ROLES OF JAK2 IN LEPTIN ACTION: FROM MOLECULAR SIGNALING TO PHYSIOLOGY

by

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CHAPTER 1

INTRODUCTION

Overview of leptin action

In mammals, the brain is a primary sensor and integrator of metabolic homeostasis. Given the multitude of specialized tissues in higher organisms, the overall metabolic status of the organism must take into account the status of several key tissues -- including adipose, liver, pancreas and gut—and the quantity of nutrients currently circulating in the bloodstream. The brain is well-suited to do this. Indeed, various neuronal populations in the CNS directly sense levels of circulating nutrients while other neurons are sensitive to circulating hormones produced by peripheral tissues. Integration of this information allows the brain to act appropriately to maintain homeostasis.

One important peripheral hormone is leptin. Leptin is adipose tissue-derived and is produced in proportion to fat stores. Circulating leptin communicates the level of energy reserves in the periphery to the central nervous system. High levels of leptin tell the brain that energy stores are sufficient. This suppresses food intake, permits energy expenditure and allows activities of high energy investment to proceed, like reproduction and growth [6,41,47,99]. Conversely, lack of leptin signaling due to mutation of leptin (e.g.

Lep$^{ob/ob}$ mice) or the leptin receptor (LepR) (e.g. LepR$^{db/db}$ mice) in rodents and humans results in increased food intake in combination with reduced energy expenditure and thus obesity, hyperglycemia and insulin insensitivity. Leptin deficiency also results in neuroendocrine dysfunction (including hypothyroidism, decreased growth, infertility and decreased immune function) [26,42,47,97]. Most of the effects of leptin are attributable to effects in the CNS, particularly in the hypothalamus, a site of high leptin receptor mRNA expression [41,99].

LEPTIN SIGNALING

The leptin receptor

Leptin possesses a four-helix-bundle structure characteristic of the class-I family of cytokines [87,136]; LepR, similarly, represents a typical class-I cytokine receptor [60]. Alternative splicing generates several isoforms of LepR with identical ligand binding domains, but which possess differing perimembrane and intracellular domains [79]. Membrane-bound LepRs consist of long (LepRb) and short (LepRa, among others) isoforms. LepRb features an approximately 300 amino acid intracellular tail that contains several docking sites for proteins critical for signal transduction. In contrast, short-form receptors contain 30-40 amino acid intracellular domains lacking these docking sites and which play no major role in signal transduction. Consistent with the unique presence of signaling moieties on LepRb, *db/db* mice that lack only LepRb phenocopy leptin-deficient *ob/ob* animals [79]. Potentially important roles for membrane bound short-form

receptors include the endocytosis and transport of leptin across the blood-brain barrier [127]. Alternative splicing and proteolytic cleavage events also produce circulating extracellular domain of LepR, which may affect the stability and/or availability of circulating leptin [52].

LepRb and Jak2

Like other class-I cytokine receptors, LepRb has no intrinsic enzymatic activity; propagation of downstream leptin signals requires the LepRb-associated tyrosine kinase, Jak2 (Fig 1) [75]. Membrane-proximal residues on the intracellular tail of LepR, including the proline-rich Box 1 motif and the downstream Box 2, mediate Jak2 interactions. While LepRa and other short forms contain Box 1, they do not possess Box2 and poorly interact with Jak2 [75].

Upon leptin binding, Jak2 is activated by autophosphorylation on two key tyrosines residues in the activation loop of the enzyme [23,44]. Activation in this manner is common throughout the kinome. Phosphorylation of the activation loop causes a shift from an inactive conformation to a conformation that locks key catalytic residues in the active state [107]. While the precise mechanism by which leptin binding triggers Jak2 activation is not completely understood, a growing body of work supports a model in which leptin binding causes LepR monomers to dimerize or oligomerize in such a way as to bring their constitutively-bound Jak2 molecules close to one another and phosphorylate each other *in trans*. Interestingly, forced dimerization of Jak2 molecules is sufficient for their activation [96]. Additionally, work with other class-I cytokine receptors

demonstrates that ligand binding brings the intracellular tails of the cytokine receptor, and their associated Jak2 molecules, in close proximity to one another [110]. Lastly, work using chimeric leptin receptors show that forced dimerization of LepRb intracellular domains is sufficient for Jak2 activation and downstream signal transduction [132].

LepRb tyrosine phosphorylation mediates important downstream signals

Once activated, Jak2 phosphorylates all three conserved tyrosine residues (Tyr_{985}, Tyr_{1077}, and Tyr_{1138}) on the intracellular tail of LepRb, promoting their recruitment of downstream signaling proteins (Fig 1). The family of signal transducers and activators of transcription (STATs) represent the canonical Jak2/cytokine receptor-dependent signaling proteins. These latent transcription factors are recruited to activated cytokine receptor/Jak kinase complexes, whereupon their phosphorylation stimulates their nuclear translocation and transcriptional activation. LepRb recruits multiple STAT isoforms, and leptin-stimulated phosphorylation and activation of STAT3 and STAT5 have been demonstrated in vivo. LepRb Tyr_{1138} lies in a consensus YXXQ binding site for STAT3 [8], and is the sole residue responsible for the activation of STAT3 during leptin action.

The physiological importance of Tyr_{1138} has been demonstrated using a knock-in mouse model in which LepR mutant for Tyr_{1138} replaces endogenous LepRb ($LepR^{Y1138S}$). These mice (*s/s*) are hyperphagic and obese but lack some of the phenotypes that characterize the *ob/ob* and *db/db* models including severe

diabetes and infertility [5,7]. Furthermore, some leptin actions, including some aspects of immune function, are enhanced in Tyr_{1138}-mutant mice compared to wild-type mice. Thus, while Tyr_{1138}→STAT3 LepRb signaling is crucial for the regulation of energy homeostasis, other LepRb signals also contribute to leptin action, and Tyr_{1138} (along with Tyr_{985}) may attenuate LepRb signaling in vivo.

Phosphorylation of Tyr_{985} recruits at least two different proteins: SHP-2 (SH2-domain containing phosphatase-2), and SOCS-3 (suppressor of cytokine signaling-3) (the consensus phosphotyrosine recognition sequences of these two proteins are very similar) [31]. SHP-2 binds to phosphorylated Tyr_{985} [22,83] to mediate the activation of ERK in cultured cells [12]. SOCS-3, a member of a family of SOCS-box containing proteins that attenuate cytokine signaling, mediates feedback inhibition of LepRb signaling by binding to Tyr_{985} [21].

In order to understand the contribution of LepRb Tyr_{985} to leptin action and inhibition in vivo, we generated mice in which LepRb was homologously replaced by a mutant containing a substitution of Tyr_{985} that abrogates phosphorylation of the site and blocks interaction with SHP2/SOCS3 ($LepR^{Y985L}$) [3,12,13] [14]. Homozygous $LepR^{Y985L}$ mice (*l/l*) have reduced feeding and adiposity, decreased orexigenic arcuate (ARC) neuropeptide expression, and increased baseline STAT3 activation in female *l/l* mice- all in the face of low leptin levels. Coupled with the increased sensitivity of *l/l* animals to exogenous leptin, these observations suggest that mutation of Tyr_{985} blocks the activation of an inhibitory Tyr_{985}-dependent LepRb signal, ultimately leading to increased leptin sensitivity in vivo. These results suggest an important role for Tyr_{985} in the attenuation of

leptin action in vivo, consistent with results from cultured cells suggesting an important role for Tyr_{985} in the inhibition of LRb signaling [13,20,128].

Since Tyr_{985} of LepRb recruits both SHP-2 and SOCS3 [13,20,83], the failure of $LepRb^{L985}$ to recruit either of these proteins could theoretically underlie the lean, leptin-sensitive phenotype of *l/l* mice. Most data from cultured cells and animals support a primary role for SOCS3 in the inhibition of LRb signaling, however [11-13,37,59,98,135]. Haploinsuffiency of SOCS3 leads to increased leptin sensitivity and attenuation of diet-induced obesity while deletion of SOCS3 from POMC neurons has similar effects [59,69].

The phosphorylation of LepRb Tyr_{1077} has been demonstrated only recently; indeed, it was not clear initially that this site was phosphorylated, since mutating Tyr_{985} and Tyr_{1138} eliminate all immunoreactivity of LepRb with standard anti-phosphotyrosine antibodies [3,83]. The demonstration of a role for Tyr_{1077} in the activation of STAT5 prompted us to re-examine this residue using a site- and phosphorylation state-specific antibody, revealing its ligand-dependent phosphorylation [55]. The role for this site in the regulation of physiology is not yet clear.

While Tyr_{1077} mediates the majority of LepRb-dependent STAT5 phosphorylation and transcriptional activation, Tyr_{1138} also contributes to the phosphorylation of STAT5- although the importance of Tyr_{1138} in the transcriptional activation of STAT5 is unclear. LepRb activation initiates feedback inhibition of leptin signaling by promoting the transcription of SOCS-3 in a Tyr_{1138}/STAT3-dependent manner. Thus, mutation of Tyr_{1138} eliminates

LepRb-mediated SOCS-3 accumulation and blocks the attenuation of Jak2 and STAT5 signaling during prolonged receptor stimulation [37].

Other downstream events

While STAT3, STAT5, and ERK activation can be conveniently ascribed to specific phosphorylation sites on LepRb, some downstream effects are, to date, less well understood. These signals include activation of phosphoinositide-3 kinase (PI3-K), the regulation of mammalian target of Rapamycin (mTOR), and the inhibition of the AMP-activated protein kinase (AMPK) in the arcuate nucleus of the hypothalamus (ARC). The integrity and activity of these pathways play a crucial role in leptin action in vivo. For instance, leptin increases PI3-K activity in the ARC, and PI3-K inhibitors administered directly to the brain abrogate leptin-mediated changes in food intake and glucose homeostasis [46,100,104]. The signaling events linking LepRb activation to PI3K activation remain unclear, though some studies suggest roles for the insulin receptor substrate- (IRS)-proteins in the regulation of PI3-K activity during leptin signaling [104]. Furthermore, at least in cultured cells, the Jak2-interacting protein SH2B1 interacts with both Jak2 and IRS-2 and promotes IRS-1/2 mediated activation of PI3-K in response to leptin [34]. While this may not be the only protein connecting LepRb/Jak2 activation to PI3-K activation, SH2B1 loss-of-function does lead to significant metabolic defects, consistent with a role for SH2B1 in leptin action. [111,112].

Recently, leptin has been linked to mTOR, a kinase known to regulate cell size and global protein synthesis by integrating a variety of cellular inputs, including nutrient availability, growth stimuli, and energy status [90]. Activation of mTOR by these signals causes mTOR to phosphorylate its substrates, including S6K and 4E-BP, events necessary for protein synthesis and cellular growth. Active forms of mTOR and its substrate, S6K, are present in many POMC and AgRP neurons in the hypothalamus [28]. mTOR substrates are sensitive to the energy state of the animal and can be regulated by direct administration of L-leucine into the brain. Leptin causes an increase in activated mTOR substrates and, interestingly, rapamycin, a specific inhibitor of mTOR, abrogates leptin-mediated decreases in food intake. This seems to suggest that leptin is an upstream regulator of mTOR and that mTOR action is necessary for some of leptin's effects. However, this data may be interpreted in other ways. mTOR is not the only kinase that is known to regulate S6K and leptin-stimulated increases in phosphorylated S6K may not be regulated through mTOR. ERK-pathway regulated ribosomal S6 kinase (Rsk) pathway may regulate S6K activation apart from mTOR [115,116]. Also, while mTOR seems necessary for the anorectic effects of centrally-administered leptin, it may be only one of a number of converging signals that are necessary for this effect. In other words, mTOR may function in a parallel pathway to leptin-signaling, not necessarily downstream of leptin.

Leptin signaling has also been linked to AMPK. In some ways AMPK is similar to mTOR. Both are evolutionarily-conserved, nutrient-sensitive kinases that integrate energy status signals. However, the regulation of AMPK is

opposite that of mTOR. AMPK is an ATP-sensor and is active in states of low energy whereas mTOR is active in states of nutrient availability. AMPK acts by phosphorylating proteins that activate ATP-generating or conserving pathways in the cell. While AMPK is well-known as an important player in cellular energy management, recent studies suggest a more global role for AMPK in managing whole-body energy status. In vivo studies using non-targeted approaches suggest AMPK may integrate a variety of energy or hormonal signals in key areas of the hypothalamus, including leptin. Leptin decreases the activity of hypothalamic AMPK. However, leptin *increases* AMPK activity in skeletal muscle [95]. These discrepancies may indicate that the effects of leptin on AMPK are not cell-autonomous and indirect. Cell culture studies also give a mixed picture of AMPK regulation by leptin. Cell studies using insulinoma cell lines expressing exogenous leptin receptor did not detect regulation of AMPK by leptin [57]. Conversely, leptin activates AMPK in a hepatocyte cell line in a Jak2-dependent fashion[126].

Jak family kinases

In mouse and human, the Jak kinase family contains four members: Jak1-Jak3 and Tyk2 [51,63]. Of these, Jak1, Jak2 and Tyk2 are ubiquitously expressed, while Jak3 is found predominantly in immune and hematopoeitic tissues. Jak1 null mice survive birth but die perinatally from severe combined immunodeficiency disorder (SCID). Jak3 null mice also have SCID, but are more viable than Jak1 null mice. Mice lacking functional Tyk2 have the most-mild

phenotype, with defects in IL-12 signaling which causes some increased susceptibility to viral infection.

Unique amongst this family, Jak2 is essential for life as total body Jak2 knockout in the mouse results in embryonic lethality at E12.5 from defective hematopoesis [102,108]. Consistent with Jak2's indispensability, Jak2 mediates signaling through a wide variety of cytokine receptor pathways. These include receptors for leptin, erythropoietin, prolactin, thrombopoetin, growth hormone, granulocyte macrophage colony stimulating factor, IL-3, and IL-5 [105]. Jak2 also mediates signaling through the interferon gamma receptor, which is in the Type II family cytokine receptor family and is important for viral immunity.

Jak family members are approximately one thousand amino acids in length and contain seven so-called Jak homology domains (JH1-7). These homology-based domains span four putative functional domains: the FERM domain, the SH2-like domain, the pseudokinase domain, and the kinase domain. Solving the structure of full-length Jak2 has proven difficult, although the structure of the kinase domain expressed alone has been determined [86]. In the absence of a full structure, one research group approximated the structure of full-length Jak2 by modeling its individual domains based on their homology with similar domains of known structure[53].

Jak2 domains

The four functional domains of Jak2 (beginning with the NH_2-terminus) are the FERM domain, the SH2-like domain, the pseudokinase domain and the

kinase domain (Fig 2). The FERM domain (an abbreviation of Band 4.1, Ezrin, Radixin, Moesin) occupies the N-terminal side of the protein and mediates binding to cytokine receptors. C-terminal to the FERM domain, the SH2-like domain lies near the center of the protein. Interestingly, the sequences of Jak kinases diverge significantly in a normally highly conserved area of the SH2 motif. Indeed, this domain does not exhibit classical phosphotyrosine binding properties in Jak family members. This domain may have important functions, however, as deletion impairs protein function by disrupting interactions with the cytokine receptor [84].

The pseudokinase domain is highly conserved among the Jak family and, while catalytically inactive, plays a critical role in kinase regulation. In the absence of cytokine stimulation, the pseudokinase domain inhibits the neighboring kinase domain. Ligand binding relieves this influence and allows full activation of the kinase. Deletion of this domain in Jak2 results in constitutively active protein. Additionally, point mutations in functionally critical residues also result in highly active Jak2, presumably by disrupting inhibitory interactions with the kinase domain.

The kinase domain lies at the C-terminus of the protein and is also highly conserved among Jak family members. The conformation of the so-called 'activation loop' controls the activity of the ATPase domain, a method of regulation shared by a substantial number of protein kinases [107]. In its unphosphorylated state, the activation loop is disordered and blocks access to the catalytic cleft. Phosphorylation on key residues ($Tyr_{1007/8}$ in the case of Jak2)

changes the loop's conformation and stabilizes the active conformation of catalytic residues. For Jak2, transphosphorylation of $Tyr_{1007/8}$ represents a critical event which must occur for Jak2 activation. Indeed, mutation of these residues results in a kinase-dead protein [44].

Regulation of Jak2 by multiple phosphorylation events

Following the initiation of kinase activity, Jak2 rapidly becomes highly phosphorylated on numerous tyrosine residues, events which mostly represent Jak2 autophosphorylation events. Jak2 contains forty-nine tyrosine residues and work over the past ten years have shown a substantial portion of these become phosphorylated on active Jak2 and are important in regulating Jak2 activity. These phosphorylation events can be stimulatory or inhibitory and some mediate binding with other proteins [2,43,49,50,54,64,76,91,93,94].

Interestingly, the phosphorylation sites that positively impact the activity of Jak2 reside only in the kinase and pseudokinase domain--perhaps indicating the importance of these domains for the full activation of kinase activity or the dispensability of the phosphorylation in the other domains of Jak2 for this purpose. Among the tyrosine sites that negatively influence Jak2 activity, each are phosphorylated in response to cytokine stimulation and are likely function as feedback inhibitors to restrain maximal kinase activity or to return activated Jak2 to its inactive state. For example, Tyr_{119} is phosphorylated in response to cytokine stimulation, and causes Jak2 to dissociate from Type I cytokine receptors [48]. Lastly, Ser_{523} is the only site of serine phosphorylation to be

mapped on Jak2. It is not regulated by cytokine stimulus but is constitutively phosphorylated on Jak2, consistent with a role in providing a basal inhibitory tone [65].

There are three phosphorylation sites that, thus far, have been shown to mediate binding with proteins containing phospho-tyrosine binding SH2 domains. Phosphorylation on Tyr_{813} mediates interaction with SH2B1 via its SH2 domain. Tyr_{813} is phosphorylated in response to cytokine stimulation and SH2B1 binding augments the activity of Jak2 [76]. Tyr_{201} of Jak2 mediates binding with SHP-2 in the context of angiotensin signaling [54]. Substitution of phenylalanine at this position abrogates angiotensin II-induced upregulation of Jak2 and STAT signaling. Though, it should be noted that the angiotensin receptor is not a Type I or Type II cytokine receptor and the regulation of Jak2 activity by Tyr_{201} might be limited to this context. Lastly, Tyr_{1007}, in addition to its critical role in Jak2 activation, also acts as a binding site for SOCS-1, an inhibitor of cytokine signaling[134].

Clinical manifestations of Jak2 dysregulation

Given Jak2's prominence in hematopoesis, it is not surprising that aberrant Jak2 activity manifests itself in the blood cell compartment. Jak2 is the causal factor in a subset of acute lymphoblastic leukemia (the earliest report of Jak2-caused disease) [78]. In these cases, a chromosomal translocation between chromosomes 9 and 12 creates a fusion protein between the homodimerization domain of TEL (an ETS family transcription factor) and the kinase domain of

Jak2. The combination of homodimerization (which promotes the transphosphorylation and activation of Jak2 monomers) and the lack of the inhibitory pseudokinase domain of Jak2, results in a constitutively active Jak2 conferring cytokine-independent growth to cells that express it.

In 2005, two research groups discovered that mutations in the Jak2 gene cause most cases of myeloproliferative disorder (MPD), which are a collection of similar diseases: polythemia vera, essential thrombocytosis and myelofibrosis [66,82]. The majority of these cases have an identical somatic mutation, a phenylalanine substitution at Val_{617}, which lies in the pseudokinase domain of Jak2. Predictive models of kinase/pseudokinase domain interactions suggest that this residue projects into the interface between the two domains and that substitution of phenylalanine is disruptive and relieves the pseudokinase domain's inhibitory influence. Interestingly, no alternative mutations have been detected for this codon even though studies in cultured cells show that other large non-polar residues are capable of transformation [38].

Other mutations involving the pseudokinase domain have been detected in patients with myeloproliferative disorder, including the T875N mutation and some exon 12 deletions, though both of these are much less common than the Phe_{617} substitution [82]. Interestingly, although these mutations always occur sporadically (they are never found in the germline), there is strong genetic influence in the occurrence of MPD. Recently, several groups identified a particular Jak2 allele that confers susceptibility to the 617 mutation from a presumed cis-acting element [67,70,106]. The mechanism by which a genetic

element could predispose toward such a specific, somatic mutation in Jak2 is not known.

LEPTIN PHYSIOLOGY

Leptin regulates physiology via LepRb in the brain

LepRb-expressing neurons in the brain mediate most leptin action as LepRb replacement in a *db/db* mice using a neuron-specific promoter completely rescues their obesity, diabetes and infertility [30]. The largest populations of LepRb neurons reside in hypothalamic nuclei, including the arcuate (ARC), dorsomedial (DMH), ventromedial (VMH), lateral hypothalamic area (LHA), and ventral premammillary (PMv) nuclei. Additional important populations of LepRb neurons reside outside the hypothalamus, however, including in the ventral tegmental area (VTA), brainstem, periaqueductal gray matter and elsewhere [4,39,40,80].

LepRb signaling modulates neuronal function through at least two major mechanisms. First, signaling modulates the expression of neuropeptides. Second, leptin can modulate the neuron's electrical activity. In the ARC, two well-characterized populations of neurons express LepRb (although others likely exist, as well): one population synthesizes orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) and the other synthesizes the anorexigenic pro-hormone pro-opiomelanocortin (POMC) [40,117]. Leptin activates/depolarizes LepRb/POMC neurons and increases POMC synthesis [29,117] to decrease appetite and increase energy expenditure by activating the melanocortin—3 and

-4 receptors [18,19,24,62,89,120]. By contrast, leptin inhibits NPY/AgRP neurons and suppress expression of these orexigenic (appetite-stimulating) neuropeptides [29,117]. Collectively, leptin/LepRb modulate these ARC neurons to respond to alterations in energy homeostasis. When energy stores are high and leptin is abundant, LepRb signaling stimulates the production of anorectic POMC and suppresses levels of orexigenic AgRP and NPY. Conversely, decreased or deficient leptin activity (e.g. during starvation and in Lep$^{ob/ob}$ and LepR$^{db/db}$ mice) stimulates appetite by suppressing synthesis of anorectic neuropeptides and increasing expression of orexigenic peptides. While the POMC and NPY/AgRP neuronal populations are important, simultaneous deletion of LepRb from both of these population results in relatively mild obesity compared to *db/db* animals demonstrating the existence of other neuron populations that remain sensitive to leptin and can compensate for POMC and NPY/AgRP leptin insensitivity [129].

Mechanisms of neuropeptide and electrical regulation

The pathways by which LepRb action regulates neuropeptide expression and electrical activity are complex and somewhat different between populations of LepRb-expressing cells. With respect to neuropeptide regulation, STAT3 and FOXO1 are major mediators. FOXO1 is a forkhead-family transcription factor and regulates gene expression in a PI3-K dependent manner. Phosphorylation of FOXO1 by Akt leads to its nuclear exclusion and proteosomal degradation [92,124]. The links between LepRb and FOXO1 are not well-understood but leptin

does activate PI3-K in the hypothalamus, the upstream regulator of FOXO1 [103,104].

A subset of neurons in the ARC coexpress LepRb and POMC and leptin increases the transcription of POMC [25]. The POMC promoter contains several STAT3 binding sites and studies both in cell culture and in vivo demonstrate STAT3 is a key POMC transcription factor [7,16,73,101]. Alternatively, FOXO1 negatively regulates the expression of POMC and deletion of Akt from POMC neurons drastically reduces POMC expression [9]. These studies suggest a model of dual-regulation of POMC by STAT3 and FOXO1 in which each transcription factor competes for binding sites on the POMC promoter [9,71,74].

