The Role of the Src-like Adaptor Protein in the Regulation of GM-CSFR Signaling

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Medical Biophysics
University of Toronto

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2014

Abstract

GM-CSF is a cytokine that regulates proliferation and survival of myeloid progenitor cells and stimulates dendritic cell (DC) differentiation. The Src-like adaptor protein (SLAP) has been implicated in the negative regulation of the GM-CSF receptor and downstream signaling in DC, although the mechanism of SLAP action remains unknown. We hypothesized that SLAP regulates GM-CSFR by recruiting CBL to the receptor, where it can ubiquitylate and target it for degradation. In this investigation we found that SLAP interacts with both SHP2 and CBL in a GM-CSF dependent manner and that SLAP and CBL over-expression alters GM-CSFR trafficking. SLAP is also important in regulating GM-CSF-stimulated DC development. In this study we determined that the role of SLAP in regulation of GM-CSF signaling is specific to differentiation in DC and we identified differentially expressed genes in Slap knock-out DC that may be important for maturation.
Acknowledgements

First and foremost I would like to thank my supervisor Dr. Jane McGlade for her support and guidance in this project and in life outside of science. I would also like to thank my committee members Dr. Dwayne Barber and Dr. Mark Minden for providing me with a different perspective and helpful advice for my research.

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<td>Aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell Receptor</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding proteins</td>
</tr>
<tr>
<td>CBL</td>
<td>Casitas B-lineage Lymphoma</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>colony forming unit - granulocyte/monocyte</td>
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<tr>
<td>CIITA</td>
<td>class II, major histocompatibility complex, transactivator</td>
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<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myeloid leukemia</td>
</tr>
<tr>
<td>CMML</td>
<td>chronic myelomonocytic leukemia</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphorylated-Guanine</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>colony stimulating factor 1 receptor</td>
</tr>
<tr>
<td>CSK</td>
<td>c-Src tyrosine kinase</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>Ephrin Receptor</td>
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<td>erythropoietin</td>
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<td>EpoR</td>
<td>erythropoietin receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signaling-regulated kinase</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>friend leukemia integration-1</td>
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<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
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<td>GM-CSFR</td>
<td>granulocyte-macrophage colony stimulating factor receptor</td>
</tr>
<tr>
<td>GMP</td>
<td>granulocyte-monocyte progenitor</td>
</tr>
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<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL-</td>
<td>interleukin</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-2-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITSN1</td>
<td>Intersectin 1</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>JMML</td>
<td>juvenile myelomonocytic leukemia</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>LAT</td>
<td>linker of activated T-cells</td>
</tr>
<tr>
<td>LCK</td>
<td>lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>long-term hematopoietic stem cell</td>
</tr>
<tr>
<td>LYN</td>
<td>Lck/Yes novel tyrosine kinase</td>
</tr>
<tr>
<td>LZ</td>
<td>leucine zipper</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEP</td>
<td>megakaryocyte/erythroid progenitor</td>
</tr>
<tr>
<td>MHCII</td>
<td>major histocompatibility complex II</td>
</tr>
<tr>
<td>MiTF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>Mtss1</td>
<td>Metastasis suppressor protein 1</td>
</tr>
<tr>
<td>NF-1</td>
<td>Neurofibrin-1</td>
</tr>
<tr>
<td>NF-Y</td>
<td>Nuclear Transcription Factor Y</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol-4,5-bisphosphate 3 kinase</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphoinositol phosphate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat Sarcoma</td>
</tr>
<tr>
<td>RFX</td>
<td>Regulatory Factor X</td>
</tr>
<tr>
<td>RING</td>
<td>really interesting new gene</td>
</tr>
<tr>
<td>RMA</td>
<td>Robust Multi-array</td>
</tr>
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<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcription</td>
</tr>
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<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylimide gel electrophoresis</td>
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<tr>
<td>SH2</td>
<td>src homology domain 2</td>
</tr>
<tr>
<td>SH3</td>
<td>src homology domain 3</td>
</tr>
<tr>
<td>SHC</td>
<td>src homology 2 domain-containing</td>
</tr>
<tr>
<td>SHP2</td>
<td>src homology 2-containing tyrosine phosphatase 2</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>SILAC</td>
<td>stable isotope labeling by/with amino acids in cell culture</td>
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<tr>
<td>SLP-76</td>
<td>src homology 2 domain-containing leukocyte protein 76</td>
</tr>
<tr>
<td>SOCS</td>
<td>supressor of cytokine signaling</td>
</tr>
<tr>
<td>SPRY2</td>
<td>Sprouty 2</td>
</tr>
<tr>
<td>SRC</td>
<td>Sarcoma (protein)</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>short term hematopoietic stem cell</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen Tyrosine Kinase</td>
</tr>
<tr>
<td>TBST</td>
<td>tris buffered saline plus tween</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TKB</td>
<td>tyrosine kinase binding</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>UBA</td>
<td>ubiquitin association</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>zeta-associated protein of 70kDa</td>
</tr>
<tr>
<td>Zhx2</td>
<td>Zinc finger and homeoboxes 2</td>
</tr>
</tbody>
</table>
Attributions

The TF-1 cell line over-expressing HA-CBL was generated by Dr. Mojib Javadi Javed in the lab of Dr. Dwayne Barber (Javadi et al., 2013). This cell line was used in both Chapter 2 and 3.

WT and SLAP KO BM-DC used to precipitate RNA for the microarray analysis was generated by Dr. Larissa Liontos.

