INVESTIGATION OF THE ROLE OF JAK2 IN SOCS1-MEDIATED GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR RECEPTOR TURNOVER

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract  

Our lab has recently shown that SOCS1 binds and degrades the granulocyte-colony stimulating factor receptor beta common subunit (GMRβc) in a proteasome-dependent manner. The focus of this project was to determine the role of JAK2 in SOCS1-mediated GMRβc turnover.  

Results: The binding of SOCS1 to GMRβc was observed in the JAK2-null γ2A fibrosarcoma cell line. In TF1s, an erythroleukemic cell line that endogenously expresses GMRβc, transient knockdown of JAK2 resulted in the marked stabilization of GMRβc levels by thirty minutes. Using the kinase-dead K882E JAK2 mutant and the Y1007F JAK2 mutant, which abrogates SOCS1-binding as well as JAK2 activation because it is a key phosphorylation site, it was determined that the kinase activity of JAK2 is required SOCS1-mediated degradation of GMRβc. Lastly, SOCS1 has been predicted to bind Y468 on GMRβc.  

Conclusions: JAK2 is not necessary for SOCS1 to bind GMRβc; however, active JAK2 is necessary for substantial GMRβc degradation.
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Chapter 1. Introduction: Literature review

1.1. Cytokines and cytokine receptor superfamily

Cytokines are small proteins (~5-20 kDA) that play critical roles in cell signaling, largely having an impact on the immune and hematopoietic systems but are also important in development; this broad family of proteins includes chemokines, interferons, interleukins, lymphokines, tumour necrosis factor (TNF) but generally not hormones or growth factors. Cytokines are produced by many different cells and some cytokines are produced by more than one cell type. Examples of immune cell types that produce cytokines are macrophages, B lymphocytes, T lymphocytes, and mast cells; other cell types that also produce cytokines include endothelial cells, fibroblasts, and some stromal cells. Upon production, cytokines act by binding to their respective cell-surface, membrane-bound receptors in an autocrine or paracrine fashion using hormone terminology. Subsequent signal transduction involves one or members from the JAK/STAT pathway through which most cytokine receptors relay messages. Functional outcomes through downstream signaling include many biological processes such as development, haematopoiesis, inflammation, immune responses, and tissue repair.

The cytokine receptor superfamily can be subdivided into the following categories: Type 1 cytokine receptors, Type II cytokine receptors, chemokine receptors...
receptors, tumor necrosis factor receptor (TNFR) superfamily, transforming
growth factor beta (TGF-β) receptors, and immunoglobulin (Ig) superfamily. These
receptors usually contain one to three subunits that assemble together into
functional, higher-order signaling complexes upon ligand-binding to its respective
receptor chain/s. According to traditional nomenclature, the ligand-binding subunit
is referred to as an alpha chain; other subunits are named beta or gamma chains.

1.1.1. **Type 1 cytokine receptors** have two pairs of cysteines linked by disulfide
bonds in a conserved ~200 amino acid N-terminal region and a characteristic
C-terminal WSXWS sequence in their proximal extracellular region (Bazan, 1990). The four cysteines are important for maintaining structural and
functional integrity of the receptors whereas the Trp-Ser-X-Trp-Ser motif
serves as a recognition site for functional protein-protein interactions. These
receptors assemble in complexes consisting of at least one signal transducing
receptor chain with membrane-proximal Box 1 and Box 2 motifs, which are
associated with JAK binding sites (Reviewed in Leonard and Lin, 2000).
Examples of Type I receptors include interleukin (IL) receptors (e.g. IL-2R,
IL-7R, and IL-9R), colony stimulating factor receptors (e.g. erythropoietin
receptor and granulocyte-macrophage colony-stimulating factor (GM-CSF)
receptor), and hormone receptors (e.g. growth factor receptor and prolactin
receptor). Another classification system of Type I receptors is based on the subunit being shared among a group of receptors as indicated in parentheses below; as per this system, there are four major families: IL-2R (common gamma), IL-3R (common beta), IL-6R (gp130), and homomeric receptors. Many pathological conditions arise from aberrancies in this family of cytokine receptors. For example, the deficiency of the common gamma chain of IL-2R is responsible for x-linked severe combined immunodeficiency (X-SCID) (Reviewed in Kovanen and Leonard, 2004). Additionally, inappropriate activation of these receptors has been found in leukemias and lymphomas whereas hyperactivating mutations in receptors have been found in many myeloproliferative diseases.
1.1.2. **Type II cytokine receptors** also have two pairs of conserved cysteines but with a different arrangement, structural similarities in their ligand-binding domains, several conserved intracellular sequences that mediate recruitment of signaling molecules, and don’t have the WSXWS sequence found in Type I receptors (Bazan, 1990). These receptors dimerize into pairs consisting of a long intracellular ligand-binding receptor and a short intracellular accessory receptor with the exception of tissue factor (TF) and IL-22 Decoy. Type II receptors are primarily involved in regulating antiviral and inflammatory responses apart from TF, which mediates the clotting process. Examples of Type II receptors are those that bind type I and type II interferons and members of the IL-10 family (e.g. IL-10, IL-20, and IL-22). The other classification system breaks this group of receptors into 10 categories as shown in Table 1.1.
Table 1.1. Type I and Type II cytokine receptors with their respective JAK/STAT signaling axes (O’Sullivan et al., 2007).
1.1.3. **Chemokine receptors** are G-protein coupled receptors with seven transmembrane domains and found largely on leukocytes; in addition, the second intracellular domain has a characteristic DRY motif and an intracellular C-terminus that contains a serine and a threonine, which act as phosphorylation sites. The role of the G-protein is to facilitate signal transduction and this is associated with an influx of intracellular calcium, a process known as chemotaxis; this allows the cell to home to a certain location within an organism. This family of receptors can be further subdivided into CC, CXC, CX3C, and C chemokine receptors (Reviewed in Allen *et al.*, 2007). Their role in disease can be highlighted with the classic example of the binding of HIV to CXCR4 and CCR5 chemokine receptors (Reviewed in De Clercq and Schols, 2001).

1.1.4. **Tumor necrosis factor (TNF) receptor superfamily** members are part of a large family of preformed timers that respond to ligands that also form trimers; they share a cysteine-rich domain formed by three disulfide bonds and a characteristic CXXCXXC motif (Reviewed in Idriss and Naismith, 2000). The TNF superfamily of receptors, which include TNFR1, TNFRII, CD40, Fas, lymphotoxin receptor, and BAFF receptors, are best known for their role to trigger apoptosis through their association with adapter proteins.
(e.g. TRADD) that activate the caspase cascade. One well heard cytokine that binds to the CD120 receptor in this family is TNFα. The TNF receptor superfamily activates numerous pathways, notably NF-κB.

1.1.5. **TGF-β receptors** are single pass with intrinsic serine/threonine kinase activity that recognize TGF-β, which is secreted by many cell types and governs proliferation and differentiation among other functions (Reviewed in Huang and Chen, 2012). The three members in this group, TGFBR1, TGFBR2, and TGFBR3, differ in structural and functional properties. Aberrant TGF-β and TGF-β receptor regulation are associated with many diseases such as cancer, diabetes, Loeys-Dietz syndrome, Marfan syndrome, and heart disease.
1.1.6. **The immunoglobulin (Ig) superfamily** of receptors are expressed throughout the body on many cell types and possess an Ig domain (Reviewed in Barclay, 2003). Members of this family include antigen receptors, antigen presenting molecules, and co-receptors/accessory molecules. Antigen receptors, such as antibodies or T cell receptor (TCR) chains, are respectively found on the surface of B and T lymphocytes in all jawed vertebrates. In humans, there are five distinct types of Ig classes or isotypes that all comprise a heavy chain with four Ig domains and a light chain with two Ig domains; they are IgA, IgD, IgE, IgG, and IgM. The TCR is composed of two chains, either the TCR-alpha and TCR-beta, or the TCR-delta and TCR-gamma. Antigen presenting molecules, such as Class I MHC, Class II MHC, and beta-2 microglobulin (β2M), are ligands for TCRs. MHC class I forms a dimer with β2M and interacts with the TCR on cytotoxic T cells where as MHC II has two chains (alpha and beta) that interact with the TCR on helper T cells. Co-receptors and accessory molecules (e.g. CD4 found on helper T cells, CD8 found on cytotoxic T cells, and CD19 on B cells) are other proteins on the surfaces of T cells that also interact with MHC molecules alongside with TCRs.
1.2. Granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor signaling pathway

Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and interleukin-5 (IL-5) belong to one family of cytokines. They are generally not essential for steady-state functions but produced in response to emergency situations (e.g. exposure to pathogens) to increase leukocyte numbers (Reviewed in Hercus et al., 2009).

GM-CSF is produced by many cells in the body unlike IL-3 and IL-5, which are mainly produced by T cells and respectively act on basophils/mast cells and eosinophils; some of the GM-CSF-producing cell types belong to the epithelium, the lung, colon, and joints. Although GM-CSF is not required for steady-state hematopoiesis (Stanley et al., 1994), it has ancillary functions such as mediating surfactant clearance by alveolar macrophages (Ikegami et al., 1996).

Known as a colony stimulating factor, GM-CSF plays a pleiotropic role by governing many different cellular processes such as growth, survival, differentiation and activation of normal myeloid cells in a concentration-dependent manner. For example, the Ser\textsuperscript{585}/14-3-3/PI3 kinase (PI3K) survival-only pathway is triggered by low pM concentrations of GM-CSF. The alternative survival and
proliferation pathway, involving JAK/STAT, Ras/mitogen-activated protein kinase (MAPK), and PI3K pathways, depends on Tyr$^{577}$ in the GMR$\beta$c and requires higher concentrations of GM-CSF (around 10 pM or more). Interestingly, Ser$^{585}$ and Tyr$^{577}$ phosphorylation are mutually exclusive and unidirectional events (Reviewed in Hercus et al., 2009).

The GM-CSF receptor is expressed at low levels, roughly around < 1500 receptors per cell (Fan et al., 1993). Comprising of a 60-80 kDA alpha chain (GMR$\alpha$) and a 120-140 kDA beta chain (GMR$\beta$c), it is expressed on the cell surface of primarily granulocytes (basophils, eosinophils, and neutrophils) and monocytes/macrophages, which are all derived from myeloid progenitors.
Figure 1.1. Schematic of hematopoietic differentiation. The hematopoietic cell bifurcates into either the myeloid or lymphoid progenitors. The myeloid cell can differentiate into megakaryocytes, red blood cells, monocytes, and granulocytes (basophils, eosinophils, or neutrophils). Monocytes can further differentiate into dendritic cells and macrophages. The GM-CSF receptor is expressed on granulocytes and macrophages.

Unlike many other cytokine receptors, the GM-CSF receptor has a significant non-redundant role in signal transduction of essential cellular processes. In the last few years, molecular and structural data on the GM-CSF receptor has further elucidated the mechanism of receptor assembly. The binding of GM-CSF to GMRα creates a low-affinity binary complex. This complex recruits pre-formed GMRβc dimers, forming a 2 GM-CSF: 2 GMRα: 2 GMRβc hexamer complex, and converting the interaction between GM-CSF and GMRα into a high-affinity interaction; interestingly, GM-CSF strictly associates with GMRα and doesn’t make contact with GMRβc. Consequently, two hexamers that come together create a dodecamer, which is the functional GM-CSF receptor signaling complex. It is only in the dodecamer, and not in the hexamer, that JAK2 molecules are able to dimerize and transphosphorylate each other (Hansen et al., 2008).
Figure 1.2. Model of GM-CSF receptor activation. Structural data provided insight into the sequence of events that lead to the signaling-competent GM-CSF receptor complex, which is a dodecamer (Hercus et al., 2009).

Apart from JAK2, which is the main kinase that transmits signals for the GM-CSF receptor, GMRβc associates with many signaling molecules with Src homology 2 (SH2) domains, Stat5 being of chief importance. Other binding partners of GMRβc include other tyrosine kinases (Lyn, Btk, Tec, Fyn, and Hck), tyrosine phosphatases (Shp1 and Shp2), adaptor proteins (Shc and Grb2) (Pazdrak
et al., 2008), and E3 ligases (Cbl and SOCS1) (Bunda et al., 2013, Bunda et al., 2014). Aside from signaling molecules that function downstream of the GM-CSF receptor, GMRβc also associates with other receptors (e.g. ICAM-1, c-Kit, FcRγ, integrin β1, EPOR, and VEGFR2) through its extracellular, transmembrane, and juxtamembrane regions (Reviewed in Broughton et al., 2012).

Figure 1.3. Receptors that interact with the GM-CSF receptor. Examples include ICAM-1, c-Kit, FcRγ, integrin β1, EPOR, and VEGFR2 (Broughton et al., 2012).