In contrast to POMC, leptin decreases the expression of NPY and AgRP, both orexigenic peptides. A subset of ARC neurons coexpress LepRb, NPY and AgRP and the down-regulation of these neuropeptides by leptin share some similarities [56]. FOXO1 binds both the NPY and AgRP promoters and stimulates them both [72]. The role of STAT3 in the regulation of AgRP and NPY is more unclear. Ablating STAT3 activation in LepRb-expressing cells results in a partial rise is AgRP expression suggesting that STAT3 partially regulates AgRP [7]. However, another report shows that STAT3 is not necessary for the normal regulation of AgRP[68]. For NPY, ablating STAT3 activation in LepRb-expressing cells does not change NPY expression suggesting that STAT3 is not necessary for NPY regulation [7].

With respect to electrical activity, leptin depolarizes POMC neurons and hyperpolarizes NPY/AgRP neurons. While the pathways linking LepRb to

electrical modulators are mostly unknown, PI3-K activity plays an important role in some leptin-responsive neurons. PI3-K activity is necessary for depolarization via a non-specific cation channel in POMC neurons[58]. However, leptin action in NPY/AgRP does not involve PI3K, and hyperpolarizes these neurons through K_{ATP} channel opening [133]. Leptin also modulates intracellular calcium concentrations in these cell populations; it causes increased Ca^{2+} concentrations in POMC neurons but lower concentrations in NPY/AgRP neurons [131].

Leptin regulation of energy homeostasis

Db/db mice have much lower energy expenditure than normal. Without leptin, the brain perceives a state of starvation and takes action to conserve energy. VO_2 is a measurement of that rate of oxygen consumption and is a direct reflection of total metabolic output. Leptin-deficient mice have lower VO_2 levels than their wild-type counterparts. Several different factors contribute to decreased energy expenditure in leptin-deficiency. Uncoupling protein 1 (UCP1) is expressed in the brown fat and is important for non-shivering thermogenesis in mice. Leptin-deficiency causes reduced expressing of this protein and lower core body temperature. Furthermore, leptin is critical for maintaining sympathetic tone and leptin's absence causes reduced sympathetic output. Leptin deficient animals also exhibit less locomotor activity, and remaining skeletal muscle movement are more efficient and energy-conserving [114]. Again, a combination of all of these factors, result in lower energy expenditure in leptin-deficient animals contributing to their obesity.

Leptin regulation of glucose homeostasis

Leptin deficiency causes impaired glucose and insulin tolerance, demonstrating the critical role of leptin in glucose homeostasis [109,119]. Leptin's effects on glycemic control are both direct and indirect. Indirectly, obesity from almost any cause is sufficient to bring about insulin resistance and hyperglycemia. However, leptin also has direct effects on insulin sensitivity and glucose production, irrespective of body weight. Submaximal doses of leptin that are insufficient to induce weight loss in *ob/ob* mice, nonetheless normalize blood glucose levels, suggesting that leptin effects on weight and glycemic control are dissociable[109]. Similarly, restoration of LepRb expression selectively in the ARC of *db/db* mice results in only a mild abrogation of obesity and hyperphagia, but completely normalizes blood glucose levels and ameliorates insulinemia [27]. Lastly, while abrogation of STAT3 action in LepRb neurons results in profound obesity, these animals have relatively intact glycemic control [5].

Though the mechanism remains unclear, LepRb/POMC and LepRb/NPY neurons of the ARC specifically contribute to glucose homeostasis [69]. Replacing LepRb specifically in POMC neurons of *db/db* mice significantly improves glycemia and insulin sensitivity [61]. ICV administration of NPY has acute effects on insulin sensitivity and glucose turnover under conditions or stable body weight [88,130]. However, while chronic blockade of melanocortin receptors –the mechanism by which AgRP acts-- also causes insulin resistance, the effects independent of body weight are not yet clear [1]. While the hypothalamic loci

responsible for these actions are still unclear, some effector pathways have been described that link nutrient sensing to compensatory action. For example, hypothalamic LepRb-expressing glucose responsive neurons send relays via the sympathetic nervous system that innervate the liver to mediate changes in glucose production and release. Both STAT3 and PI3K have important roles in this brain-liver connection [17,100].

Leptin action in reproduction

Leptin levels communicate sufficient energy available to undertake energy-demanding physiological functions, including reproduction. Accordingly, leptin deficient *ob/ob* mice can not reproduce, but leptin treatment fully restores reproductive capacity [125]. Leptin regulates the reproductive system via gonadotropin-releasing hormone (GnRH)-secreting neurons in the preoptic areas and these neurons in turn facilitate release of pituitary gonadotropins into the circulation. GnRH is not expressed in LepRb neurons, however, indicating an indirect effect of leptin on GnRH neurons [45]. Several LepRb-expressing regions send projections to GnRH neurons and play roles in reproduction, including the ARC, medial preoptic area (MPOA) and the PMv. Recently, our research group showed leptin and sexual odorant-responsive neurons in the PMv project to the MPOA and synapse on GnRH neurons establishing a novel circuit linking LepRb and GnRH neurons [81].

Leptin also regulates expression of hypothalamic kisspeptin-1 (Kiss1) and galanin-like peptide (GALP), both of which regulate GnRH secretion; it remains

unclear whether Kiss1 and GALP neurons co-express LepRb or are indirectly modulated by LepRb neurons [36,125]. CART is also a facilitator of GnRH expression, and the large population of CART neurons in the ARC and PMv that project onto GnRH neuron-containing regions are potential sites of direct or indirect leptin regulation [113].

Roles of leptin in other systems

Beyond leptin's classical roles in energy and glucose homeostasis, leptin also has effects elsewhere, including the immune system, the skeletal system, and the cardiovascular system. Given that serum leptin conveys a metabolically important signal, it is not surprising that a variety of systems throughout the body are sensitive to it. Interestingly, the leptin resistance in the hypothalamus that is a hallmark of obesity does not necessarily develop in other leptin-responsive tissue in the periphery. This suggests tissue-specific responses to chronically elevated leptin levels.

With respect to the immune system, leptin deficiency results in thymic hypoplasia and immunodeficiency, similar to what occurs during starvation[85]. Leptin is generally pro-inflammatory and promotes T_H1-cell differentiation [77]. These effects occur even in isolated cell culture systems demonstrating these effects are cell autonomous and not mediated primarily through the CNS. Also, leptin deficient animals have more numerous T regulatory cells; these cells suppress immune function, allow tolerance to self-antigens and are critical for preventing autoimmune disease[32,123]. Consequently, leptin deficiency or leptin

neutralization is protective against the development of some autoimmune diseases [33,77].

Leptin is also anti-osteogenic [35]. States of low circulating leptin are correlated with high bone mass and the incidence of osteoporosis is low in obese individuals, possibly related to their leptin insensitivity [121]. Interestingly, leptin's effects on bone mass are regulated centrally. Leptin regulates sympathetic outflow from the brain to the bone marrow compartment that, in turn, regulates bone formation[122]. Thus far, it is difficult to ascertain the teleological purpose of this regulation, though, continuing work examining these brain/bone connections might reveal this information.

Leptin also has effects in the cardiovascular system. High circulating levels of leptin are correlated with higher risk of myocardial infarction and stroke independent of body weight[118]. Leptin's effects are proatherogenic as it stimulates oxidative stress, endothelial dysfunction, platelet aggregation, smooth muscle cell proliferation, endothelial inflammation and neointimal hyperplasia [10,15]. Crossing lipodystrophic mice (a murine model of atherogenesis) onto an *ob/ob* background significantly ameliorates the number and severity of atherosclerotic lesions in these animals [123].

SUMMARY

Since the identification of the leptin and its receptor fifteen years ago, a massive body of work has expanded on this discovery and revealed many of the details explaining to role of this hormone is mammalian physiology [137]. Work in

cultured cells and mice has given us a mechanistic understanding of leptin signaling: how the leptin receptor and Jak2 work together the initiate a signaling cascade in response to ligand binding, how these signaling events mediate changes in gene expression and membrane polarization, and how these events ultimately affect the brain's neuroendocrine and neurological activities. Furthermore, work in vivo has shown us the physiological importance of leptin. The leptin signal is essential to communicate the levels of energy stored in the body's fat depots and this signal's absence causes serious metabolic defects. The work presented in this dissertation adds to the body of knowledge regarding leptin's activities from molecular signaling events to whole-body physiology.

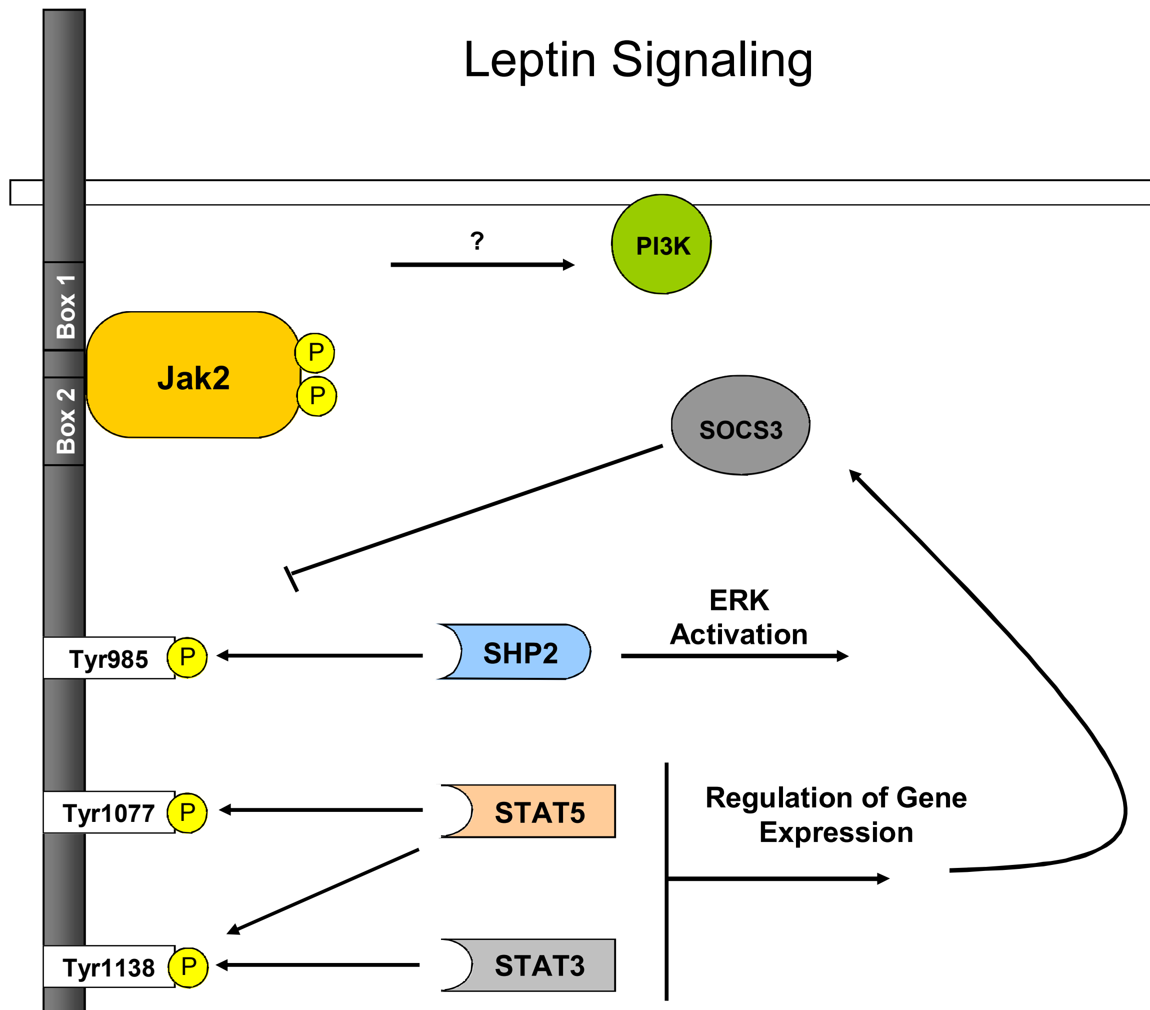
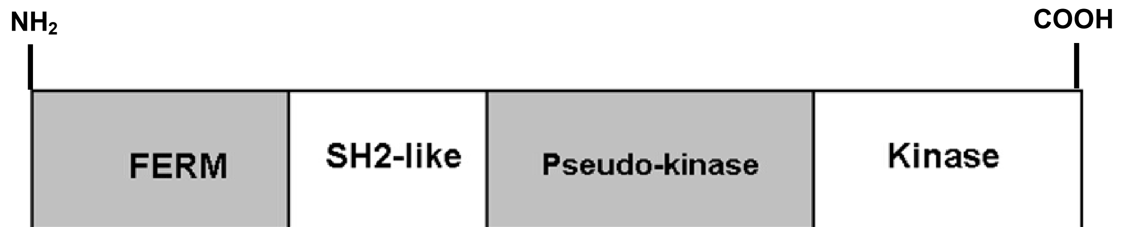


Figure 1.1 The role of functional sites in LepRb signaling. Leptin binding to LRb activates the associated Jak2 tyrosine kinase bound at the Box 1 and Box 2 motifs. Activated Jak2 undergoes robust autophosphorylation and phosphorylates Tyr985, Tyr1077 and Tyr1138 on the LepRb intracellular tail. These phosphorylated residues act as docking sites for SH2-domain containing proteins. Phosphorylated Tyr985 mediates docking with SHP2 and subsequent activation of ERK through the MAPK signaling cascade. Phosphorylated Tyr1077 mediates STAT5 activation. Phosphorylated Tyr1138 mediates both STAT3 and STAT5 activation. STAT3 activation ultimately leads to increased expression of SOCS3 which acts as a feedback inhibitor and negatively regulates LRb signaling. Activation of the pathway also leads to activation of PI3K signaling, though the intermediate steps for this process remain to be fully elucidated.



FERM: (1-380) Interacts with the cytokine receptor

SH2-Like: (400-520) Function unclear

Pseudokinase: (520-840) Regulates kinase activity

Kinase: (840-1129) Active site of protein

Figure 1.2 Schematic of Jak2 functional domains. There are four putative functional domains on murine Jak2. The functions of these domains, and their approximate boundaries, are indicated in the figure.

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CHAPTER 2

REGULATION OF JAK2 FUNCTION BY PHOSPHORYLATION OF TYR317 AND TYR637 DURING CYTOKINE SIGNALING

Abstract

Jak2, the cognate tyrosine kinase for numerous cytokine receptors, undergoes multisite phosphorylation during cytokine stimulation. To understand the role of phosphorylation in Jak2 regulation, we used mass spectrometry to identify numerous Jak2 phosphorylation sites and characterize their significance for Jak2 function. Two sites outside of the tyrosine kinase domain, Tyr_{317} in the FERM domain and Tyr_{637} in the JH2 domain, exhibited strong regulation of Jak2 activity. Mutation of Tyr_{317} promotes increased Jak2 activity, and the phosphorylation of Tyr_{317} during cytokine signaling requires prior activation loop phosphorylation, consistent with a role for Tyr_{317} in the feedback inhibition of Jak2 kinase activity after receptor stimulation. Comparison with several previously-identified regulatory phosphorylation sites on Jak2 revealed a dominant role for Tyr_{317} in the attenuation of Jak2 signaling. In contrast, mutation of Tyr_{637} decreased Jak2 signaling and activity and partially suppressed the activating JH2 V617F mutation, suggesting a role for Tyr_{637} phosphorylation in the release of JH2 domain-mediated suppression of Jak2 kinase activity during

cytokine stimulation. The phosphorylation of Tyr_{317} and Tyr_{637} act in concert with other regulatory events to maintain appropriate control of Jak2 activity and cytokine signaling.

Introduction

Type I cytokines act via cell surface receptors on target cells to mediate a plethora of physiologic processes, ranging from hematopoeitic and immune functions (such as those controlled by erythropoietin and the interleukins), to growth and neuroendocrine responses (such as those modulated by growth hormone and leptin) [1-5]. Cytokine receptors contain an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular domain that, although devoid of enzymatic activity, transmits intracellular signals by means of an associated Jak family tyrosine kinase. Ligand binding activates the receptor-associated intracellular Jak kinase, resulting in Jak kinase autophosphorylation and activation, as well as the subsequent tyrosine phosphorylation of the intracellular domain of the cytokine receptor. These tyrosine phosphorylation events mediate downstream signaling by the cytokine receptor/Jak kinase complex [1,2,5,6].

The Jak kinase family contains four members: Jak1-Jak3 and Tyk2 [1,2]. Of these, Jak1-2 and Tyk2 are ubiquitously expressed, while Jak3 is found predominantly in immune and hematopoeitic tissues. Jak kinases contain four conserved domains: The NH_2-terminal FERM domain mediates cytokine receptor interactions [7,8], while function of the adjacent (non-phosphotyosine binding) SH2-

like fold remains unclear. The COOH-terminal region of Jak kinases contains a kinase-like JH2 domain that is devoid of enzymatic activity, but which regulates the activity of the COOH-terminal JH1 tyrosine kinase domain [9-13].

Our laboratory studies signaling by LepRb, which regulates energy balance, neuroendocrine homeostasis, and immune function in response to leptin- a hormonal signal of long-term energy stores [4,5,14,15]. Leptin binding to LepRb promotes the activation and tyrosine phosphorylation of the LepRb-associated Jak2, resulting in the phosphorylation of tyrosine residues on Jak2 and the intracellular tail of LepRb Jak2 [5,16,17]. Tyrosine phosphorylation sites on LepRb recruit signal transducers and activators of transcription (STATs) and SHP-2 to mediate downstream signaling, as well as the suppressor of cytokine signaling-3 (SOCS3) to attenuate LepRb signaling [5,16,18].

Several sites of Jak2 tyrosine phosphorylation have also been identified, and functions for some of these sites have been elucidated: Within the FERM domain, phosphorylation of Tyr_{119} disrupts Jak2-cytokine receptor interactions [19]. Within and adjacent to the JH2 domain, the phosphorylation of Ser_{523} and Tyr_{570} inhibits Jak2 kinase activity [20,21]. Within the kinase domain itself, phosphorylated Tyr_{813} mediates binding of SH2-B/SH2B1 to increase Jak2 signaling [6], phosphorylation of the activation loop residues Tyr_{1007} and Tyr_{1008} plays an essential role in kinase activation [22], and the phosphorylation of Tyr_{913} inhibits Jak2 signaling [23]. Other sites of Jak2 phosphorylation also exist (some known, while others have remained undefined), although the function(s) for many of these remain unknown [21,24]. Here, we report the MS analysis of Jak2 protein,

which revealed several novel sites of phosphorylation. We also report the in-depth analysis of two cytokine-regulated Jak2 phosphorylation sites outside of the kinase domain- Tyr_{317} and Tyr_{637}. Phosphorylation of Tyr_{317} mediates negative feedback regulation for Jak2, while phosphorylation of Tyr_{637} is necessary for maximal Jak2 kinase activity. We propose an integrated model for how these and other phosphorylation sites orchestrate the activity of Jak2.

Results

Identification of Jak2 Phosphorylation Sites. We used mass spectrometric (MS) analysis of Jak2 protein to identify phosphorylation sites on Jak2. We prepared murine Jak2 by overexpression in HEK293 cells. To enable cytokine-mediated activation, we cotransfected plasmids encoding Jak2 and ELR (a chimeric receptor containing the extracellular domain of the erythropoietin receptor and the intracellular domain of LepRb, which effectively places the LepRb intracellular domain under the control of erythropoietin) into HEK293 cells. ELR was utilized because it is expressed more highly and allows more robust activation of Jak2 compared to native LepRb. HEK293 cells expressing Jak2 protein were stimulated with Epo for 15 min before lysis and immunoprecipitation to isolate Jak2. Immunoprecipitates were resolved by SDS-PAGE and stained with Coomassie Blue to identify Jak2 protein. Jak2 bands were subjected to proteolytic cleavage and analyzed by two MS instruments, a linear ion trap mass spectrometer and a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer. Analysis of MS/MS spectra revealed numerous precursor ions that represented

phosphorylated Jak2 peptides, each of which was observed in multiple runs and conformed to strict standards of confidence by software analysis. Some of these had previously been noted in the literature or on scientific websites [6,20-22,24-27](www.phosphosite.org), while others (3 Tyr and 2 Ser/Thr residues) were novel; most (11 sites overall) had not been functionally characterized (Table 1).

With respect to Tyr_{317} and Tyr_{637}, which represent the focus of this report, analysis of MS/MS spectra from the linear trap instrument using TurboSequest revealed a 3+ charged species corresponding to a Tyr_{317}-containing Jak2 tryptic peptide ESETLTEQDVQL(P)YCDFPDIIDVSIK with Xcorr 3.74. The spectra showed an ion consistent with the neutral loss of 80 Da resulting from the loss of HPO_3 from a triply charged phosphotyrosine-containing peptide (Fig 1A). Analysis of Q-TOF MS/MS spectra using Mascot revealed a 2+ charged precursor corresponding to a Tyr_{637}-containing Jak2 tryptic peptide FGSLDT(P)YLK with score 51. The spectrum also shows an ion consistent with neutral lost of 80 Da resulting from the loss of HPO_3 from the doubly charged phosphotyrosine-containing peptide (Fig 1B). Manual inspection and interpretation of fragmentation spectra confirmed peptide sequences in these and the other noted cases.

Screening Candidate Phosphorylation Sites for Functional Relevance. In order to determine which of the identified Jak2 phosphorylation sites merited in-depth study, we mutated each site to a non-phosphorylateable residue (using either a Tyr→Phe or Ser→Ala substitution) to create a panel of phosphorylation site-defective Jak2 molecules, and examined the basal and hormone-stimulated

tyrosine phosphorylation of each Jak2 mutant following co-expression with ELR in HEK293 cells (Fig 2). While anti-phosphotyrosine (αPY) immunoreactivity in immunoblotting represents an indirect measure of Jak2 function, multiple studies have revealed a correlation with Jak2 activity [22,28], and this analysis should reveal any alterations in Jak2 activity resulting from the loss of a specific phosphorylation site. This analysis revealed two mutants for phosphorylation sites lying outside of the kinase domain that differed significantly from wild-type Jak2 with respect to αPY immunoreactivity. $Jak2^{Y317F}$ exhibited high αPY immunoreactivity in both stimulated and unstimulated states suggesting that phosphorylation at Tyr_{317} (a novel site of Jak2 phosphorylation) inhibits Jak2 activity (Fig 2). $Jak2^{Y637F}$, conversely, exhibited reduced αPY immunoreactivity in both stimulated and unstimulated states suggesting that phosphorylation at Tyr_{637} (a previously identified phosphorylation site that has not been functionally studied [24]) is required for maximal Jak2 activity (Fig 2). Based on these screening data, Tyr_{317} and Tyr_{637} were chosen for further study. A number of sites within the kinase domain also modulate Jak2 tyrosine phosphorylation; the in-depth analysis of these sites will be reported elsewhere (Carter-Su and Argetsinger, in preparation).

Phosphorylation of Jak2 Tyr_{317} and Tyr_{637} during cytokine stimulation. To examine the phosphorylation status of these sites under different conditions, we generated antibodies against phosphorylated Tyr_{317} and phosphorylated Tyr_{637} (αpY317 and αpY637, respectively). To examine the phosphorylation of Tyr_{317} and Tyr_{637} and the function of the Jak2 molecules mutated at these sites more

thoroughly, we coexpressed ELR with either Jak2, Jak2^{Y317F} or Jak2^{Y637F} in HEK293 cells, and stimulated them with Epo for various times before lysis. Cell lysates or Jak2 immunoprecipitates were immunoblotted with αPY, αpY1007/8 (which recognizes the phosphorylated activation loop of Jak2), αJak2, αPY, and αpY317 or αpY637 (Fig 3).