Validation of differentially expressed genes in SLAP KO BM-DC by qPCR (Table 3-1) was performed by Dr. Donna Berry, including primer design. Analysis of ZHX2 expression by western blot was also performed by Dr. Donna Berry (Fig. 3-5).
Chapter 1 - Introduction

1.1 Hematopoiesis

Hematopoiesis is the process by which all types of blood forming cells are produced. In adult humans, this process occurs in the bone marrow and begins with the hematopoietic stem cell (HSC). HSCs are unique cells with self-renewing capacity and the potential to divide and produce progenitor cells of specific lineages. Upon receiving cues from the cellular environment, HSCs will divide and differentiate into more mature progenitor cells that decrease in self-renewal ability and increase in commitment to a specific lineage. Figure 1-1 depicts the process by which an HSC can divide and differentiate into any hematopoietic cell type. First, Long-term HSCs (LT-HSC) will divide into Short-term HSCs (ST-HSC) that have limited self-renewing capability but retain the ability to differentiate into multiple lineages (Morrison et al., 1997). The first phase of lineage commitment is the division of the ST-HSC to multipotent progenitors which include the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) (Akashi et al., 2000; Kondo et al., 1997; Metcalf, 1991). CMP cells can further differentiate into the Megakaryocyte-Erythroid progenitor (MEP), which can produce erythrocytes and megakaryocytes, and the Granulocyte-Macrophage progenitor (GMP), which will produce eosinophils, basophils, neutrophils, and macrophages (Akashi et al., 2000). CLP cells can differentiate into T-cells, B-cells, and Natural Killer (NK) cells which function in adaptive immunity (Kondo et al., 1997). Dendritic cells, which are antigen presenting cells that act as a link between innate and adaptive immunity, can be formed from both the myeloid and lymphoid lineages (Liu, 2001).
Figure 1-1 Hematopoiesis
Hematopoiesis begins with the hematopoietic stem cell, which undergoes multiple cell differentiation stages to become a mature cell from any lineage. LT-HSC (Long-Term Hematopoietic Stem Cell), ST-HSC (Short-Term Hematopoietic Stem Cell), CMP (Common Myeloid Progenitor), CLP (Common Lymphoid Progenitor), MEP Megakaryocyte/Erythroid Progenitor, GMP (Granulocyte/Monocyte Progenitor), CDP (Common Dendritic Cell Progenitor)
1.2 Hematopoietic Growth Factor and Cytokine Receptors

In order to orchestrate hematopoiesis, HSCs and immature progenitor cells are influenced by the combination of growth factors in their environment. These hematopoietic growth factors and cytokines play many roles in hematopoiesis including regulating survival and proliferation of early progenitors, promoting cell differentiation, and functional activation of mature blood cells (Smithgall, 1998). They function by binding to their specific membrane receptors, leading to activation of downstream molecules which turn on expression of genes that dictate cell fate. These receptors can be divided into two broad categories based on the mechanism of signaling activation: Receptor tyrosine kinases (RTK) and Type I cytokine receptors (Figure 2). RTKs have intrinsic tyrosine kinase activity, thus, upon growth factor binding, the receptor will dimerize, bringing their catalytic regions in close proximity. This allows for the trans-phosphorylation of the receptor itself, which results in a fully active kinase with the ability to phosphorylate downstream substrates (Fantl et al., 1993). The type I cytokine receptors do not possess intrinsic tyrosine kinase activity, but instead interact with and activate non-receptor tyrosine kinases of the JAK family in order to initiate activation of downstream signaling pathways (Ihle et al., 1995). These cytokine receptors can be further subdivided based on different types of receptor dimerization, examples of which can be seen in Figure 1-2. Future discussion of the details of cytokine signaling activation will be described in the context of Granulocyte-Macrophage Colony Stimulating Factor receptor (GM-CSFR) signaling.
**Figure 1-2 Hematopoietic Growth Factors and Cytokine Receptors**

This schematic shows representatives of the 5 major types of hematopoietic growth factors and cytokines.

1. **Type III Receptor Tyrosine Kinases (RTK)** — These receptors have intrinsic kinase domains where upon ligand binding, receptors homodimerize and trans-phosphorylate tyrosine residues of opposite receptor subunits. This includes CSF-1R, Kit, Flt3, and PDGFR

Type I Cytokine Receptors — These receptors do not contain intrinsic kinase activity and instead require JAK family tyrosine kinases to phosphorylate the receptor subunits and downstream molecules

2. **Single Chain Type** — A single polypeptide chain homodimerizes upon ligand binding. This includes EpoR, GHR, PrlR, and G-CSF

3. **βc linked** — The common β subunit is shared between the α subunits of GM-CSFR, IL-3R, and IL-5R to form a heterodimer. JAK phosphorylation occurs on the βc subunit

4. **Yc linked** — The common Y subunit is shared between the α and β subunits of IL2R, IL4R, IL7R, IL9R, IL15R, and IL21R to form heterodimers (α/γ) or heterotrimers (α/β/γ). JAK phosphorylation occurs on the Y subunit

5. **gp130 linked** — The common gp130 subunit is shared between α subunits of IL6R, IL11R, IL27R, LIF, and OSM to form heterodimers. JAK phosphorylation occurs on the gp130 subunit
1.3 Granulocyte-Macrophage Colony Stimulating Factor Receptor (GM-CSFR) Signaling

1.3.1 Physiological Role of GM-CSF

GM-CSF is a cytokine that is secreted by T-cells, B-cells, mast cells, macrophages, and endothelial cells (Baldwin, 1992). It plays a role in regulating proliferation and survival of myeloid progenitor cells and the production and activation of mature granulocytes and macrophages. For this reason, recombinant GM-CSF is approved for use in the clinic to help myeloid cell recovery after bone marrow transplantation and chemotherapy treatment (Armitage, 1998). In spite of its role in the production of myeloid cells, GM-CSF is not essential for steady-state hematopoiesis, since knock-out (KO) of either GM-CSF or GM-CSFR in mouse models does not result in altered number of myeloid cells (Stanley et al., 1994). More recent studies suggest that GM-CSF may function in signaling during an emergency state, such as infection, to regulate dendritic cell and macrophage survival and activation at sites of insult (Hirata et al., 2010; Levine et al., 1999; Paine et al., 2000).

