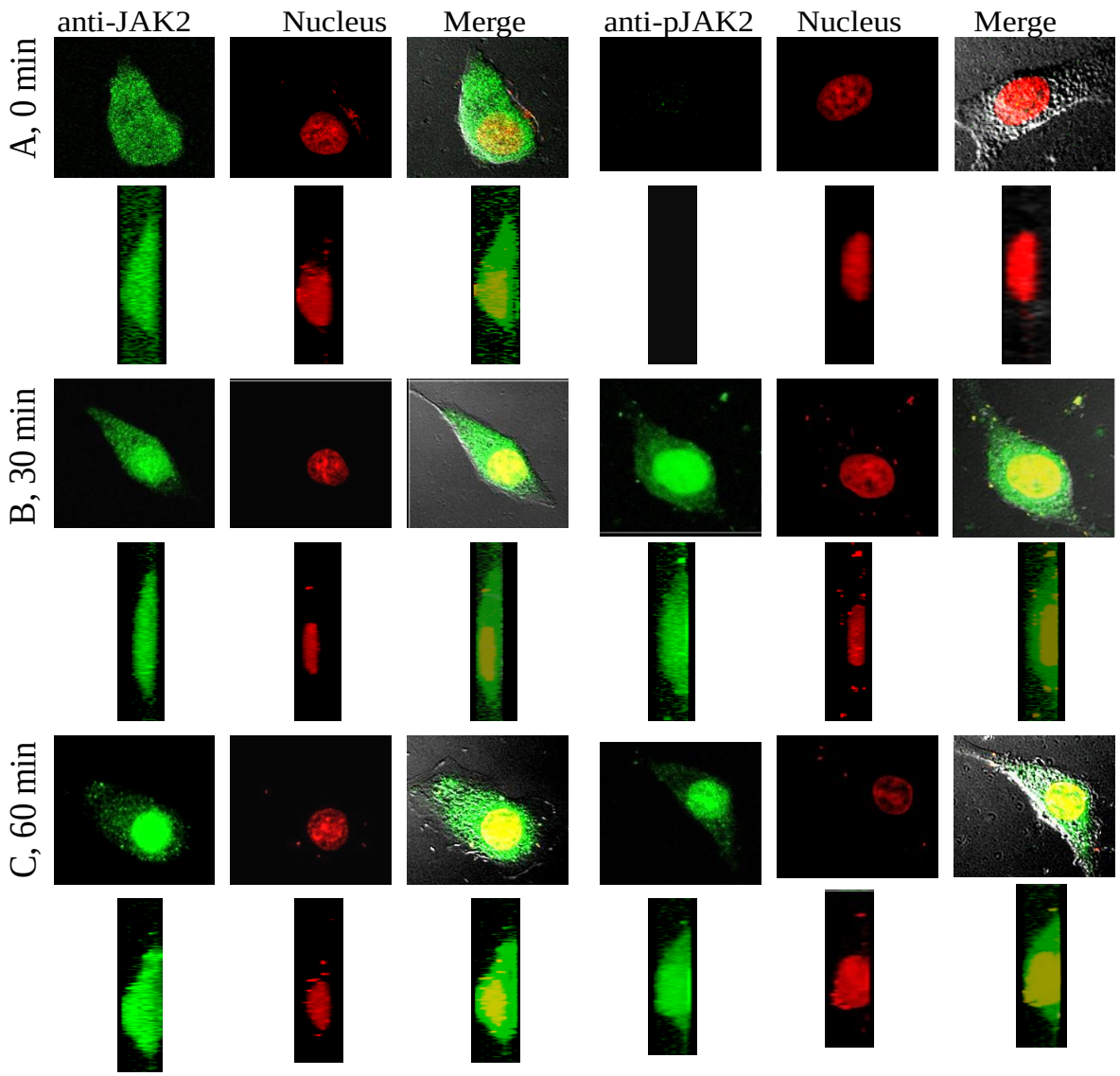

Figure 3-3. JAK2 is constitutively present in nuclei, while pJAK2 appears in nuclei following IFNγ treatment. 2D and 3D volume reconstruction of JAK2 and pJAK2 in WISH cells either left A) untreated or with IFNγ (250 ng/ml) for B) 30 or C) 60 minutes. Following immunofluorescence, images of cells were obtained via confocal microscopy. 3D reconstruction of images from 2D images was done using 17 merged image sections from the stack, 4 μm above and below the focal plane through the nucleus of the cells (a 0.5 μm displacement each along the Z-axis). Sections were merged to render a 3D reconstruction of the cells. The resulting images were projected by rotation at 0° along the X-axis, 90° along the Y-axis, and presented below each un-rotated image. All image processing was done using Pascal (Microsoft) software attached to a LSM 5 Pascal workstation.