With respect to Jak2^{Y317F}, we found high baseline αPY- and αpY1007/8-immunoreactivity, which increased only slightly upon stimulation and remained elevated for the duration of the experiment (Fig 3A). This contrasts with Jak2, in which αPY- and αpY1007/8-immunoreactivity rose quickly and peaked in the first 30 minutes of stimulation before slowly decreasing over the course of several hours. The minimal regulation of the tyrosine phosphorylation of Jak2^{Y317F} by Epo may suggest that this Jak2 mutant is close to maximal tyrosine phosphorylation at baseline, limiting further increases with cytokine stimulation. The failure of αpY317 to react with Jak2^{Y317F} suggests the specificity of this antibody preparation for phosphorylated Tyr_{317}. Phosphorylation at Tyr_{317} on Jak2, detected by αpY317 immunoblotting, peaked later than tyrosine phosphorylation or activation loop phosphorylation and remained elevated for at least 8 hours following stimulation, suggesting a potential role for the phosphorylation of Tyr_{317} in the feedback inhibition of Jak2 activity following cytokine stimulation

With respect to Jak2^{Y637F}, overall αPY- and αpY1007/8-immunoreactivity was decreased compared to Jak2 at baseline and over the entire time-course of stimulation (Fig 3B). The stimulation of αpY637-reactivity on Jak2 by cytokine

treatment and the failure of αpY637 to react with $Jak2^{Y637F}$ revealed the specificity of this antibody for phosphorylated Tyr_{637}. The phosphorylation of Tyr_{637} increased rapidly during the first thirty minutes of stimulation before declining (as for overall αPY and αpY1007/8 immunoreactivity), suggesting a potential role for the phosphorylation of Tyr_{637} in the activation of Jak2 during cytokine signaling. We also observed IL-3-stimulated phosphorylation of Tyr_{317} and Tyr_{637} on endogenous Jak2 purified from 32D myeloid progenitor cells [26,29] (Fig 3C-D), suggesting the phosphorylation of these sites in response to multiple cytokines.

Transphosphorylation of Tyr_{317} and Tyr_{637} during cytokine stimulation. Protein autophosphorylation is often ordered and can occur by intramolecular (cis) or intermolecular (trans) mechanisms. To determine the potential the mechanisms underlying phosphorylation at Tyr_{317} and Tyr_{637} and their timing relative to the activating $Tyr_{1007/1008}$ phosphorylation, we generated hemagglutinin (HA) epitope-tagged Jak2 (Jak2-HA) and two different inactive variants of Jak2-HA. The mutation in Jak2-HA^{K882E} blocks ATP binding to the kinase domain, while the alteration in Jak2-$HA^{Y1007/8F}$ blocks the phosphorylation of the activation loop in the Jak2 kinase domain. We co-expressed these Jak2-HA constructs with ELR plus a FLAG-epitope tagged wild-type Jak2 (Jak2-FLAG) and used αHA to immunoprecipitate the Jak2-HA isoforms in order to assess their phosphorylation status with phosphospecific antibodies (Fig 4). The finding of similarly insignificant amounts of αHA-precipitable αFLAG-reactive Jak2 under each condition (including without Jak2-HA isoforms- see overexposed αFLAG

panel in Fig 4) revealed that the signal from each phosphospecific antibody is attributable to the precipitated Jak2-HA isoform. While the phosphorylation of Jak2-HA on $Tyr_{1007/8}$, Tyr_{317} and Tyr_{637} was consistently greater than for Jak2-HA^{K882E}, each of these sites was phosphorylated on the ATP-binding mutant, consistent with the transphosphorylation of these sites by Jak2-FLAG (Fig 4). The decreased phosphorylation of Jak2-HA^{K882E} relative to Jak2-HA could reflect a combination of absent cis-autophosphorylation of Jak2-HA^{K882} (which might occur in Jak2-HA), decreased total kinase activity in Jak2-HA^{K882}-expressing cells, and/or an altered conformation of the inactive Jak2-HA^{K882E}. Interestingly, the decreased phosphorylation of Tyr_{317} and Tyr_{637} on Jak2-HA^{K882E} and Jak2-$HA^{Y1007/8F}$ was more pronounced than the decrease in total αPY-reactivity. While αPY-reactivity per unit of HA-immunoreactive protein on stimulated Jak2-HA^{K882E} was decreased to aproximately 40% of Jak2-HA levels, phosphorylation at Tyr_{317} and Tyr_{637} on stimulated Jak2-HA^{K882E} was decreased to approximately 5 and 10% of Jak2-HA levels, respectively. This difference suggests a stricter requirement for Jak2 kinase activity in order to phosphorylate Tyr_{317} and Tyr_{637} compared to other major sites of tyrosine phosphorylation of Jak2. Additionally, in Jak2-$HA^{Y1007/8F}$, which cannot undergo the conformation change associated with activation loop phosphorylation, phosphorylation of both Tyr_{317} and Tyr_{637} was further decreased to approximately 1% compared to Jak2-HA (relative to HA-immunoreactive protein and relative to total αPY-immunoreactivity). Thus, these data are consistent with the trans-phosphorylation of Tyr_{317} and Tyr_{637} and also with the requirement for $Tyr_{1007/8}$- and activity-dependent conformational

changes to enable the phosphorylation of Tyr_{317} and Tyr_{637} during Jak2 activation- placing the phosphorylation of these sites downstream of the initial phosphorylation event of kinase activation.

Effects of Tyr_{317} and Tyr_{637} on cytokine signaling. LepRb/Jak2 signaling promotes the activation of STAT3 via the phosphorylation of Tyr_{1138} on LepRb, and mediates the activation of ERK both via the phosphorylation of Tyr_{985} on LepRb and via Jak2-mediated signals that are independent of LepRb phosphorylation sites [30,31]. To determine the potential roles for Tyr_{317} or Tyr_{637} on these downstream pathways, we measured the activation of ERK and STAT3 by assessing their phosphorylation in cells expressing Jak2, $Jak2^{Y317F}$ or $Jak2^{Y637F}$ in the presence or absence of ELR (Fig 5). Consistent with its increased αpY1007/8 immunoreactivity, $Jak2^{Y317F}$ mediated increased downstream signaling to STAT3 and ERK compared to Jak2 in the presence of ELR. Across multiple experiments, $Jak2^{Y317F}$ mediated approximately a two-fold increase in phosphorylated STAT3 and phosphorylated ERK in unstimulated cells (N=3, $p<0.05$) Levels of these activated proteins were not significantly different in stimulated cells. In contrast to $Jak2^{Y317F}$, $Jak2^{Y637}$ exhibited lower αpY1007/8 immunoreactivity and downstream STAT3 and ERK signaling than Jak2. Across multiple experiments, $Jak2^{Y637F}$ mediated approximately a forty percent decrease in phosphorylated STAT3 and phosphorylated ERK in stimulated cells (N=3, $p<0.05$) Levels of these activated proteins were not significantly different in unstimulated cells. Taken together, these data suggest that alterations in STAT3 and ERK activation are likely the result of changes in Jak2 kinase activity rather

than any pathway-specific interactions that phosphorylation at Tyr_{317} or Tyr_{637} might have on these pathways.

Failure of negatively charged mutations of Tyr_{317} and Tyr_{637} to permit normal Jak2 activity. Tyrosine phosphorylation may regulate protein function in many ways, including by the alteration of protein conformation by the addition of negative charge that accompanies the addition of a phosphate group. In order to determine whether this mechanism might operate for Tyr_{317} and/or Tyr_{637}, we replaced these residues with the negatively charged amino acid Glu to generate $Jak2^{Y317E}$ and $Jak2^{Y317E}$, and studied their activity relative to Jak2 and their respective Tyr→Phe mutants (Fig 6). We found that $Jak2^{Y317E}$, like $Jak2^{Y317F}$, was highly active in both stimulated and unstimulated states (Figure 6A). Furthermore, $Jak2^{Y637E}$ was less active than Jak2 and exhibited similar or decreased activity compared to $Jak2^{Y637F}$ (Figure 6B). Thus, the addition of a negative charge at these sites is not sufficient to mimic the effects of tyrosine phosphorylation. Neither are the altered activities of $Jak2^{Y317F}$ and $Jak2^{Y637F}$ likely due to nonspecific effects resulting from the substitution of hydrophobic Phe in place of Tyr, as both Glu and Phe produce the same phenotype at each of these sites.

Regulation of Jak2 kinase activity by Tyr_{317} and Tyr_{637}. While αPY and αpY1007/8 immunoreactivity generally correlate well with Jak2 kinase activity, this relationship breaks down to some extent at high levels of overall Jak2 phosphorylation, where further increases in phosphorylation become difficult to detect. To gain a more accurate indication of enzymatic activity, we directly

measured the *in vitro* kinase activity of immunoaffinity-purified Jak2 and Jak2^{Y317F} toward a target peptide derived from STAT5B- a known substrate of Jak2 [21,32] (Fig 7A). These data demonstrated increased *in vitro* kinase activity for purified Jak2^{Y317F} compared to Jak2. Importantly, these data also show that the Jak2^{Y317F} tyrosine kinase, while highly active at baseline, remains responsive to Epo stimulation, suggesting that the mutation of this site does not uncouple kinase function from receptor-mediated regulation. Jak2^{Y637F} displayed reduced kinase activity compared to Jak2 in the stimulated state (Fig 7B). Jak2^{Y637F} also undergoes activation upon ligand stimulation in the presence of ELR, however. These data confirm the ability of ELR stimulation to increase kinase activity and downstream signaling by Jak2^{Y317F} and Jak2^{Y637F}, suggesting that neither of these sites is required for functional regulation by cytokine receptors.

Regulation of Jak2 by Tyr$_{317}$ and Tyr$_{637}$- Role for cytokine receptor interaction. In addition to the modulation of Jak2 activity by ligand binding to Jak2-associated receptors, the association of Jak2 with cytokine receptors promotes Jak2 activity (baseline Jak2 activity increases in the receptor-bound, relative to the unbound, state), presumably by modulating the conformation of Jak2 [19,33]. The phenotypes of Jak2^{Y317F} and Jak2^{Y637F} are independent of ELR expression in 293 cells (Fig 5), but it is not clear whether endogenous cytokine receptors in HEK293 cells bind to Jak2 in the absence of ELR to permit the manifestation of potential receptor-dependent effects on signaling.

To determine whether the roles for Tyr$_{317}$ and Tyr$_{637}$ in the regulation of Jak2 function might be independent of cytokine receptor/FERM domain

interactions, we examined Jak2^{Y317F} and Jak2^{Y637F} activity in the context of Jak2^{Y119E} (Jak2$^{Y119E/Y317F}$ and Jak2$^{Y119E/Y637F}$). Phosphorylation of Tyr$_{119}$ disrupts Jak2/FERM domain interaction to most Jak2-binding cytokine receptors and a Tyr→Glu substitution at Tyr$_{119}$ mimics this effect [19,33]. We thus compared the kinase activity of Jak2^{Y119E}, Jak2$^{Y119E/Y317F}$ and Jak2$^{Y119E/Y637F}$ to determine whether FERM domain/cytokine receptor interactions were required for modulation of Jak2 activity by Tyr$_{317}$ or Tyr$_{637.}$ We found that the activating effect of Tyr$_{317}$ mutation was eliminated in the context of Jak2^{Y119E}, while inhibitory response to alteration of Tyr$_{637}$ remained in Jak2$^{Y119E/Y637F}$ (Fig 7C). This suggests that the FERM domain-mediated interaction between Jak2 and cytokine receptors is necessary for the negative regulation of Jak2 by phosphorylation at Tyr$_{317}$ (presumably reflecting a requirement for FERM domain conformation), while the effect of Tyr$_{637}$ on Jak2 kinase activity occurs independently of FERM/receptor binding. Since Jak2^{Y119E} is still capable of binding interferon-gamma receptors, it is possible that there may be intact interferon-gamma receptor/Jak2^{Y119E} binding which could contribute to Tyr$_{637}$ mutation induced decrements in kinase activity [34]. However, the ability of the Tyr$_{119}$→Glu$_{119}$ substitution to abrogate the activating effect of Tyr$_{317}$ mutation suggests the likely independence of Tyr$_{637}$ function from FERM domain requirement

Functional interaction of Tyr$_{317}$ and Tyr$_{637}$ with other regulatory sites on Jak2. Tyr$_{317}$ represents the third inhibitory phosphorylation site that we have identified on Jak2, as phosphorylation at Ser$_{523}$ and Tyr$_{570}$ also inhibits Jak2 [20,21,26,27]. To determine the relative contributions of these sites to Jak2 activity,

we compared the kinase activity of $Jak2^{Y317F}$, $Jak2^{S523A}$, $Jak2^{Y570F}$ and several Jak2 isoforms containing various combinations of mutations at these inhibitory sites (Fig 8A-C). The cytokine-stimulated activity of each of these mutants was near maximal and relatively similar (data not shown), and we therefore focused on the kinase activity at baseline in the absence of cytokine stimulation. $Jak2^{S523A}$, while more active than Jak2 [26,27], was less active than $Jak2^{Y317F}$, suggesting a stronger effect of Tyr_{317}-mediated inhibition than of Ser_{523}-inhibition (Fig 8A). Mutation at both sites produced additive effects, however, suggesting the independent effects of these phosphorylation sites on Jak2 activity. The activities of $Jak2^{Y317F}$ and $Jak2^{Y570F}$ were increased relative to Jak2 and similar in magnitude; the additive effects of mutation at these sites also suggested independent effects on Jak2 activity (Fig 8B). Comparing the kinase activity of each double mutant with the $Jak2^{Y317F/S523A/Y570F}$ triple mutant revealed that that the most active Jak2 species contain the Tyr_{317} mutation, and the triple mutant demonstrated no increased activity compared to Y317F-containing double mutants, underlining the importance of Tyr_{317} for the appropriate suppression of Jak2 activity (Fig 8C).

Somatic mutation that substitutes Phe in place Val_{617} of Jak2 ($Jak2^{V617F}$) mediates the constitutive activation of Jak2 that underlies most cases of polycythemia vera and some portion of other myeloproliferative disorders [35]. Val_{617}, like Tyr_{637}, lies in the JH2 pseudo-kinase domain of Jak2, and their close proximity suggested the potential for functional interactions between the two residues in the regulation of JH2-mediated control of kinase activity. To

determine the ability of Tyr_{637} mutation to suppress the activity of $Jak2^{V617F}$, we compared the kinase activities of Jak2, $Jak2^{V617F}$, $Jak2^{Y637F}$, and $Jak2^{V617F/Y637F}$. Interestingly, $Jak2^{V617F/Y637F}$ is less active than $Jak2^{V617F}$ demonstrating that Tyr_{637} influences kinase activity in $Jak2^{V617F}$ (Fig 8D). While $Jak2^{V617F/Y637F}$ is less active than $Jak2^{Y617F}$, however, it remains more active than Jak2 at baseline, suggesting that Tyr_{637} contributes to only a portion of Val_{617}-mediated Jak2 activation.

Discussion

Although many sites of Jak2 phosphorylation have been identified, previous analyses have suggested the presence of others; the function of most Jak2 phosphorylation sites has remained unclear, as well. As the analysis of *in vitro* autophosphorylated Jak2 may not reveal the full complement of physiologically important phosphorylation sites (especially Ser/Thr phosphorylation sites), we utilized MS analysis of Jak2 protein prepared from mammalian cells following cytokine receptor activation in order to identify phosphorylation sites on Jak2. This analysis revealed 18 phosphorylation sites on Jak2, including 15 Tyr phosphorylation sites and three Ser/Thr phosphorylation sites. Of these, 12 sites had been previously published by ourselves or others [6,20-22,24-27], and two had been presumptively identified by MS analysis reported on www.phosphosite.org. Thus, our present analysis identified 3 novel sites of Tyr phosphorylation and 2 novel sites of Ser/Thr phosphorylation on Jak2. The MS data for each of the sites we report here possessed high

confidence scores by initial software analysis and their MS/MS spectra were manually-verified and generally showed specific evidence of phosphorylation, such as fragment ions consistent with neutral phosphate loss. Our analysis did not identify some previously-reported sites of phosphorylation, including Tyr_{119} in the FERM domain and Tyr_{972} (as well as a number of other presumptive sites) in the kinase domain: Although spectra potentially consistent with many of these sites were noted, they did not pass our rigorous criteria for inclusion, presumably due to the behavior of the peptides on MS/MS analysis.

We initially assayed the potential importance of these sites by analyzing the basal and ligand-stimulated tyrosine phosphorylation of Jak2 molecules mutant for each identified site, and focused our subsequent functional analysis on the two sites outside of the kinase domain that altered Jak2 activity when mutated: Tyr_{317} and Tyr_{637}. Additional sites within the kinase domain also regulate Jak2 kinase activity (including Tyr_{868}, Tyr_{966}, and Tyr_{972}); these represent the topic of another study (Carter-Su and Argetsinger). Since our screening assay most sensitively detects large changes in Jak2 activity, we cannot rule out more modest roles in Jak2 regulation for additional sites. Since this screen focused on Jak2 phosphorylation, it is also possible that some of the identified sites may play other, unknown, roles in downstream signal transmission for which no assays currently exist.

While cytokine receptor stimulation mediates the phosphorylation of both Tyr_{317} and Tyr_{637}, these residues oppositely regulate Jak2-dependent signaling: The mutation of Tyr_{317} enhances Jak2 function, suggesting a role for the

phosphorylation of Tyr_{317} in the inhibition of Jak2. Conversely, mutation of Tyr_{637} reduces Jak2 signaling, suggesting a role for the phosphorylation of this residue in the activation of Jak2. Tyr_{637} lies in the highly conserved pseudokinase domain of Jak2 and a corresponding residue is also present in Jak3, suggesting potential partial conservation of the function of this site across the Jak kinase family. In contrast, Tyr_{317} is not conserved with any other member of the Jak family of kinases suggesting the mechanism by which Tyr_{317} affects protein function is Jak2 specific.

With respect to both Tyr_{317} and Tyr_{637}, the functional equivalence of Glu substitution of either of these sites compared to Phe indicates that the negatively-charged phosphate in the context of Tyr is required for the function of both Tyr_{317} and Tyr_{637}, suggesting that criteria stricter than a simple negative charge must be met to mediate the presumptive intramolecular interactions that underlie the function of these sites. The finding that Glu and Phe substitutions mediate similar affects and the mutants are regulated by cytokine stimulation in a manner proportional to their baseline activity also suggests that non-specific disruption of Jak2 domain structure does not underlie the phenotype of these mutants.

Our data suggest that the interaction of Jak2 with cytokine receptors and/or the conformation of the FERM domain is required for the modulation of Jak2 activity by Tyr_{317}, consistent with the location of Tyr_{317} in the FERM domain. The decreased activity of free Jak2 (e.g. $Jak2^{Y119E}$) compared to receptor-associated Jak2 [19,36,37], the ability of receptor stimulation to promote the increased activity of $Jak2^{Y317F}$, and the placement of Tyr_{317} in the extreme

COOH-terminus of the FERM domain (beyond the presumptive receptor interaction motifs) suggest that Tyr_{317} is unlikely to act by altering the stability of the receptor/Jak2 complex. It is more likely that receptor interaction and/or the conformation of the FERM domain regulates the phosphorylation or function of Tyr_{317}; this is consistent with the dependence of Tyr_{317} phosphorylation on the prior phosphorylation of $Tyr_{1007/1008}$ in the activation loop of the kinase domain, which suggests the requirement for a specific, activated, conformation of Jak2 for the phosphorylation of Tyr_{317}. Indeed, we did not detect Tyr_{317} phosphorylation on $Jak2^{Y119E}$ (data not shown), although we cannot rule out the possibility that any Tyr_{317} phosphorylation on this poorly active protein was below our detection threshold.

Our data are thus consistent with a role for Tyr_{317} in the attenuation of Jak2 activity following receptor activation, rather than in suppressing inactive Jak2. Indeed, the phosphorylation of Tyr_{317} is very low in unstimulated Jak2, increases significantly with stimulation, and is highest following full activation of Jak2. Furthermore, the phosphorylation of $Tyr_{1007/1008}$ and the adoption of an activated conformation are required even for the transphosphorylation of Tyr_{317} by an activated Jak2 molecule. Hence, the increased baseline activity of $Jak2^{Y317F}$ in cells prior to cytokine stimulation may result from the inability to inactivate the normally small portion of Jak2 that becomes activated in quiescent HEK293 cells; the failure to return $Jak2^{Y317F}$ to the pool of inactive Jak2 would eventually lead to the accumulation of Jak2 protein that is locked in the active state.

Mutation of Tyr_{637} inhibits kinase activity under most of the conditions we tested: The activity of $Jak2^{Y637F}$ was decreased in the absence or presence of receptor and/or ligand activation, and in the context of a receptor-insensitive Jak2. This is consistent with its position in the JH2 domain of Jak2, which constitutes a major regulator of Jak2 kinase activity [9-13]. The phosphorylation of Tyr_{637} during cytokine stimulation may thus act to alleviate JH2-mediated inhibition to promote the appropriate robust activation of Jak2 kinase activity. Indeed, while the phosphorylation of Tyr_{637} requires the prior phosphorylation of $Tyr_{1007/1008}$ (which promotes kinase activity by altering the conformation of the kinase domain), the phosphorylation and dephosphorylation of Tyr_{637} progresses with a grossly similar time-course as that of $Tyr_{1007/1008}$. That mutation of Tyr_{637} partially suppresses the activating V617F JH2 mutation is also consistent with a role for Tyr_{637} in reducing JH2 –mediated inhibition of kinase function.

The activated phenotype of Jak2 molecules mutant for Ser_{523}, Tyr_{317}, and Tyr_{570} suggests that the phosphorylation of each residue acts to inhibit the activity of Jak2. Ser_{523} exhibits several differences compared to Tyr_{317} and Tyr_{570}, however. Not only is the Ser_{523}-mediated inhibition weaker than that mediated by these other two sites (as shown by the relatively modest activation exhibited by its cognate Jak2 mutant), but also Ser_{523} is highly (stoichiometrically) phosphorylated in the baseline state and not further phosphorylated during cytokine stimulation, at least in rapidly proliferating cells such as HEK293 and 32D cells [26]. In contrast, Tyr_{317} and Tyr_{570} are phosphorylated upon cytokine receptor/Jak2 activation and more strongly inhibit Jak2 kinase activity.

Interestingly, the phosphorylation of Tyr_{317} is delayed compared to that of activating sites such as Tyr_{637} and $Tyr_{1007/1008}$, depends upon prior phosphorylation of $Tyr_{1007/1008}$ and an active conformation of Jak2, and remains highly phosphorylated even after these activating sites become relatively dephosphorylated.

Together, these observations suggest a model of phosphorylation-dependent regulation of Jak2 (Fig 8) in which the baseline phosphorylation of Ser_{523} may serve to preserve the relative inactivity of Jak2 in the absence of cytokine stimulation. Since few forces promote the activation of Jak2 in the absence of stimulation, the relatively modest Ser_{523}-mediated inhibition suffices to suppress Jak2 activation in the absence of ligand binding, while the strong cytokine-mediated stimulus can overcome the weak inhibition by Ser_{523}. Upon cytokine stimulation, the rapid phosphorylation of $Tyr_{1007/1008}$ alters conformation of the JH1 domain activation loop and partially activates the kinase, permitting the phosphorylation of Tyr_{637}, which promotes further Jak2 activation. Other sites in the kinase domain, such as Tyr_{868}, Tyr_{966}, and Tyr_{972}, may also be involved in fully activating Jak2 [38](and Carter-Su and Argetsinger, in preparation).