1.3.2 GM-CSF in Dendritic Cell Development

Dendritic cells (DCs), along with macrophages and some B-cells, are professional antigen presenting cells of the innate immune system which process and present antigen to T-cells to activate an adaptive immune response. There are 3 major subsets of DCs recognized by their unique combination of cell surface markers, localization, and function. These are resident DCs which are located in the spleen, migratory DCs which are located in tissues that are exposed to pathogens such as lung, skin, and intestine, and monocyte-derived DCs which are found circulating in the blood (Zhan et al., 2012). At steady state, all types of dendritic cells are
in an immature form, where they sample their environment to sense pathogen. Stimulation of DC maturation occurs upon pathogen uptake. This results in the up-regulation of MHCII molecules, digestion of the internalized antigen in the lysosome and presentation of an antigen peptide at the cell surface by MHCII (Thomas and Lipsky, 1996). Peptide bound MHCII interacts with the T-cell receptor (TCR) to initiate CD4+ T-cell activation. Co-stimulatory molecules CD80 and CD86 are also up-regulated and promote T-cell survival and activation through binding to CTLA-4 and CD28 (Krummel and Allison, 1995). Mature DCs also produce the inflammatory cytokine IL-12, which stimulates the production of interferon-gamma in T-cells to help fight viral and bacterial infections (Trinchieri, 1994). In vitro, DC maturation can be stimulated by several factors including CpG and lipopolysaccharide (LPS), unmethylated DNA sequences, and TLR agonists, all which mimic pathogens (Mellman et al., 2001).

Both GM-CSF and the FLT3 ligand FL play a role in DC development in vivo and in vitro. FL is a regulator of resident DCs in vivo, as KO of Flt3 in mice leads to reduced numbers of DC in the spleen (Kingston et al., 2009). It can also be used to generate resident DCs in vitro from mouse bone marrow cells (Naik et al., 2007). GM-CSF has been shown to promote development of monocyte DCs in vivo, and loss of gm-csf or gm-csf receptor in mice results in significant reduction of monocyte DCs in the skin and gut (Bogunovic et al., 2009; King et al., 2010; Zhan et al., 2012). GM-CSF is very efficient at generating DCs in vitro from bone marrow and monocyte cells that functionally and phenotypically resemble monocyte DC (Zhan et al., 2012). These DCs are more immunogenic than those generated by FL, making them better candidates for vaccine therapy (Van de Laar et al., 2012). GM-CSF stimulates DC development through activation and tight control of JAK/STAT, PI3-K/AKT and RAS/MAPK pathways that regulate the transcription factors IRF8, IRF4, PU.1, RelB, C/EBPα, and CIITA.
(Van de Laar et al., 2012). How GM-CSF activates GM-CSFR and down-stream signaling is discussed in more detail below.

1.3.3 GM-CSF Signal Activation

GM-CSF transmits signals for multiple biological activities through interaction with its corresponding receptor, GM-CSFR. GM-CSFR is part of the type I cytokine receptor family, and is composed of an alpha subunit (GMRα) specific to GM-CSF and a beta subunit (β common, βc) that is shared with the IL-3 and IL-5 receptors. GMRα forms a low affinity interaction with GM-CSF, leading to the formation of a high affinity GM-CSF/GMRα/βc heterotrimer. This trimer can assemble into a hexamer that fully matures into a unique dodecamer complex (Hercus et al., 2009). This unique structure allows signal activation by localizing JAK2, a non-receptor tyrosine kinase which is pre-associated with βc, in close proximity to another JAK2 molecule, thus allowing the trans-phosphorylation of JAK2 (Hercus et al., 2009). Phosphorylated JAK2 can activate signaling in a number of ways, one of which is through tyrosine phosphorylation of the βc cytoplasmic tail. There are 8 tyrosine residues within the tail, Tyr577 being important for SHC binding, phosphorylation, and subsequent activation of the ERK signaling pathway (Okuda et al., 1997). JAK2 is also responsible for the phosphorylation of STAT5, which then dimerizes and translocates to the nucleus to turn on gene expression (Van Dijk et al., 1997; Feldman et al., 1997). GM-CSFR activation can also promote cell survival through phosphorylation of serine 585 on βc. This leads to recruitment of 14-3-3 and PI3-K to the receptor and activation of the AKT pathway (Guthridge et al., 2004). Together these pathways can promote cell proliferation by initiating transcription of genes such as c-FOS, c-MYC, and c-JUN (Sato et al., 1993)(Fig. 1-3). SRC is another non-receptor tyrosine kinase that is activated by GM-CSF stimulation and has been shown to interact with GMRα,
phosphorylate βc, and may be involved in promoting GM-CSF induced cell proliferation in disease states (Bunda et al., 2013a; Perugini et al., 2010).

1.3.4 Negative Regulation of GM-CSF Signaling

In order to maintain tight control of GM-CSF signal activation, several proteins are responsible for inactivation of the molecules driving this signal. The SH2-containing inositol 5’-phosphatase, SHIP, dephosphorylates the phospholipid PIP₃ at the 5’ position, producing PI(3,4)P₂ (Liu et al., 1997). Removal of this lipid phosphorylation leads to termination of PI3-K/AKT signaling, although the mechanism of how this occurs is not well understood.

The JAK/STAT pathway works in a negative feedback loop in which GM-CSF stimulation initiates expression of SOCS family proteins which function to terminate JAK2/STAT signaling in several ways (Kubo et al., 2003). SOCS1 can act as a pseudosubstrate by associating with the JAK2 activation loop via its SH2 domain, preventing substrates from entering the JAK2 catalytic pocket (Yasukawa et al., 1999). It can also regulate JAK2 degradation by recruiting the ubiquitin system components through its SOCS box (Kamizono et al., 2001). A recent study provided evidence that SOCS1 can regulate ubiquitylation and degradation of βc, although the role of JAK2 in this process has not been defined (Bunda et al., 2013b).