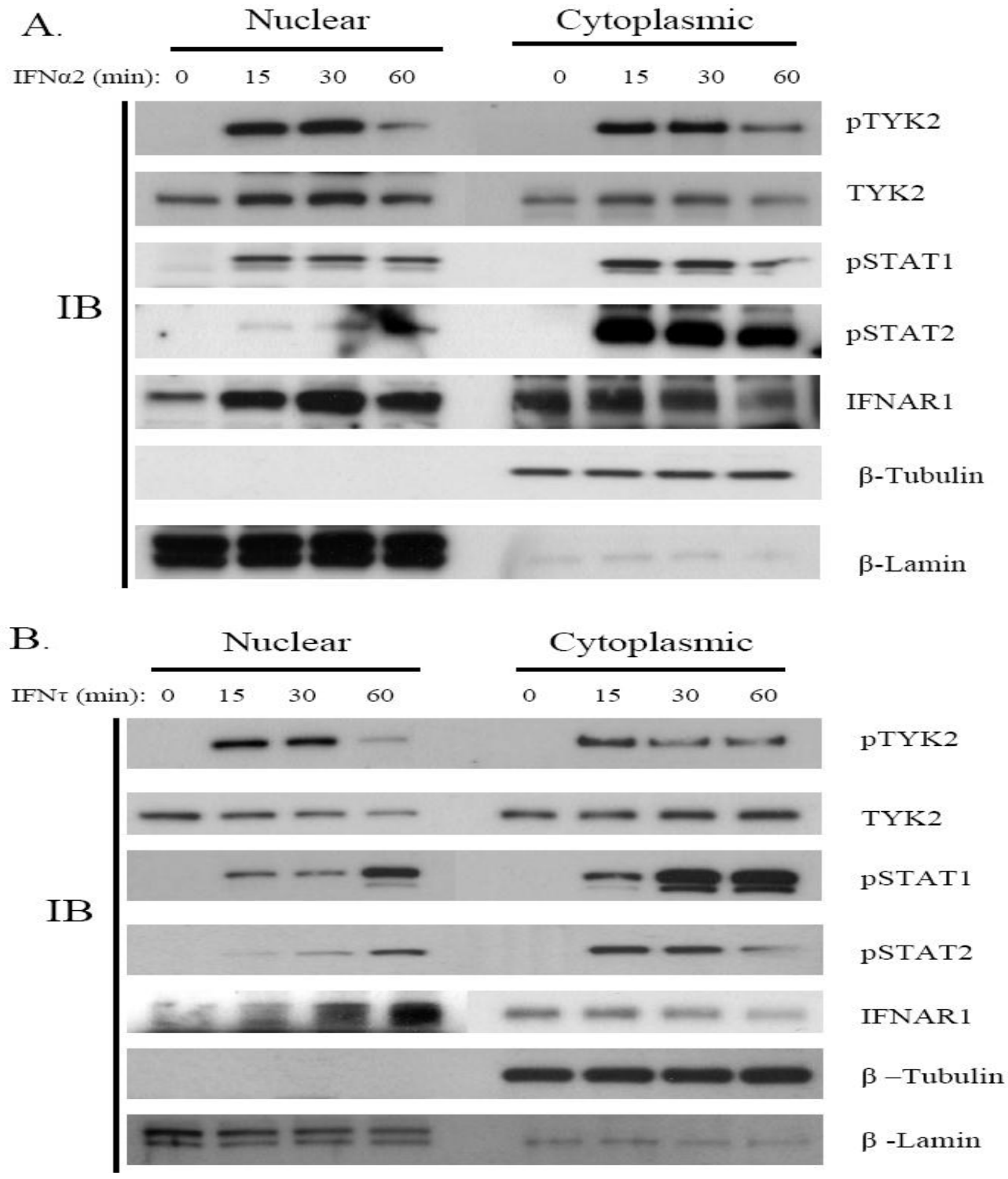

Figure 3-4. TYK2 is constitutively present in the nucleus and is activated upon type I IFN treatment. WISH cells were incubated with A) 10 kU/ml of IFNα2 or B) 500 ng/ml IFNτ for the indicated times, then cytoplasmic and nuclear lysates were obtained and analyzed by Western blot with the indicated antibodies. Results are representative of at least three experiments.