Although GMRβc has a longer cytoplasmic domain than GMRα and is the primary signal transducing unit, thereby having many binding sites for signaling
molecules, GMRα too has been reported to bind p85 of PI3K (Dhar-Mascareno et al., 2003), inhibitor of nuclear factor kB kinase β (IKkβ) (Ebner et al., 2003), GRAP (Tu et al., 2000), Lyn (Perugini et al., 2010), Src (Perugini et al., 2010), Src-like adapter protein (SLAP) (Liontos et al., 2011), and SLAP2 (Liontos et al., 2011). Despite being essential for receptor function, the exact contributions of GMRα in association with its binding partners remains to be established. Deletion of the short cytoplasmic domain of GMRα eliminates ligand-induced signaling but retains its ligand-binding abilities (Polotskaya et al., 1994). Although GMRβc is the main signaling unit, the membrane-proximal SBP motif in GMRα is required for GM-CSF-induced receptor activation (Reviewed in D’Andrea et al., 2004).
Figure 1.4. The GM-CSF signaling axis. Signaling proteins that bind to GMRα and GMRβc are illustrated in addition to the major signaling pathways that get activated downstream of the GM-CSF signaling axis (Hercus et al., 2012).
Many studies have been undertaken to identify specific residues and domains in GMRβc that are required for certain functional outcomes. Some of the earliest studies have identified that the box 1 region is required for JAK2 activation. Additionally, mutating all eight tyrosine residues to phenylalanines in the cytoplasmic region resulted in the activation of JAK2 but not Shp-2, MAPK cascades, STAT5, or the c-fos promoter in BA/F3 cells and subdued proliferation; additionally, it was revealed in this study that every site is a possible STAT5 activation site (Itoh et al., 1998). A more recent study illustrated that FI-delta, which contains a 37-amino acid duplication within the extracellular membrane-proximal region of GMRβc, resulted in ligand-independent activation with a requirement for GMRα; on the other hand, V449E promotes ligand and GMRα-independent aggregation and activation. Using the FDB1 cell line, it was shown that the V449E mutant was able to induce factor-independent proliferation whereas the FI-delta mutant resulted in granulocyte-macrophage differentiation (Perugini et al., 2010).

Aberrant activity along the GM-CSF axis is associated with many hematological and immune diseases. For example, the absence of GM-CSF activity due to either genetic alterations or neutralizing antibodies results in pulmonary alveolar proteinosis (PAP). Other GM-CSF-related pathologies include inflammatory diseases such as asthma (Sun et al., 1999, reviewed in Lampinen et
al., 2004, Yamashita et al., 2002), Crohn’s disease (Goldstein et al., 2011, Nylund et al., 2011), rheumatoid arthritis (Cook et al., 2001, Campbell et al., 1998, reviewed in Hamilton, 2008), multiple sclerosis (Marusic et al., 2002, Ponomarev et al., 2007), and autoimmune glomerulonephritis (Kitching et al., 2002, Timoshanko et al., 2005). The GM-CSF receptor signaling axis is also quite significant in cancer biology as it is associated with three of the major pathways that get deregulated in this disease: JAK/STAT, MAPK, and PI3K/Akt. GM-CSF functions as a growth and survival factor for several myeloid diseases such as acute myeloid leukemia (AML) (Young et al., 1987), chronic myeloid leukemia (CML) (Lajmanovich et al., 1993), chronic myelomonocytic leukemia (CMML) (Ramshaw et al., 2002), and juvenile myelomonocytic leukemia (JMML) (Bernard et al., 2002). Different mechanisms might lead to aberrant regulation of the GM-CSF signaling axis. For example, βc is constitutively phosphorylated on S585 in AML, possibly giving clonal cell populations a survival advantage.

In the clinic, GM-CSF is used as an adjuvant to enhance the body’s immune response to antigens. Further importance of the GM-CSF pathway is delineated in Table 1.2, which highlights that it is being modulated with antibodies in several ongoing clinical trials.
Table 1.2. Ongoing clinical trials that are modulating the GM-CSF signaling pathway (Hamilton et al., 2012).

<table>
<thead>
<tr>
<th>Target</th>
<th>Molecule</th>
<th>Type</th>
<th>Company</th>
<th>Indication</th>
<th>Phase</th>
<th>Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>MOR103</td>
<td>Ab</td>
<td>MorphoSys AG</td>
<td>RA</td>
<td>III</td>
<td>NCT01023256,</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>MT-203</td>
<td>Ab</td>
<td>Takada Nycomed/Amgen (formerly Micromet)</td>
<td>MS</td>
<td>I</td>
<td>NCT01517292</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>KB003</td>
<td>Ab</td>
<td>KaloBios Pharmaceutical</td>
<td>RA</td>
<td>II</td>
<td>NCT00995449</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>MORAb-022</td>
<td>Ab</td>
<td>Morphotek/Eisai</td>
<td>RA</td>
<td>I</td>
<td>NCT01367789</td>
</tr>
<tr>
<td>GM-CSFR</td>
<td>Mavrilumab(CAM-3001)</td>
<td>Ab</td>
<td>MedImmune</td>
<td>RA</td>
<td>II</td>
<td>NCT01950998</td>
</tr>
<tr>
<td>M-CSF</td>
<td>PD 0360324</td>
<td>Ab</td>
<td>Pfizer</td>
<td>CLE</td>
<td>I</td>
<td>NCT01470313</td>
</tr>
<tr>
<td>M-CSF-R</td>
<td>AMG-820</td>
<td>Ab</td>
<td>Amgen</td>
<td>Cancer</td>
<td>I</td>
<td>NCT01444404</td>
</tr>
<tr>
<td>M-CSFR</td>
<td>IMC-CS4</td>
<td>Ab</td>
<td>ImClone/Eli Lilly</td>
<td>Cancer</td>
<td>I</td>
<td>NCT01346568</td>
</tr>
<tr>
<td>M-CSFR</td>
<td>PLX5622</td>
<td>Chemical</td>
<td>Plexxikon/Daiichi Sankyo</td>
<td>RA</td>
<td>I</td>
<td>NCT01329991</td>
</tr>
<tr>
<td>M-CSFR</td>
<td>ARRY-382</td>
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<td>Array BioPharma</td>
<td>Cancer Metastasis</td>
<td>I</td>
<td>NCT01316822</td>
</tr>
</tbody>
</table>

*Abbreviation: Ab, antibody.

1.2.1. Juvenile myelomonocytic leukemia (JMML)

Juvenile myelomonocytic leukemia (JMML) is part of the group of myeloproliferative disorders (Figure 1.6), which are cancers of the blood that result from elevated numbers of red blood cells, white blood cells, or platelets. JMML falls under the subcategory of myelodysplastic/myeloproliferative neoplasms, along with chronic myelomonocytic leukaemia (CMML), which result from a clonal expansion of immature and mature myeloid cell types. In particular, JMML is an aggressive haematopoietic cancer in children that is characterised by the overproduction of monocytic cells that infiltrate the spleen, lung and liver. Haematopoietic stem cell transplant (HSCT) is the only curative therapy available
for JMML patients; however, relapse is as high as 30% (Locatelli et al., 2005) and the long-term morbidities of HSCT for survivors are severe.

A hallmark cellular feature of JMML and CMML is the formation of a marked increase in granulocyte-macrophage colony forming units in the presence of low concentrations of GM-CSF. Although the precise mechanism for this hypersensitivity to GM-CSF is unknown, several mutations that involve the Ras signaling pathway have been identified as key initiating events in JMML; specifically, mutations in PTPN11 (Shp-2, 35%), NRAS, or KRAS comprise 60% of the cases. Additionally, NF1 (Side et al., 1998, Loh et al., 2004) and homozygous CBL mutations have each been found to account for roughly 15% of the cases (Loh et al., 2009, Makishima et al., 2009); these findings are summarized in Figure 1.5. Recently, a publication to which our lab contributed, Niemeyer et al. identified that germline CBL mutations predispose patients to JMML. Despite JAK2 being mutated in CMML (Levine et al., 2005, Onida et al., 2002) and Philadelphia chromosome-negative disorders, it has not been reported to be mutated in JMML.
Figure 1.5. Proteins that are most frequently mutated in JMML. As indicated by the dashed lines, Shp-2, Cbl, NF1, and Ras have been reported to be mutated in JMML among other diseases (Chan et al., 2007).
As indicated with the above mutations, it is likely that there are different mechanisms that lead to GM-CSF-hypersensitivity in JMML. Another mechanism that our lab identified is that CBL-mediated loss of negative regulation of Src is crucial for the hyperactivation of GM-CSF receptor signaling in JMML cells (Bunda et al., 2013). Constitutively activating mutations in GMRβc or failure of a clearance mechanism that results in prolonged receptor turnover time might also be at work. To further investigate the mechanism that underlies GM-CSF receptor turnover, we investigated whether CBL was also the E3 ligase that targets it for proteasome-mediated degradation. However, preliminary data from our lab surprisingly found that CBL is not the E3 ligase that targets GMRβc for degradation. Lastly, our lab has also identified a mechanism that confers chemoresistance in JMML patients by way of enhanced CBL function, leading to increased Lyn-dependent PI3K/Akt pro-survival signaling (Bunda et al., 2014).
Figure 1.6. Classification of myeloproliferative disorders (Source: National Cancer Institute, NIH).
1.3. JAK-STAT pathway

A notable characteristic of most cytokine receptors is the activation of the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway, which primarily signals in hematopoietic and immune responses. Offering an alternative to the second messenger system, the JAK/STAT axis is significant as it is evolutionarily conserved from slime molds to mammals although not present in fungi and plants.

Mammalian genomes encode 4 JAKs: JAK1, JAK2, JAK3, and Tyk2. Initially named “Just Another Kinase” (Wilks et al., 1989), the first two JAK family members (JAK1 and JAK2) were initially discovered by a polymerase chain reaction (PCR)-based screen of kinases. They were later renamed Janus Kinases after the two-faced Roman god Janus because of their nearly identical kinase and pseudokinase domains. Tyk2 was subsequently discovered in a T-cell cDNA library using a probe against the c-fms tyrosine kinase domain (Krolewski et al., 1990), followed by the discovery of JAK3 due to its association with IL-2 and IL-4 signaling in myeloid and T cells (Kawamura et al., 1994, Witthuhn et al., 1994).

Functionally important domains that are common to all JAKs are a C-terminal JH1 kinase domain, a JAK homology (JH)2 pseudokinase domain, and an N-terminal Four-point-one/Ezrin/Radixin/Moesin (FERM) domain. JH1 is the
catalytic domain that confers kinase activity. Also called the pseudokinase domain because of its nearly identical phosphate transferring domain as JH1 but with no catalytic activity, JH2 is important because it negatively regulates the kinase domain. The FERM domain mediates the interaction between JAKs and their respective cytokine receptors (Reviewed in LaFave and Levine, 2012).

As non-receptor tyrosine kinases, JAKs are ubiquitously expressed in many cell types and relay proliferation, differentiation, survival, and apoptotic signals for cells that largely have hematological and immune functions. Examples of specific roles of each JAKs are as follows. JAK1 propagates signals for inflammatory cytokines such as IL-6 and TNFα (Miscia et al., 2002). JAK2 is important for the growth and differentiation of hematopoietic stem cells (Reviewed in Delhommeau et al., 2010, Verstovsek, 2010). JAK3, whose role is more restricted to the hematopoietic system, plays an important role in cytoskeletal remodeling and mucosal wound repair (Kumar et al., 2007). Tyk2 plays an important role in interferon signaling (Velazquez et al., 1992).

Apart from their signaling roles, JAKs have also been implicated to play a role in receptor stabilization at the cell-surface, endocytosis, and ubiquitination of receptors. For example, JAK2-bound to the growth hormone receptor has been shown to prevent endocytosis (Putters et al., 2011). On the other hand, JAK2 has been shown to be required for ligand-dependent internalization of EPOR (Sulahian
et al., 2009) as well as partially required for internalization of IL-5Rβc and ubiquitination (Martinez-Moczygemba et al., 2007).

When it comes to the mechanism of JAK activation, JAKs are either already pre-bound or recruited to cytoplasmic domains of receptors upon cytokine (or ligand)-binding to the extracellular portion of its respective receptor subunit/s; the ligand may or may not bind to the JAK-associated subunit, which may not be every subunit in the entire receptor complex as is the case for the GM-CSF receptor. Ligand-binding is usually one of the initiating steps that triggers subsequent receptor subunit dimerization or oligomerization. JAKs then either autophosphorylate or transphosphorylate each other, transforming them from an inactive to an active state. This process of JAK activation by way of transphosphorylation is thought to occur not only by the juxtaposition of JAKs brought about by spatial proximity from receptor subunit assembly but also through a conformational change that induces their reorientation. The YY motif in the activation loop of all JAKs is essential for their activation. JAKs can phosphorylate the same type of JAK or another JAK family member as well as other tyrosine kinases. Once activated, JAKs consequently phosphorylate tyrosine residues on the cytoplasmic portion of receptors to create docking sites for signaling molecules with phosphotyrosine-binding SH2 domains such as STAT proteins, Src-kinases, Shp phosphatases, and the Shc adaptor protein; they also
consequently phosphorylate other proteins such as STATs (Reviewed in Rane and Reddy, 2000).

STAT proteins, of which there are seven (Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6), are the prototypical substrates of JAKs as indicated by the name of the JAK/STAT pathway. STATs comprise a carboxy-terminal SH2 domain, an SH3-like domain, several conserved amino-terminal regions, and a conserved region in the middle of the protein that binds DNA. Tyrosine phosphorylation of a carboxy-terminal site by JAKs results in homo or heterodimerization of STATs via their SH2 domains and subsequent translocation to the nucleus where the bind DNA and initiate transcription of signal-specific genes (Reviewed in Aaronson and Horvath, 2002).