Another mechanism many RTK and cytokine receptors use to control length of signaling is by degradation of the activated receptor. Following GM-CSF stimulation, part of the cytoplasmic tail of βc is degraded by the proteasome (Martinez-moczygemba and Huston, 2001). In the
Figure 1-3 Signaling Downstream of GM-CSFR
Upon binding of GM-CSF to GMRA, GM-CSF, GMRA, and βc form a dodecamer complex (not shown). This brings JAK2 kinases in close proximity to allow for trans-phosphorylation and activation of the kinases followed by phosphorylation of tyrosine residues on βc. Phosphorylation of Y577 recruits the SHC/GRB2/SOS complex, allowing it to activate RAS. This leads to ERK1/2 phosphorylation which can activate transcription factors to alter gene expression. RAS can also activate the PI3-K and AKT pathway. Phosphorylation of S585, which occurs when low levels of GM-CSF are present, allows for binding of 14-3-3, also leading to activation of the PI3-K/AKT pathway. Activated JAK2 also directly phosphorylates STAT5 leading to translocation of STAT5 to the nucleus where it takes part in gene transcription. SRC kinase can also promote RAS signal activation although the mechanism of how this occurs is poorly understood. Negative signaling molecules SHIP and SOCS1 (shown in red) can down-regulate signaling by dephosphorylating PI3-K and inhibiting JAK2 activity respectively.
context of IL-5 signaling, ubiquitylation of βc at lysine 457, 461, and 467 was shown to be important for receptor endocytosis and degradation, although the proteins involved in these processes are yet to be revealed (Lei et al., 2011).

1.3.5 Deregulation of GM-CSF Signaling in Leukemia

Although no mutations in the GM-CSFR gene have been identified, deregulation of the GM-CSF pathway has been implicated in leukemia. Particularly, deregulation of GM-CSF survival signals through constitutive phosphorylation of serine 585 on the βc cytoplasmic tail is observed in some AML, CML, and CMML patients (Guthridge et al., 2006). Additionally, in CML and other myeloproliferative diseases, presence of GM-CSFR is important in promoting survival and proliferative signals through abnormal expression of downstream proteins such as JAK2 (Coue et al. 2005). The most frequent occurrence of GM-CSF signal deregulation is seen in Juvenile Myelomonocytic Leukemia (JMML), where bone marrow and peripheral blood mononuclear cell samples from patients show a significant hypersensitivity to GM-CSF in CFU-GM colony forming assays (Emanuel et al., 1991). JMML is characterized by a BCR-ABL negative genetic background and overproduction of monocytic cells which infiltrate into organs (De Vries et al., 2010). The most frequent mutations seen in JMML patients are in regulators of the RAS signaling pathway which include activating mutations in RAS (25 %), and SHP2 (35 %), and inactivating mutations in CBL (17 %), and the RAS GTPase activating protein, NF-1 (10-25%) (De Vries et al., 2010).

1.4 SHP2

SHP2, the most commonly mutated protein in JMML, is a non-receptor protein tyrosine phosphatase that is a positive regulator of RAS signaling downstream of GM-CSFR. It contains two N-terminal SH2 domains (N-SH2, C-SH2), a phosphatase domain, and a C-terminal tail
with two tyrosine phosphorylation sites (Y542, Y580) surrounding a proline rich region (PRR) (Fig. 1-4A). In an unstimulated state, SHP2 remains in a catalytically inactive conformation due to interactions between the N-SH2 and phosphatase domain (Hof et al., 1998). Tyrosine phosphorylation of βc and downstream proteins creates docking sites for the N-SH2 domains of SHP2, releasing the phosphatase domain from the inactive conformation (Cunnick et al., 2001; Neel et al., 2003). In JMML, mutations in SHP2 are found in the N-SH2 and phosphatase domains, which disrupt the inhibitory conformation of SHP2 thus producing a constitutively active phosphatase (Neel et al., 2003). Many different roles of SHP2 in promoting RAS/ERK signaling have been studied, some of which include: dephosphorylation of the CSK regulators Pag/Cbp and Paxillin which prevents CSK from phosphorylating SRC at its inhibitory site (Zhang et al., 2004), activation of SRC kinases through direct dephosphorylation of the SRC inhibitory tyrosine (Peng and Cartwright, 1995), dephosphorylation of the RAS inhibiting Sprouty proteins (Hanafusa et al., 2002), and dephosphorylation of RAS GAP protein binding sites on tyrosine phosphorylated receptors (Cleghon et al., 1998) (Fig. 1-4B/C).

1.5 CBL

Another mutation found in JMML patients is in the gene coding for the protein c-CBL (subsequently referred to as CBL). CBL is a RING type E3 ubiquitin ligase, which works together with E1 ubiquitin activating and E2 ubiquitin conjugating enzymes to covalently link ubiquitin molecules to lysine residues on substrate proteins (Schmidt and Dikic, 2005). Ubiquitin, a 76 amino acid protein, can link to target proteins by attachment of a single ubiquitin molecule (monoubiquitination), attachment of multiple individual ubiquitin molecules
Figure 1-4 Function of SHP2 in RAS Signaling

A. Schematic of SHP2 protein containing 2 N-terminal SH2 domains, a phosphatase domain, and 2 phosphorylated residues, Y542/Y580, surrounding a proline rich region (PRR). B. SHP2 can promote RAS signaling by dephosphorylating the GRB2 inhibitor SPRY so that it can no longer bind to GRB2. This allows GRB2 to be recruited to tyrosine phosphorylated residues on activated receptors, where it can bind SOS and activate RAS (left). SHP2 can also dephosphorylate the tyrosine residue on activated receptors that is responsible for recruiting RAS GTPase activating proteins (GAP). This prevents RAS GAPs from hydrolyzing GTP in the activated form of RAS, thus promoting RAS activation (right) C. SHP2 can also regulate RAS signaling through regulating the activation of SRC. SHP2 can dephosphorylate the tyrosine residue on cbp/PAG or Paxillin which is essential for recruitment of the protein tyrosine kinase CSK. This prevents CSK from phosphorylating SRC at the inhibitory tyrosine 527 residue, thus allowing SRC to become active (left). SHP2 is also able to directly dephosphorylate tyrosine 527 on SRC to relieve it from its inhibitory state (right).
(multimonoubiquitylation), or attachment of a chain of ubiquitin molecules (polyubiquitylation) (Hicke, 2001; Li et al., 2003). Different types of polyubiquitylation are possible based on which of the 7 lysine residues of ubiquitin forms these linkages. All of these forms of ubiquitylation can impart different fates to the target protein including changes in localization, activity, and protein-protein interactions, as well as endocytosis, and degradation via the proteosomal or lysosomal pathways (Komander and Rape, 2012).