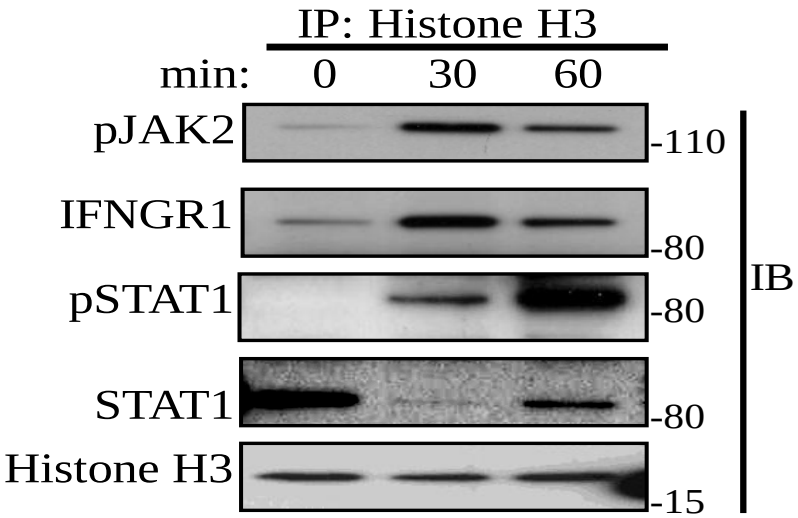

Figure 3-5. pJAK2, IFNGR1, and pSTAT1, are induced to associate with histone H3 in response to IFNγ stimulation while the association of unphosphorylated STAT1 is constitutively associated. Equal amounts of whole cell lysates obtained from WISH cells treated with IFNγ (250 ng/ml) for 0, 30, and 60 minutes were subjected to immunoprecipitation against histone H3 antibody. The bound proteins were washed, eluted, electrophoresed, and probed sequentially with antibodies to pJAK2, IFNGR1, pSTAT1, STAT1, and histone H3. The results are representative of at least two experiments.

CHAPTER 4
DISCUSSION

Our previous studies of IFNγ signaling have focused on events that were critical for the specificity of gene activation. One of the reasons for such studies is the lack of obviousness for specific gene activation in the classical model of the mechanism of JAK/STAT signaling. The STAT family of transcription factors has seven members, which are thought to be the mediators of the unique functions associated with greater than 60 different ligands/receptor systems (Ahmed and Johnson, 2006; Johnson et al., 2004). Activated STAT in the phosphorylated state, pSTAT, uniquely forms homodimers that bind to the response elements in the promoter region of activated genes. Heterodimers are the exception of which pSTAT1/pSTAT2, pSTAT1/pSTAT3, and pSTAT5α/pSTAT5β function as mediators of type I IFN, IL-6, and growth hormone activity, respectively. The sole function of the ligand in this view, is to interact with the extracellular domain of the receptor complex. This in turn results in activation of receptor-associated JAK kinases, whose sole function is activation of the STATs on the cytoplasmic domain of the receptor complex. The receptor complex is ascribed no other relevance to the signaling beyond a platform for STAT activation and activated JAKs are ascribed no other function beyond that in the cytoplasm. Disassociation of the pSTATs from the receptor and subsequent nuclear translocation results in the specific transcriptional events that are associated with the ligand (Borden et al., 2007; Horvath, 2000).

It has recently been acknowledged that the classical model of JAK/STAT signaling was over simplified in its original form (Gough et al., 2008). In the case of IFNγ, complexity beyond simple JAK/STAT activation in signal transduction is indicated

in the relatively recent demonstration that other pathways, including MAP kinase, PI3 kinase, Cam kinase II, and others cooperate with or act in parallel to JAK/STAT signaling to regulate IFNγ effects at the level of gene activation and cell phenotypes (Gough et al., 2008). All of these pathways are generic in the sense that a plethora of cytokines with functions different from those of IFNγ also activate them. Thus, for IFNγ and other cytokines, uniqueness of function would seem to depend on cytokine control of complex and unique qualitative, quantitative, and kinetic aspects of the activation of these pathways. We are not aware that this has been demonstrated for any cytokine.