The subsequent dephosphorylation of these stimulatory sites coincides with the relatively slower phosphorylation of strong inhibitory sites, such as Tyr_{317} (and Tyr_{570}) to mediate feedback inhibition to limit the duration of the signal. Prolonged phosphorylation of the strong inhibitory sites also protects against continued high activity. The phosphorylation of Tyr_{119} (which promotes receptor/Jak2 dissociation) and the inhibitory Tyr_{913} in the kinase domain is also

slightly delayed and more sustained compared to $Tyr_{1007/1008}$ [19,23], and the phosphorylation of Tyr_{119} and Tyr_{913} likely functions together with Tyr_{317} and Tyr_{570} to limit the amplitude and duration of Jak2-dependent cytokine signaling.

Thus, a substantial subset of phosphorylation sites on Jak2 function to regulate Jak2 kinase activity. Each of these phosphorylation sites plays a specific role in the choreography of Jak2 activation and deactivation, and together they ensure appropriate levels of Jak2 activity under a variety of circumstances. The functions of many other phosphorylation sites on Jak2 remain unknown. While certain of these may possess relatively minor functions, others presumably contribute substantially to the regulation of Jak2 kinase activity and/or mediate as yet undiscovered downstream signals.

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Materials and Methods:

Antibodies, Growth Factors and Reagents: Antibodies recognizing phosphorylated Tyr_{317} and phosphorylated Tyr_{637} were generated by raising rabbit polyclonal antibodies against these sites. Synthetic peptides corresponding to phosphorylated Tyr_{317} (CQDVQLY*CDFPD) and Tyr_{637} (CGSLDTY*LKKNK) motifs were generated in the MDRTC Peptide Core (University of Michigan), conjugated to keyhole limpet hemocyanin and inoculated into rabbits. Antisera were affinity purified using a 1:1 mixture of Affigel-10 and Affigel-15 coupled to phosphorylated synthetic peptide. The resulting antibody was further purified by incubating it with Affigel coupled to the non-phosphorylated variant of the peptide in combination with an irrelevant phosphorylated peptide. αJak2(476) antisera for immunoprecipitation were generated by injection into rabbits of keyhole limpet hemocyanin-couple synthetic peptide with the following sequence: DSQRKLQFYEDKHQLPAPK. Generation of αJak2(1139) for immunoblotting has been described previously (previously referred to as αJak2(NT)) [26]. Antibodies against phosphorylated (activated) ERK and phosphorylated (activated) STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Antibody directed against the phosphorylated activation loop of Jak2 (phospho-$Tyr_{1007/8}$) was purchased from Upstate Biotechnology (Lake Placid, NY). Antibody against the hemagglutinin (HA) epitope was purchased from Covance (Denver, PA). Antibody against the FLAG epitope was purchased from Sigma. Synthetic erythropoietin (Epogen) was obtained from Amgen (Thousand Oaks, CA).

Generation of Jak2 Constructs: The generation of ELR has been described previously [16]. The generation of Jak2/pCDNA3 has been described previously [17]. To make epitope-tagged Jak2, DNA encoding either a double HA epitope or double FLAG epitope was added to the 3` end of the Jak2 open reading frame using a two-step strategy. In the first stage, PCR was conducted employing an oligo that primed upstream of Jak2's internal XhoI site in conjunction with a long downstream oligo that contained the epitope tag, an NheI site and a region homologues to the 3' end of Jak2. The PCR product was inserted into TOPO (Invitrogen). An NheI/XhoI digest was used to move the epitope tag containing fragment back into the original Jak2 plasmid. Quikchange mutagenesis (Stratagene) was used to generate $Jak2^{Y317F}$, $Jak2^{Y637F}$, $Jak2^{Y317E}$, $Jak2^{Y637E}$, $Jak2^{K882E}$, $Jak2^{Y1007/8F}$, $Jak2^{Y119E\text{-}HA}$, $Jak2^{Y119E/Y317F\text{-}HA}$, $Jak2^{Y119E/Y637F\text{-}HA}$, $Jak2^{Y317F/S523A}$, $Jak2^{Y317F/Y570F}$, $Jak2^{S523A/Y570F}$, and $Jak2^{Y317F/S523A/Y570F}$. The presence of the desired mutations and the absence of adventitious mutations were confirmed by DNA sequencing for all plasmids generated.

Cell Lines and Transfection: HEK293 cells were grown at 37°C in humidified air with 5% CO_2. Cells were grown in DMEM supplemented with 10% FBS, penicillin and streptomycin. HEK293 cells were transfected using Lipofectamine (Invitrogen). 3-5 hours following transfection, the cells were serum-starved overnight in DMEM supplemented with 0.5% BSA. 32D cells were grown in RPMI 1640 media supplemented with 10% FBS and 5% IL-3 conditioned media. IL-3 conditioned media was generated using WEHI cells

grown in RPMI 1640 media supplemented with 10% FBS. Cells were grown to confluence and media was harvested then filtered (0.2 μm) and stored at 4°C.

Cytokine Stimulation, Lysis and Immunoprecipitation: HEK293 cells were stimulated with Epogen at 12.5 U/mL. 32D cells were stimulated with undiluted IL-3 conditioned media from WEHI cells. Cells were harvested with lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate) and cleared by centrifugation. Lysates used for direct immunoblotting were mixed 1:1 with Laemmli buffer. Total Jak2 was immunoprecipitated using a polyclonal antibody against Jak2 and protein-A agarose (Invitrogen) overnight at 4°C. Immunoprecipitates were washed three times with lysis buffer before elution with Laemmli buffer. HA-epitope tagged Jak2 was immunoprecipitated using a monoclonal antibody against HA and protein-G sepharose (GE Healthcare) overnight at 4°C. Immunoprecipitates were washed three times with lysis buffer before elution with Laemmli buffer.

Mass Spectrometry: For preparation of protein for MS analysis, material was immunoprecipitated from 5-10 15 cm dishes of HEK293 cells and resolved by SDS-PAGE. Jak2 protein was visualized by Coomassie brilliant blue G-250 stain. Jak2 protein was subject the mass spectrometry using two different instruments and methods. In one method, gel slices containing Jak2 were digested with 5 ng/μl sequencing grade modified trypsin (Promega) in 25 mM ammonium bicarbonate containing 0.01% n-octylglucoside for 18 hours at 37°C. Peptides were eluted from the gel slices with 80% acetonitrile, 1% formic acid.

Tryptic digests were separated by capillary HPLC (C18, 75μM i.d. Picofrit column, New Objective) using a flow rate of 100 nl/min over a 3 hour reverse phase gradient and analyzed using a LTQ 2-dimensional linear ion trap mass spectrometer (ThermoFinnigan). Resultant MS/MS spectra were matched against mouse Jak2 sequence using TurboSequest (BioWorks 3.1) with fragment ion tolerance <0.5 and amino acid modification variables including phosphorylation (80 Dal) of Ser, Thr, and Tyr, oxidation (16 Dal) of Met, and methylation (14 Dal) of Lys.

For the second method, gel slices containing Jak2 were destained, reduced and alkylated as described elsewhere [39]. Proteolytic digestion was carried out with 13 ng/μl sequencing-grade trypsin (Promega) in 25mM ammonium bicarbonate for 18 hours at 37°C. Resulted peptides were eluted with solution containing 60% methanol/5% acetic acid and peptide mixture was lyophilized (Labconco, Kansas City, MO). Esterification and IMAC enrichment were performed with "optimized" buffers described previously [40]. Phosphopeptide enriched peptide mixture was loaded off-line onto precolumn packed with an 8-cm bed length of 5-15 μm spherical C18 beads (Applied Biosystems, Foster City, CA) in 75 μm i.d. fused silica capillary tubing. HPLC was performed with high-performance analytical column (50 μm × 8 cm) packed with 5 μm-diameter C18 reversed-phase beads (YMC, Wilmington, NC) [40] at estimated eluent flow rate 20-50 nL/min. HPLC solvent A was 0.2 M acetic acid and solvent B was 70% acetonitrile/0.2 M acetic acid. LC-MS analysis of phosphopeptide mixture was performed using a solvent gradient of 0-5% B in 5

min and 5-100% B in 35 min. Mass spectrometry data acquisition was performed on QSTAR XL in data-dependent mode (MS scan, $300 \leq m/z \leq 2000$, top 5 most abundant MS/MS scans using low resolution for precursor isolation and using 1.5 s accumulation with enhance all mode, 1.8 kV ESI voltage). The resulting spectra were compared against NCBI mouse database using search engine Mascot Daemon (Matrix Science, Inc London, United Kingdom). The search parameters allowed for 2 missed cleavages for trypsin, a fixed modification of +14 for d_0-methyl esters, for aspartic acid, glutamic acid, and peptide C-terminus, and variable modification of + 80 for serine, threonine, and tyrosine phosphorylation and +16 for methionine oxidation. Mass tolerance was 1.0 Da for precursors and 0.35 Da for fragment ions. MS/MS spectra corresponding to phosphorylated peptides of Jak2 were manually verified.

Immunoblotting: SDS-PAGE gels were transferred to nitrocellulose membranes (Whatman) in Towbin buffer containing 0.02% SDS and 20% methanol. Membranes were blocked for 1 hour at room temperature or overnight at 4°C in buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, and 0.01% Tween 20 (wash buffer) supplemented with 3% bovine serum albumin (block buffer). Membranes were incubated in primary antibody in block buffer for 2 hrs at room temperature or overnight at 4°C. Membranes were rinsed three times with wash buffer then incubated with a secondary antibody conjugated to either horseradish peroxidase (Santa Cruz) or a fluorescent tag (Molecular Probes, Licor). For horseradish peroxidase detection, membranes were rinsed three times in wash buffer before being treated with chemiluminescence reagent (Roche) and

exposed to film (Kodak, Denville). For fluorescence detection, membranes were rinsed three times in wash buffer before detection using an Odyssey Imaging system manufactured by Licor Biosciences (Lincoln, NE). Immunoreactive bands were identified and quantified using Odyssey application software version 2.1.

In Vitro Kinase Assays: HEK293 cells were transfected with plasmids encoding ELR and either mutant or wild-type Jak2. Following transfection, the cells were serum-starved overnight in DMEM supplemented with 0.5% BSA. Prior to lysis, cells were treated with either vehicle or erythropoietin for 15 min. Cells were harvested with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EGTA, 0.2% Triton X-100, 0.5 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and cleared by centrifugation. Lysates were immunoprecipitated using an antibody against Jak2 or an antibody against the HA epitope. Immunoprecipitates were washed twice with lysis buffer then twice with kinase buffer (50mM HEPES pH 7.5, 100 mM NaCl, 1 mM Na_3VO_4, 5 mM $MnCl_2$, 0.5 mM DTT). Washed immunoprecipitates were split into two fractions. One fraction was used in the kinase assay. The kinase reaction was initiated by adding kinase buffer containing 500 mM STAT5 target peptide (AKAADGYVKPQIKQVV), 125 μM ATP, and 10μCi ^{32}P-labeled γATP to each immunoprecipitate. Aliquots were taken from the kinase reaction at various time-points and blotted on P81 papers. The P81 paper was washed in 75 mM H_3PO_4, scintillation fluid was added (Cytoscint (BP Biomedical)) and bound ^{32}P counted using a Packard scintillation counter. The second immunoprecipitate fraction was used to measure Jak2 content used to normalize kinase assay data. This

fraction was resolved by SDS-PAGE, transferred to nitrocellulose, then immunoblotted with a monoclonal antibody directed against total Jak2 or a monoclonal antibody against the HA epitope. Fluorescent secondary antibody was used and immunoreactive bands were imaged and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Jak2 Phosphorylation Sites Identified by MS in Present Study			
	Site	**Previous Identification**	**Function**
Tyr	134	Novel	NS
	201	(25)	SHP-2 Binding?
	206	(25)	NS
	221	(20,21,24)	Weakly activating?
	317	Novel	NS
	372	Novel	NS
	423	Psite*	NS
	435	Psite*	NS
	570	(20,21,24)	Inhibitory
	637	(24)	NS
	813	(6,24,)	Activating/SH2B binding
	868	(24)**	NS
	966	(24)**	NS
	1007/8	(22)	Activating
Ser/Thr	523	(26,27)	Inhibitory
	668	Novel	NS
	904	Novel	NS

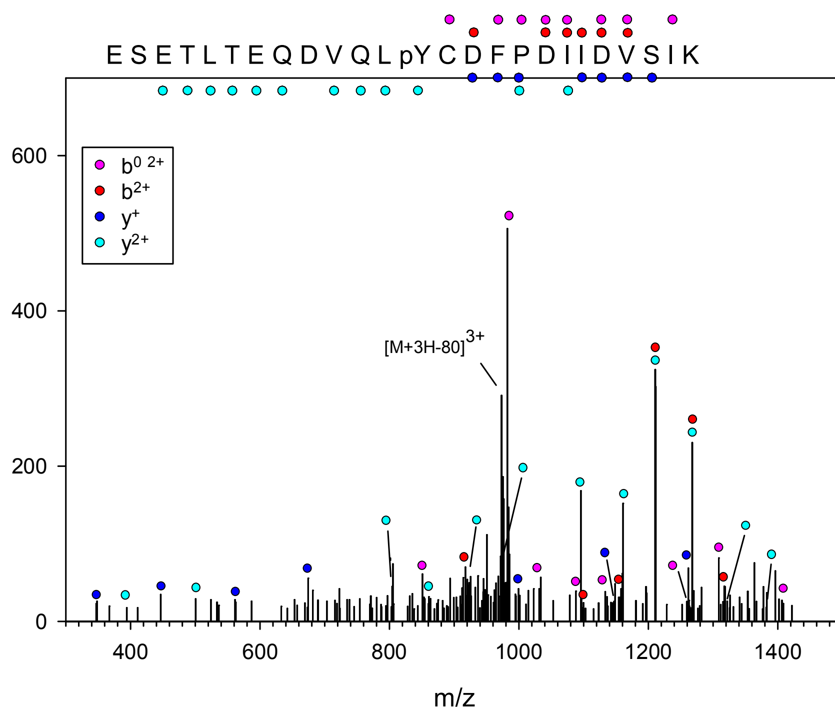
Table 2.1 Jak2 phosphorylation sites identified by MS/MS in the present study. Shown are the sites identified with high confidence (each site has been observed in multiple runs, conforms to strict confidence standards, and was manually confirmed). Also noted is any previous description of the site (or its novelty), along with any known or presumptive function for the site.

PSite*- Reported but not otherwise studied on www.phosphosite.org.

**The subject of a separate study (Larry Argetsinger and Christin Carter-Su)

NS- not previously studied.

A. pY317



B. pY37

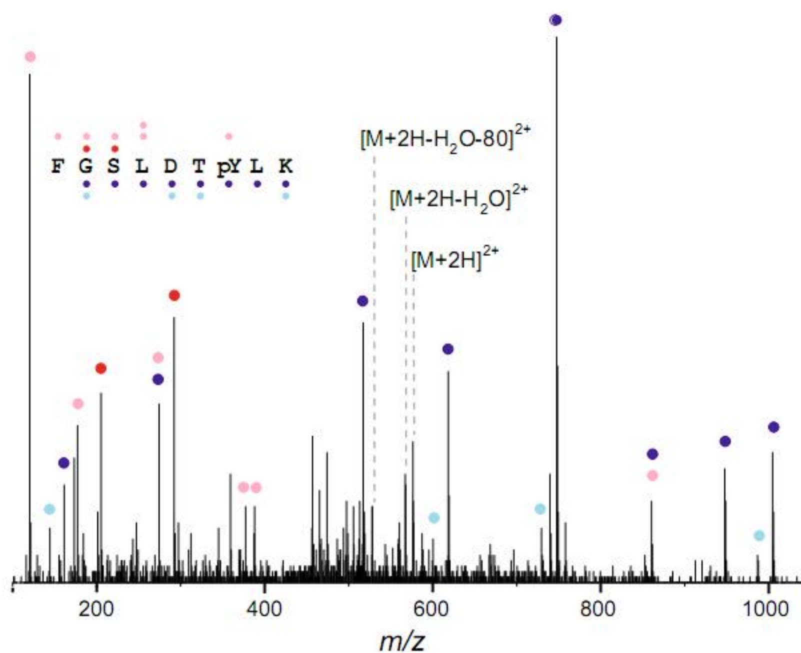


Figure 2.1 Representative MS/MS Spectra (for pY317 and pY637). MS/MS spectra resultant from Jak2 peptides containing pY317 (A) and pY637 (B). Sequence assignments of y+ and y0+ ions are shown in blue and light blue, respectively. The assignments of b+ ions, and derivative b0+ and a+ ions are presented in red and pink, respectively. An ion corresponding to neutral loss of 80 Da from each precursor is shown.

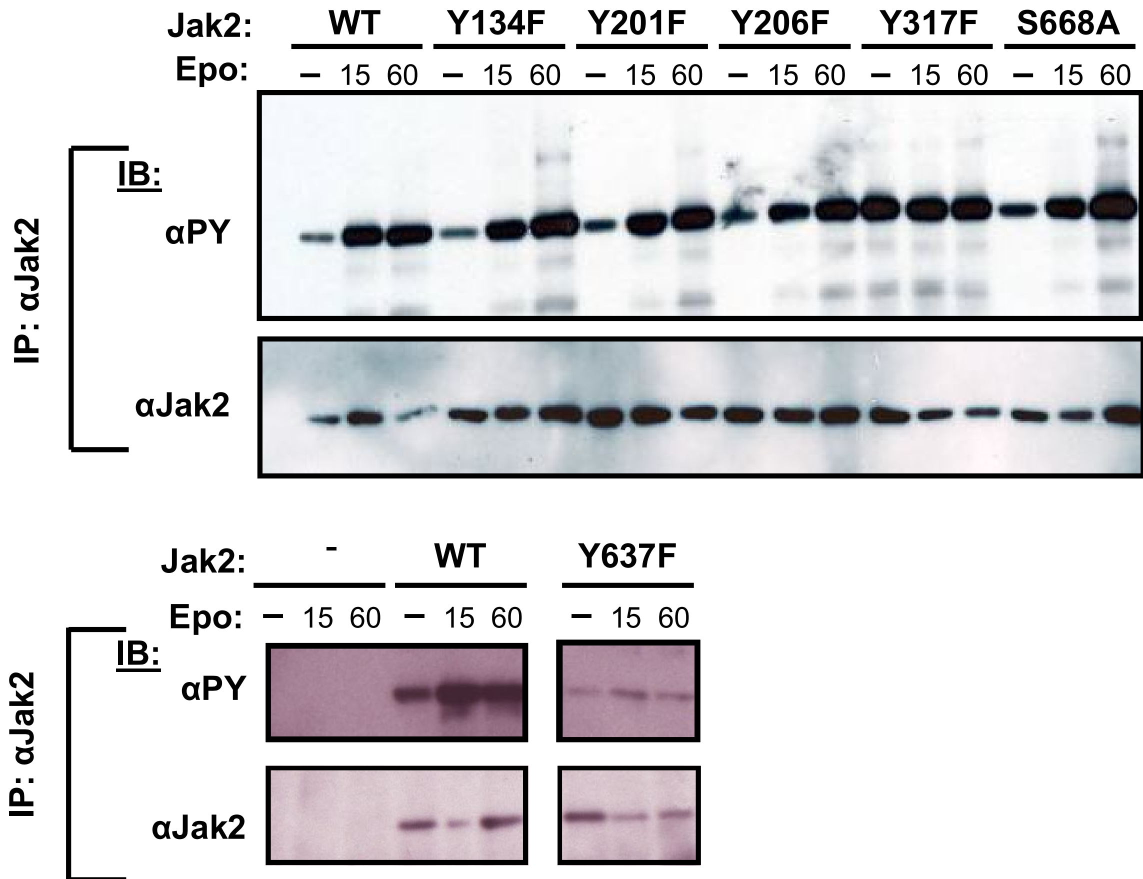


Figure 2.2 Representative examples of mutant screening data. HEK293 cells were transfected with the indicated Jak2 or Jak2 mutant plasmid. Cells were made quiescent overnight. Cells were either lysed without treatment (0) or incubated in the presence of EPO (12.5 U/ml) for either 15 or 60 minutes and then lysed. Lysates were immunoprecipitated with an antibody against total Jak2. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with the indicated antibodies. The figures shown are typical of three independent experiments.

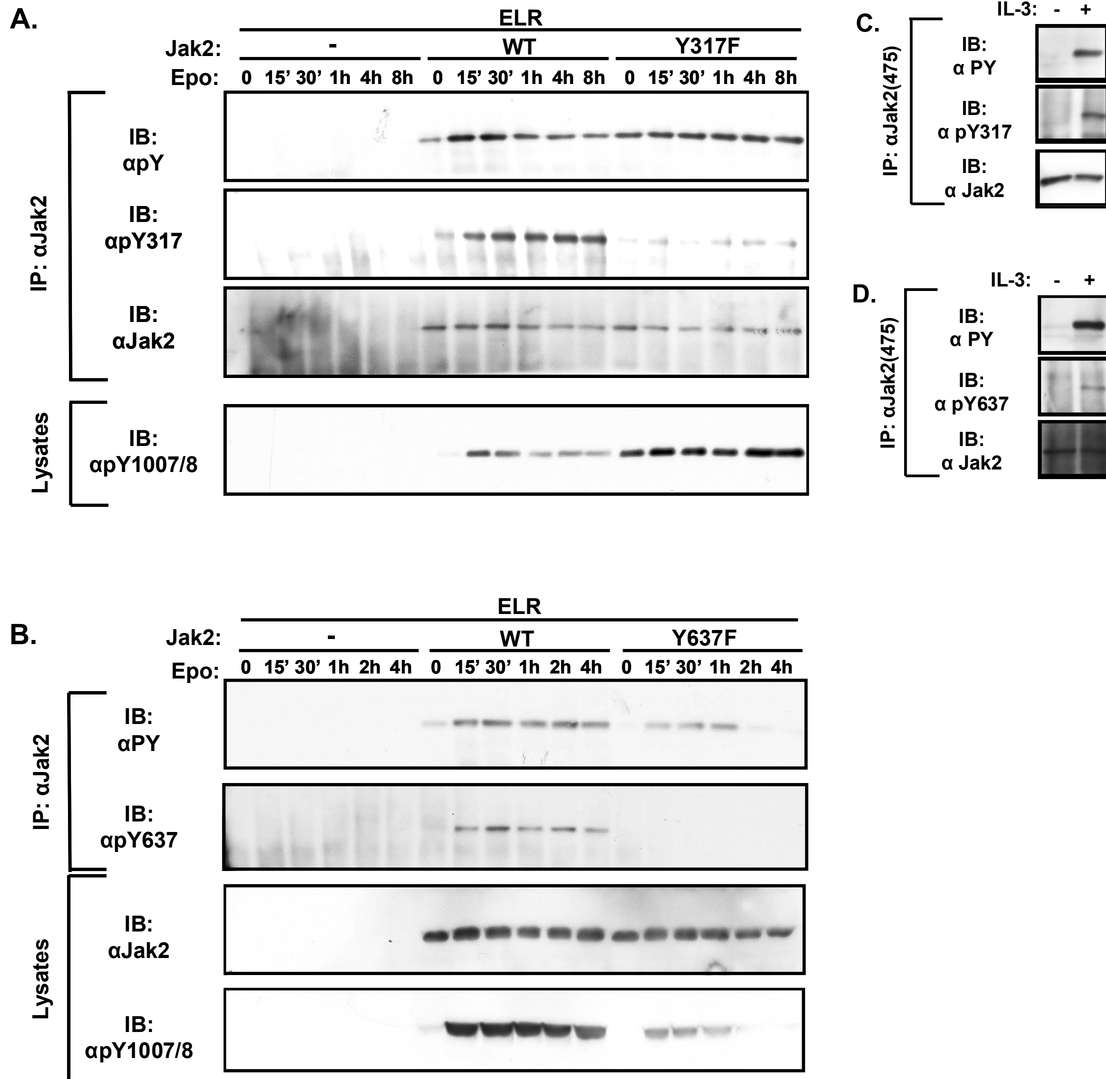


Figure 2.3 Time-course of phosphorylation of Tyr317 and Tyr637 following cytokine stimulation. A-B) HEK293 cells were transfected with ELR and the indicated Jak2 isoform, made quiescent, and incubated with EPO (12.5 U/ml) for the indicated amount of time before being lysed. Lysates were immunoprecipitated with the indicated antibodies. Lysates or immunoprecipitated proteins (as indicated) were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting with the indicated antibodies. The figures shown are typical of multiple independent experiments. C-D) 32D cells were made quiescent for 4 hours then incubated with either serum-free media or undiluted IL-3 containing WEHI-cell conditioned media for 1 hour (C) or 30 minutes (D) before lysis. Lysates were immunoprecipitated with an antibody against total Jak2. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with the indicated antibodies. The figures shown are representative of multiple independent experiments.