This pathway is negatively regulated by several different proteins. For example, protein tyrosine phosphatases (e.g. Shp proteins) remove phosphates from cytokine receptors, JAKs, and activated STATs (Reviewed in Hebenstreit et al., 2005). STATs are negatively regulated by protein inhibitors of activated STAT (PIAS), which exert their activity in the nucleus by blocking STAT access to DNA-binding sites and through SUMOylation (Shuai, 2006). A more recent discovery and central to my thesis are SOCS proteins, which terminate signaling either by directly inhibiting JAK activity, competing with STAT binding sites on receptors, or targeting proteins for proteasome-mediated degradation.
1.3.1. Aberrant JAK regulation

Disrupted JAK/STAT signaling is most often seen in immune-related disorders and hematopoietic malignancies. For example, mutations and translocations in JAKs that result in constitutive activation are found in several blood disorders. Examples, with the JAK members that are aberrant in parentheses, include myeloproliferative disorders (JAK1, JAK2), cancers (e.g. acute lymphoblastic leukemia [JAK2], acute myeloid leukemia [JAK1, JAK2], and acute megakaryoblastic leukemia [JAK2, JAK3]), and immune-specific disorders (e.g. T-cell precursor acute lymphoblastic leukemia [JAK1]). Disruption of JAK/STAT signaling may occur from: 1) JAK mutations (e.g. loss-of-function mutations in JAK3 and TYK2, leading to immunodeficiency; JAK2V617F confers constitutive activity); 2) mutations in receptors; 3) increased JAK1 signaling (as seen with JAK1 overexpression in cancer cells, which allows individual cells to contract and possibly promote metastasize); 4) an excess of cytokines; 5) damage of intracellular mechanisms that act on the JAK/STAT pathway (e.g. prevention of appropriate JAK2 degradation as seen in chuvash polycythemia-associated VHL mutants whereby SOCS1 has altered affinity to JAK2 (Russell et al., 2011)) (Reviewed in Laurence et al., 2012).
1.3.2. JAK2

JAK2 is of particular importance to this thesis because it is the primary kinase that transmits signals for the GM-CSF receptor. Experiments with dominant-negative JAK2 revealed that the activity of JAK2 is necessary for all the biological functions expressed by GM-CSF (Watanabe et al., 1996). Other receptor systems for which it also relays signals are the IL-3R and IL-5R, which share the βc subunit with the GM-CSF receptor, EPOR, TPOR, GHR, and PRLR.

Over the last decade, many groups have identified either activating or deactivating mutations involving tyrosine or serine residues in JAK2 in different receptor systems (Figure 1.7). Specific residues that are used in experiments in this report are K882 (renders JAK2 to be kinase-dead) and Y1007 (required phosphorylation site for the activation of JAK2 and for SOCS1-mediated negative regulation). As mentioned earlier, the JAK2V617F mutation is notorious in the clinical world because it is found in the following Philadelphia Chromosome-negative myeloproliferative disorders with the indicated percentages: 50% of primary myelofibrosis patients, 90% of the polycythemia vera patients, and 50% of the essential thrombocythemia patients.
Significant effort has been made to develop JAK2 inhibitors against these myeloproliferative disorders (Reviewed in LaFave and Levine, 2012, Tibes et al., 2012). Table 1.3 highlights some of the newer inhibitors that have entered Phase I and II clinical trials. There is still much interest to optimize these inhibitors to overcome current challenges such as non-specificity to other tyrosine kinases, toxicity from high-doses, and competition from high intracellular concentrations of ATP as all JAK inhibitors to date are ATP-competitive. In order to develop these next-generation inhibitors, experts have suggested to consider inhibitor-resistance
that is likely to develop with prolonged usage. Furthermore, several groups have also underscored the importance of better understanding the mechanism of JAK activation and deactivation in association with receptors when developing more efficacious inhibitors.

Table 1.3. JAK2 inhibitors that are currently being evaluated in the clinic (LaFave et al., 2012).

<table>
<thead>
<tr>
<th>JAK2 inhibitor</th>
<th>Inhibitor specificity</th>
<th>Clinical development</th>
<th>Toxicities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruxolitinib</td>
<td>JAK1, JAK2</td>
<td>FDA approved for MF</td>
<td>Thrombocytopenia, anemia, cytokine rebound phenomenon</td>
</tr>
<tr>
<td>SAR302503</td>
<td>JAK1, JAK2</td>
<td>Phase I/II</td>
<td>Increased amylase and lipase, increased transaminases, diarrhea, nausea and vomiting, GI symptoms, thrombocytopenia, anemia</td>
</tr>
<tr>
<td>CYT387</td>
<td>JAK2</td>
<td>Phase I/II</td>
<td>Thrombocytopenia, increased transaminases, peripheral neuropathy, transient loss of blood pressure and lightheadedness as first-dose effect</td>
</tr>
<tr>
<td>SB1518</td>
<td>JAK2</td>
<td>Phase II</td>
<td>GI symptoms, diarrhea, nausea, thrombocytopenia</td>
</tr>
<tr>
<td>CEP701</td>
<td>JAK2</td>
<td>Phase II</td>
<td>GI, diarrhea nausea and vomiting, anemia, thrombocytopenia, thrombosis, leukocytosis, thrombocytosis</td>
</tr>
<tr>
<td>LYZ784544</td>
<td>JAK2</td>
<td>Phase I ongoing</td>
<td>N/A</td>
</tr>
<tr>
<td>NS018</td>
<td>JAK2</td>
<td>Phase I/II ongoing</td>
<td>N/A</td>
</tr>
<tr>
<td>AZD1480</td>
<td>JAK1, JAK2</td>
<td>Phase I/II ongoing</td>
<td>N/A</td>
</tr>
<tr>
<td>BMS911543</td>
<td>JAK2</td>
<td>Phase I/II ongoing</td>
<td>N/A</td>
</tr>
</tbody>
</table>
1.4. Suppressors of cytokine signaling (SOCS) proteins

Exerting their effects on over 30 cytokines, SOCS proteins are important negative regulators of growth and cytokine signaling and best known for their role to terminate JAK/STAT signaling. SOCS expression can be induced by many different proteins, STATs being the most notable activators (Reviewed in Krebs and Hilton, 2000).

There are eight SOCS family members, SOCS1-7 and CIS. The common anatomical features that are conserved among all SOCS family members are a C-terminal SOCS box (40 amino acid), an SH2 domain, and an extended SH2 domain (ESS). One other important region that has been historically significant is an N-terminal kinase inhibitory region (KIR) (40 amino acid region) that is present in SOCS1 and SOCS3. Fig 1.8 illustrates the location of these domains in each of the eight SOCS proteins.
**Figure 1.8.** SOCS proteins with annotated domains (Linossi et al., 2013).
1.4.1. Roles of SOCS proteins

Transgenic models of CIS-deficient mice resemble STAT5-deficient mice, displaying inhibited JAK-STAT5 signaling. These mice also exhibit defects in growth and lactation, due to decreased growth hormone and prolactin signaling, as well as enhanced TCR signaling and defective IL-2 signaling (Reviewed in Croker et al., 2008).

Mice lacking SOCS1 display: 1) reduced growth 2) a reduced lymphocyte count in the thymus, spleen, and bone marrow 3) monocytic/macrophage and granulocyte inflammation in the heart, pancreas, and lungs. Furthermore, death in SOCS1 -/- mice occurs by 21 days and has been attributed to adipose degeneration, necrosis, and monocytic inflammation in the liver largely due to unchecked IFNγ signaling as SOCS1 -/- IFN -/- mice survive until adulthood. However, mice with the latter condition do exhibit problems such as polycystic kidneys and inflammation at 6 months of age. The increase in IFNγ, which is normally produced upon encountering pathogens, is most likely a product from T cells and natural killer (NK) cells; thus, it is unclear why SOCS1 -/- mice produce IFNγ (Reviewed in Croker et al., 2008).
Figure 1.9. Phenotype of a homozygous knockout of SOCS1 in mice (Metcalf et al., 1999).

SOCS2-deficient mice have an enlarged phenotype due to increased activity in growth hormone signaling as well as reduced neuronal density and aberrant neuronal differentiation (Reviewed in Croker et al., 2008). SOCS3-deficient mice have enhanced leptin receptor signaling, leading to the development of gastric tumors (Inagaki-Ohara et al., 2014); furthermore, several studies have found tissue-specific effects in the absence of SOCS3 such as the promotion of M1 polarization in SOCS3-deficient macrophages (Qin et al., 2012). Whereas the
effects of SOCS4-deficient mice have not been investigated, SOCS5 and SOCS6-deficient mice have no overt phenotype (Reviewed in Croker et al., 2008). SOCS7-deficient mice display increased insulin signaling, resulting in hypoglycemia in an insulin-tolerance test; these mice also correspondingly have enlarged islets of Langerhans, which contain the insulin-secreting pancreatic beta cells (Banks et al., 2005).

It is possible that a given SOCS protein can compensate for the function of another; this may explain why there was no detectable phenotype in SOCS5 and SOCS6-deficient mice. Identifying functionally redundant SOCS proteins through double knockouts in mice remains to be studied (Reviewed in Croker et al., 2008).

1.4.2. Mechanism of inhibition

The diverse mechanisms of inhibition that different SOCS members employ make this family of proteins interesting from a biochemical standpoint.

All eight proteins contain a SOCS Box motif, which is part of a complex with elongin B, elongin C, cullin-2 or cullin-5, RING-box-2 (RBX2), and an E2 ligase. The SOCS Box is what defines the greater SOCS family, which encompasses more than 40 proteins that can be subdivided into further categories based on the presence of other protein-interaction domains such as SPRY domains, ankyrin
repeats, WD-40 domains, and GTPases; there is no evidence that these extended SOCS family members also inhibit cytokine signaling. The SOCS proteins, as well as the other SOCS Box-containing proteins, target SH2-bound substrates for proteasome-mediated degradation. The repertoire of proteins that SOCS bind and target for clearance has been expanded over the last decade to include receptors and many other proteins from their initial discovery as JAK-binding proteins (Table 1.4 and Table 1.5). Examples of such substrates include the GM-CSF receptor, IFNγR1 receptor, Vav, and IRS1/2. Although the SOCS Box is not necessary for binding to substrates such as receptors, it appears to confer a higher affinity interaction (Reviewed in Croker et al., 2008).
**Table 1.4. Receptor substrates of SOCS1.** Residues in parentheses are SOCS1-binding sites predicted from sequence homology with experimentally-derived SOCS1-binding sites, indicated in parentheses with *.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Receptor substrates of SOCS1</th>
<th>References</th>
</tr>
</thead>
</table>
| IFNγ    | IFNGR1 (Y441)*              | Qing et al., 2005  
Starr et al., 2009 |
| IFNα/β  | IFNAR1                      | Fenner et al., 2006  
Piganis et al., 2011 |
| IL-2    | IL-2-Rβ (330-350 within the A region)* | Sporri et al., 2001 |
| IL-2, IL-4, IL-7, IL-9, IL-15 | IL-2-Ryc (note to self: check references again) | Marine et al., 1999  
Chong et al., 2003  
Cornish et al., 2003  
Davey et al., 2005  
Zhan et al., 2009  
Ilangumaran et al., 2003 |
| IL-12   | IL-12-R                     | Chong et al., 2005  
Mooney et al., 2001  
Ueki et al., 2004 |
| Insulin, IGF-I, IGF-II | IR (Y1150) | |
| GM-CSF  | GM-CSF-R (Y468)             | Bunda et al., 2013 |
| PDGF    | Kinase domain of PDGF-R     | De Sepulveda et al., 1999 |
| M-CSF   | Kinase domain of M-CSF-R/CSF1-R | De Sepulveda et al., 1999 |
| Flt3    | Kinase domain of CD135/Flt3 | De Sepulveda et al., 1999 |
| Angiopoietin-1, 2 | Kinase domain of Angiopoietin-1-R/Tek | De Sepulveda et al., 1999 |
| SCF/steel factor | Mast/SCF-R/c-kit | De Sepulveda et al., 1999 |
| Antigens bound to MHC | CD3ζ/T-cell receptor zeta chain | Matsuda et al., 2000 |
| FGF     | FGF receptor                | Masuhara et al., 1997 |
### Table 1.5. Non-receptor substrates of SOCS1.

Residue in parentheses with * is a SOCS1-binding site based on experimental evidence.