There is evidence of a functional interaction between STATs in gene activation/suppression, which provide more insight into STAT mediation of cytokine signaling. The induction of IL-17 by activated STAT3, for example, was countered by IL-2 activation of STAT5 (Yang et al., 2011). It was demonstrated by ChIP sequencing that STAT3 and STAT5 bound to multiple common sites across the IL-17 gene locus, including non-coding sequences. Activation of STAT5 by IL-2 resulted in more binding of STAT5 and less binding of STAT3 at these sites, whereas activation of STAT3 by IL-6 induced the opposite; the combination of the two STATs resulted in dynamic regulation of the IL-17 gene locus by the opposing effects of IL-2 (STAT5) and IL-6 (STAT3) (Yang et al., 2011). A similar complementarity was observed with STAT4 and STAT6 with respect to Th1 and Th2 cell development, but with much less competition for binding sites at coding and non-coding regions of the gene (Wei et al., 2010). These Yin-Yang interactions of STAT transcription factors are referred to as specification with respect to lymphocyte phenotypes. Important questions, however, are not addressed with respect to claims of specification and signaling specificity. For example, IL-6, IL-23,

and IL-27 all activate STAT3 and are all involved in Th17 induction/differentiation and function (Batten et al., 2006; Colgan and Rothman, 2006; Stumhofer et al., 2006). Additionally, it has been shown that IL-23 receptor is required for terminal differentiation of IL-17-producing effector T helper cells (McGeachy et al., 2009). Thus, STAT3 does not seem to be the only factor required for activation and generation of Th-17 cells. Rather, the requirements of IL-6 and IL-23 for Th17 induction/differentiation and IL-27 for suppression all involve activated STAT3 mediated through multiple unique ligand/receptor interactions. Interesting and contrary to the above report, it has been demonstrated that IL-2 participates in expansion of Th-17 cells in uveitis and scleritis (Amadi-Obi et al., 2007).

We have previously shown that IFNγ, its receptor subunit IFNGR1, and pSTAT1α are transported to the nucleus as a complex where IFNγ provides a classical polycationic NLS for such transport (Ahmed and Johnson, 2006). The C-terminus of IFNγ, represented by the mouse IFNγ peptide, IFNγ (95-132), was capable of also forming a complex with IFNGR1 and pSTAT1α when introduced intracellularly and provided the NLS signal for nuclear transport. Importantly, mouse IFNγ (95-132) and human IFNγ (95-134) mimetics both induced an antiviral state and upregulation of MHC class II molecules in cells similar to that of full length IFNγ. Both IFNγ and its peptide mimetics bind to an intracellular site, IFNGR1 (253-287), on the cytoplasmic domain of receptor subunit IFNGR1. This binding plays a key role in movement of JAK2 from IFNGR2 to IFNGR1 as it enhances the binding affinity of JAK2 and IFNGR1 (Szente et al., 1995). The activation of JAK2 and JAK1 as a result of this is important for the binding and activation of STAT1α. ChIP assays and receptor gene studies of IFNγ and

IFNγ mimetic treated cells indicate that they, along with IFNGR1 and pSTAT1α, bind to the GAS element of IFNγ activated genes and participate in STAT1α transcription (Ahmed and Johnson, 2006).

It has been suggested that JAK tyrosine kinases play an important role in the epigenetics of gene activation in addition to STAT activation in the cytoplasm (Dawson et al., 2009; Griffiths et al., 2011; Nilsson et al., 2006). Leukemic cells with a JAK2V617F gain-of-function mutation have constitutively active JAK2V617F in the nucleus. This leads to tyrosine phosphorylation on Y41 on histone H3, which results in disassociation of heterochromatin protein 1α, HP1α. The heterochromatin remodeling was associated with exposure of euchromatin for gene activation. Although present in the nucleus, wild-type JAK2 was only activated when K562 cells were treated with PDGF or LIF, or when BaF3 cells were treated with IL-3. The question of how a ligand/receptor interaction resulted in the presence of pJAK2 in the nucleus was not addressed, nor its targeting mechanism to discrete genomic sites and promoters.

We felt that our discovery of the IFNγ-IFNGR1-pSTAT1 complex and its movement to the nucleus provided a logical mechanism for transport of pJAK1 and pJAK2 not only to the nucleus, but also to histone H3 regions of genes activated by IFNγ. Thus, ChIP followed by PCR in IFNγ treated cells showed the association of pJAK1, pJAK2, IFNGR1, and STAT1 on the same DNA sequence of the IRF1 gene promoter. Similar to the pJAK2 findings above, pJAK1 has recently been shown to phosphorylate Y41 on histone H3 in *in vitro* experiments (Griffiths et al., 2011). The β-actin gene, which is not activated by IFNγ, did not show the above associations. These findings were confirmed by biotinylated GAS promoter binding and confocal microscopy.