CBL has multiple domains and has many tyrosine and serine residues which are typical of adaptor proteins (Fig. 1-5). CBL consists of an N-terminal tyrosine-kinase binding (TKB) domain which interacts with phosphorylated tyrosine residues on substrate proteins, followed by a small linker and RING domain which are important for activating E3 ligase function and binding E2 conjugating enzymes respectively. CBL also contains a proline-rich region that is important for interactions with the SH3 domains of GRB2, SRC and CIN85 (Dombrosky-ferlan and Corey, 1997; Odai et al., 1995; Take et al., 2000), followed by a region rich in serine residues which contain the motif for binding to 14-3-3 (Liu et al., 1996) and tyrosine residues which interact with the SH2 domains of VAV, CRKL and PI3-K (Beckwith and Jorgensen, 1996; Marengere et al., 1997; Uemura and Griffin, 1999). Finally, CBL contains a carboxy-terminal Ubiquitin Associated (UBA) domain overlapping with a leucine zipper (LZ) motif, although the function of this domain is not well understood. CBL is part of a family of proteins with 2 other homologues, CBL-b and CBL-c, which all contain the TKB, linker, and RING domains but differ in length of the C-terminus allowing specificity in adaptor function for each protein (Schmidt and Dikic, 2005).
Figure 1-5 CBL Family Proteins
Schematic of c-CBL, CBL-b, and CBL-c domain structure. All 3 proteins contain a conserved N-terminal tyrosine kinase binding domain (TKB), linker region RING domain, and proline rich region. The linker regions contain tyrosine residues which are important for activating E3 ligase function upon phosphorylation. CBL-c has a shortened proline rich region and truncated C-terminus. c-CBL and CBL-b both have regions rich in serine and tyrosine residues that become phosphorylated and act as docking sites for other proteins with SH2 domains. Both c-CBL and CBL-b also contain a Ubiquitin association (UBA) domain overlapping with a Leucine Zipper (LZ) motif.
The function of CBL is best characterized in the down-regulation of activated RTKs. The epidermal growth factor receptor (EGFR) is one example of an RTK that is regulated by CBL. Upon EGFR activation and trans-phosphorylation, CBL is recruited to EGFR via interactions with the SH3 domain of GRB2 (Jiang et al., 2003). At this time, CBL can act as an adaptor protein to recruit several proteins involved in receptor endocytosis such as CIN85 to EGFR (Soubeyran et al., 2002). CBL also interacts with EGFR through its TKB domain allowing for ubiquitylation of the receptor, which is necessary to target it to the lysosome for degradation (Grøvdal et al., 2004). In this way CBL promotes clathrin-mediated endocytosis and lysosomal degradation of EGFR to attenuate signaling. CBL also functions in the regulation of other activated receptors such as the T-cell receptor (TCR). CBL is important for ubiquitylation and degradation of TCRζ in activated TCR-CD3 complexes, which is mediated by both Src-like Adaptor protein (SLAP) and ZAP-70 (Myers et al., 2005; Wang et al., 2001). In agreement, Cbl KO mice have increased TCR surface levels at the double positive (DP) stage of T-cell development (Naramura et al., 1998). In JMML, the majority of mutations in CBL reside in the linker region which disrupts its E3 ligase function (Niemeyer et al., 2010), however, it is not clear whether CBL acts to down-regulate GM-CSFR or down-stream signaling molecules.

1.6 SLAP

1.6.1 Domain Structure and Interacting Proteins

SLAP was cloned in yeast 2 hybrid screens by two independent groups using the cytoplasmic domain of the EphA family receptor Eck and the N-terminus of Cbl (Pandey et al., 1995; Tang et al., 1999). A related family member, SLAP2, was identified using bioinformatics and functional screening approaches (Holland et al., 2001; Loreto and McGlade, 2003; Pandey
et al., 2002). SLAP2 has 2 additional isoforms (Fig. 1-6), which are generated from an alternative translation start site at Methionine 27 and by alternative splicing between exon 5 and 6 which leads to a frameshift that results in a truncated C-tail (Loreto and McGlade, 2003; Loreto et al., 2002).

Both SLAP and SLAP2 contain an N-terminal myristolation motif, SH3 and SH2 domains that share high sequence identity with those of the Src family kinases, and a unique C-terminal tail (Fig. 1-6). The Src family kinase LCK shares the highest sequence identity to SLAP SH3 and SH2 domains with 55 % and 50 % identity respectively (Sosinowski et al., 2000). SLAP SH3 and SH2 domains are unique as the connector sequence between the SH3 and SH2 domains is shortened compared to Src family kinases (Wybenga-Groot and McGlade, 2013). Additionally, the structure of the SH3 and SH2 domain module of SLAP2 reveals continuous beta sheet formation between the SH3 and SH2 domains which is important for domain stability, thus explaining the inability of these domains to be purified as isolated recombinant proteins (Wybenga-Groot and McGlade, 2013). SLAP is not soluble in mild detergents, which appears to be dependent on its C-tail, as removal of the carboxy terminus 94 amino acids increases solubility of murine slap (Sosinowski et al., 2000) It has also been suggested that the myristolation motif of SLAP localizes the protein to lipid rafts, also affecting it solubility (Kim et al., 2010).