<table>
<thead>
<tr>
<th>Other SOCS1 substrates</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK2 (nRTK in JAK family) (Y1007)*</td>
<td>Ungureanu et al., 2002</td>
</tr>
<tr>
<td>Tel-JAK2</td>
<td>Monni et al., 2001; Frantsve et al., 2001</td>
</tr>
<tr>
<td>JAK1</td>
<td>Sporri et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Diao et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Krebs et al., 2000</td>
</tr>
<tr>
<td>JAK3 (nRTK in JAK family)</td>
<td>Sporri et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Krebs et al., 2000</td>
</tr>
<tr>
<td>Tyk2 (nRTK in JAK family)</td>
<td>Piganis et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Krebs et al., 2000</td>
</tr>
<tr>
<td>Vav</td>
<td>De Sepulveda et al., 1999 and 2000</td>
</tr>
<tr>
<td>Onco-VAV</td>
<td>De Sepulveda et al., 2000</td>
</tr>
<tr>
<td>Vav2</td>
<td>De Sepulveda et al., 1999</td>
</tr>
<tr>
<td>Grb2</td>
<td>De Sepulveda et al., 1999</td>
</tr>
<tr>
<td>p85</td>
<td>De Sepulveda et al., 1999</td>
</tr>
<tr>
<td>Nck</td>
<td>De Sepulveda et al., 1999</td>
</tr>
<tr>
<td>ITK (nRTK in TEC family)</td>
<td>De Sepulveda et al., 1999</td>
</tr>
<tr>
<td>Fyn (nRTK in SRC family)</td>
<td>De Sepulveda et al., 1999</td>
</tr>
<tr>
<td>Mal/TIRAP</td>
<td>Mansell et al., 2006</td>
</tr>
<tr>
<td>P65/RelA</td>
<td>Ryo et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Strebovsky et al., 2011</td>
</tr>
<tr>
<td>VHL</td>
<td>Russell et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Metcalf et al., 2013</td>
</tr>
<tr>
<td>p53</td>
<td>Calabrese et al., 2009</td>
</tr>
<tr>
<td>ATM (nuclear kinase in PIKK family)</td>
<td>Calabrese et al., 2009</td>
</tr>
<tr>
<td>ATR (nuclear kinase in PIKK family)</td>
<td>Calabrese et al., 2009</td>
</tr>
<tr>
<td>PTK2/FAK (FAK family) (Y397)*</td>
<td>Liu et al., 2003</td>
</tr>
<tr>
<td>DCN1</td>
<td>Heir et al., 2013</td>
</tr>
<tr>
<td>IRS1/IRS2</td>
<td>Rui et al., 2002</td>
</tr>
<tr>
<td>Elongin C</td>
<td>Kamura et al., 1998</td>
</tr>
<tr>
<td>Elongin B</td>
<td>Kamura et al., 1998</td>
</tr>
<tr>
<td>Pericentrin</td>
<td>Srsen et al., 2006</td>
</tr>
<tr>
<td>SHC1</td>
<td>Trinei et al., 2002</td>
</tr>
<tr>
<td>TRIM28/KAP1</td>
<td>White et al., 2006</td>
</tr>
<tr>
<td>NT5C2</td>
<td>Yamauchi et al., 2009</td>
</tr>
<tr>
<td>BCLAF1</td>
<td>Letsas et al., 2005</td>
</tr>
<tr>
<td>PC4</td>
<td>Wang et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Mortusewicz et al., 2008</td>
</tr>
<tr>
<td>Syk (Syk family)</td>
<td>Matsuda et al., 2000</td>
</tr>
<tr>
<td>Tec</td>
<td>Ohya et al., 1997</td>
</tr>
<tr>
<td>HPV-E7</td>
<td>Kamio et al., 2004</td>
</tr>
<tr>
<td>Pyk2</td>
<td>Masuhara et al., 1997</td>
</tr>
</tbody>
</table>
A SOCS Box-independent mechanism of inhibition involves the ability of CIS to compete with STAT5 recruitment sites on receptors such as the IL-3βc, EPOR, and IL-2R. Apart from this mechanism, SOCS1 and SOCS3 have an additional inhibitory mechanism involving the 12 amino acid KIR region found in only these two SOCS proteins. Although this region is dispensable for SOCS1 and SOCS3 binding to JAKs, it confers a higher-affinity interaction and is necessary for inhibiting kinase activity. SOCS1 and SOCS3 have been shown to directly bind JAKs and inhibit their kinase function although the contributions of homologous regions are different. The SH2 domain of SOCS1 has a stronger affinity for pY1007 in JAK2 than the SH2 domain of SOCS3. On the other hand, the KIR of SOCS3 has a stronger affinity to JAK2 than the KIR of SOCS1 (Reviewed in Croker et al., 2008). The requirement of the SOCS Box when it comes to KIR-mediated inhibition of JAKs has yet to be determined; although deletion of the SOCS Box in SOCS1 is not required for inhibiting JAK/STAT signaling in vitro, mice that lack the SOCS Box in SOCS1 demonstrate prolonged JAK/STAT signaling (Zhang et al., 2001).

It was initially proposed that the KIR inhibited JAK activity by acting as a pseudosubstrate by way of lodging in the region that substrates would normally bind. However, it was recently shown that the KIR of SOCS3 doesn’t bind in the catalytic cleft that substrates bind but rather to a GQM motif that results in a
conformational change in the catalytic pocket, preventing the transfer of a phosphate group onto the substrate (Babon et al., 2012). It has yet to be established whether the KIR of SOCS1 also binds to the GQM motif and prevents phosphate transfer.

Since both SOCS1 and SOCS3 interact with receptors and JAKs, it was initially proposed that they bound to the receptor and subsequently detached from the receptor to bind JAKs in a sequential fashion (Figure 1.10). However, recent crystal and biochemical experiments have shown that SOCS3 simultaneously binds to gp130 and JAK2 in a 1:1:1 ratio through the dual nature of its SH2 domain; one side, termed the phosphotyrosine face, binds to a phosphotyrosine residue on a receptor whereas its opposite hydrophobic face contacts JAK2 (Figure 1.11) (Babon et al., 2012). Whether SOCS1 also simultaneously binds to a receptor subunit and a non-receptor kinase such as JAK2 in a 1:1:1 ratio has yet to be determined. It is questionable that SOCS1 uses the same simultaneous mechanism as SOCS3 because SOCS1 has been reported to bind a phosphotyrosine on receptors as well as pY1007 in JAK2 (Ungureanu et al., 2002).
Figure 1.10. Proposed sequential model for the interplay between SOCS proteins, JAKs, and receptors (O’Sullivan et al., 2007).
Figure 1.11. Simultaneous model for the interaction between SOCS3, gp130, and JAK2 (Linossi et al., 2013).
1.4.3. Discovery and regulation of SOCS1

Discovered second among the SOCS family members after CIS, SOCS1 was independently identified by three groups: 1) as a JAK-binding protein (JAB) using a yeast two-hybrid screen with the JAK kinase domain as bait (Endo et al., 1997); 2) suppressor of IL-6 signaling (SOCS1) from a retroviral expression screen of IL-6 inhibitors that recovered cDNA that encodes an SH2 domain with homology to the known SH2-domain-containing CIS protein and to related ESTs (Starr et al., 1997); 3) protein with antigenic similarity with the STAT3-SH2 domain using a monoclonal antibody that recognized this region (STAT-induced STAT inhibitor: SSI) (Naka et al., 1997).

Interestingly, SOCS1 is able to inhibit the activity of cytokines that signal through the JAK/STAT pathway as well as cytokines that signal independently of the JAK/STAT pathway (e.g. LPS and insulin). Although SOCS1 can be induced by many cytokines (Reviewed in Kile et al., 2002), SOCS1 is primarily expressed in the thymus, spleen, lung, and testes (Starr et al., 1997, Naka et al., 1997).

SOCS1 is regulated at the transcriptional, translational, and post-translational level. In many cases, its gene expression is induced by STAT translocation to the nucleus, whereby STATs can bind the SOCS1 promoter (Naka et al., 1997, Saito et al., 2000). It has also been shown that SOCS1 expression can
be induced indirectly by STAT1 by way of inducing interferon regulatory factor-1 (IRF-1) transcription factor, which then induces SOCS1 expression (Saito et al., 2000). At the translational level, SOCS1 can be regulated through its 5’-untranslated region (Gregorieff et al., 2000). Post-translationally, SOCS1 can be phosphorylated by the Pim family of kinases, which prolongs the half-life of SOCS1 and as a result its inhibitory effect (Chen et al., 2002).

1.4.4. Aberrant regulation of SOCS1 in immunity

SOCS1 plays a significant role in attenuating the immune response to prevent a hyper-response to pathogenic stimulus. As mentioned earlier, SOCS1 -/- mice die within 3 weeks of birth and display stunted growth with activation of peripheral T cells, necrosis in the liver, and macrophage infiltration in many organs. The primary reason attributed for their death is unchecked IFNγ signaling since SOCS1 -/- mice that also lack the IFNγ or IFNγ receptor gene do not die neonatally. The lethality of the SOCS1 knockout can also be rescued by the deletion of STAT6, indicating that IL-4 and IL-13 signaling contribute to the fatal inflammation seen in SOCS1-deficient mice (Reviewed in Croker et al., 2008).

SOCS1-deficient dendritic cell (DC) s were hyperactivated in response to IL-4 and IFNγ, resulting in an autoimmune phenotype; additionally, SOCS1-deficient
dendritic cells (DCs) signify lupus-like autoimmune disease phenotypes. Liver-specific SOCS1 knockout mice demonstrated that SOCS1 deletion in hepatocytes enhanced concanavalin A (ConA)-induced hepatitis due to enhanced pro-apoptotic signals. SOCS1 depletion in natural killer (NK)T cells also enhanced sensitivity to ConA-induced hepatitis. SOCS1 deficiency in macrophages resulted in hyper-responsiveness to LPS. SOCS1 has also been shown to play an important role in intestinal immune homeostasis by regulating prostaglandin E2 (PGE2)-mediated DC and macrophage suppression (Reviewed in Croker et al., 2008).

SOCS1 also has a significant role in T cell activation, by regulating IL-4, IL-12, and IL-15 signaling, and in coordinating T helper (Th) 1/Th2 cellular responses. For example, it has recently been shown to be an essential regulator for T cell differentiation. Deletion of SOCS1 in CD4 naive T cells promoted differentiation into Th1 cells, inhibiting Th2 by suppressing IL-4 signaling and Th17 differentiation in an IFNγ-dependent manner. Since SOCS1 -/- mice display both Th1 and Th2 responses, it is unknown whether SOCS1 is a molecular switch that mediates differentiation into one cell type over the other (Reviewed in Croker et al., 2008).

SOCS1 also plays an important role in the regulation of regulatory T cells (Tregs). T cell-specific SOCS1 -/- mice had higher numbers of Tregs in the thymus and spleen. SOCS1 deletion in Tregs induced the development of spontaneous
dermatitis, splenomegaly, and lymphadenopathy; loss of SOCS1 expression resulted in loss of Foxp3 expression, which lead to severe colitis. SOCS1 also prevents inflammatory cytokine production from T_{regs}. Normally, T_{regs} do not secrete inflammatory cytokines; however in the absence of SOCS1, they secrete IFNγ and IL-17 by hyperactivation of STAT1 and STAT3, respectively (Reviewed in Croker et al., 2008).

Microbial activation of TLRs results in the induction of many different pathways including NFκB, IRF-3, IRF-7, MAPK, and interferon. While a role for SOCS1 in regulating IFNγ signaling has been well-defined, a definite role for its impact on TLR signaling remains to be established. What is known is that SOCS1 degrades mal/TIRAP, an adaptor associated with TLRs to prevent excessive p65/RelA phosphorylation and production of IL-6 and TNFα (Reviewed in Croker et al., 2008).

SOCS1 has also been shown to be upregulated by M. tuberculosis infection, impairing IFNγ secretion; its expression in macrophages hampered M. tuberculosis clearance early after infection in an IFNγ-dependent manner. Similarly, SOCS1 is highly induced by Toxoplasma gondii infection and Ebola virus infection in macrophages. Taken together, SOCS1 is upregulated by several pathogens in macrophages and is accompanied with the inhibition of TLR signaling, IL-12 production, and IFNγ responses (Reviewed in Croker et al., 2008).
SOCS1 may also prevent atherosclerosis, which can be classified as an inflammatory condition, by suppressing IFNγ signaling because blocking IFNγ by overexpressing soluble IFNγ receptor prevented atherosclerotic plaque formation in apolipoprotein E -/- mice (Reviewed in Croker et al., 2008).

1.4.5. Dysregulation of SOCS1 in cancer

SOCS1 has been reported to be aberrantly regulated largely by way of hypermethylation in many cancers such as acute myeloid leukemia (AML), glioblastoma multiforme, and chronic myeloid leukemia (CML). In AML, it has been reported to be inactivated in 60% of newly diagnosed cases. In CML patients, SOCS1 has been shown to revert from a methylated to an unmethylated state during remission. SOCS1 has also been reported to be mutated in mediastinal lymphoma, impacting its ability to degrade JAK2 (Reviewed in Croker et al, 2008).

SOCS1 hypermethylation is frequently accompanied with constitutive STAT phosphorylation, which has been found in many cancers such as hepatocellular carcinoma (HCC), Barrett’s adenocarcinoma, and myeloproliferative diseases. In addition, SOCS1 methylation has been associated with transformation of liver cirrhosis to HCC (Reviewed in Croker et al, 2008).
Overexpression of SOCS proteins reduces STAT activity as well as inhibits the transforming activity of certain haematopoietic-specific oncogenes such as TEL-JAK and oncogenic Vav by targeting them for proteasomal degradation. These data suggest that SOCS1 is a tumor suppressor (Reviewed in Croker et al, 2008).

On the other hand, SOCS1 has also been found to be constitutively expressed in cancers like melanoma and chronic myeloid leukemia (CML) and this corresponds with constitutive JAK/STAT activation (Roman-Gomez 2004). In this case, it is possible that excessively constitutive JAK/STAT activation prevents SOCS1 from exerting its ability to negatively regulate this pathway (Reviewed in Croker et al, 2008).
Table 1.6. Dysregulation of SOCS proteins in cancer (Trengove et al., 2013).