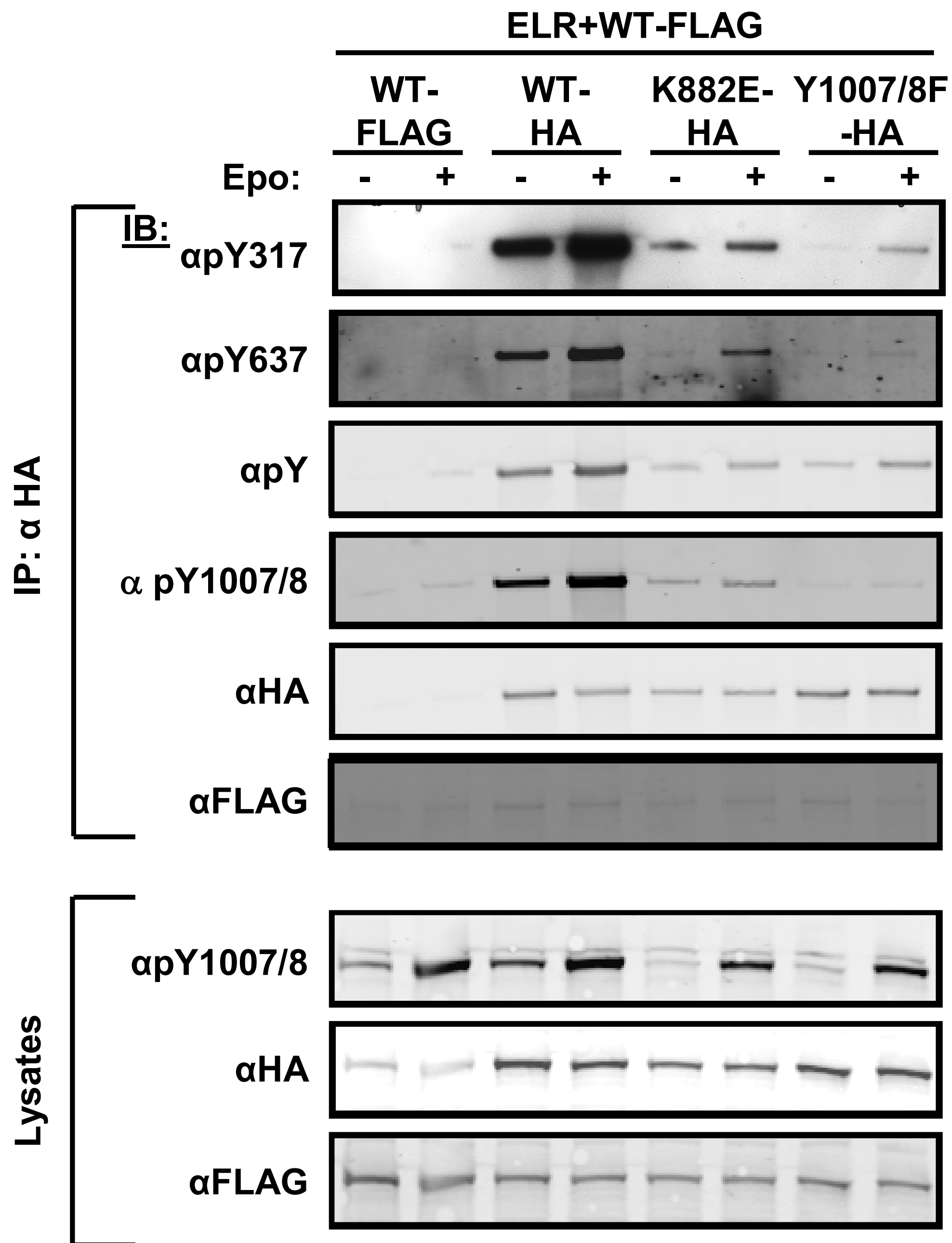


Figure 2.4 Transphosphorylation of Tyr317 and Tyr637 in response to cytokine stimulus. HEK293 cells were transfected with the indicated plasmids, made quiescent overnight, and incubated in the absence (-) or presence (+) of EPO (12.5 U/mL) for 15 minutes before lysis. Lysates were immunoprecipitated with aHA antibody. Immunoprecipitates or lysates (as indicated) were resolved by SDS-PAGE, transferred to nitrocellulose and blotted with the indicated antibodies. The figures shown are typical of multiple independent experiments.

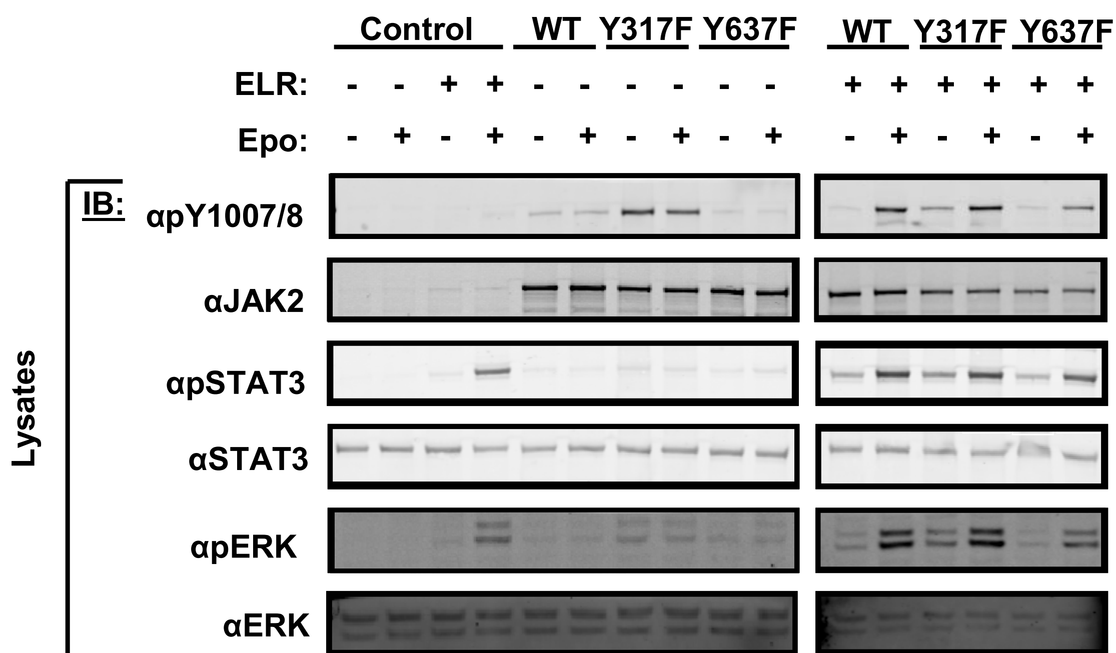
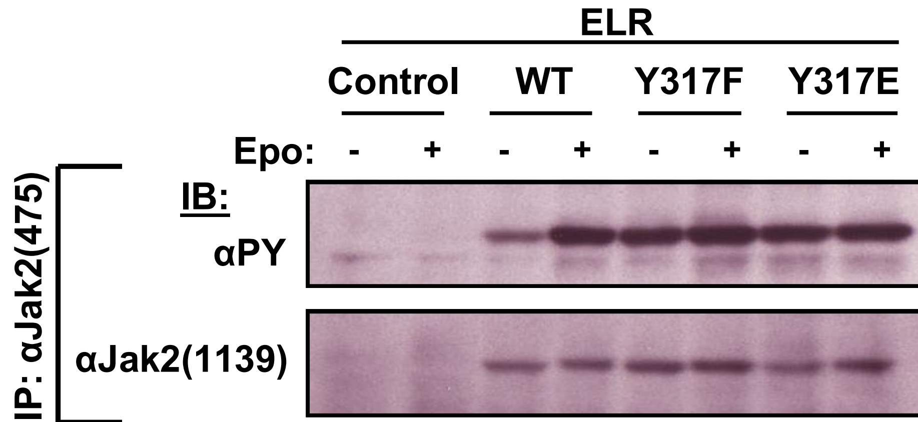


Figure 2.5 Effects of Tyr317 and Tyr637 on downstream signaling. HEK293 cells were transfected with the indicated plasmids, made quiescent overnight, and incubated in the absence (-) or presence (+) of EPO (12.5 U/mL) for 15 minutes before lysis. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with the indicated antibodies. The figures shown are typical of multiple independent experiments.



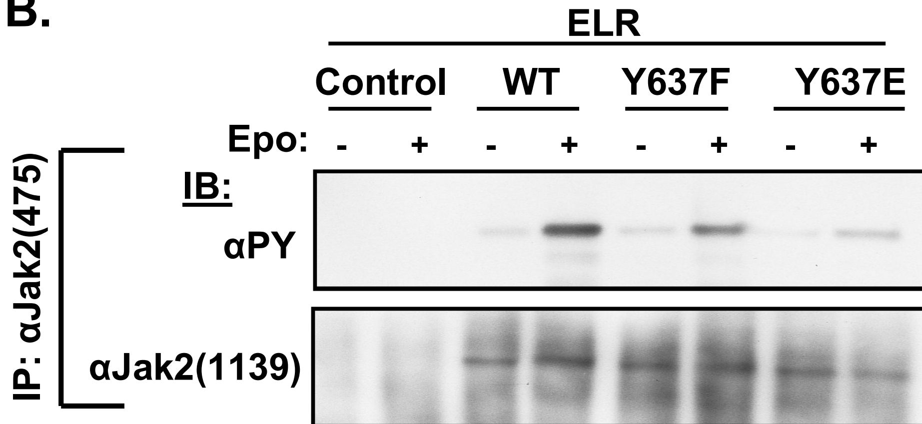


Figure 2.6 Similar phenotypes of Phe and Glu substitution mutants for Tyr317 and Tyr637. A-B) HEK293 cells were transfected with the indicated plasmids, made quiescent overnight, and incubated in the presence or absence of EPO (12.5 U/ml) before lysis. Lysates were immunoprecipitated with the indicated antibody. Immunoprecipitates or lysates (as indicated) were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with the indicated antibodies. The figures shown are typical of multiple independent experiments.

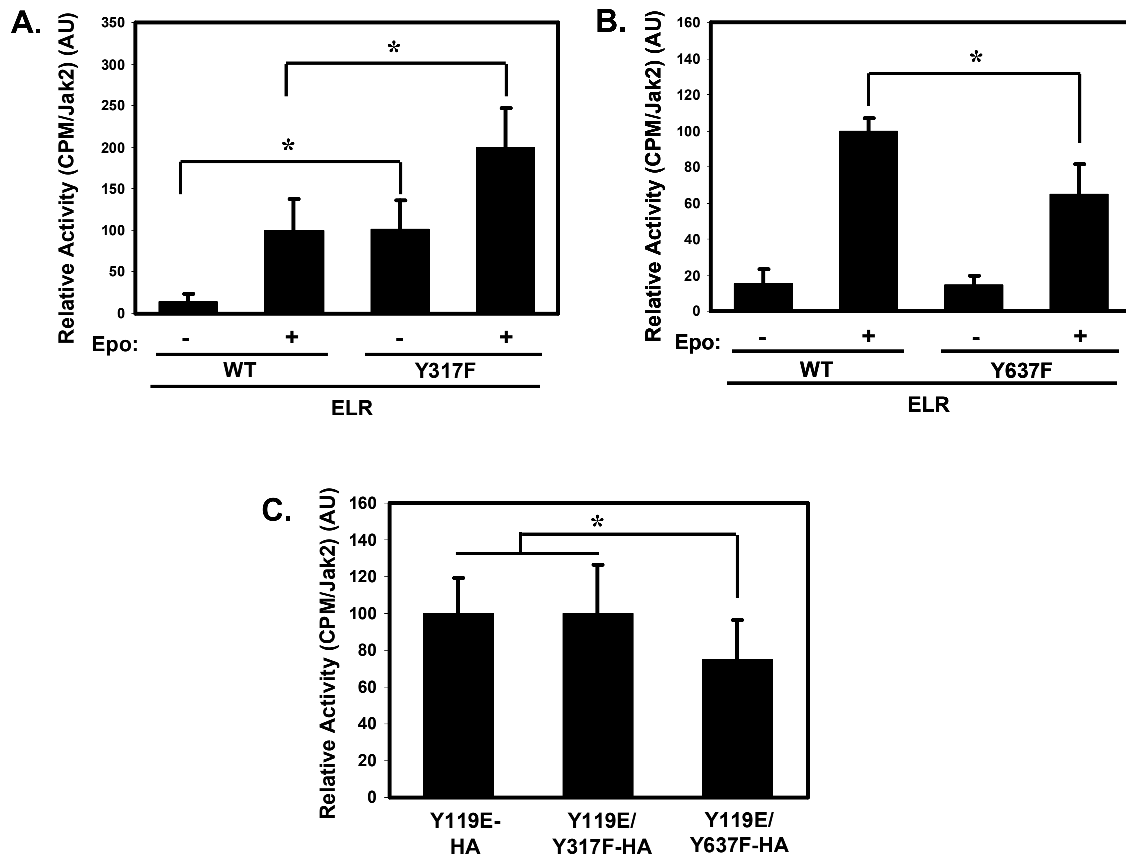
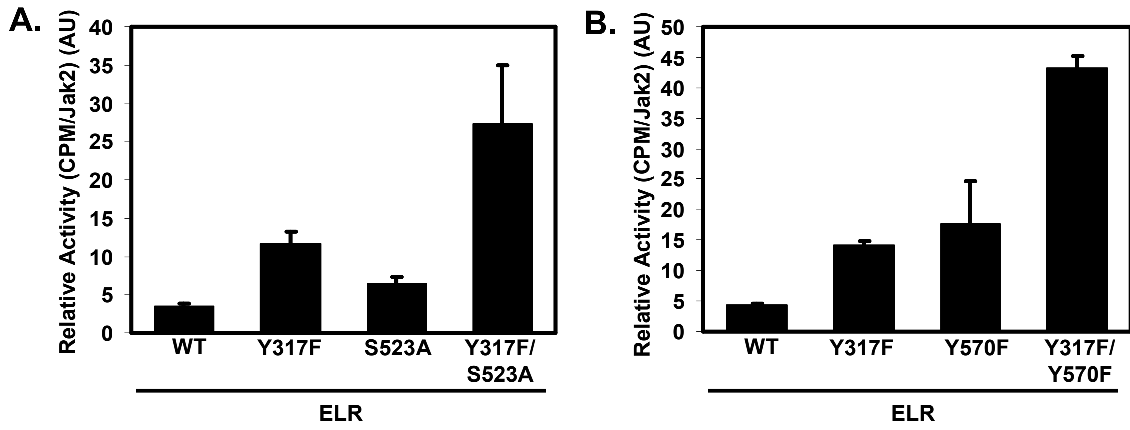
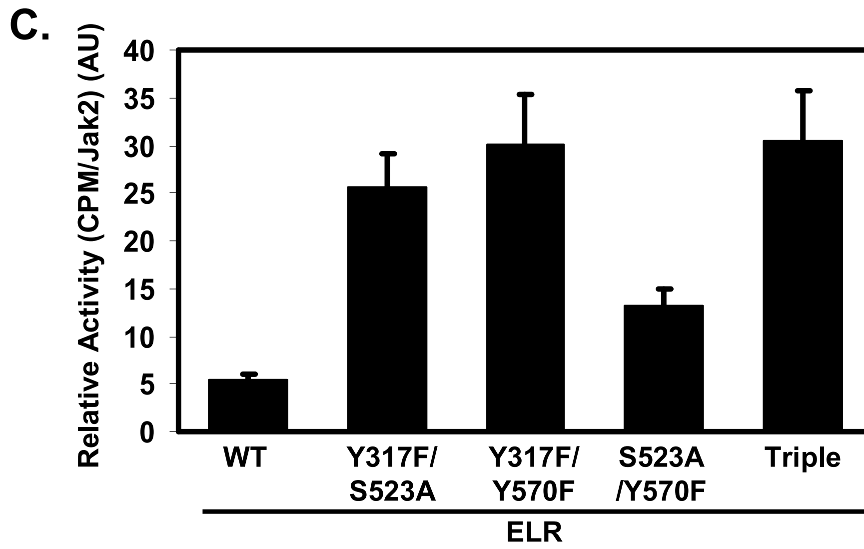
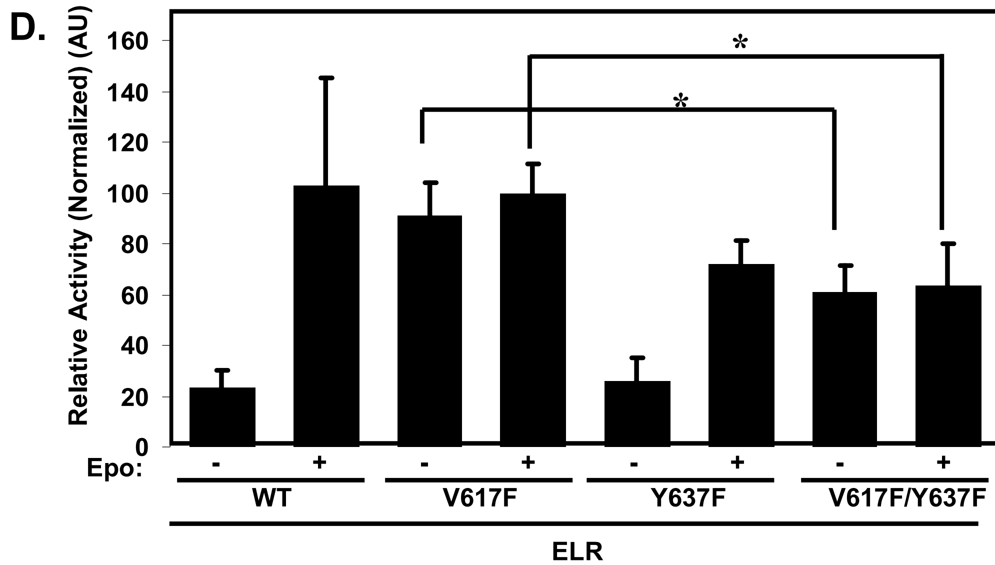


Figure 2.7 Modulation of Jak2 kinase activity by Tyr317 and Tyr637. A-B) HEK293 cells were transfected with the indicated plasmids made quiescent overnight before treatment with EPO or vehicle and lysed. Lysates were immunoprecipitated with an antibody against total Jak2. Immunoprecipitates were subjected to in vitro kinase assays using a target peptide derived from STAT5B. Scintillation counts were normalized by Jak2 content (determined by immunoblotting aliquots of each sample in parallel with the kinase assay) and are plotted in arbitrary units (AU). Bars represent the mean value derived from two independent experiments with two replicates each (n=4, total), +/- standard deviation. Values from each experiment were normalized by the mean value of the Jak2 stimulated samples which was set at 100 for each experiment. *Student's unpaired t-test, $p<0.05$. C) HEK293 cells were transfected with the indicated plasmids made quiescent overnight then lysed. Lysates were immunoprecipitated with aHA . Immunoprecipitates were subjected to in vitro kinase assays using a target peptide derived from STAT5B. Scintillation counts were normalized by HA content in each immunoprecipitate and are plotted in arbitrary units (AU). Bars represent the mean value derived from three independent experiments with four replicates each (n=12, total), +/- standard deviation. Values from each experiment were normalized by the mean value of the Jak2Y119E samples which was set at 100 for each experiment. *Student's unpaired t-test, $p<0.05$.

Figure 2.8 Functional interaction of Tyr317 and Tyr637 with other regulatory sites on Jak2. A-C) HEK293 cells were transfected with the indicated plasmids made quiescent overnight then lysed. Lysates were immunoprecipitated with an antibody against total Jak2. Immunoprecipitates were subjected to in vitro kinase assays using a target peptide derived from STAT5B. Scintillation counts were normalized by Jak2 content in each immunoprecipitate and are plotted in arbitrary units (AU). Bars indicate the mean of duplicate samples (N=2). Error bars indicate standard deviation. D) HEK293 cells were transfected with the indicated plasmids, made quiescent overnight, and incubated in the presence or absence of EPO (12.5 U/ml) before lysis. Lysates were immunoprecipitated with an antibody against total Jak2. Immunoprecipitates were subjected to in vitro kinase assays using a target peptide derived from STAT5B. Scintillation counts were normalized by Jak2 content in each immunoprecipitate and are reported in arbitrary units. Bars represent the mean value derived from two independent experiments with two replicates each (n=4, total), +/- standard deviation. Values from each experiment were normalized by the mean value of the Jak2V617F stimulated samples which was set at 100. *Student's unpaired t-test, $p<0.05$.