SLAP contains protein interaction domains but does not have intrinsic enzymatic activity, making it a classic adaptor protein that functions to mediate protein-protein interactions. SLAP can interact with a number of signaling molecules via its modular domains (Fig. 1-6). Specifically, the SH2 domain of SLAP is important for interaction with phosphorylated ITAMs in TCRζ as well as proximal TCR signaling components including Syk,
**Figure 1-6 SLAP Family Proteins**

Schematic of human SLAP, SLAP2, and SLAP2-v. All 3 proteins have myristolation motifs at the N-terminus, followed by SH3 and SH2 domains that share high sequence identity (36 %, 59 %), as well as a unique C-tail of varying lengths. M27 indicates the alternate transcription initiation site at Methionine 27 in SLAP2. Arrows extending from the SH2 and C-tail of SLAP indicate proteins which interact with SLAP through these regions. The amino acid length of each SLAP family member is indicated to the right of each protein schematic.
18

ZAP-70, LAT, Vav, SLP-76 (Sosinowski et al., 2000; Tang et al., 1999). The SH2 domain of SLAP can also interact with other membrane receptors including the B-cell receptor (BCR), CSF-1R, Flt3, and Kit (Dragone et al., 2006a; Kazi and Rönnstrand, 2012; Kazi et al., 2014; Pakuts et al., 2007). The unique C-tail of SLAP also plays a role in protein interactions as it is important for binding to the N-terminus of CBL (Loreto et al., 2002; Swaminathan et al., 2007; Tang et al., 1999).

1.6.2 SLAP Expression in Hematopoietic Cells

SLAP is expressed primarily in hematopoietic cells, but expression is also seen in tissues including the brain, lung, liver, skeletal muscle, and kidney (Pandey et al., 1995). Of the hematopoietic cells, SLAP is expressed mainly in lymphoid cells and is differentially expressed at particular stages of T and B cell development (Dragone et al., 2006b; Sosinowski et al., 2001). During T and B cell development, SLAP expression is highest in the DP thymocyte stage and in B-cell subsets that express BCR respectively (Dragone et al., 2006b; Sosinowski et al., 2001). SLAP plays a role in developing T-cells, as Slap KO mice have increased TCR expression on DP thymocytes and enhanced positive T-cell selection (Sosinowski et al., 2001). In B-cell development, Slap KO mice have increased splenic B-cell numbers which are hyporesponsive to BCR stimulation (Dragone et al., 2006b). Other than effects on lymphocyte development, SLAP KO mice are born at Mendelian frequencies, are fertile, and show no obvious physical abnormalities (Sosinowski et al., 2001).

Expression of SLAP can be induced by a number of factors in different cell types. For example, phorbol myristate acetate, ionomycin, and CD3 stimulation can all induce expression of Slap in single positive thymocytes (Sosinowski et al., 2001). SLAP is also up-regulated by retinoic acid treatment of HL60 and U937 myeloid cell lines, as well as by dexamethasone and antigen stimulation in RBL-2H3 mast cells (Hiragun et al., 2006; Ohtsuki et al., 1997; Park et
al., 2009). Additionally, in FLI-1 transformed erythroblasts, SLAP is up-regulated after treatment with EPO (Lebigot et al., 2003).

1.6.3 Role of SLAP in Regulation of Hematopoietic Receptors

The function of SLAP is best characterized in the regulation of the TCR. As mentioned above, loss of Slap expression in mice leads to an increase in mature TCR-CD3 complexes at the cell surface of DP thymocytes (Sosinowski et al., 2001). Further study of these Slap deficient thymocytes revealed that loss of Slap expression results in an increase in total TCRζ levels due to a decrease in receptor degradation (Myers et al., 2005). These results suggested that Slap plays a role in regulating TCR levels through degradation of TCRζ.

The Weiss group was the first to study the mechanism of TCR regulation by SLAP (Myers et al., 2006). As SLAP interacts with CBL and CBL functions to ubiquitylate and target activated receptors for lysosomal degradation, it was a good candidate for regulating TCR degradation. Additionally, Cbl KO mice also have increased TCR surface expression in DP thymocytes (Naramura et al., 1998). Slap and Cbl doubly deficient mice showed no additive or synergistic effects in regulation of TCR levels, which supported the idea that Cbl and Slap work together in the same pathway (Myers et al., 2006). In the same study, over-expression of SLAP and CBL together, but not individually, led to a decrease in CD3ε at the cell surface as well as an increase in TCRζ ubiquitylation and degradation in Jurkat cells. Altered TCR trafficking depended on the myristolayion motif, SH2 domain, and C-tail of SLAP as well as the RING domain of CBL (Myers et al., 2006). Since the SH2 domain is needed to interact with TCRζ, and the C-tail of SLAP interacts with CBL, it was proposed that SLAP could recruit CBL to the receptor thus allowing it to ubiquitylate TCRζ and target it for degradation.
The model that SLAP functions to couple CBL to activated receptors was further supported by studies in Bal-17 mature B-cells, where overexpression of SLAP and CBL together but not separately resulted in a decrease in surface and total BCR levels as well as altered BCR recycling (Dragone et al., 2006a). Similarly, the SH2 domain and C-tail of SLAP were required for this function. Additionally, in the regulation of CSF-1R, a point mutation in the SLAP2 SH2 domain which disrupts its interaction with CSF-1R resulted in a failure of Cbl to be recruited to the receptor as well as a decrease in ubiquitylation and internalization of CSF-1R (Pakuts et al., 2007). SLAP also regulates the stability of other membrane receptors including FLT3 and c-KIT, although whether this involves recruitment of CBL has not been elucidated (Kazi and Rönnstrand, 2012; Kazi et al., 2014). It is possible that SLAP may also function to recruit other E3 ligases to their substrates as SLAP can also interact with the E3 ligases RNF126 and RABRing7 (unpublished data). Additionally, SLAP has been reported to regulate the RTK EPHA2 in a CBL independent manner, but is dependent on interactions with the ubiquitin factor UBE4a, suggesting SLAP may function to recruit other E3 ligases to RTKs (Naudin et al., 2014).