Table 2. Association of SOCS proteins with disease

<table>
<thead>
<tr>
<th>SOCS protein</th>
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<th>Mechanism</th>
<th>Reference</th>
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<tbody>
<tr>
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<tr>
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<tr>
<td></td>
<td>Glioblastoma multiforme</td>
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<td></td>
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<td>Hypermethylation</td>
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<td></td>
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<td>Hypermethylation</td>
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<tr>
<td></td>
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<td>Constitutive expression</td>
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<td>Tuberculosis</td>
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<td>5’ SNP</td>
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<td>Breast cancer with lymph node metastasis</td>
<td>Reduced expression</td>
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<td></td>
<td>Breast cancer with lymph node metastasis</td>
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<tr>
<td></td>
<td>Liver cancer</td>
<td>Decreased expression</td>
<td>[239]</td>
</tr>
<tr>
<td></td>
<td>Thyroid gland cancer</td>
<td>Decreased expression</td>
<td>[239]</td>
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<tr>
<td>SOCS7</td>
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<tr>
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<td>Metabolic syndrome</td>
<td>Specific haplotypes</td>
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1.5. **Hypothesis and aims**

The overall goal of this project was to understand the role of JAK2 in SOCS1-mediated GMRβc turnover. The rationale behind this investigation was to uncover molecular insight into the hypersensitivity to GM-CSF that is observed in JMML. Aim 1 of this project was to validate SOCS1 to be the E3 ubiquitin ligase that degrades GMRβc and assess its impact on downstream signaling. The working hypothesis in the lab when I started the project was that SOCS1 is the E3 ligase that degrades GMRβc and not Cbl, which also encodes an E3 ligase and targets proteins along the GM-CSF receptor signaling axis for degradation. Aim 2 of my project was to determine whether JAK2 is necessary for SOCS1 to bind and degrade GMRβc. My hypothesis was that JAK2 is necessary for both SOCS1 to bind and degrade GMRβc. Since JAK2 was necessary for substantial GMRβc degradation, Aim 3 of my project was to determine whether the kinase activity of JAK2 is necessary for proteasome-directed clearance; this question was answered using the K882E JAK2 mutant, which renders JAK2 kinase-dead, and the Y1007F JAK2 mutant, which abrogates phosphorylation on a key site and prevents SOCS1-binding to JAK2. My hypothesis was that both K882 and Y1007 in JAK2 are required for GMRβc degradation. Taken together, these aims were designed to better understand the mechanism behind JAK2’s involvement in SOCS1-mediated GMRβc turnover.
Chapter 2. Results

2.1. Knockdown of endogenous SOCS1 in TF-1 cells promotes GMRβc stabilization and GM-CSF-induced downstream signaling.

In order to determine the impact of SOCS1 on GM-CSF-induced downstream signaling of the GM-CSF receptor, SOCS1 was stably knocked down in the human erythroleukemic TF-1 cell line and protein expression was analyzed in a time-course experiment. TF-ShSOCS1 cells, which were depleted of endogenous SOCS1 as confirmed by western blotting (Figure 2.1.A) and qRT-PCR (Figure 2.1.B), showed a marked stabilization in GMRβc levels when compared with TF-ShScr cells upon treatment with GM-CSF for 30 minutes. In other words, GMRβc levels in TF-ShSOCS1 cells were similar at 0, 15, and 30 minutes post-GM-CSF stimulation whereas GMRβc levels in TF-ShScr cells slightly decreased by 15 minutes after treatment with GM-CSF and significantly decreased by 30 minutes.

In addition to GMRβc, total JAK2, phosphorylated STAT5 (pSTAT5), and phosphorylated ERK (pERK) levels were higher in TF-ShSOCS1 cells when compared with TF-ShScr cells while total STAT5 and total ERK levels remained constant over time.
Taken together with other findings from Bunda et al. (2013), these results demonstrate that SOCS1 is the E3 ligase that degrades GMRβc and its absence upregulates downstream signaling along the GM-CSF receptor axis.

2.1. A.
2.1. B.

Figure 2.1.A. **Time-course analysis of downstream signaling upon GM-CSF induction.** Lentiviral-mediated stable knockdown of endogenous SOCS1 was generated by transducing human erytholeukemic TF-1 cells with shSOCS1 or non-targeting scrambled shRNA (shScr), followed by selection with puromycin. These cells were serum and cytokine-starved prior to no treatment or treatment with GM-CSF for 15 and 30 minutes prior to lysis. Protein expression from whole cell extract (WCE)s was analyzed by western blotting with the indicated antibodies. Results are representative of three independent experiments.

Figure 2.1.B. **SOCS1 mRNA expression post-lentiviral transduction.** Knockdown of endogenous SOCS1 was confirmed with qRT-PCR using SOCS1 primers or β-actin control primers. Values were normalized to β-actin mRNA and expressed relative to shScr samples (arbitrarily set to 1.0). Results are the average of three independent experiments +/- SD. Reproduced from Bunda et al., 2013, “SOCS1 Mediates Ubiquitylation and Degradation of GM-CSF Receptor”, PLoS ONE 8(9): e76370.”

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Figure 2.1.A. Time-course analysis of downstream signaling upon GM-CSF induction. Lentiviral-mediated stable knockdown of endogenous SOCS1 was generated by transducing human erytholeukemic TF-1 cells with shSOCS1 or non-targeting scrambled shRNA (shScr), followed by selection with puromycin. These cells were serum and cytokine-starved prior to no treatment or treatment with GM-CSF for 15 and 30 minutes prior to lysis. Protein expression from whole cell extract (WCE)s was analyzed by western blotting with the indicated antibodies. Results are representative of three independent experiments.

Figure 2.1.B. SOCS1 mRNA expression post-lentiviral transduction. Knockdown of endogenous SOCS1 was confirmed with qRT-PCR using SOCS1 primers or β-actin control primers. Values were normalized to β-actin mRNA and expressed relative to shScr samples (arbitrarily set to 1.0). Results are the average of three independent experiments +/- SD. Reproduced from Bunda et al., 2013, “SOCS1 Mediates Ubiquitylation and Degradation of GM-CSF Receptor”, PLoS ONE 8(9): e76370.”
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2.2. JAK2 is not necessary for SOCS1 to bind GMRβc.

Since previous findings from our lab showed that SOCS1 co-immunoprecipitated (IPed) with GMRβc in the presence of exogenously-transfected and endogenous JAK2, we then asked whether SOCS1 can bind and degrade GMRβc in the absence of JAK2. We tested this question in two different cell lines, HEK293 and JAK2-null γ2A.

HEK293 cell line

In HEK293 cells, which only have trace amounts of endogenous JAK2, GMRα, GMRβc, and Flag-tagged SOCS1 plasmids were transfected with and without JAK2. Figure 2.2 shows that SOCS1 co-IPed with GMRβc (using an anti-GMRβc antibody) in the absence of exogenously-transfected JAK2 (Figure 2.2: lane 1 vs. 3 and 2 vs. 4). The same result was obtained when SOCS1 was IPed (using an anti-FLAG antibody) and immunoblotted (IBed) for GMRβc (Supplementary Figure 2.9: lane 1 vs. 5 and 2 vs. 6). This interaction was stabilized in the presence of MG132 (Figure 2.2: lane 5 vs. 7 and 6 vs. 8/9). [Note: The hyphen between 8 and 9 indicates that lane 9 is the same condition as lane 8 but serves as an immunoprecipitation control. This hyphenated format is used in the rest of the report to represent identical experimental conditions between lanes other than the latter]
serving as an immunoprecipitation control.). Treatment with GM-CSF for 15
minutes appears to enhance SOCS1-recruitment to GMRβc without exogenously-
transfected JAK2, most clearly seen with MG132 pretreatment (Figure 2.2: lane 6
vs. 5) as well as in the presence of JAK2 (Figure 2.2: lane 4 vs. 3).

Lastly and importantly, a greater amount of SOCS1 co-IPed with GMRβc in
the presence of exogenously-transfected JAK2 compared to lanes without
exogenously-transfected JAK2 (Figure 2.2: lane 3 vs. 1 and 4 vs. 2). Preliminary
densitometry analysis suggested that twice as many SOCS1 molecules co-IPed with
GMRβc when JAK2 was present (Table 2.1).
**Figure 2.2. GMRβc-immunoprecipitation and degradation profile in the absence or presence of exogenous JAK2.** HEK293A cells were co-transfected with plasmids encoding GMRα, GMRβc, and Flag-tagged SOCS1 either in the absence of JAK2 (Lanes: 1, 2, 5, and 6) or in the presence of exogenously transfected JAK2 (Lanes: 3, 4, 7, 8, and 9); empty pcDNA3 vector was additionally transfected in required lanes to ensure than an equal amount of exogenous DNA was present. Lanes 5-9 were pretreated with 5µg/mL MG132 (+) and lanes 1-4 were pretreated with DMSO (control,-) for 4 hours before GM-CSF stimulation (+) for 15 minutes in the indicated lanes. The cells were lysed 48 hours later, IPed with the anti-GMRβc antibody (rabbit polyclonal) or a rabbit IgG control, and IB with the indicated antibodies. The results here are representative of three independent experiments. WCE, whole-cell extract.
Table 2.1. Densitometry analysis performed with Image Studio Lite Ver 4.0. The signal for lanes 1-8 in Figure 2 for the amount of co-IPed SOCS1 and GMRβc were calculated. The column titled “IPed: S1/GMRβc” (column 7) was calculated by dividing SOCS1 signals/GMRβc for each respective lane to normalize the amount of GMRβc receptor being IPed so that SOCS1 values could be compared. The last lane titled “JAK2/No JAK2” has divided values from column 7 with JAK2/without JAK2 to determine the amount of SOCS1 co-IPed in either condition. For example, the value from column 7, lane 3 (+ JAK2, - GM-CSF) was divided with the value in column 7, lane 1 (- JAK2, - GM-CSF). N = 1 for this densitometry analysis.

<table>
<thead>
<tr>
<th>Image Name</th>
<th>Lanes</th>
<th>Signal</th>
<th>Total</th>
<th>Area</th>
<th>Bkgrnd.</th>
<th>S1/GMRβc</th>
<th>JAK2/ No JAK2</th>
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</thead>
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<th>Image Name</th>
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<th>Area</th>
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JAK2-null γ2A cell line

The above results in HEK293 cells were confirmed using our standard immunoprecipitation/immunoblotting method in the JAK2 -/- γ2A fibrosarcoma cell line to eradicate doubt about SOCS1-binding to GMRβc due to trace amounts of JAK2 present in the cells.

SOCS1 bound to GMRβc in γ2A cells, implying that JAK2 is not necessary for the interaction between SOCS1 and GMRβc (Figure 2.3). This interaction occurred both in the absence and presence of GM-CSF stimulation as seen in HEK293 cells. Similarly, an increased amount of SOCS1 also co-IPed with GMRβc in the presence of GM-CSF in γ2A cells (Figure 2.3: lane 2 vs. 1) and the interaction between GMRβc and SOCS1 was stabilized with the proteasomal-inhibitor MG132 (Figure 2.3: lane 6 vs. 1 and lane 7 vs. 2).
Figure 2.3. Immunoprecipitation of GMRβc in JAK2-null γ2A fibrosarcoma cells in the presence of proteasomal inhibitor MG132. γ2A cells were co-transfected with GMRβc and Flag-tagged SOCS1 (Lanes: 1, 2, 3, 6, 7, and 8), Flag-tagged SOCS1 alone (Lanes: 4 and 9), or GMRβc alone (Lanes: 5 and 10). Lanes 6-10 were pretreated with 5µg/mL MG132 (+) and lanes 1-5 were pretreated with DMSO (-) as a control for 4 hours before treatment with GM-CSF for 15 minutes in the indicated lanes. The cells in lanes 1, 2, 4, 5, 6, 7, 9, and 10 were then lysed and IPed with an anti-GMRβc antibody (rabbits polyclonal); lanes 3 and 8 were IPed with a rabbit IgG control. The IPed samples and respective WCEs from each condition were IB with the indicated antibodies. The results here are representative of three independent experiments. WCE, whole-cell extract.
2.3. JAK2 enhances SOCS1-mediated degradation of GMRβc.

Since SOCS1 can bind GMRβc in the absence of JAK2, we then asked whether it can degrade this receptor subunit without its primary kinase. Figure 2.3 shows that GMRβc was tenuously degraded in the WCEs of γ2A cells when SOCS1 was co-expressed with and without GM-CSF stimulation (Figure 2.3: lanes 1 and 2/3 vs. 5). Furthermore, this modest degradation could be rescued by MG132 (Figure 2.3: lane 6 vs. 1 and 7/8 vs. 2/3).