A.
Relative Activity (CPM/Jak2) (AU)
40
35
30
25
20
15
10
5
0
WT
Y317F
S523A
Y317F/
S523A
ELR
B.
Relative Activity (CPM/Jak2) (AU)
50
45
40
35
30
25
20
15
10
5
0
WT
Y317F
Y570F
Y317F/
Y570F
ELR


C.
Relative Activity (CPM/Jak2) (AU)
40
35
30
25
20
15
10
5
0
WT
Y317F/
S523A
Y317F/
Y570F
S523A
/Y570F
Triple
ELR


D.
Relative Activity (Normalized) (AU)
160
140
120
100
80
60
40
20
0
*
*
Epo:
-
+
-
+
-
+
-
+
WT
V617F
Y637F
V617F/Y637F
ELR

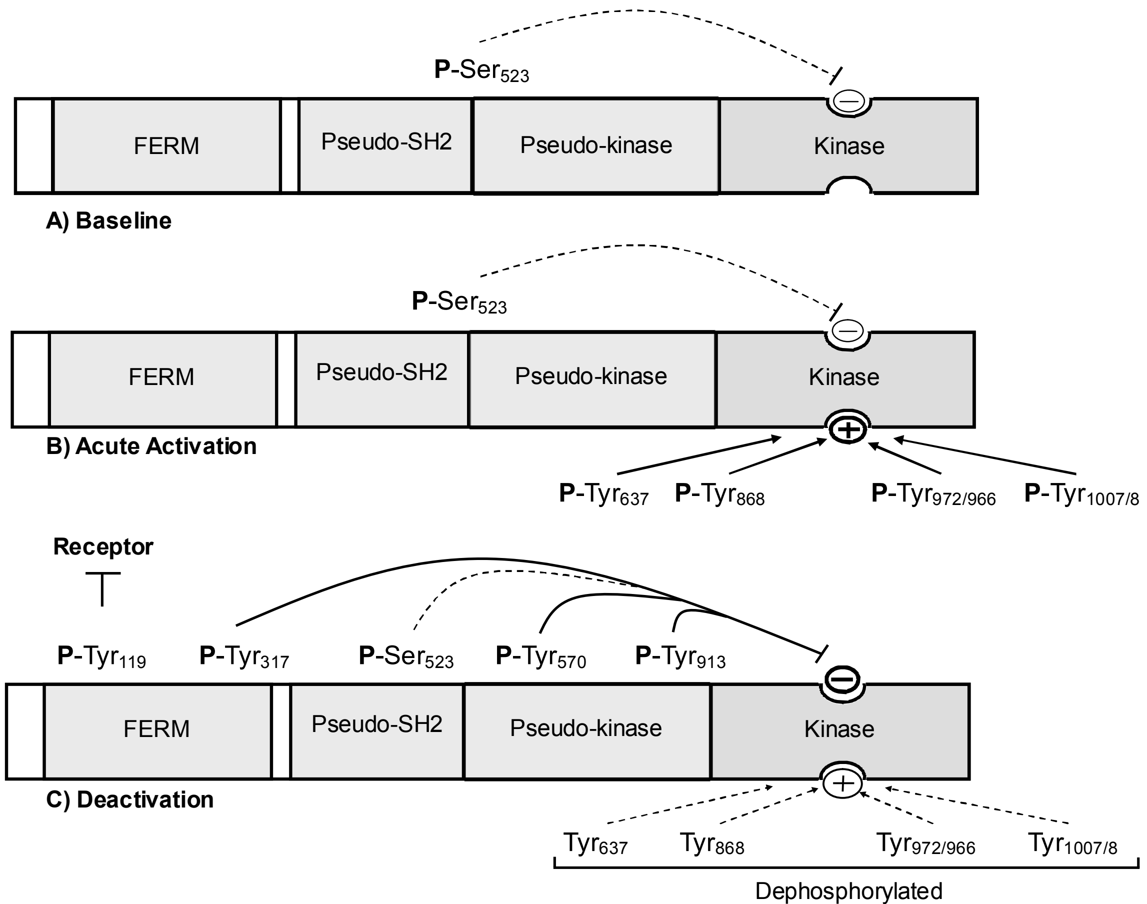


Figure 2.9 Model of phosphorylation-dependent regulation of Jak2 activity. A) Without stimulation, Jak2 is essentially unphosphorylated on most sites, although Ser523 is constitutively phosphorylated, providing a baseline of negative feedback to block kinase activity in the absence of stimulation. B) Upon cytokine stimulation, Tyr1007/8 (the activation loop sites) are rapidly phosphorylated to promote partial activation of the kinase domain. The phosphorylation of Tyr1007/1008 permits the phosphorylation of additional activating sites, including Tyr637 as well as other sites in kinase domain, leading to full activation of Jak2. C) Following acute activation, strongly inhibitory sites including Tyr317, Tyr570, Tyr913, and Tyr119 are phosphorylated to limit the extent of Jak2 activity, concomitant with the dephosphorylation of activating sites.

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CHAPTER 3

INSUFFICIENCY OF JAK2-AUTONOMOUS LEPTIN RECEPTOR SIGNALS FOR MOST PHYSIOLOGIC LEPTIN ACTIONS

Abstract

The adipose-derived hormone leptin regulates energy and glucose homeostasis as leptin deficiency results in obesity and diabetes. The intracellular domain of the major signaling form of the leptin receptor, LepRb, has a proximal Jak2 binding domain, and three distal tyrosine phosphorylation sites. To determine if the Jak2 binding site is sufficient to mediate any of leptin's actions, we replaced the endogenous LepR gene with one that encodes a receptor that is truncated immediately following the Jak2-binding site (LepRΔ65). We show that mice homozygous for this gene are phenotypically similar to *db/db* mice in most respects. They are infertile and develop similar amounts of adiposity and hyperphagia. However, while these mice are diabetic, their progression to hyperglycemia is delayed compared to *db/db* animals. Therefore, while the Jak2-binding site of LepRb is sufficient to delay the onset of diabetes, the majority of leptin action requires the remainder of the LepRb's intracellular tail.

Introduction

The adipose tissue-derived hormone leptin is produced in proportion to fat stores. Circulating leptin communicates the level of energy reserves in the periphery to the central nervous system in order to suppress food intake, permit energy expenditure and maintain euglycemia [4,12,14,26]. Adequate leptin levels permit energy expenditure and the processes of reproduction and growth, and similarly regulate other elements of the endocrine and immune systems [1,4,23]. Conversely, lack of leptin signaling due to mutation of leptin (e.g. *ob/ob* mice) or the leptin receptor (LepR) (e.g. *db/db* mice) in rodents and humans results in increased food intake in combination with reduced energy expenditure (and thus obesity) plus hyperglycemia and insulin insensitivity (even when leptin's effects on body weight are controlled for). Leptin deficiency also results in neuroendocrine dysfunction (including hypothyroidism, decreased linear growth, infertility and decreased immune function) [10,13,14,24]. Many of the effects of leptin are attributable to effects in the CNS, particularly in the hypothalamus, a site of high LepRb mRNA expression [12,26].

LepR represents a typical class-I cytokine receptor [15]. Alternative splicing generates several isoforms of LepR with identical ligand-binding domains, but which possess differing perimembrane and intracellular domains [21]. Membrane-bound LepRs consist of long (LepRb) and short (LepRa, among others) isoforms. LepRb features an approximately 300 amino acid intracellular tail that contains several docking sites for proteins critical for signal transduction. The most

membrane-proximal functional site mediates Jak2 binding and consists of Jak-binding consensus Box 1 and Box 2 motifs, both of which are necessary for LepRb-mediated Jak2 activation[17]. LepRb has no intrinsic enzymatic activity and LepRb-mediated activation of Jak2 is necessary for leptin signaling. The remaining length of LepRb contains three tyrosine sites (Tyr_{985}, Tyr_{1077} and Tyr_{1138}) which are phosphorylated by Jak2 in response to leptin stimulation and mediate binding with SH2-domain containing effector proteins.

Several studies have determined the individual contributions of LepRb's functional sites on leptin physiology in vivo. To determine the importance of Tyr_{1138} we generated a knock-in mouse model in which LepR mutant for Tyr_{1138} replaced endogenous LepRb and abrogates STAT3 activation by LepRb (*s/s* mouse model). These mice are hyperphagic and obese but lack some of the phenotypes that characterize the *ob/ob* and *db/db* models including severe diabetes and infertility [3,5] Similarly, we generated mice in which LepRb was homologously replaced by a mutant containing a substitution of Tyr_{985} that abrogates phosphorylation of the site and blocks SHP2/SOCS3 association (*l/l* mouse model) [2,6,7] [8]. Mutation of Tyr_{985} in vivo results in reduced feeding and adiposity, and decreased orexigenic arcuate nucleus (ARC) neuropeptide expression. Coupled with the increased sensitivity of *l/l* animals to exogenous leptin, these observations suggest that mutation of Tyr_{985} blocks the activation of an inhibitory Tyr_{985}-dependent LRb signal, ultimately leading to increased leptin sensitivity in vivo. Lastly, mutating all three intracellular tyrosines on LepRb results in an obese, diabetic and infertile animal (Y123F mouse model) [16].

However, in contrast to *db/db* mice, Y123F animals are slightly less obese and diabetic demonstrating a functional role for LepRb independent of its intracellular tyrosine residues.

To determine if the Jak2-binding site of LepRb is sufficient to account for any of leptin effects in vivo, we replaced the endogenous LepRb allele with one encoding a leptin receptor truncated immediately following the Jak2 binding site at the 65th residue of the intracellular domain (LepRbΔ65). We found the mice homozygous for this truncated receptor were similar to *db/db* mice overall, though with some improvements in glucose homeostasis.

Results

Model validation and gene targeting. Previous studies showed that LepRb truncated after the 65th amino acid of the intracellular domain retains the ability to dock with Jak2 and mediate its activation in response to leptin[17]. In contrast, LepRa (which is identical to the *db* allele encoded leptin receptor) contains only the first 29 amino acids of LepRb and does not mediate Jak2 activation (at physiological levels of Jak2). To confirm that LepRbΔ65 could facilitate activation of Jak2 in our hands, we expressed it in cultured HEK293 and compared it to wild-type LepRb and a short-form LepR (LepRa). Leptin stimulation caused Jak2 activation in LepRbΔ65 and LepRb-expressing cells but not in control or LepRa-expressing cells (Fig 1A). This confirms that LepRbΔ65, but not LepRa, mediates Jak2 activation in response to leptin stimulation.

In order to understand the role that the Jak2-binding site in leptin physiology, we replaced the endogenous LepR allele with one encoding LepRbΔ65 (*Δ* allele) using homologues recombination (Fig 1B). Correct targeting was confirmed by Southern blotting (Fig 1C). This strategy allowed LepRbΔ65 expression from the native LepR locus and similar models have resulted in similar gene expression patterns as wild-type LepRb [5,9]. Heterozygous animals (*+/Δ*) were backcrossed with +/+ C57BL/6J mice obtained from Jackson Laboratories to facilitate direct comparison to *db/db* animals on this background. We confirmed equivalent hypothalamic expression of leptin receptor mRNA in *Δ/Δ* animals compared to wild-type animals using RT-PCR (Fig 1D).

Effect of Jak2-binding site on energy homeostasis and linear growth. We initially studied the energy homeostatic characteristics of *Δ/Δ* mice. Wild-type, *Δ/Δ* and *db/db* mice were weaned at four weeks of age and individually housed with a normal chow diet. Body weight and food intake were monitored weekly from four to eight weeks. We found that *Δ/Δ* mice were obese and hyperphagic (Fig 2A-D). Compared to age and sex-matched *db/db* animals, *Δ/Δ* mice had similar body weights and levels of food intake over the four week study period. This shows that LepRbΔ65 confers no improvement to obesity in *db/db* mice and that the Jak2-binding site on the leptin receptor is not sufficient to mediate leptin-induced prevention of hyperphagia and obesity.

To determine if the relative levels of fat mass and lean mass of *Δ/Δ* mice were similar to those of *db/db* mice; we used an NMR Minispec to analyze the

body composition of *Δ/Δ*, *db/db* and wild-type animals. We found that age and sex-matched *Δ/Δ* and *db/db* mice had similar proportions of fat and lean mass (Table 1). Leptin also regulates energy expenditure by managing autonomic outflow and regulating non-shivering thermogenesis. We measured core body temperature in wild-type, *Δ/Δ* and *db/db* animals. Both *Δ/Δ* and *db/db* were significantly cooler than wild-type animals but were no different from each other (Table 1).

Lastly, leptin plays a role in regulating linear growth as leptin-deficiency causes reduced body length. We observed that *Δ/Δ* males were shorter than wild-type animals but no different from *db/db* animals (Table 1). Overall, we were unable to detect any differences in energy homeostasis or linear growth between *Δ/Δ* and *db/db* mice, suggesting that the Jak2-binding site on LepRbΔ65 is not sufficient to regulate these functions.

Effect of Jak2-binding site on fertility. Leptin action is required for reproduction and *db/db* and *ob/ob* animals are both infertile. Obesity alone, however, is not sufficient to explain this defect as *s/s* mice ($LepR^{Y1138S}$) are almost as obese as *db/db* mice (10% less body weight on average compared to *db/db*) but they retain their fertility[5]. This finding suggests that a signal emanating from a different location on LepRb could be responsible for leptin's role in fertility. To determine if the Jak2-binding site of LepRb could mediate fertility, we monitored estrous cycling in female mice from four to eight weeks. We also attempted to mate females with wild-type males and monitor for pregnancy and the production of offspring. We compared *Δ/Δ*, *db/db*, wild-type

and *s/s* mice. We found that *Δ/Δ* mice were infertile (Table 2). No litters were produced by crossing *Δ/Δ* females with wild-type males. Additionally, monitoring the vaginal cytology of *Δ/Δ* females revealed these mice did not cycle normally and did not attain estrous. Gross examination revealed the reproductive organs of *Δ/Δ* females were atrophic and similar to those of *db/db* females (data not shown). These findings show that the Jak2-binding site in LepRΔ65 is not sufficient to account for leptin's permissive role in fertility.

Effect of Jak2-binding site on hypothalamic neuropeptide regulation. In the hypothalamus, leptin regulates key neuropeptides that mediate changes to energy and glucose homeostasis. The most well-characterized of these are POMC, NPY and AgRP. POMC is anorexigenic and is up-regulated by leptin. Conversely, NPY and AgRP (which are coexpressed in the same neurons) are orexigenic and are suppressed by leptin. To determine if the Jak2-binding site of the leptin receptor is sufficient to regulate changes in neuropeptide expression, we measured the levels of POMC, NPY and AgRP in the hypothalamus of male *Δ/Δ*, *db/db* and wild-type mice. We found similarly high levels of NPY and AgRP in *Δ/Δ* and *db/db* mice compared to wild-type mice (Fig 3A-C). With respect to POMC, *Δ/Δ* and *db/db* exhibited significantly lower levels compared to wild-type. Interestingly, POMC levels in *Δ/Δ* mice were even lower than those in *db/db* mice. The significance of this change is unclear and may suggest a minor role for the Jak2-binding site in POMC suppression that is overshadowed in the context of the full-length leptin receptor and strong STAT3-induced up-regulation of POMC mediated by the intracellular domain of LepRb.

Effect of Jak2-binding site on regulation of neuronal activity. Leptin modulates neuronal activity in cells expressing LepRb. Leptin up-regulates activity in POMC-expressing cells and down-regulates activity in AgRP and NPY-expressing cells. c-fos like immunoreactivity (CFLIR) is an indicator of neuronal activity and is present in AgRP neurons in *db/db* mice as leptin is unable to suppress neuronal firing in these cells[28]. To determine if *Δ/Δ* mice exhibit similar patterns of CFLIR, we intercrossed *+/+*, *Δ/+* and *db/+* animals with mice heterozygous for an allele which expresses LacZ from the AgRP locus (*a/+*). We performed immunohistochemistry on *+a/++, Δa Δ+* and *dba/db+* animals and determined CFLIR in the ARC in relation to AgRP neurons. We found many AgRP neurons in *Δ/Δ* and *db/db* animals were CFLIR positive while CFLIR was minimally present in the AgRP neurons of +/+ animals (Fig 3D). The proportion of AgRP neurons colocalized with CFLIR was not different between *Δ/Δ* and *db/db* animals indicating the Jak2-binding site of LepRΔ65 is not sufficient to mediate leptin-mediated suppression of AgRP-neuron firing (Fig 3E).

Effect of Jak2-binding site on regulation of immune cell function. Leptin deficiency results in thymic hypoplasia, reduced T cell function, and consequently, immunodeficiency [20,23,23]. To survey these parameters in our mouse models, we isolated spleens from wild-type, *ob/ob*, *Δ/Δ*, *s/s*, and *l/l* animals, determined the number of total white cells, determined the number of CD4+ cells, then assessed the proliferative capacity of CD4+ cells ex vivo. *s/s* and *l/l* animals were included in these analyses because immune function has not been previous assessed in these models. We found that the total number of

white cells and CD4+ T cells was lower in ob/ob and *Δ/Δ* spleens compared to wild type but the quantity of these cells from *l/l* and *s/s* animals was not (Fig 4A-B). Similarly, the proliferative capacity of CD4+/CD25- T effector cells (the majority of CD4+ cells from the spleen) stimulated ex vivo was also reduced in cells isolated from ob/ob and *Δ/Δ* animals compared to the other mouse strains (Fig 4C). These data suggest a necessary role for the tyrosine-containing domain of LepRb in both maintenance of white blood cells and T cells in the spleen and the function of T effector cells stimulated ex vivo, outside of their natural environment. Data from *s/s* and *l/l* mice were no different than wild type and may suggest that either Tyr_{985} or Tyr_{1138} is sufficient for immune function. Alternatively, maybe a different aspect of the intracellular domain of LepRb is responsible, independent of these phosphorylation sites.

Effect of Jak2-binding site on glucose homeostasis. Given leptin's prominence in regulation of glucose homeostasis, we examined glucose homeostasis in *Δ/Δ* mice. Glucose levels in free-fed, individually housed *Δ/Δ*, *db/db* and wild-type mice were measured at four, six and eight weeks. Interestingly, we found that at four weeks of age, the glucose levels of male and female *Δ/Δ* mice were normal while *db/db* mice were already significantly diabetic (Fig 5A-B). At later time-points, though, *Δ/Δ* progressed to diabetes and had glucose levels similar to *db/db* mice. Fasted blood glucose was measured at six and nine weeks. Male *Δ/Δ* mice had significantly lower glucose levels than *db/db* mice at six weeks of age (Fig 5C). Female *Δ/Δ* glucose levels were not significantly different from *db/db* levels, though they trended down in comparison.

However, at nine weeks, fasted blood glucose of both male and female *Δ/Δ* mice were both elevated compared to wild-type and not significantly different *db/db* (Fig 5D). These data suggest that the Jak2-binding site present in LepRbΔ65 is sufficient to delay the onset of diabetes compared to *db/db* mice. Though, following this early age, *Δ/Δ* mice become just as diabetic as *db/db* mice.

To explore glucose homeostasis in these mice more completely, and to differentiate between potential glucose and insulin sensitivity, we performed glucose and insulin tolerance tests. Since *Δ/Δ* and *db/db* mice seemed to be most different at young ages, we performed glucose and insulin tolerance tests at five and six weeks, respectively. We found that female *Δ/Δ* mice at five weeks of age mounted a more robust response to glucose challenge that *db/db* mice, though still less so than wild-type mice. We detected no differences in glucose tolerance in male mice at this age (Fig 6A-B). With respect to insulin tolerance, both *db/db* and *Δ/Δ* mice at six weeks of age were similarly insensitive to insulin challenge (Fig 6C-D). Taken together, *Δ/Δ* females are able to mitigate a high glucose challenge better than *db/db* females while being similarly insulin resistant. This may suggest that *Δ/Δ* mice secrete more insulin in response to glucose challenge and are, therefore, able to return more quickly to euglycemia. However, it is also possible that the observed euglycemia in young mice may result from better insulin sensitivity, but that it decays prior to our measurement of insulin tolerance at six weeks of age.

To explore the possibility that differences in insulin secretion could explain the differences in glucose homeostasis that we observed between *db/db* and *Δ/Δ*

mice, we measured the serum concentrations of insulin and C-peptide at four, six, and eight weeks of age from free-fed animals. Interestingly, we observed significant differences between male and female *Δ/Δ* mice (Fig 7). For female mice, there was no difference in serum insulin or C-peptide levels at any age between *db/db* and *Δ/Δ* mice. In male mice, insulin and C-peptide were also similar at four weeks of age. However, *Δ/Δ* males at later ages were able to more robustly secrete insulin than age-matched *db/db* males.

These data suggest that the relative euglycemia of *Δ/Δ* compared to *db/db* animals at four weeks of age is not due to differences in insulin secretion. Alternatively, it may be accounted for by superior insulin sensitivity or attenuated hepatic glucose production at this age. Interestingly, male *Δ/Δ* mice beyond four weeks of age secrete significantly more insulin than *db/db* males. However, given the similar levels of blood glucose at these ages this robust secretion is unable to overcome insulin insensitivity.

Discussion

The aim of this work was to determine if the Jak2-binding site of the leptin receptor is sufficient to mediate any of leptin's effects on physiology, independent of the remaining length of LepRb. To do this, we created a mouse model to express a LepR mutant with the minimum requirements for Jak2 binding. LepRbΔ65 is longer than the *db*-encoded leptin receptor by only 30 amino acids but these residues are essential for Jak2 binding. Not surprisingly, the phenotype of LepRbΔ65 homozygous mice was very similar to that of *db/db*

mice, demonstrating that other regions of LepRb (including its tyrosine phosphorylation sites) are required for most leptin action. However, we did detect some small differences between *Δ/Δ* and *db/db* mice demonstrating a role for Jak2 action independent of the remaining intracellular tail of the LepRb.

To summarize our data, *Δ/Δ* mice are obese, hyperphagic, diabetic and infertile. We detected no differences between *Δ/Δ* and *db/db* mice with respect to energy homeostasis. Body weight, food intake and body composition were all tightly matched between these mouse strains. Other phenotypes associated with leptin-deficiency were also very similar between *Δ/Δ* and *db/db* mice. Both models were similarly short, hypothermic and infertile. They failed to produce offspring and we did not observe estrus cycling in either mouse model. These findings demonstrate the necessity of the tyrosine-containing region of LepRb for these functions of leptin.

While we did not detect any differences in energy homeostasis between *Δ/Δ* and *db/db* animals, there were some differences in glucose homeostasis; which is consistent with the fact that the brain is more sensitive to the glucose lowering effects of leptin than its body weight lowering effects[29]. Overall, both male and female *Δ/Δ* mice exhibited a delayed progression to diabetes compared to *db/db* mice. At four weeks of age, *Δ/Δ* mice were euglycemic while their *db/db* counterparts were already hyperglycemic. Blood insulin and C-peptide levels were similar between *Δ/Δ* and *db/db* animals at this age suggesting that the euglycemia in *Δ/Δ* mice was the result of better insulin sensitivity compared to sex-matched *db/db* animals. However, the euglycemia in *Δ/Δ* mice required

highly elevated insulin release compared to wild-type controls and demonstrated severely stressed glycemic control in these young animals. Following weaning, *Δ/Δ* mice progressed quickly to diabetes. While we did observe better glucose tolerance in five week old females and lower fasted blood glucose in six week old males, beyond this point, we observed no differences between *Δ/Δ* and *db/db* animals with respect to blood glucose levels (fasted or free-fed) or insulin tolerance.

Hypothalamic neuropeptides mediate some of leptin's effects on energy and glucose homeostasis and perhaps changes in POMC, NPY or AgRP expression underlie the difference in diabetes progression between *Δ/Δ* and *db/db* mice. However, at 10-11 weeks of age, the expression of these leptin-regulated neuropeptides in *Δ/Δ* and *db/db* mice was very similar. AgRP and NPY levels were highly elevated in *Δ/Δ* and *db/db* mice while POMC levels were suppressed. While these data do not explain the delayed onset of diabetes in *Δ/Δ* mice, we cannot rule out the possibility that neuropeptide differences between *Δ/Δ* and *db/db* mice exist at earlier ages and dissipate quickly following weaning. Neuropeptide levels in our studies were measured at 10-11 weeks of age, by which time *Δ/Δ* mice had become fully diabetic.

Surprisingly, the levels of POMC in *Δ/Δ* mice were lower than they were in *db/db* mice. This may suggest a minor role for the Jak2-binding region in the suppression of POMC expression, though, a mechanistic explanation for this phenomenon is difficult to ascertain. The known signaling pathways linking LepRb to POMC gene regulation (STAT3 and FOXO1) cause POMC up-

regulation in response to leptin[5,34]. However, the regulation of neuropeptides in the ARC is not completely cell-autonomous and involves a complex network of neural connections[11]. Therefore, the expression of LepRbΔ65 in a different population of neurons could send increased inhibitory signals to POMC neurons resulting in the very low POMC expression observed in *Δ/Δ* mice.