1.6.4 SLAP in GM-CSF Signaling

Recently our lab identified GMRα as a Slap interacting protein in bone marrow derived dendritic cells (BM-DC) generated in vitro with GM-CSF (Liontos et al., 2011). The role of SLAP in regulation of GM-CSFR was studied using BM-DC from Slap/Slap2 KO mice. When Slap KO BM-DC were stimulated with GM-CSF, we showed a failure to down-regulate βc of GM-CSFR compared to WT DC, suggesting that Slap plays a role in regulating GM-CSFR levels (Fig. 1-7). Interestingly, an increase in levels of Cbl in Slap KO BM-DC was also
Figure 1-7 Model of SLAP Function in GM-CSF Generated BM-DC

In SLAP KO BM-DC βc levels fail to be down-regulated after stimulation with GM-CSF. This leads to increased and prolonged activation of ERK1/2 and AKT downstream of RAS and PI3-K. SLAP KO BM-DC also had a decrease in MHCII surface levels as well as other maturation markers, indicating these DC have impaired maturation.
observed. Since the coupling of CBL to membrane receptors leads to self-ubiquitylation and targeting of CBL for degradation, this suggested that increased Cbl levels in Slap KO BM-DC could be a consequence of failure to be coupled to the activated receptor. Downstream of GM-CSFR there was enhanced and prolonged activation of Akt and Erk1/2 in Slap KO BM-DC compared to WT, indicating that failure to down-regulate GM-CSFR results in deregulation of downstream signaling pathways. Slap KO BM-DC also had impaired DC development as expression of the maturation markers MHCII, CD80, and CD86, as well as production of IL-12 was decreased compared to WT BM-DC. These SLAP-deficient DC failed to stimulate T-cells in mixed leukocyte reactions and had decreased ability to induce Interferon-gamma secretion in DO11.10 cells. Taken together, these experiments suggest that loss of Slap expression leads to impaired BM-DC maturation and function. Whether this phenotype is linked to impairment of Cbl-dependent regulation of GM-CSFR remains to be tested.

1.7 Rationale and Thesis Objectives

As SLAP is involved in the down-regulation of several hematopoietic receptors in a CBL-dependent manner, we postulated that SLAP may be regulating GM-CSFR in a similar fashion. The fact that CBL is also deregulated in SLAP KO DC and mutations impairing the E3 ligase function of CBL are found in JMML patients with deregulated GM-CSF signaling, further supports this idea. Therefore, we hypothesized that SLAP down-regulates GM-CSFR in a CBL dependent manner which is important for regulating cellular processes such as DC maturation. The objectives of this study are:

1. To investigate the mechanism of SLAP regulation of GM-CSFR

2. To investigate the effects of SLAP on cellular processes downstream of GM-CSF, such as proliferation and differentiation
Chapter 2
Investigation of SLAP Protein Interactions in GM-CSF Signaling Pathway and Regulation of GM-CSFR

2.1 Introduction

GM-CSF is a cytokine that plays a role in the proliferation and survival of myeloid progenitor cells and the production and activation of mature granulocytes and macrophages. Regulation of GM-CSF signaling is vital for normal cell function, as overactive signaling can lead to myeloproliferative disease and leukemia. SLAP is an adapter protein that has been implicated in the negative regulation of GM-CSFR and downstream signaling in dendritic cells, although the mechanism of SLAP action remains unknown. SLAP has also been shown to regulate other hematopoietic receptors by promoting their lysosomal degradation through a CBL dependent mechanism. Therefore, we hypothesized that SLAP regulates GM-CSFR by recruiting CBL to the receptor, where it can ubiquitylate and target it for degradation. In this investigation, we examined GM-CSF dependent SLAP interacting proteins and GM-CSFR trafficking in cells over-expressing SLAP and CBL. We found that SLAP interacts with both SHP2 and CBL in a GM-CSF dependent manner and that SLAP and CBL over-expression together, but not separately, alters GM-CSFR trafficking. These data support the hypothesis that SLAP regulates GM-CSFR in a CBL dependent manner and reveal a novel interaction of SLAP with SHP2, which may provide further insight into the mechanism by which SLAP regulates GM-CSFR and downstream signaling.
2.2 Materials and Methods

2.2.1 Cloning and Plasmids
To clone SLAP into pEF4 myc/his C, SLAP was PCR amplified from the human SLAP sequence in the pENTR233.1 vector (human ORFeome library) with the primers listed below.

For pFLAG-CMV SHP2 and tyrosine mutants (pFLAG-CMV SHP2 Y542F, pFLAG-CMV SHP2 mutant, pFLAG-CMV SHP2 Y542/580F), human SHP2 was PCR amplified from pBABE puro SHP2 constructs given to us by Dr. Benjamin Neel. Primers with the restriction sites used are listed below. The constructs used to generate the TF-1 SLAP inducible cell line were purchased from Clontech (Lenti-X Tet-On 3G Inducible Expression System, EF1α version). SLAP was PCR amplified and ligated into the pLVX TRE3G vector using the primers below.

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<thead>
<tr>
<th>VECTOR</th>
<th>RESTRICTION SITES</th>
<th>PRIMERS</th>
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<tr>
<td>pEF4 SLAP myc/his C</td>
<td>BamHI, EcoRI</td>
<td>Fwd 5’ ccaactttgccttgcatgcggaac</td>
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<tr>
<td></td>
<td></td>
<td>Rev 5’ ccaactttgacagagatcgggctcc</td>
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<tr>
<td>pFLAG-CMV SHP2 and mutants</td>
<td>HindIII, BamHI</td>
<td>Fwd 5’ cgcaagctttctgacatgcggagatgttttc</td>
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<tr>
<td></td>
<td></td>
<td>Rev 5’ gcagggatccgagttggcaggttctctatc</td>
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<tr>
<td>pLVX TRE3G SLAP</td>
<td>BamHI, MluI</td>
<td>Fwd 5’ gctcggatccggtggcatg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev 5’ ccaacgcgttcagcgggttaaatcaatgg</td>
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2.2.2 RNA Isolation and RT-PCR
TF-1 cells were harvested and RNA was isolated using Qiagen RNeasy kit as per manufacturer’s protocol. RNA was reverse transcribed into cDNA using the Qiagen Omniscript Reverse Transcription Kit. cDNA was amplified using primers against human SLAP (Fwd
5′atgggaacagcatgaatcc, Rev 5′ ggctagtcctcaaagtaaggt) and human β actin (Fwd 5’ aagatcaagatcattgcctc, Rev 5’ ggtgttaacgcaactagtc).