When GMRβc degradation in γ2A cells was compared with GMRβc degradation in JAK2-reconstituted γ2AR cells, it was noted that significantly lower levels of GMRβc were present with and without GM-CSF stimulation for 15 minutes in the presence of JAK2 (Figure 2.4: lanes 6, 7/8, and 10 vs. 1, 2/3, and 5, respectively) as seen in HEK293 cells (Figure 2.2: lane 3 vs. 1 and 4 vs. 2). This was even the case when there was no exogenously-transfected SOCS1, possibly suggesting that other E3 ligases might also degrade GMRβc in the presence of JAK2 (Figure 2.4: lane 10 vs. 5).
Figure 2.4. Immunoprecipitation of GMRβc in JAK2-null γ2A and stably-reconstituted JAK2 in γ2AR cells. γ2A cells (lanes 1-5) and γ2AR cells (lanes 6-10) were co-transfected with GMRα/βc and Flag-tagged SOCS1 (Lanes: 1, 2, 3, 6, 7, and 8), Flag-tagged SOCS1 alone (Lanes: 4 and 9), or GMRα/βc alone (Lanes: 5 and 10); empty pcDNA3 vector was additionally transfected in required lanes to ensure than an equal amount of exogenous DNA was present. Lanes 2-5 and 7-10 were treated with GM-CSF (+) for 15 minutes whereas lanes 1 and 6 were left untreated (-). The cells in lanes 1, 2, 4, 5, 6, 7, 9, and 10 were then lysed and IPed with an anti-GMRβc antibody (rabbit polyclonal); lanes 3 and 8 were IPed with a rabbit IgG control. The IPed samples and respective whole cell extracts from each condition were IBed with the indicated antibodies. The results here are representative of three independent experiments.
2.4. Transient knockdown of JAK2 stabilizes GMRβc levels in TF-1s.

To confirm the above overexpression findings of the requirement of JAK2 for substantial SOCS1-mediated GMRβc degradation, a transient knockdown of endogenous JAK2 was generated in TF-1s as indicated by decreased levels of total JAK2 in si-JAK2 lanes compared to si-Scr lanes (Figure 2.5). Most importantly, this led to a stabilization of GMRβc levels in si-JAK2 cells; the effect was most noticeable after 30 minutes post-GM-CSF stimulation. The downstream signaling events when JAK2 was knocked down resembled the reverse protein expression profile as that seen in TF-ShSOCS1 cells; decreased pSTAT5 and pERK levels were observed whereas total STAT5 and ERK levels remained similarly unchanged.

These results support the above findings that although SOCS1 can bind GMRβc in the absence of JAK2, it requires the presence of JAK2 for its E3 ligase activity to degrade GMRβc in a rapid manner.
Figure 2.5. Transient knockdown of endogenous JAK2 in human erythroleukemic TF-1 cells. TF-1 cells were transfected with siScr or siSOCS1 plasmids and lysed 72 hours later upon GM-CSF stimulation for the indicated time points. SDS-PAGE and western blot analysis, with the indicated panel of antibodies, was performed on equal amounts of WCEs. The results here are representative of three independent experiments.
2.5. Kinase activity as determined by K882 in JAK2 is required for GMRβc degradation.

Since our data indicated that the presence of JAK2 is necessary for expediting GMRβc degradation, we then asked whether the kinase activity of JAK2 is necessary for SOCS1 to degrade GMRβc using the kinase-dead K882E JAK2 mutant. The abrogation of JAK2 kinase activity was confirmed in HEK293 cells by western blot analysis with a pJAK2 antibody (Figure 2.6: lanes 3, 4, 9, and 10); although negligible phosphorylated JAK2 levels were detected (Figure 2.6: lanes 4 and 10) upon GM-CSF stimulation for 15 minutes, this K882E JAK2 mutant was not able to be phosphorylated by and large as was the WT JAK2 mutant in the presence of GMRβc.

In the WCE samples, GMRβc levels were significantly elevated in the presence of K882E JAK2 compared to WT JAK2 (Figure 2.6: lanes 3 vs. 5 and 4 vs. 6). Interestingly, although GMRβc was able to be rescued both in the presence of K882E JAK2 and WT JAK2, a greater amount of GMRβc was able to be rescued when JAK2 was not active (Figure 2.6: lanes 9, 10 vs. 3, 4 compared to lanes 11, 12 vs. 5, 6).

Lastly, a lower amount of SOCS1 co-IPed with GMRβc in the presence of K882E JAK2 compared with WT JAK2 in MG132-pretreated samples (Figure 2.6:...
lane 9 vs. 11 and lane 10 vs. 12); MG132-pretreated samples were compared instead of non-MG132 treated samples because GMRβc levels were relatively stabilized, allowing for a more standardized assessment. Although the levels of IPed GMRβc were lower in the presence of WT JAK2, as reflected by lower levels in the WCE, the amount of co-IPed SOCS1 was greater than the amount that co-IPed with higher amounts of GMRβc in the presence of K882E JAK2; therefore, the differing amount of IPed GMRβc is not a major issue when making this conclusion.
Figure 2.6. GMRβc-IPed and degradation profile in the presence of mutant K882E JAK2. HEK293A cells were co-transfected with plasmids encoding GMRα, GMRβc, and Flag-tagged SOCS1 either in the presence of no JAK2, kinase-dead K882E JAK2, or WT JAK2; empty pcDNA3 vector was additionally transfected in required lanes to ensure than an equal amount of exogenous DNA was present. Lanes 7-12 were pretreated with 5µg/mL MG132 (+) and lanes 1-6 were pretreated with DMSO (control, -) for 4 hours before GM-CSF stimulation (+) for 15 minutes in the indicated lanes. The cells were lysed 48 hours later, IPed with the anti-GMRβc antibody (rabbit polyclonal), and IB with the indicated antibodies. The results here are representative of three independent experiments. WCE, whole-cell extract.
2.6. SOCS1-docking site Y1007 in JAK2 is required for GMRβc degradation.

Since it has been shown that phosphorylation of Y1007 is a key activation site as well as a SOCS1-docking site for JAK2 degradation (Ungureanu et al., 2002), we wanted to confirm if this residue was also necessary for GMRβc degradation. As with the K882E JAK2 mutant, Y1007F was unable to be phosphorylated (Figure 2.7: lanes 3, 4, 9, and 10).

In support of the kinase-dead JAK2 findings, Y1007 in JAK2 is required for GMRβc degradation as GMRβc levels in the WCE were elevated in the presence of Y1007F JAK2 compared to WT JAK2 (Figure 2.7: lane 3 vs. 5 and 4 vs. 6) and similar to the levels with no exogenously-transfected JAK2 (Figure 2.7: lane 3 vs. 1 and 4 vs. 2). As with the K882E JAK2 mutant, a greater amount of GMRβc was able to be rescued with MG132 in the presence of Y1007F JAK2 than WT JAK2 (Figure 2.7: lanes 9, 10 vs. 3, 4 compared to lanes 11, 12 vs. 5, 6, respectively). As with the K882E samples, a decreased amount of SOCS1 co-IPed with GMRβc in the presence of Y1007F JAK2 compared with WT JAK2 in MG132-pretreated samples (Figure 2.7: lane 9 vs. 11 and lane 10 vs. 12).
Figure 2.7. GMRβc-immunoprecipitated and degradation profile in the presence of mutant Y1007F JAK2. HEK293A cells were co-transfected with plasmids encoding GMRα, GMRβc, and Flag-tagged SOCS1 either in the presence of no JAK2, Y1007F JAK2 lacking the SOCS1 binding site, or WT JAK2; empty pcDNA3 vector was additionally transfected in required lanes to ensure an equal amount of exogenous DNA was present. Lanes 7-12 were pretreated with 5µg/mL MG132 (+) and lanes 1-6 were pretreated with DMSO (control, -) for 4 hours before GM-CSF stimulation (+) for 15 minutes in the indicated lanes. The cells were lysed 48 hours later, IPed with the anti-GMRβc antibody (rabbit polyclonal), and IBed with the indicated antibodies. The results here are representative of three independent experiments. WCE, whole-cell extract.
2.7. SOCS1 is predicted to bind Y468 in GMRβc and GMRβc is predicted bind Y114 in JAK2 based on sequence homology.

Preliminary bioinformatics analysis indicates that SOCS1 binds Y468 in GMRβc (Figure 2.8.A) and GMRβc binds Y114 in JAK2 (Figure 2.8.B). Multiple sequence alignment was performed using ClustalW. Figure 2.8.A illustrates an alignment with a GYR motif in GMRβc that was also found in the cytokine receptor IFNGR1, which is an experimentally-shown SOCS1-binding site. Figure 2.8.B is an alignment of human JAK family members, centered on tyrosines that have been reported to prevent interaction with receptors when mutated; based on significant sequence homology to the right of the tyrosines, it is predicted that GMRβc interacts with Y114 in JAK2.
Figure 2.8.A. **Alignment of SOCS1-binding sites in GMRβc, IFNGR1, FAK, JAK2, and the insulin receptor.** One letter abbreviations of amino acid residues are colour-coded as follows: red (SOCS1-binding tyrosine residues), blue (moderately polar), purple (polar and neutral), yellow (polar and charged), and green (nonpolar). The italicized tyrosine residues are predicted from sequence homology.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMRβc (Y468)</td>
<td>CG I Y<strong>G</strong> Y RL R R K</td>
</tr>
<tr>
<td>IFNGR1 (Y441)</td>
<td>E S L I <strong>G</strong> Y R P T E D</td>
</tr>
<tr>
<td>FAK (Y397)</td>
<td>S E T D D <strong>Y</strong> A E I I D</td>
</tr>
<tr>
<td>JAK2 (Y1007)</td>
<td>P Q D K E Y Y K V K E</td>
</tr>
<tr>
<td>Insulin Receptor</td>
<td>I Y E T D Y Y R K G G</td>
</tr>
</tbody>
</table>

1. Mutation Y107A on JAK1 leads to loss of binding to gp130 (Haan et al., 2001).
2. Mutating Y114 on JAK2 is predicted to inhibit interaction with GMRβc based on sequence homology.
3. Mutation Y100C on JAK3 leads to loss of binding to IL-2-Rβ (Cacalano et al., 1999).

Figure 2.8.B. **Alignment of human JAK family members.** Boxed region in red indicates tyrosine residues on JAKs that are necessary for binding to receptors. The residue for JAK2 was predicted based on sequence homology from experimentally shown evidence with the inability of Y107A JAK1 and Y100C JAK3 to bind to gp130 and IL-2-Rβ.
Supplementary Figure 2.9. Immunoprecipitation of Flag-tagged SOCS1 in HEK293A cells with and without MG132. HEK293A cells were co-transfected with the following plasmid-combinations: GMRα, GMRβc, and Flag-tagged SOCS1 (Lanes: 1, 2, 7, and 8); JAK2 and Flag-tagged SOCS1 (Lanes: 3, 4, 9, 10); GMRα, GMRβc, and JAK2 (Lanes: 5, 6, 11, and 12); empty pcDNA3 vector was additionally transfected in required lanes to ensure an equal amount of exogenous DNA was present. Lanes 7-12 were pretreated with 5µg/mL MG132 (+) and lanes 1-6 were pretreated with DMSO (control, -) for 4 hours before GM-CSF stimulation (+) for 15 minutes in the indicated lanes. The cells were lysed 48 hours later, IPed with the anti-Flag antibody (mouse monoclonal), and IBed with the indicated antibodies. WCE, whole-cell extract.
Chapter 3. Discussion

My project began with validating SOCS1 to be the E3 ligase that targets GMRβc for proteasome-mediated degradation. The results obtained from the stable knockdown analysis of endogenous SOCS1 in TF-1 cells were consistent with other data in our lab, highlighting the role of SOCS1 to be a negative regulator of the GM-CSF receptor and its downstream signaling as removal of SOCS1 increased pSTAT5 and pERK levels (Figure 2.1.A). This increase in phosphorylation of downstream signaling proteins can be explained by the elevated levels of GMRβc and JAK2 in TF-ShSOCS1 cells. Since more JAK2-bound GMRβc complexes are available at 15 and 30 minutes post-GM-CSF stimulation, more JAK2 molecules can transphosphorylate each other and in turn phosphorylate tyrosine residues on a greater number of GMRβc chains than the number in TF-ShScr cells at 15 and 30 minutes. Since more GMRβc molecules can serve as STAT5 docking sites, JAK2 then is able to phosphorylate more STAT5 proteins. Increased pSTAT5 levels results in increased pERK levels since ERK signaling has been reported to occur downstream of the GM-CSF receptor.

The next part of my project, and the majority of my Master’s thesis, hinges on elucidating the role of the primary, non-receptor tyrosine kinase JAK2 in GM-CSF receptor signaling. Our investigation thus far has demonstrated that although SOCS1
retains its ability to bind GMRβc in the absence of JAK2, as best shown in the JAK2-null γ2A cells (Figure 2.3), it requires JAK2 for rapid degradation of GMRβc. It is not that surprising that SOCS1 can bind cytokine receptors, such as GMRβc, because it contains an SH2 domain like STAT5 and other signaling proteins that dock at cytokine receptors. Even though the GMRβc/SOCS1 interaction occurred both in the absence and presence of GM-CSF stimulation, a greater amount of SOCS1 co-IPed with GMRβc in the presence of GM-CSF; it is plausible that the requirement for SOCS1 to be recruited to cytokine-activated receptor is greater than to unstimulated and therefore not actively signaling receptor.