Overall, these data suggest a minor, but measurable role for the Jak2-binding region of LepR in the leptin's regulation of glycemic control. While the molecular mechanisms underlying this action are unclear, it is possible that PI3-K activation could fill this role. Phosphoinositide-3 kinase (PI3-K) activation is critical for leptin action in the hypothalamus[25]. However, the molecular events linking LepRb to PI3-K have not been established in vivo. Perhaps, Jak2 activation in *Δ/Δ* mice is sufficient to alter glucose homeostasis through this signaling cascade. A potential intermediate linking Jak2 activation to PI3-K activation is SH2B1, a SH2-domain containing protein that binds phosphorylated Tyr_{813} on Jak2[18,19]. Cell culture studies show that SH2B1 binds directly to Jak2, augments its kinase activity and couples leptin stimulation to insulin receptor substrate (IRS) activation, a well-known activator of PI3-K[22]. Furthermore, whole body knockout of SH2B1 results in a hyperphagia, obesity and diabetes[30]. Alternatively, while we presume that Jak2 activity accounts for the differences in phenotype between *Δ/Δ* and *db/db* animals, we acknowledge the possibility that other, unidentified factors could interact with the Jak2-binding site and mediate these changes.

Intriguingly, our model of LepRb differs significantly from a related model featuring a replacement of wild-type LepRb with one in which all three tyrosines in the intracellular domain are mutated ($LepR^{Y123F}$)[16]. Like *Δ/Δ* animals, $LepR^{Y123F}$ homozygous animals are obese, diabetic and infertile. However, in comparison to *db/db* mice, $LepR^{Y123F}$ are different in several important ways. First, $LepR^{Y123F}$ animals are less hyperphagic and less obese than *db/db* mice, demonstrating improved energy homeostasis. Second, the levels of NPY, AgRP and POMC in these animals are intermediate between those in *db/db* and wild-type animals. Third, glucose homeostasis in $LepR^{Y123F}$ animals is better than in *db/db* mice and sustained until at least twelve weeks of age. Lastly, these mice are longer than their wild-type controls (as measured by snout-anus length).

Subtle differences in background strain could explain these differences. While both and $LepR^{\Delta 65}$ and $LepR^{Y123F}$ mice were backcrossed onto a C57BL/6 background, wild-type animals were obtained from different mouse repositories. However, a more interesting possibility is that the tyrosine-containing domain of LepRb, independent of phosphorylation, can mediate energy and glucose homeostatic signals. While the current list of proteins known to bind LepRb in this region are exclusively SH2-domain containing, these findings hint at the possibility of additional effector proteins that can interact with LepRb independent of its phosphorylation sites. Further work will necessary to confirm the importance of these proteins and determine their identity.

This work provides an in vivo model of the potential sufficiency of LepRb's Jak2-binding site for leptin action and demonstrates a small, but measurable role

for this region in leptin's regulation of glucose homeostasis. Precise genetic manipulation of the LepRb has provided key insights into the roles and responsibilities of its discreet functional units and this current work fits well within this paradigm. While these findings, taken together with those of other LepRb mouse models, show that the Jak2-binding region is not sufficient to account for most leptin action, they do suggest a previously unappreciated role for the tyrosine-containing region of Jak2, independent of phosphorylation. More work will be necessary understand this phenomenon, identify the molecular players that enable it, and determine its role in leptin physiology.

Acknowledgements

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Methods and Materials

Cell Culture Studies: Plasmids encoding LepRbΔ65 and LepRa were created by Quikchange Mutagenesis (Stratagene) using LepRb as template. The absence of adventitious mutations was confirmed by DNA sequencing for all plasmids in use. HEK293 cells were grown at 37°C in humidified air with 5% CO_2. Cells were grown in DMEM supplemented with 10% FBS, penicillin and streptomycin. HEK293 cells were transfected using Lipofectamine (Invitrogen).

3-5 hours following transfection, the cells were serum-starved overnight in DMEM supplemented with 1% BSA.

Prior to lysis, cells were stimulated with leptin (625 ng/mL) (Amylin Pharmaceuticals) for 15 min. Cells were harvested with lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate) and cleared by centrifugation. Total Jak2 was immunoprecipitated using a polyclonal antibody against Jak2 (used previously [31]) and protein-A agarose (Invitrogen) overnight at 4°C. Immunoprecipitates were washed three times with lysis buffer before elution with Laemmli buffer.

Gel electrophoresis was performed using precast 4-12% Bis-Tris gels (Invitrogen). Following transfer to nitrocellulose, membranes were blocked for 1 hour at room temperature in buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, and 0.01% Tween 20 (wash buffer) supplemented with 3% bovine serum albumin (block buffer). Membranes were blotted with primary antibodies against Jak2 phospho-$Tyr_{1007/8}$ (Upstate) and total Jak2 (Millipore). Membranes were rinsed three times with wash buffer then incubated with a secondary antibody conjugated to a fluorescent tag (Molecular Probes, Licor). Membranes were rinsed three times in wash buffer before detection using an Odyssey Imaging (Licor Biosciences).

Mouse Model Generation: A targeting vector encoding LepRbΔ65 (Δ) was created and transfected into murine ES cells. Correctly targeted clones were identified and confirmed with real-time PCR and Southern Blotting as performed previously for other LepRb-targeted projects[5,9]. LepRΔ65/+ intercrosses

generated +/+, *Δ/+* and *Δ/Δ* animals at expected Mendelian frequencies. *Δ/+* animals were backcrossed with C57BL/6J +/+ animals (Jackson Laboratories) for six generations prior to data collection.

Experimental Animals: *Δ/+* intercrosses were used to generate +/+ and *Δ/Δ* mice. For *db/db* mice, *db/+* were obtained from Jackson Laboratories and intercrossed. For body weight, food intake, ad libitum glucose monitoring and estrous monitoring, animals were weaned at four weeks and housed individually. Body weight and food intake were recorded weekly from four to eight weeks. Glucose was measured and blood was collected for hormone measurement at four, six and eight weeks. In females, vaginal lavage was used to assess estrous cycling daily from four to eight weeks. Animals intended for glucose tolerance tests, insulin tolerance tests and body composition were weaned at four weeks and group housed. Following weaning, all mice were maintained on 5011 Labdiet chow. Snout-anus length and bone length were measured with a micrometer. Body composition was determined using a NMR Minispec LF90II scanner (Bruker Optics). Mice were housed in accredited animal facilities of the University of Michigan with ad libitum access to chow and water. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Michigan.

Glucose and Insulin Tolerance Tests: For glucose tolerance tests, mice at five weeks of age were fasted overnight for 20 hours. 2g/kg glucose was administered and blood glucose was monitored using an Ascensia Elite device prior to glucose administration and following administration at 15 min, 30 min, 60

min and 120 min using whole venous blood from the tail vein. For insulin tolerance tests, mice at six and nine weeks of age were fasted for five hours. 4U/kg insulin (Novolin) was administered and blood glucose was monitored prior to glucose administration and following administration at 15 min, 30 min and 60 min using whole venous blood from the tail vein.

Hormone Measurement: Blood was collected at four, six and eight weeks by tail vein nick. A terminal bleed was also collected after euthanasia with pentobarbital. Blood was centrifuged and serum was frozen to – 20 ºC prior to analysis. Commercial ELISA kits were used to determine insulin (Crystal Chem), leptin (Crystal Chem) and C-peptide concentrations (Millipore).

Analysis of hypothalamic RNA. Hypothalami were isolated from random-fed mice and snap-frozen. Total RNA was isolated using Trizol RNA reagent (Invitrogen) and converted to cDNA using SuperScript reverse transcriptase (Invitrogen). For comparison of relative LepRb expression, total hypothalamic cDNA was subjected to PCR with LepRb-specific primers and subjected to gel electrophoresis. For determination of relative neuropeptide expression, total hypothalamic cDNA was subjected to automated fluorescent RT-PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). GAPDH primers and probes were as supplied by Applied Bio-systems. POMC, AgRP, and NPY primers and probes were as previously described (26). Each predicted RT-PCR product spanned an intron/exon junction.

Immunonological Cell Analysis: For counting total spenocytes and T cells in the spleen, splenocytes and T cells were magnetically separated from the

spleen by AutoMACS as previously described and counted by flow cytometry[32]. For proliferation assays, T cells were separated using CD90 microbeads and 2 × 105 of these were incubated with 2 × 10^4 of B6 bone marrow–derived dendritic cells for 48, 72, and 96 hours. Cells were stimulated with soluble anti-CD3e (1 mg/mL). Incorporation of ^{3}H-thymidine (1 μCi/well) by proliferating cells during the last 12 hours of culture was measured.

Immunohistochemical Analysis of Hypothalamic Brain Sections: +/+, db/+ and *Δ/*+ were intercrossed with heterozygous mice expressing LacZ from the AgRP locus [28,33]. For immunohistochemicalanalysis, ad libitum-fed animals remained with food in the cage until the time of death. Perfusion and immunohistochemistry (IHC) procedures were performed essentially as described previously[27]. Briefly, mice were deeply anesthetized with an overdose of intraperitoneal pentobarbital (150 mg/kg) and transcardially perfused with sterile PBS, followed by 10% formalin. Brains were removed, postfixed, and cryoprotected before sectioning. For IHC, sections were pretreated in ice-cold methanol, 0.3% glycine, and 0.3% SDS and then blocked and incubated in the primary antibodies [rabbit anti-c-Fos (1:40,000) and/or goat anti-ß-Gal (1:3000)]. Detection of primary antibodies was done by either immunofluorescence (anti-rabbit-Alexa 488 and anti-goat-biotin followed by streptavidin-Alexa 564 conjugate, all 1:200 dilution) or using the avidin–biotin/diaminobenzidine method.

Genotype	WT	Db/Db	Δ/Δ
Fat Content (%)			
Male	**6.0 ± 0.7**	**47.0 ± 1.1***	**47.8 ± 0.9***
Female	**6.4 ± 0.5**	**51.0 ± 0.9***	**54.1 ± 0.7***
Lean Content (%)			
Male	**80.0 ± 0.4**	**43.7 ± 1.1***	**42.6 ± 0.8***
Female	**76.0 ± 3.0**	**39.5 ± 0.7***	**37.5 ± 0.6***
Body Temperature (°C)			
Male	**34.6 ± 0.2**	**33.0 ± 0.2***	**33.4 ± 0.2***
Female	**34.4 ± 0.3**	**33.8 ± 0.2**	**33.5 ± 0.3**
Snout-Anus Length (mm)			
Male	**88.5 ± 0.8**	**82.6 ± 2.7***	**83.0 ± 1.0***
Femur Length (mm)			
Male	**13.9 ± 0.1**	**11.8 ± 0.1***	**12.0 ± 0.1***
Femur Mass (mg)			
Male	**42.4 ± 0.6**	**34.8 ± 0.8***	**35.3 ± 0.9***

Table 3.1 Phenotypic Data for Mice Expressing Mutant LepRb. Fat and lean content were determined at 10 weeks using NMR Minispec and are expressed as a percentage of body weight. Body temperature was determined at 11 weeks. Snout-anus length, femur length and femur weight were determined a 9 weeks. Error indicates standard error of mean and astericks indicate $P<0.05$ compared to WT by Student's unpaired, two-tailed t test.

Genotype	WT	Db/Db	Δ/Δ	s/s
Estrus	8/9	0/9	0/8	3/8
Litters	9/9	0/7	0/8	5/8

Table 3.2 Fertility Data for Mice Expressing Mutant LepRb. Single-housed females were examined daily from 4 to 8 weeks of age. Cytological examination of vaginal lavage was used to monitor estrus. Females were paired with one wild-type male and monitored daily for pregnancy and production of offspring for 6 weeks.

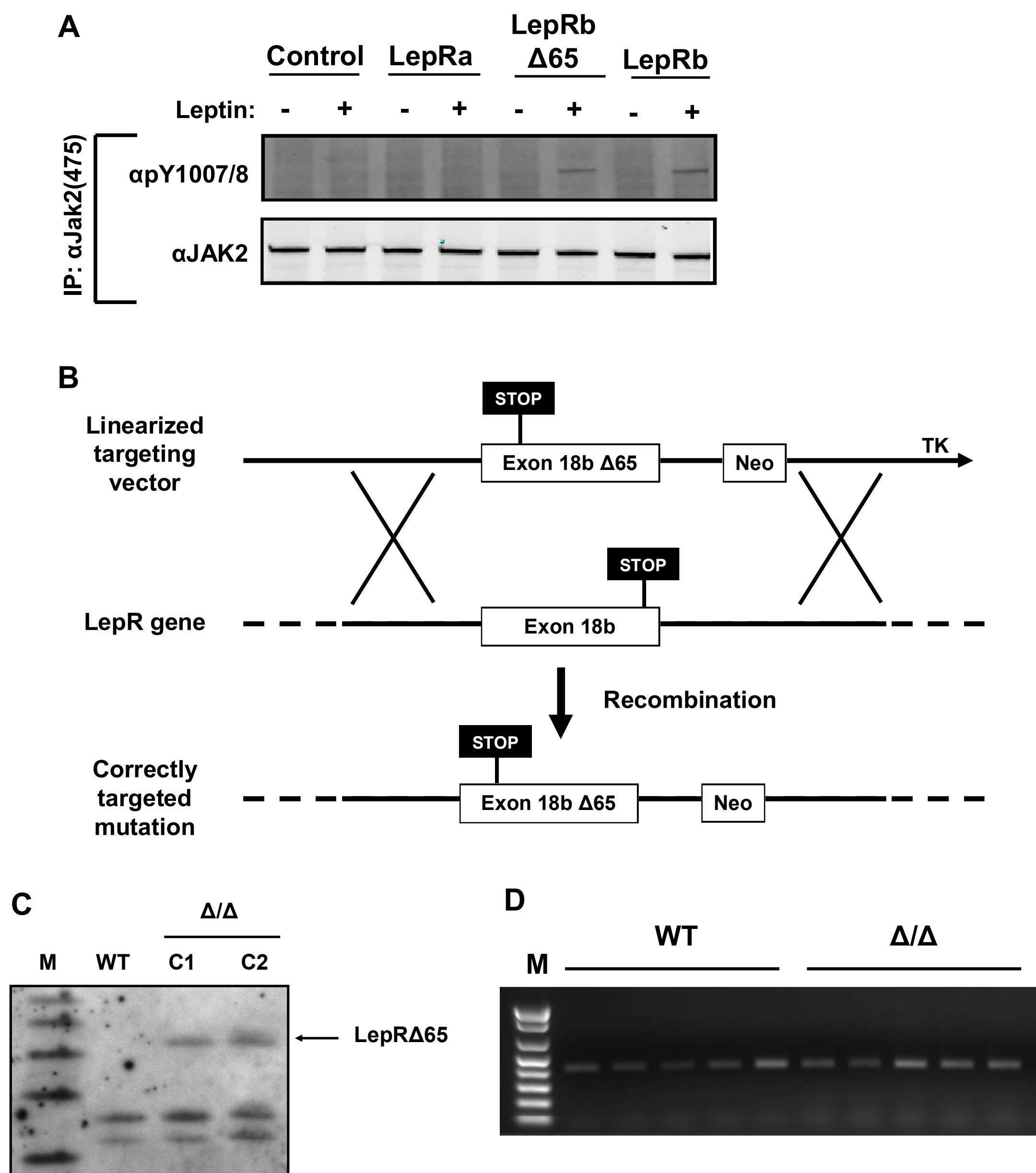


Figure 3.1 Generation of mice expressing LepRbΔ65. A) HEK293 cells were transfected with the indicated plasmids, made quiescent overnight, and incubated in the absence (-) or presence (+) of leptin (625 ng/mL) for 15 minutes before lysis. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with the indicated antibodies. The figures shown are typical of multiple independent experiments. B) Diagram of gene-targeting strategy to replace wild-type exon 18b with one encoding a premature truncation. C) Southern blotting of using LepR locus specific probe demonstrating correct targeting of vector. Labeled lanes are marker (M), wild-type, and two correctly targeted clones (C1, C2). D) PCR using LepR-specific primers on hypothalamic mRNA from 5 wild-type and 5 Δ/Δ animals.

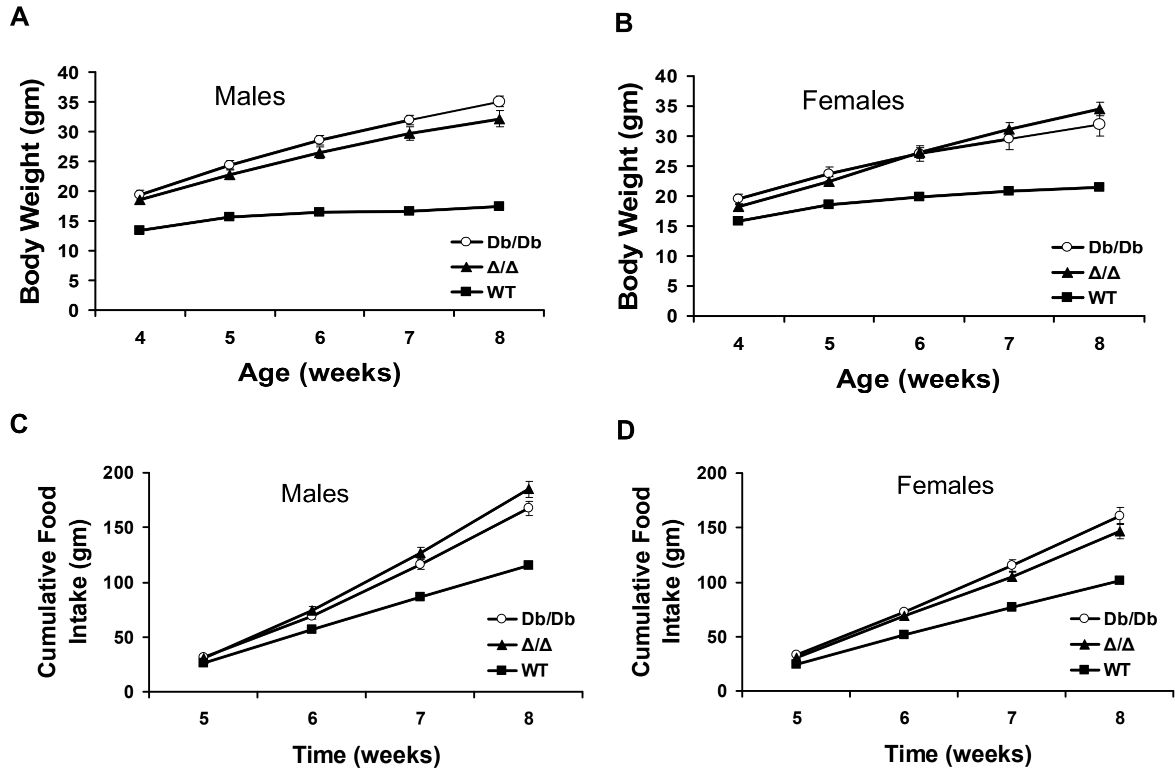
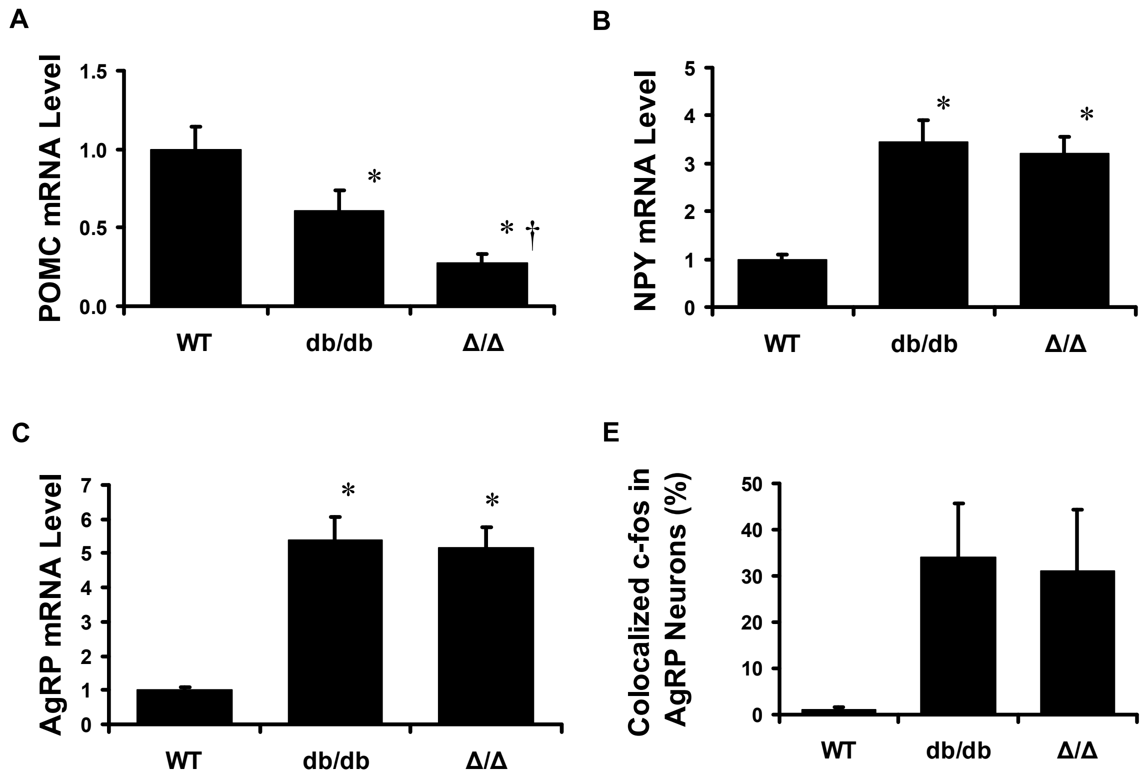
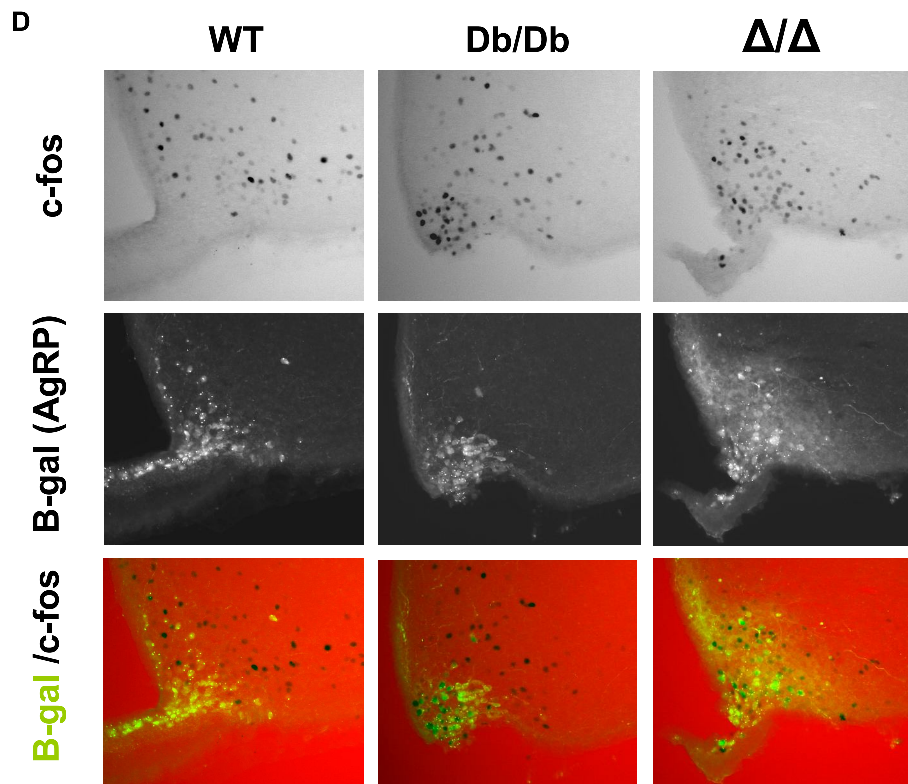


Figure 3.2 Δ/Δ mice are obese and hyperphagic. Mice of the indicated age and genotype were weaned at 4 weeks. (A-B) Body weight and (C-D) food in-take were monitored weekly from 4 to 8 weeks of age. Food intake represents cumulative food intake over the time-course. Error bars indicate standard error.