Consistent with leukemic cell studies, the presence of activated JAKs in the nucleus was associated with phosphorylated Y41 on histone H3 in the region of the GAS promoter. Cumulatively we present a model in which the IFNγ-IFNGR1-pSTAT1-pJAK1-pJAK2 complex translocates to the nucleus and targets the histone H3 surrounding the GAS element (see Figure 4-1).

Recent studies in *Drosophila* have shown that unphosphorylated STAT is associated with HP1α and plays a role in heterochromatin stability (Yan et al., 2011). Further, activation/phosphorylation of STAT to pSTAT causes it to disassociate from heterochromatin and bind to cognitive sites in euchromatin. Moreover, these events correlate with unphosphorylated STAT association with stable heterochromatin and gene silencing, while pSTAT was associated with heterochromatin destabilization and gene expression. It has been reported that unphosphorylated STATs 1 and 3 function as transcription factors by mechanisms distinct from those of phosphorylated STATs (Cheon et al., 2011). The unphosphorylated STATs have been proposed to be involved in prolonged transcriptional events of several days. The relationship of these unphosphorylated STAT transcriptional activities, unlike the results reported here and in Drosophila, were not studied in the context of epigenetic events. Similar to the *Drosophila* results, we showed that unphosphorylated STAT1α was associated with histone H3 in untreated WISH cells. Treatment of the cells with IFNγ resulted in the disassociation of unphosphorylated STAT1 from histone H3 and its return, possibly at a different site in the activated pSTAT1 form. These observations are consistent with regulated epigenetic events in the region of genes that are activated by IFNγ. Thus, we propose that the complex of IFNγ-IFNGR1-pSTAT1-pJAK1-pJAK2 contains the

transcription/co-transcription signals for specific gene activation as well as the activated JAK activity for the associated epigenetics of histone H3 phosphorylation. We estimate that the aggregate molecular weight of the complex to be approximately 530 kDa, a feasible cargo size for active transport through the nuclear pore complex (Lyman et al., 2002). The results of our study provide insight into the mechanism of IFNγ signaling including the role of the JAK/STAT pathway in the specificity of such signaling.

We were further interested to see if other ligands and JAKs beside IFNγ, JAK1, and JAK2 were involved in the nuclear translocation of activated JAKs. Towards this end we used the type I IFNs, specifically IFNα2 and IFNτ, and explored the sub-cellular activation of TYK2. We found that TYK2 was constitutively present in the nuclear and cytoplasmic fractions. However, stimulation with either type I IFN induced activation of TYK2 to pTYK2 in the nucleus and cytoplasm in agreement with previously published results (Ragimbeau et al., 2001).

Because TYK2 is known to associate with IFNAR1 before and after type I IFN stimulation (Ragimbeau et al., 2003) we also examined its nucleocytoplasmic distribution. We found that upon ligand activation, IFNAR1 translocated to the nucleus while its cytoplasmic presence decreased. This is in agreement with our previous finding that IFNAR1 possess a functional NLS and undergoes nuclear translocation upon IFNβ treatment (Subramaniam and Johnson, 2004). The association of IFNAR1 and TYK2 within the nuclear compartment was not addressed in this study, though we speculate its existence together with activated STAT1 and STAT2. Altogether our findings suggest that type I IFN activation of TYK2 to pTYK2 within the nuclear compartment are analogous to type II IFN nuclear activation of JAK2 to pJAK2.

Cumulatively, these results support the notion that ligands that activate JAK/STAT signaling in general cause their respectively activated JAKs to localize in the nuclear compartment where they may play roles in specific epigenetic translational responses.