A comparison of GMRβc degradation rates at 15 minutes post-GM-CSF stimulation in JAK2-null γ2A cells and their JAK2-reconstituted γ2AR counterparts indicates that JAK2 has a substantial effect on expediting receptor degradation (Figure 2.4). The modest GMRβc degradation that took place in the γ2A cells, which could be visualized by both a decrease in the presence of exogenous SOCS1 when compared to GMRβc without transfected SOCS1 as well as with rescue of the degraded GMRβc with MG132, can possibly be attributed to the presence of other kinases that relay signals for βc such as JAK1 in other receptor systems. For example, JAK1 has been reported to bind IL-5Rβc, contributing to its phosphorylation, and possibly in helping mount an optimal ubiquitination signal
against IL-5Rβc in partnership with JAK2 (Martinez-Moczygemba et al., 2007 and Lei et al., 2011).

Our overexpression experiments were confirmed with a transient knockdown analysis of endogenous JAK2 in TF-1 cells, which showed a marked stabilization of GMRβc by 30 minutes upon GM-CSF stimulation and suppression of downstream signaling (Figure 2.5). Taken together, these results indicate that JAK2 is a principal component of GM-CSF receptor signaling.

A simplistic and unlikely explanation of the degradation of unstimulated GMRβc seen in every figure is that it is an artifact due to the self-activating effect of overexpressing plasmids since phosphorylation of JAK2 is also seen without GM-CSF stimulation in HEK293 cells, and we and others have shown that JAK2 transphosphorylation and GM-CSF receptor downstream signaling occurs only upon GM-CSF induction in an endogenous system or cell lines with stably-transfected vectors. However, we think it is unlikely that the degradation of GMRβc is due to the overexpression system because in the system that Lei et al. investigated IL-5R signaling, which involved stable transduction of both receptor subunits into HEK293 cells, they showed that GMRβc is basally ubiquitinated without IL-5 induction in the presence and absence of both JAK1 and JAK2. It is possible that kinases other than JAK1 (e.g. Lyn) that bind to GMRβc also contribute to its ubiquitination, however unlikely in the context of the GM-CSF receptor. Quelle et al. reported that
GM-CSF induction specifically results in JAK2 phosphorylation and could not detect the phosphorylation of JAK1 or Lyn. Therefore, it is possible that SOCS1-mediated GMRβc turnover occurs even without GM-CSF induction; whether SOCS1 degrades GMRβc that is not actively signaling \textit{in vivo} has yet to be confirmed.

We also showed that not only is the presence of JAK2 required for GMRβc degradation but also JAK2 that is capable of being phosphorylated, and hence activated. In the presence of both K882E (Figure 2.6) and Y1007F JAK2 (Figure 2.7), GMRβc degradation was significantly hindered when compared to its degradation with WT JAK2. These residues have been shown to be necessary for SOCS1-mediated ubiquitination and degradation of JAK2 in a study that was conducted without a receptor (Ungureanu \textit{et al.}, 2002). Whereas K882E JAK2 abrogates kinase activity as it contains a mutation in the ATP binding loop that is involved in a phosphotransfer reaction, Y1007F JAK2 prevents the interaction between SOCS1 and JAK2 (Yasukawa \textit{et al.}, 1999); Y1007F has an additional function by serving as a key phosphorylation site in JAK2. Therefore, we are proposing that the mechanism for GMRβc degradation is similar to that of solely JAK2 degradation as degradation of both require K882 and Y1007 in JAK2. These findings are consistent with those from Lei \textit{et al.}, who demonstrated a correlation
between βc-ubiquitination and JAK2-association with the subunit in the context of IL-5 signaling.

Correlating with hindered GMRβc degradation in whole cell extract (WCE)s, a decreased amount of SOCS1 bound to GMRβc in the presence of K882E JAK2 (Figure 2.6) and Y1007F JAK2 (Figure 2.7) compared to WT JAK2. Samples pretreated with MG132 were compared instead of untreated samples so that relatively uniform levels of GMRβc could be evaluated. In addition, although SOCS1 is not able to bind K882E and Y1007F in JAK2, as shown by Ungureanu et al., it can still bind to GMRβc as it can in the absence of exogenously-transfected JAK2.

Apart from its effect on GMRβc-degradation kinetics, our study indicates that JAK2 with intact kinase activity also serves as a signal for lysosomal degradation to take place as a secondary clearance pathway to complement proteasomal degradation. In the WCEs of both K882E JAK2 and Y1007F JAK2, a greater amount of GMRβc was recovered with the proteasomal inhibitor MG132 than was recovered in the presence of WT JAK2. This implies that the amount of GMRβc that wasn’t able to be rescued in the presence of WT JAK2 was probably cleared through the alternative lysosomal degradation pathway. This hypothesis supports the model proposed by Martinez-Moczygemba et al., who reported that a truncated cytoplasmic portion of βc in IL-5R signaling is degraded by proteasomes and the
remainder of the receptor is consequently degraded by lysosomes. This is also consistent with Walrafen et al. who reported that both proteasomes and lysosomes degrade the activated erythropoietin receptor.

When it comes to the sequence of events that regulate GM-CSF receptor signaling, it has been well-established that GM-CSF binds GMRα to form a low-affinity binary complex. Two binary complexes assemble with preformed JAK2-bound GMRβc dimers, creating a hexamer (2 GM-CSF: 2 GMRα: 2 GMRβc). Recent structural data on the extracellular portion of the GM-CSF receptor indicates that two hexamers come together to form a dodecamer, which is the functional receptor. This higher-order signaling complex brings JAK2 molecules close enough together to allow them to transphosphorylate each other; this process possibly involves reorientation of JAK2 molecules through structural changes to prevent kinase activation in the absence of a ligand. We and others have additionally shown that JAK2 is largely phosphorylated when its kinase activity is intact, as determined by K882 and Y1007 in JAK2, as well as when it is bound to GMRβc versus when it does not associate with a receptor (Supplementary Figure 2.9). Lastly, it is possible that some aspect of GM-CSF receptor/JAK2 activation, signal propagation, or signal termination involves cleavage of JAK2 since K882E JAK2 ran at a higher molecular weight than WT JAK2 as can be seen in the WCEs in Figure 2.6. (The degradation
product of JAK2 has also been visualized at higher exposures in other experiments (data not shown).

The most interesting aspect of this study is the additional insight revealed about the mechanism involved in the interplay between SOCS1, JAK2, and GMRβc. Figure 2.2 and its corresponding densitometry analysis in Table 2.1 suggests that SOCS1 might also assemble into a higher-order complex since it appears that two molecules of SOCS1 are co-immunoprecipitating with GMRβc and JAK2 versus one in the presence of just GMRβc. Our current explanation is that SOCS1 is independently binding to both JAK2 and GMRβc as it has been reported that SOCS1 can bind JAK2 on pY1007 and we have shown that it binds GMRβc independently of JAK2. Therefore, the stoichiometry of this E3 ligase/receptor/kinase complex is at least 2 SOCS1: 1 JAK2: 1 GMRβc. This is different from the sequential model proposed a few years ago that proposed that SOCS proteins first interacted with the receptor and then associated with JAK.

Although it is possible that SOCS1 emulates the mechanism employed by SOCS3, which was reported to simultaneously bind JAK2 and the gp130 receptor subunit in a 1:1:1 stoichiometric ratio, our preliminary data indicates otherwise (Babon et al., 2012). A subsequent study by Kershaw et al. ascribed one SOCS3 molecule’s ability to bind two different proteins to the dual nature of its SH2 domain; one side has a preference for phosphotyrosine motifs whereas the other engages in
hydrophobic interactions. In this case, the phosphotyrosine interface of SOCS3’s SH2 domain interacted with the tyrosine on gp130 whereas its hydrophobic face, along with the extended SH2 domain (ESS) and KIR interacted with the JH1 domain of JAK2.

As mentioned earlier, the problem with inferring that SOCS1 employs a similar mechanism of inhibition is that SOCS1 has been shown to interact with a tyrosine in JAK2 and has also been shown to interact with a tyrosine in another cytokine receptor (Y441 in IFNGR1 (Starr et al., 2009); in addition, it was predicted that SOCS1 binds Y1150 in the insulin receptor (Ueki et al., 2004). Although SOCS1 has been reported to bind proteins such as Vav (De Sepulveda et al., 2000) and p53 (Calabrese et al., 2009) in a phosphotyrosine-independent manner, indicating that its SH2 domain is also capable of making phosphotyrosine-independent interactions, an alignment surrounding the experimentally-shown SOCS1-binding site Y441 in IFNGR1 and GMRβc resulted in a similar GYR motif that corresponded to Y468 in GMRβc. Therefore, we predict that Y468 is the SOCS1-binding site in GMRβc. Since SOCS1 requires its phosphotyrosine interface for binding both GMRβc and JAK2, it is thought at least two SOCS1 molecules are found in this receptor/kinase complex.

It is possible that the two SOCS1 proteins in the above complex interact with each other at the hydrophobic face of their SH2 domains. This interaction between
two SOCS1 proteins, which are also independently binding GMRβc and JAK2, might lead to their stability as SOCS1 levels seem to be stabilized in WCEs of HEK293 cells when both GMRβc and JAK2 are present compared to SOCS1 levels in the presence of GMRβc without JAK2 (Figure 2.2).

Our model does not preclude the concept of specificity for SOCS1 being able to terminate signaling for certain receptor/JAK2 pairs, as proposed by the model in which SOCS3 simultaneously bound gp130 and JAK2, since each protein SOCS1 binds is targeted for proteasome-mediated degradation most likely at the same time. It is also likely that SOCS1 itself is degraded in this complex since MG132 can rescue SOCS1 levels in HEK293 cells. This notion is supported in the literature by the fact that proteasomal inhibitors LLnL and LLM can rescue the expression of SOCS1 (Narazaki et al., 1998). Furthermore, SOCS3 has been reported to be degraded by proteasomes in a SOCS Box-dependent manner (Sasaki et al., 2003).

Although unlikely, it is possible that SOCS1 might interact with more than one tyrosine residue in GMRβc (the other site being Y466, which is two residues away from Y468) as implicated with other signaling molecules such as STAT5 (Itoh et al., 1998); this is not expected since no other group to our knowledge has implicated multiple SOCS1 binding sites in a receptor. Also, the presence of a second tyrosine residue near a SOCS1-binding site in GMRβc is not unlike that seen in the insulin receptor (predicted SOCS1-binding site Y1150 and the adjacent
Y1151) and JAK2 (experimentally-shown SOCS1-binding site Y1007 and the adjacent Y1008 (Ungureanu et al., 2002)). In the case of the insulin receptor, Y1150 was chosen as the SOCS1-binding site over Y1151 because SOCS1 is thought to share that site with the adapatator protein IRS2 for contrasting signaling effects (Ukei et al., 2004). As mentioned earlier, Y468 in GMRβc was chosen over Y466 because the former residue aligned with an experimentally-shown SOCS1-binding site in IFNGR1. It is interesting to note that all five of the proteins aligned in Figure 2.8.A (GMRβc, IFNGR1, FAK, JAK2, and the insulin receptor) have a polar, charged amino acid (E/D/R) adjacent to the tyrosine that SOCS1 is predicted to bind.

The alignment of the four human JAKs in Figure 2.8.B. revealed a putative GMRβc-binding residue in JAK2. It has been experimentally shown that Y107A in JAK1 fails to interact with gp130 (Haan et al., 2001) and Y100C in JAK3 led to loss of binding to IL-2-Rγ (Cacalano et al., 1999) or reduced binding as reported by another group (Zhou et al., 2001). Y114 in JAK2 aligned with Y107 in JAK1 and Y100 in JAK2, with significant homology to the right of these tyrosines. The motif R-(M/I)-RFYF-(T/P/R)-(N/R)-W was identified with variations at sites indicated in parentheses, revealing more homology between JAK2 and JAK3 in this region (8 out of 9 residues were identical). The reason this interaction is important is that preventing JAK-association with a cytokine receptor may lead to an aberrant clinical phenotype as that seen with Y100C JAK3, which was found in a patient with severe
combined immunodeficiency (SCID). Although Y114 in JAK2 has not been linked to a pathological process, it adds to the repertoire of existing JAK2 residues that can be exploited for novel therapies in illnesses that have constitutively-activated JAK2 for example.

What is interesting is that it is actually Y119E in JAK2, a phosphorylation mimic mutation, that doesn’t interact with EPOR (Funakoshi-Tago et al., 2006); it was proposed that phosphorylation of Y119 in JAK2 results in its disassociation from EPOR and therefore is a mechanism for downregulation of EPOR signaling. In addition, this study showed that Y119E failed to interact with thrombopoietin, prolactin, and growth hormone receptors but it interacted with the IFNGR2 receptor; this suggested that there are differences between how JAK2 interacts with the former set of receptors versus the IFNGR2 receptor. Funakoshi-Tago et al. also reported that Y114 in JAK2 did not become phosphorylated, so they did not investigate mutations at this site even though it corresponded with Y100C in JAK3. Since Y119 in JAK2 corresponds with Y105 in JAK3, and Y105A or Y105F did not have any effect on IL-2Rγ receptor association (Zhou et al., 2001), we question whether Y119 is the residue that interacts with GMRβc.

Lastly, a study that investigated the role of amino acid residues in and near the Box 1 and Box 2 regions of gp130, for their impact on signaling and JAK1-association found that W652 in the Box 1 region is necessary for JAK1
phosphorylation as well as downstream signaling (Haan et al., 2002). Mutating W652 to alanine in only one gp130 dimer prevented phosphorylation of STAT1 in a dominant-negative manner. Based on these results, the tryptophan in the Box 1 region of GMRβc is thought to prevent phosphorylation of JAK2 and possibly JAK1 as well as inhibit downstream signaling (Figure 3.3).