Figure 3.3 Hypothalamic neuropeptide expression in WT, db/db and Δ/Δ mice and c-fos like immunoreactivity in AgRP neurons. mRNA was prepared from the hypothalami of 10-11 week old male mice. QPCR was used to determine (A) POMC, (B) NPY and (C) AgRP levels. Error bars indicate standard deviation. Asterisks indicate $P<0.05$ compared to WT (*) while dagger (†) indicates $P<0.05$ compared to db/db by one-way ANOVA with Tukey's post test. D) CFLIR in AgRP neurons of ad libitum-fed WT, db/db, and Δ/Δ animals. All mouse groups were bred onto a background expressing LacZ under the AgRP promoter, enabling the identification of AgRP neurons by staining for β-Gal. Representative images showing immunofluorescent detection of c-Fos (top), β-Gal (middle), and merged c-Fos/ β-Gal (bottom). E) Quantification of double-labeled c-Fos/AgRP neurons. Total AgRP neurons were normalized to 100% for the different mouse groups, and double-labeled AgRP neurons are plotted as percentage of total AgRP neurons. Error bars indicate standard deviation.


A
POMC mRNA Level
1.5
1.0
0.5
0.0
WT
db/db
Δ/Δ
B
NPY mRNA Level
5
4
3
2
1
0
WT
db/db
Δ/Δ
C
AgRP mRNA Level
7
6
5
4
3
2
1
0
WT
db/db
Δ/Δ
E
Colocalized c-fos in AgRP Neurons (%)
50
40
30
20
10
0
WT
db/db
Δ/Δ


D
WT
Db/Db
Δ/Δ
c-fos
B-gal (AgRP)
B-gal /c-fos

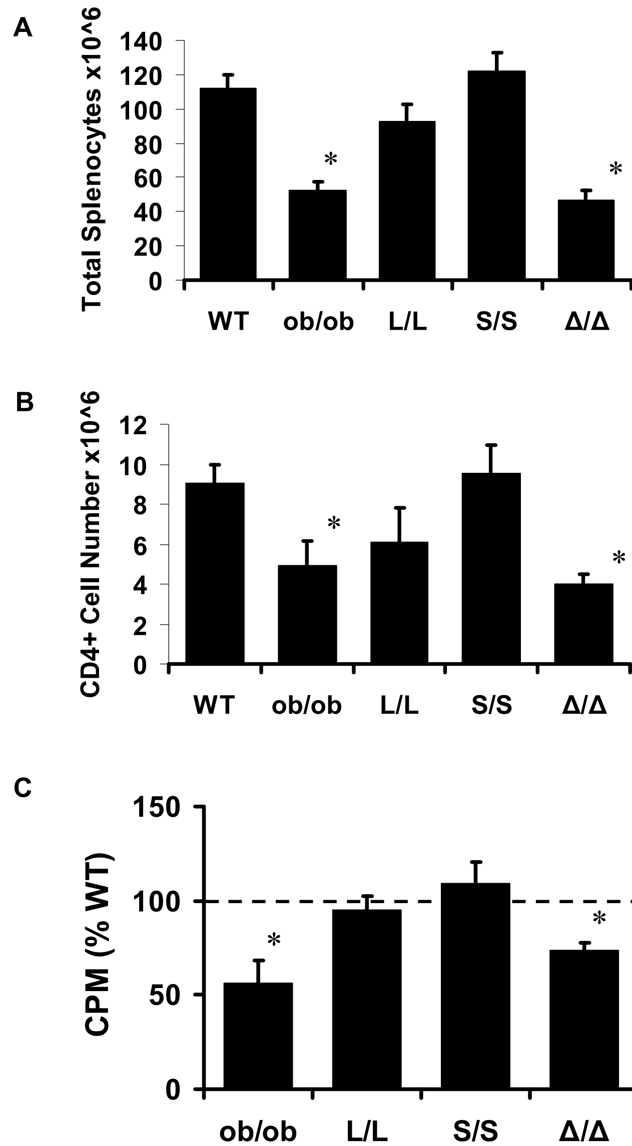


Figure 3.4 Reduced immune cells numbers and proliferation in Δ/Δ and ob/ob spleens but not from s/s or l/l spleens. Spleens were isolated from the indicated genotypes of male mice, separated using autoMACS and counted for (A) total splenocytes and (B) CD4+ cells using a flow cytometer. Error bars indicate standard error and asterisks indicates P<0.05 compared to WT by one-way ANOVA and Tukey's post test. C) For proliferation assays, CD4+CD25- Naïve T cells were isolated by autoMACS and incubated in the presence of bone marrow–derived dendritic cells from C57BL/6J mice for 48, 72, and 96 hours and stimulated with anti-CD3e. Incorporation of H3-thymidine (1 mCi/well) by proliferating cells was measured during the last 6 hours of culture. Proliferation is expressed as a percentage of a paired WT sample analyzed concurrently (dashed line). Error bars indicate standard error and asterisks indicate P<0.05 compared to s/s and l/l by one-way ANOVA and Tukey's post test.

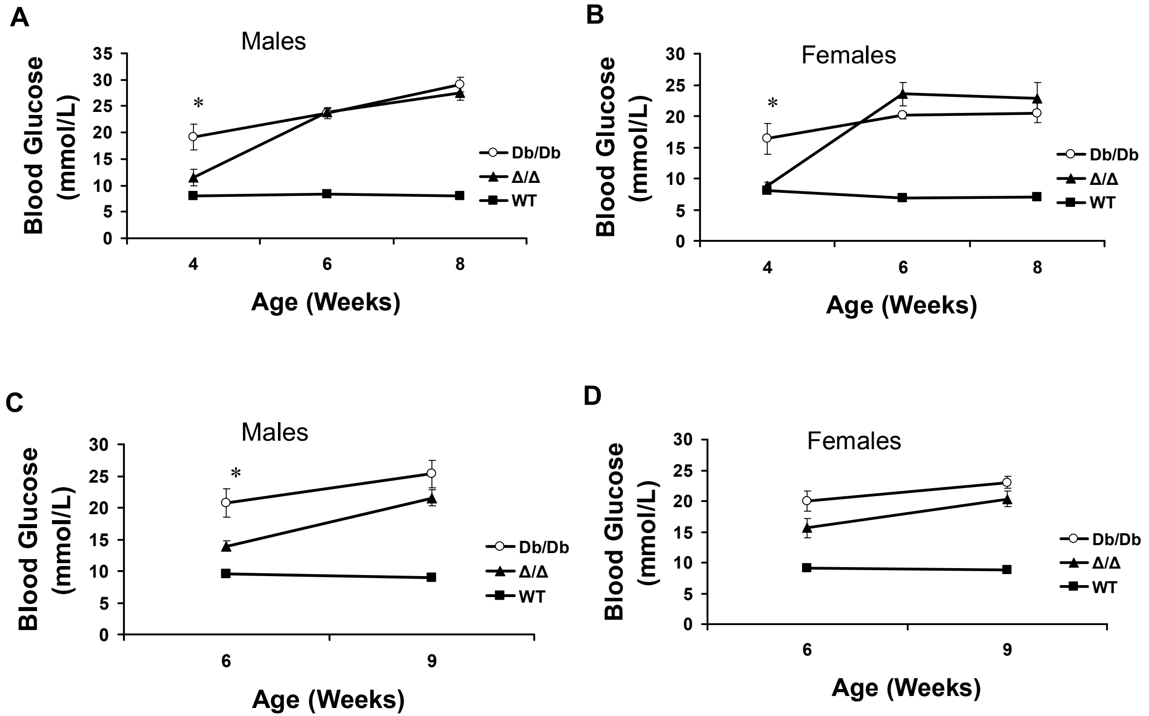


Figure 3.5 Δ/Δ mice are more euglycemic than db/db mice at young ages. A-B) Blood glucose from the indicated sex and genotypes was measured at 4, 6 and 8 weeks of age from free-fed mice. Error bars indicate standard error and asterisks indicates $P<0.05$ at the indicated time-points compared to Δ/Δ using Student's unpaired, two-tailed t test. C-D) Blood glucose from the indicated sex and genotypes was measured at 6 and 9 weeks of age from fasted mice. Error bars indicate standard error and asterisks indicate $P<0.05$ at the indicated time-points compared to Δ/Δ using Student's two-tailed, unpaired t-test.

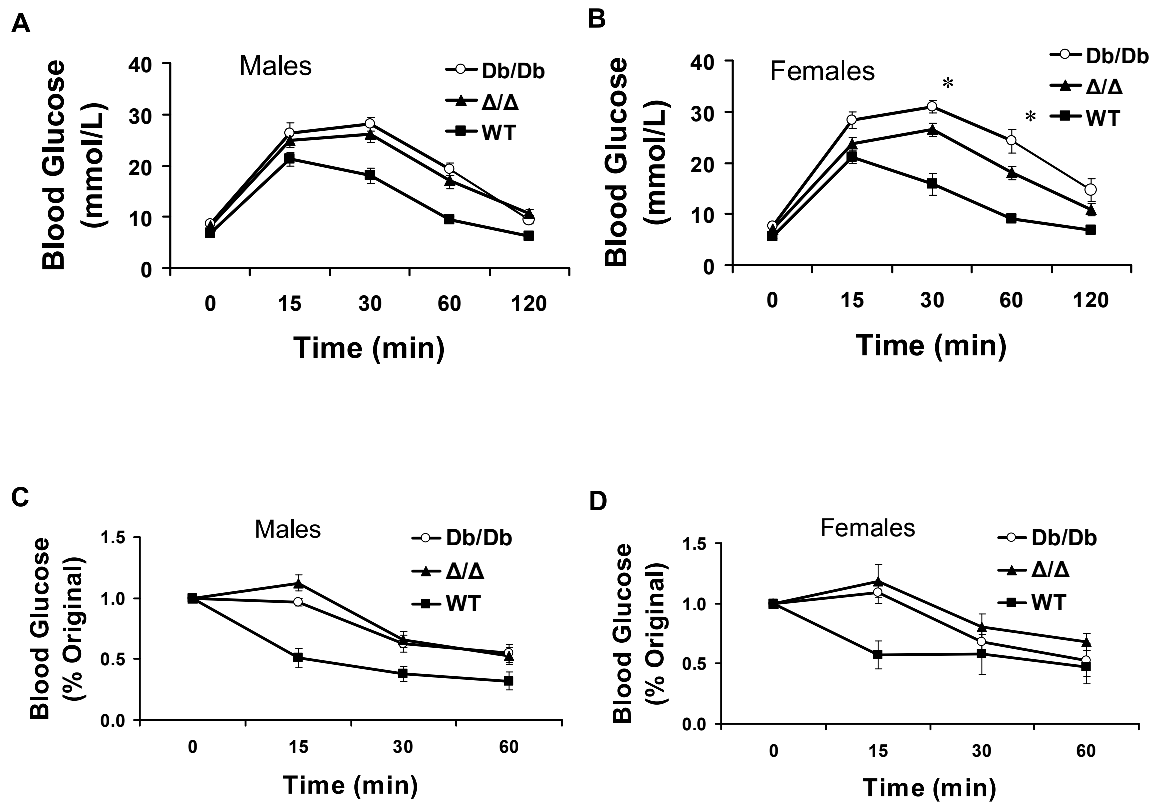


Figure 3.6 Glucose and insulin tolerance in WT, db/db and Δ/Δ mice. A-B) Mice of the indicated sex and genotype at 5 weeks of age were fasted overnight and injected with 2g/kg glucose. Blood glucose was monitored prior to injection and post-injection at 15, 30, 60 and 120 min. Error bars indicate standard error. Asterisks indicate $P<0.05$ compared to Δ/Δ at the indicated time-points by one-way ANOVA and Tukey's post test. C-D) Mice of the indicated sex and genotype at 6 weeks of age were fasted for 5 hours and injected with 4U/kg insulin. Blood glucose was monitored prior to injection and post-injection at 15, 30, and 60 min. Error bars indicate standard error.

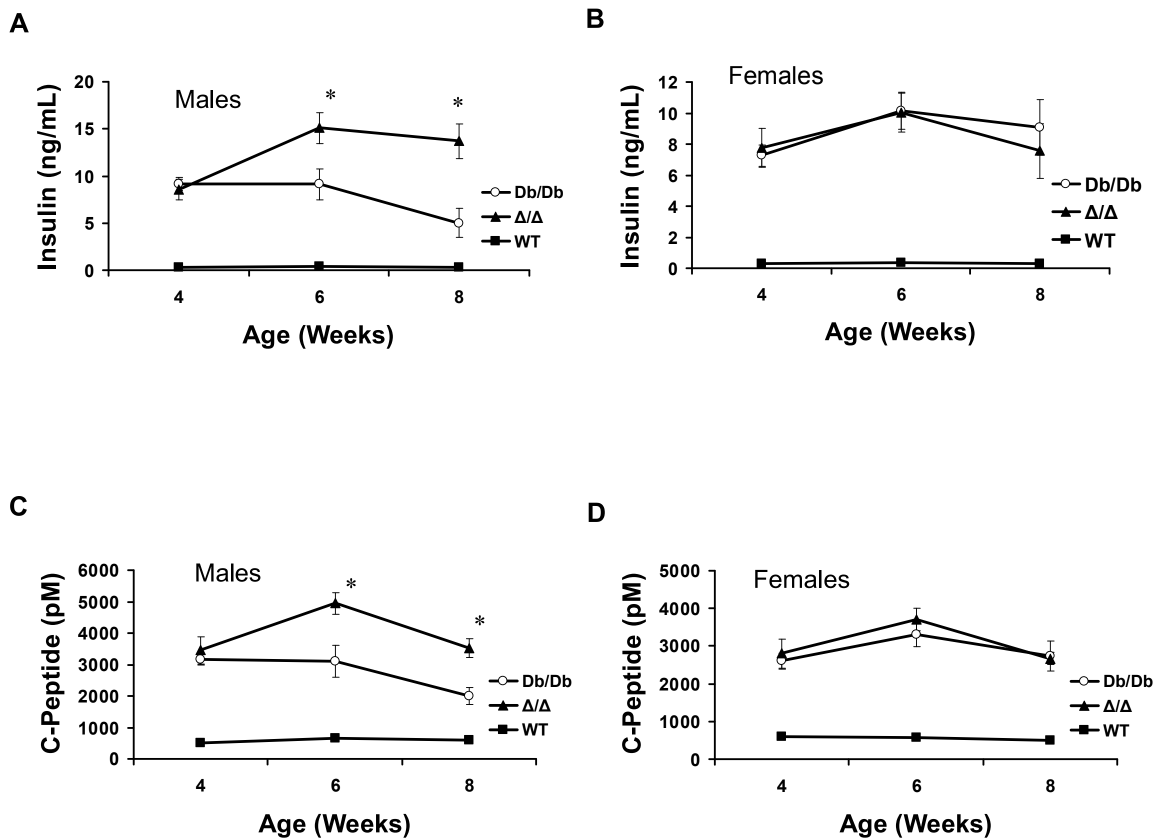


Figure 3.7 Insulin and C-peptide levels in WT, db/db and Δ/Δ mice. Serum was collected from free-fed mice at the indicated genotype and sex at 4, 6 and 8 weeks of age. (A-B) Insulin or (C-D) C-peptide was measured in the collected serum. Error bars indicate standard error and asterisks indicate $P<0.05$ compared to Δ/Δ at the indicated time-points by one-way ANOVA and Tukey's post test.

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The role of Jak2 in leptin signaling

The work presented in Chapter 2 of this dissertation furthers our understanding of the dynamics of Jak2 activation and deactivation in response to cytokine stimulus. The phosphorylation sites on Jak2 that we identified and characterized are important in modulating the activity of Jak2 through feedback inhibition, in the case of Tyr_{317}, or positive regulation, in the case of Tyr_{637}. Furthermore, since the initiation of these studies, many other novel phosphorylation sites on Jak2 have been described by our collaborators and other research groups. Given the number of researchers working in this area, and the diversity of techniques and instruments used to detect phosphorylation events on Jak2, it seems likely that the full complement of Jak2 phosphorylation modification is close to fully determined. However, many of these studies, including our own, were conducted in cell culture with supraphysiologic levels of Jak2 and cytokine stimuli. Therefore, further work will be necessary to determine the physiologic importance of these sites, in the context of normal physiology.

Determining the physiologic contribution of individual phosphorylation sites could be the subject of further study and would be immensely interesting. For

example, Tyr_{813} of Jak2, when phosphorylated, mediates binding with SH2B1 and work in cultured cells and in vivo has suggested that SH2B1 can couple Jak2 activation with IRS1 and PI3-K activation[2]. To determine the significance of these events to leptin physiology, it would be interesting to knock-out Tyr_{813} function specifically in leptin receptor expressing cells. However, this would be difficult given Jak2's indispensability for a variety of other cytokine signaling cascades. Consequently, while our research group is particularly interested in Jak2's activities in the context of leptin, it would be challenging to specifically disable individual phosphorylation sites on Jak2 in the context of a restricted population of cells.

However, one potential way to do this would be to use a cre recombinase, double-flox strategy in mice. Three different gene alleles would be needed to do this. First, an allele expressing Cre from the endogenous LepR locus would be needed to target Cre expression. Our laboratory has developed these animals and their Cre-expression has been well-validated to study leptin physiology in the brain[1]. Second, a floxed Jak2 allele would be needed to turn off wild-type Jak2 in LepRb-expressing cells. These mice have also been created and have been used for conditional Jak2 loss-of-function studies [9]. The third allele, that does not yet exist, would encode a Jak2 phosphorylation site mutant that would express only in the presence of Cre. This gene could be delivered through a traditional transgenic approach. Or, more elegantly, the sequence encoding the mutant phosphorylation site could be knocked into the endogenous Jak2 locus by homologous recombination. Importantly, mice that are heterozygous for a Jak2

loss-of-function allele are viable and normal[4,8]. Therefore, Jak2 driven from one allele should be sufficient to maintain normal activity. While complex, strategies like this would be necessary to study the individual contributions of Jak2 phosphorylation sites in subpopulations of cytokine-sensitive cells.

Further Jak2 studies

Beyond the study of Jak2 phosphorylation sites and their significance to leptin signaling, there are other intriguing questions about Jak2 that require more study and could be the subject of future work. First, the sequence of events that occurs to transform inactive Jak2 to fully active Jak2 is largely unknown. Jak2 activity requires phosphorylation on its activation loop ($Tyr_{1007/8}$) and this is an autophosphorylation event. This creates a paradox: how does a kinase that requires prior activation loop phosphorylation for activity, autophosphorylate these residues?[5] This mystery has been largely ignored in Jak2 research and could be an interesting research project.

Fortunately, many kinases are activated by activation loop autophosphorylation and work on other kinases provides a model for determining the details of Jak2 activation. Specifically, recent work on Chk2 kinase (checkpoint kinase 2) has revealed a detailed mechanism for its activation [6]. Activation occurs in the following order. First, two inactive, unphosphorylated kinase domains from different kinase molecules are brought in close proximity to one another. Second, the unphosphorylated activation loops are exchanged between opposing kinase domains. Third, the unphosphorylated activation loops

interact with the catalytic domain of the opposing kinase molecule and temporarily stabilize the active conformation. Lastly, key activation loop phosphorylation sites are phosphorylated, thus, turning on the kinase. There are a few additional details to point out here. First, an unphosphorylated kinase domain can only phosphorylate the activation loop of another inactive kinase. As a corollary, once active, the substrate specificity of the kinase domain changes and can no longer phosphorylate the activation loop of an inactive kinase. Indeed, presenting an inactive kinase as a substrate for a preactivated kinase does not result in activation of the inactive kinase[7]. It would be interesting to conduct similar experiments with Jak2 and determine if activation of Jak2 proceeds through similar mechanisms.

Lastly, the field of Jak2 research is in great need of a solved structure of full-length Jak2. Years of work on the molecular biology of Jak2 has yielded a host of insights regarding the discreet functions of nearly every part of Jak2. These findings include the contributions of individual domains, sub-domains and phosphorylation sites. However, an integrated understanding of Jak2 will almost certainly require the complete structure of Jak2. While loss-of-function studies can tell us what happens when individual parts of Jak2 are deleted, a mechanistic understanding to explain these changes is often elusive. More Jak2 structural information would certainly cast light upon these questions. Currently, only the structure of the kinase domain has been solved [3]. Hopefully, new techniques in protein structural determination will pave the way for these breakthroughs.

Further leptin receptor studies in vivo

The work presented in Chapter 3 of this dissertation describes the insufficiency of the Jak2-binding site for the majority of leptin's physiological effects. While, in and of itself, this finding may seem somewhat trivial, it is a valuable piece of information to the larger field of leptin study. There are now several genetic models addressing the individual contributions of different LepRb functional sites to leptin physiology. Our laboratory has described $LepRb^{Y985L}$, $LepRb^{Y1138F}$ and $LepRb^{\Delta 65}$ while phenotypic description of $LepRb^{Y1077F}$ is currently underway. Another laboratory has published a knock-in model featuring mutation of all three tyrosines on LepRb's intracellular tail ($LepRb^{Y123F}$). An integration of data from all of these models serves to narrow down the contributions from each part of the leptin receptor to physiologic effector pathways.

Perhaps most interesting are the differences between $LepRb^{\Delta 65}$ mice and $LepRb^{Y123F}$ mice. In theory, one would expect very similar phenotypes from these two models. Of the four known functional motifs on LepRb's intracellular tail (Jak2 binding site, Tyr_{985}, Tyr_{1077} and Tyr_{1138}) these models contain only an intact Jak2 binding site. However, the phenotypes of these mice are substantially different. While both models are obese, $LepRb^{Y123F}$ mice have improvements in energy homeostasis and neuropeptide regulation over *db/db* mice, while $LepRb^{\Delta 65}$ animals do not. This points to a potential role for the distal region of LepRb in leptin physiology independent of its phosphorylation sites. It

suggests that other, unknown factors are interacting with this region to transduce important signals.

To narrow down the identity of these signals, it would be interesting to create a mouse model featuring targeted mutations of both the Jak2-binding site and all three tyrosines on LepRb's intracellular tail. This should reveal if the tyrosine-independent signals observed in $LepRb^{Y123F}$ mice are Jak2-dependent, like all currently known signals emanating from LepRb. Alternatively, we could conditionally knock-out Jak2 function in LepRb-expressing cells. While we would expect that Jak2 loss-of-function in LepRb-expressing cells would phenocopy total leptin-deficiency, perhaps these tyrosine-independent signals do not require phosphorylation by Jak2 and would be intact in Jak2 null cells. As discussed earlier, both LepRb-cre and floxed Jak2 mouse models already exist and it would be possible to breed them together to create such a mouse model.

Lastly, although $LepRb^{\Delta 65}$ mice are mostly similar to *db/db* mice, their glycemic control is somewhat improved. While minor, this suggests an intact signal emanating from Jak2 that is independent of the remainder of Jak2. Determining the identity of this signal would be highly interesting and could be the subject of further studies. The most likely candidate is PI3-K activation. To determine if the Jak2-binding site on Jak2 can modulate the PI3-K activity, we could compare the ability of $LepRb^{\Delta 65}$ or *db/db* mice to upregulate PI3-K in the CNS in response to leptin stimulus. Unfortunately, efforts to detect neuronal PI3-K activity in response to leptin stimulation have not been successful in our hands

and we are currently working with a novel system to assay for PI3-K activity in LepRb-expressing neurons in the brain.

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