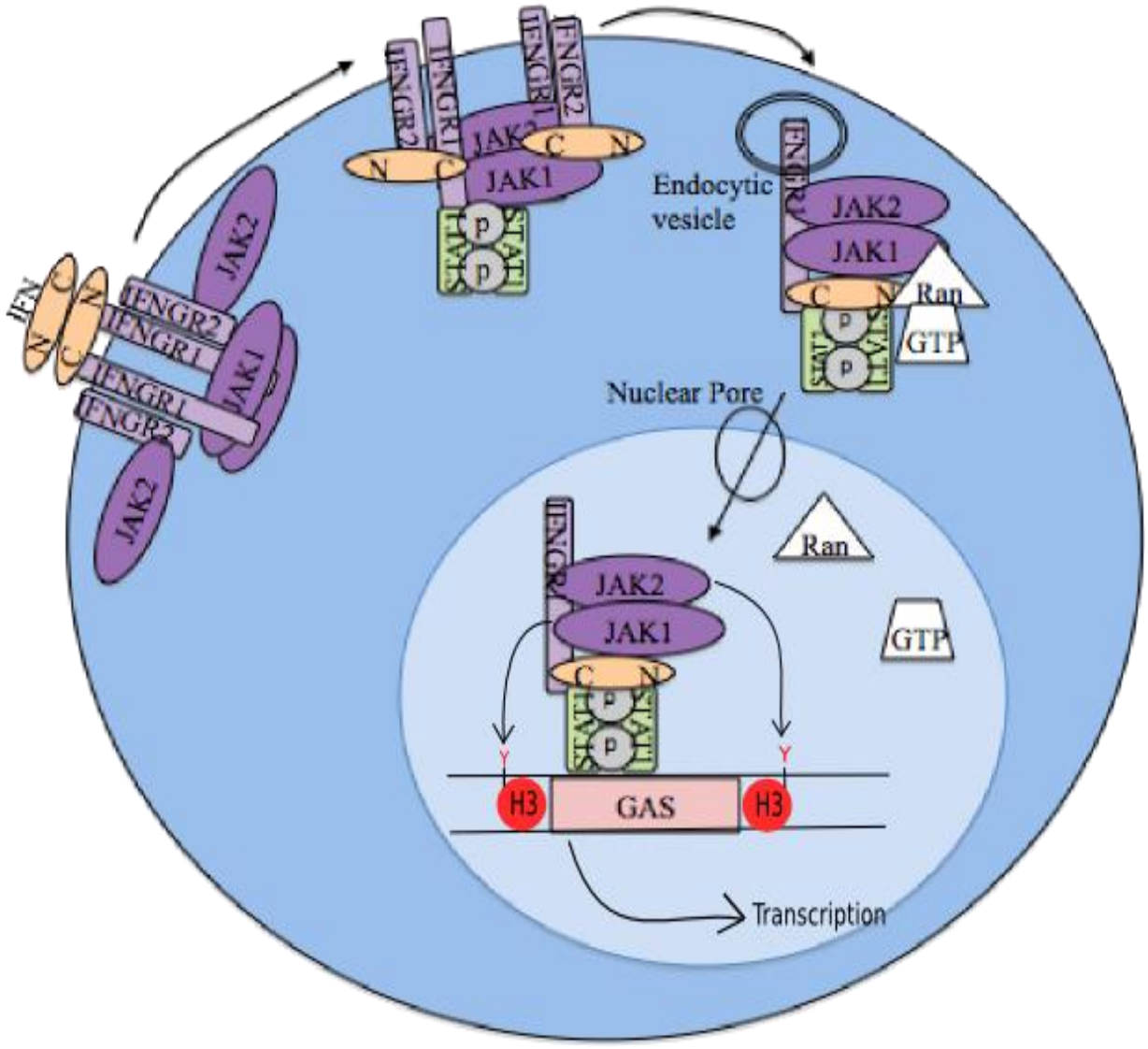

Figure 4-1. Model for the mechanism of IFNγ signaling. IFNγ binds as a dimer to two IFNGRs. Activated JAK1, activated JAK2, activated (P) STAT1 and IFNγ then bind to the intracellular domain of IFNGR1. IFNGR1 is internalized with these cofactors as a single macromolecular complex, and is imported into the nucleus via the Ran/importin pathway using the NLS found on IFNγ. Within the nucleus the receptor complex targets the GAS element using the DNA binding domain of the STATs. Finally, JAK1 and JAK2 phosphorylate adjacent histone H3 on residue 41 allowing for gene activation.

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BIOGRAPHICAL SKETCH

Ezra Neptune Noon-Song was born in Chicago, Illinois on February 28, 1984, three hours before the 29th. He lived the first four years of his life there before moving to Miami for the next fourteen years. After waiting tables for several years, he earned his High School diploma and went to Vassar College where he earned a Bachelors of Science in chemistry. Feeling a pull towards the biological sciences, he decided to follow his mother's advice and go to gradate school under Dr. Howard Johnson. After several painful years, he learned that suffering is part of the process that makes us stronger, smarter, and better people. Now that his PhD is acquired, he can venture forth into the postdoctoral wilderness to satisfy his scientific wanderlust.