Figure 3.3. Key residues and motifs in GMRβc for JAK-binding, JAK-mediated phosphorylation, and receptor ubiquitination.
Chapter 4. Concluding remarks

4.1. Summary of results

The overall goal of this study was to understand the role of JAK2 in SOCS1-mediated turnover of the GM-CSF receptor. One of the main findings is that JAK2 is not necessary for SOCS1 to bind GMRβc, contradicting my initial hypothesis. Although tenuous degradation of GMRβc is observed in the absence of JAK2 probably due to other kinases such as JAK1, JAK2 is required for substantial GMRβc degradation. Furthermore, kinase activity as determined by K882 in JAK2 and the SOCS1-docking site Y1007, which is also a key phosphorylation site for the activation of JAK2, are both required for GMRβc degradation as initially hypothesized; this indicates that JAK2 needs to be in an active state for SOCS1 to degrade GMRβc. Mechanistically, it is thought that SOCS1 inhibits GMRβc and JAK2 signaling in a different stoichiometric ratio (2 SOCS1: 1 GMRβc: 1 JAK2) than how SOCS3 inhibits gp130 and JAK2 signaling (1 SOCS1: 1 gp130: 1 JAK2). Both receptor/JAK2 pairs can be said to be “simultaneously” inhibited, using the terminology by Babon et al., referring to the SOCS protein simultaneously inhibiting signaling emanating from the receptor as well as from the JAK protein. Lastly, it is
predicted that SOCS1 binds to pY468 in GMRβc and that GMRβc binds to Y114 in JAK2 based on sequence homology. These findings are summarized in Figure 4.1.

The impact of these findings is that the mechanism of inhibition for SOCS1 is possibly different in the context of the GM-CSF receptor and JAK2 than it is for SOCS3, gp130, and JAK2. Biochemically, this is really interesting because the KIR is found in only SOCS1 and SOCS3, suggesting that they employ a similar mechanism of inhibition. The data here also offers another explanation for the pathogenesis of JMML, which is the disease of immediate relevance to this project; the failure of SOCS1, possibly by way of hypermethylation, to timely degrade the GM-CSF receptor could explain the marked hypersensitivity to GM-CSF.
Figure 4.1. Proposed model for the interplay between GMRβc, JAK2, and SOCS1. Please refer to figures 9.2 and 9.3 for corresponding alignments from which predicted residues were determined.
4.2. Future directions

An immediate future direction is to perform densitometry analysis on two other co-transfection experiments as already done so that statistical significance can be achieved with the values generated.

One potential experiment is to perform similar immunoprecipitation and western blotting experiments upon treating the γ2A cells with Ruxolitinib (INCB018424), a small molecule ATP mimetic that inhibits JAK1 and JAK2, to eliminate JAK1 activity. This will confirm whether JAK1 kinases are playing a role in degrading GMRβc. A separate experiment that will also be interesting involves assessing the stability of SOCS1 in the presence of GMRβc and JAK2, which can be answered by treating cells with the protein synthesis inhibitor cycloheximide.

One future direction involves treating GM-CSF receptor co-transfected HEK293 cells with a lysosomal inhibitor to determine the contribution of this degradation pathway to this receptor system and how this is affected in the presence or absence of JAK2 and SOCS1. As indicated by EPOR and IL-5R signaling, lysosomes are also important for receptor turnover. Performing colocalization experiments, by way of immunohistochemistry, to determine whether JAK2 and SOCS1 are simultaneously degraded by proteasomes prior to
lyosomal degradation will be informative towards understanding the sequence of events in receptor downregulation.

One of the most interesting experiments will be to validate whether the predicted-site Y468 in GMRβc is the actual site to which SOCS1 binds. This is interesting because many groups are making an effort to identify the sites of interaction between SOCS family members and cytokine receptors to better understand the mechanism of receptor turnover. This predicted site can be validated experimentally by creating a point mutation to phenylalanine (F) through a PCR-based site-directed approach, followed by co-transfecting the Y468F GMRβc and SOCS1 plasmids into either HEK293 or γ2A/R cells, and performing immunoprecipitation/immunoblotting experiments as done in this study.

If this is the actual SOCS1-binding site, this GMRβc mutant will also be helpful in determining whether SOCS1 requires a receptor docking site to prevent JAK2-mediated phosphorylation of substrates. From a biochemical standpoint, it is interesting because although SOCS1 can be induced by EPO, it hasn’t been reported to my knowledge to bind EPOR but still downregulates its signaling in a JAK2-dependent manner; furthermore, overexpression of SOCS1 negatively regulates EPO-mediated cell proliferation and STAT5 phosphorylation (Jegalian et al., 2002). On the other hand, SOCS1 has been shown to bind to all the other receptors of cytokines that induce its expression to my knowledge. This raises the
possibility that SOCS1 employs distinct mechanisms of inhibition for different receptors if it doesn’t always bind to the receptor whose signaling it affects.

Once the SOCS1-binding site in GMRβc is determined, using this mutant in co-transfection experiments with SOCS1 and JAK2 will allow us to determine whether SOCS1 is able to inhibit JAK2 and STAT5 phosphorylation independently of binding to the receptor and just by binding to JAK2. If SOCS1 does require a GMRβc-docking site for either of those activities, possible explanations are that specificity for receptors is achieved or that it competes with a STAT5-binding site (as CIS, another SOCS family member, does) since all the tyrosines in GMRβc have been implicated to be STAT5-binding and activation sites (Itoh et al., 1998). This GMRβc mutant will also help confirm that SOCS1 doesn’t detach from the receptor upon initially binding to it (sequential model) before binding JAK2, which was an older model put forth by other groups. If SOCS1 is unable to inhibit JAK2 activation or the phosphorylation of its substrate STAT5 in the presence of the GMRβc mutant that prevents SOCS1-binding, then it can be concluded that SOCS1 is probably not coming off the receptor to later bind JAK2 during this signal termination process. Finding appropriate cell lines that are transfectable and capable of STAT5 expression are key to this line of investigation. The 2C4 cell line has been indicated to have STAT5 expression so transfections in this cell line, which we have in the lab, is a possible avenue. Alternatively, a stable transduction
will have to be made with the SOCS1-binding site on GMRβc in HEK293 cells since other groups have studied STAT5 activation in this fashion as currently, transient co-expression of plasmids encoding the GM-CSF receptor in HEK293s does not induce STAT5 expression.

Another interesting interaction to experimentally validate is the one between GMRβc and JAK2; identifying whether Y114 in JAK2 is the site of association with GMRβc through a similar methodology as mentioned above will be interesting since mutations in other JAK family members at sites of homology prevented those JAKs from interacting with their respective cytokine receptors (Figure 2.8.B).

In the study by Babon et al., mutating either the Gly or the Met within the JAK2 GQM motif (1071-1073) to the corresponding residues in JAK3 (Asp and Pro, respectively) resulted in an active JAK2 that was not responsive to inhibition by SOCS3. The crystal structure put forth by these authors additionally indicated that the GQM motif is important for the SOCS/JAK interaction. It would be interesting to experimentally confirm whether this region is also necessary for interaction between SOCS1 and JAK2 and for SOCS1’s inhibitory activity. Other related experiments will involve biochemistry to validate whether SOCS1 is preventing the transfer of a phosphate group onto substrates downstream of JAK2, as it has been proposed for SOCS3, or whether it is preventing JAK2 activation.
(i.e. preventing JAK2 from transphosphorylating another JAK2 protein).

Understanding the inhibitory mechanism SOCS1 employs will be beneficial from a therapeutic standpoint as it has the potential to help us develop better JAK inhibitors that resemble the natural inhibitory action of SOCS1. Some of the problems with current JAK inhibitors are that they can: 1) be outcompeted by high intracellular concentrations of ATP 2) be prone to off-target effects because their site of interaction is similar to the common ATP-binding pocket in other kinases 3) result in toxicity when used at high doses without reducing disease burden 4) lead to the development of inhibitor-resistance upon prolonged use of the drugs.

The associations that GMRβc makes with other receptors such as ICAM-1, CD117, FcRγ, integrin β1, EPOR, as well as VEGFR2 and the functional outcomes of these interactions is another direction of study (Reviewed in Broughton et al., 2012). Another angle for future investigations could involve exploring the regulation of other SOCS1 substrates that are listed in Tables 1.4 and 1.5. Lastly, since EPOR has three E3 ligases, it would be interesting to test whether GMRβc also has more than one E3 ligase that binds and targets it for proteasome-mediated degradation; although our lab has tested SOCS2 and SOCS3 for activity against GMRβc, we didn’t test CIS. It would be worthwhile to test CIS because GM-CSF stimulation results in its induction; moreover, CIS has been shown to bind βc, which is shared with IL-5R and the GM-CSF receptor, of IL-3 (Yoshimura et al., 1995).
Appendix

Apx.1. Materials and methods

Cells

HEK293 and TF-1 cells were obtained from the American Type Culture Collection. HEK293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Wisent, St-Bruno, QC, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent, St-Bruno, QC, Canada) at 37°C in a humidified 5% CO2 atmosphere. TF-1 cells were maintained similarly in RPMI-1640 (Wisent, St-Bruno, QC, Canada) medium supplemented with 10% FBS and 1 ng/ml GM-CSF (Invitrogen, Burlington, ON, Canada). The γ2A and γ2AR cells were kindly provided by Dr. George Stark (Cleveland Clinic) and were grown in DMEM with 10% FBS, 1% sodium pyruvate, and 1% penicillin-streptomycin; additionally, the γ2A cells were selected with 400ug/mL of G418.

Antibodies

Antibodies against GMRβc (monoclonal and polyclonal) and pERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against STAT5 was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal antibodies against SOCS-1 were purchased from Novus Biologicals (Oakville, ON, Canada). JAK2, pJAK2 and pSTAT5 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal FLAG, β-actin and total ERK antibodies were obtained from Sigma (Oakville, ON, Canada).
Plasmids

pSG5-GMRα and pSG5-GMRβc constructs were generously provided by Dr. Timothy R. Hercus. The plasmid encoding Flag-SOCS1 was kindly provided by R. Rottapel. The JAK2 plasmid was provided by Dr. Dwayne Barber. The K882E and Y1007F JAK2 mutants were generated with the QuikChange site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing.

Immunoprecipitation and immunoblotting

Immunoprecipitation and Western blotting were performed as described previously [12]. In brief, cells were lysed in EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P [NP]-40) supplemented with protease and phosphatase inhibitors. Equal amount of protein was immunoprecipitated in same volume using indicated antibodies and immobilized on protein A–Sepharose beads. Immunoprecipitates were subsequently washed four times with NETN (20 mM Tris [pH 8.0], 900 mM NaCl, 1 mM EDTA, and 0.5% NP-40), boiled in SDS-containing sample buffer for 5 min, resolved on SDS-PAGE, and immunoblotted with the indicated antibodies.

SOCS1 knockdown via lentivirus-shRNA

The following pGIPZ plasmids from Thermo Scientific (Billerica, MA, USA) were used to generate lentivirus for indicated shRNA-mediated knockdown: RHS4346 non-silencing control and V2LHS_23983 human SOCS1 shRNA. TF-1-shSOCS-1 and TF-1-shScr cells were generated by infection with lentivirus and selection in puromycin. The knockdown of SOCS-1 was confirmed by Western blot analysis and real-time quantitative PCR (qPCR).
RNA purification and real-time qPCR

Total RNA was extracted using the Qiagen RNeasy extraction kit (Qiagen, Hilden, Germany) as per the provided protocol. cDNA was synthesized using random hexamer primer Thermo Scientific (Billerica, MA, USA), 5X First-Strand Buffer, 0.1 M DTT, 100 mM dNTP set, and SuperScript II Reverse Transcriptase (Invitrogen). qPCR was performed using the CFX384 Real-Time PCR Detection System from Bio-Rad, (Mississauga, Ontario, Canada) and expression levels were determined with the Bio-Rad CFX Manager 3.0 software Bio-Rad (Mississauga, Ontario, Canada). Each qPCR reaction totaled 10 uL and consisted of 1:4 diluted cDNA, 400 nM of each primer, and 2x SYBR Green Supermix Bio-Rad (Mississauga, Ontario, Canada). Amplification conditions were as follows: 95°C (3 min); 40 cycles of 95°C (10 s), 55°C (30 s); 65°C (5 s). Values were normalized to β-ActinmRNA and expressed relative to shScr samples (arbitrarily set to 1.0). The primer sets used were: β-Actin (5’-TTCTACAATGAGCTGCTGTG-3’ and 5’-GGGGTGTTGAAGGTCTCAA-3’); SOCS1 (5’-CGATTACCGCGCATCACGC-3’ and 5’-TGTCGCGCACCAGGAAGGTG-3’).

Chemicals

MG132 was obtained from Peptides International (Louisville, Kentucky, USA).

SiRNA-mediated knockdown of JAK2 in TF-1

ON-TARGETplus SMART pool siRNA targeted to JAK2 (Thermo Scientific, Billerica, MA, USA) or nontargeting scrambled siRNA (Thermo Scientific) was transfected with X-Treme Gene siRNA transfection reagent (Roche, Mississauga, ON, Canada) in TF-1 cells as per the manufacturer’s instructions.
Apx.2. References


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