2.2.3 Antibodies and Reagents
Antibodies against SLAP (49.53), βc (K-17), and SHP2 (C-18) were purchased from Santa Cruz Biotechnology. Phosphotyrosine antibody (4G10) was purchased from Millipore. Phospho-CBL (Y774) and phospho-SHP2 (Y542) antibodies were purchased from Cell Signaling Technology. c-CBL antibody was purchased from BD Sciences. FLAG, HA, and Transferrin antibodies were purchased from Sigma, Roche, and Zymed respectively.

2.2.4 Cell lines and Culture
TF-1 cells were grown in complete RPMI 1640 (Wisent) with 10% FBS (Wisent) and 2ng/mL GM-CSF (R&D Systems). TF-1 cells stably over-expressing HA-CBL were generated by Dr. Mojib Javadi Javed under the supervision of Dr. Dwayne Barber (Javadi et al., 2013). HEK293T cells were maintained in DMEM (Wisent) with 10 % FBS. SLAP inducible cell lines were generated by transfecting the pLVX EF1α Tet-On plasmid into TF-1 and TF-1 HA-CBL cells by electroporation (25 µg, 240V, 950 µF). Transfected cells were selected using 800ug/mL G418 (Wisent). These cells were then transfected with the pLVX- TRE3G SLAP Myc/His vector and selected with 2 ug/mL puromycin (Wisent). Cells were maintained in RPMI, with 10% FBS, 2ng/mL GM-CSF, 80ug/mL G418, and 1 ug/mL puromycin. SLAP expression was induced by addition of 1ug/ml doxycycline.

2.2.5 Purification of GST fusion proteins
Full-length SLAP, SLAP2, and SLAP mutants were cloned into the pGEX4T1 vector as previously described (Loreto et al., 2002). Recombinant GST-fusion proteins were expressed in BL21 bacteria after induction with IPTG. Protein was purified by cell lysis in PLC lysis buffer.
(50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA), followed by sonication, and incubation of lysate with glutathione-sepharose beads for 30 minutes at 4°C. Bound GST-fusion proteins and glutathione-sepharose beads were washed 3 times in lysis buffer and resuspended in equal volume of PLC buffer and 1 mM DTT.

2.2.6 Cell Stimulation
TF-1 cell lines were starved in complete RPMI media containing 10 % FBS but without addition of GM-CSF. After 24 hours, cells were harvested and resuspended in RPMI at a concentration of 10 x 10⁶ cells/mL. Cells were incubated at 37°C for 5 minutes, followed by stimulation with either 100 ng/mL or 10 ng/mL GM-CSF as indicated. To terminate stimulation, cells were removed at multiple time points and placed on ice, followed by an addition of equal volume of cold PBS containing 10mM sodium pyrophosphate, 100mM NaF, and 1mM Na₃VO₄. Cells were washed once more in PBS, and then lysed in PLC lysis buffer containing 10mM sodium pyrophosphate, 100mM NaF, and 1mM Na₃VO₄, and complete protease inhibitors (Roche). Lysates were cleared by centrifugation for 20 minutes at 21 000 g at 4°C.

2.2.7 GST Pull-down assays, Immunoprecipitation, and Western Blotting
For GST Pull-down assays and Immunoprecipitation, 1 mg of total cell lysate was incubated with 5 µg of GST-fusion protein bound to glutathione sepharose beads (GE Healthcare) or 2 µg of antibody bound to Protein G Sepharose beads (Sigma) and mixed at 4°C for 2 hours. Samples were washed 3 times in PLC lysis buffer and beads were resuspended in 2x SDS sample buffer (125 mM Tris-HCL pH6.8, 4 % SDS, 20 % glycerol, 0.7 M β-Mercaptothanol), and resolved by SDS-PAGE. For western blotting, resolved proteins on poly-acrylamide gels were transferred to PVDF membranes and blocked for 30 minutes in 5% skim milk powder in Tris-buffered saline with 0.05% Tween 20 (TBST) or 1% bovine serum albumin in TBST (for
phospho-tyrosine blotting). After blocking, membranes were incubated with primary antibodies for 1 hour at room temperature or 18 hours at 4°C, and then washed 3 times in TBST. This was followed by 1 hour incubation of membranes with corresponding secondary antibodies conjugated to horseradish peroxidase, and then 3 additional washes with TBST. Antibody binding was detected using enhanced chemiluminescence reagent (Perkin Elmer).

2.2.8 Cell Surface Biotinylation
Cell surface biotinylation was performed as described (Liontos et al., 2011). Briefly, SLAP expression was induced by addition of doxycycline at 1 µg/mL in TF-1 and TF-1 HA-CBL cells followed by cytokine starvation for 18 hours. TF-1 and TF-1 HA-CBL cells with or without SLAP expression were stimulated for 2, 5, and 10 minutes with 10ng/ml of GM-CSF at 37°C. Cells were placed on ice for 5 minutes, washed with cold PBS, and then surface proteins were labelled by mixing cells with 0.5mg/ml of EZ-link-sulfo-NHS-LC-Biotin (Pierce) in biotinylation buffer (10mM HEPES, 154mM NaCl, 3mM KCl, 1mM MgCl2, 0.1mM CaCl2 and 10mM glucose) for 1 hour at 4°C. Following another wash with cold PBS, biotin was quenched by incubating cells in RPMI + 100 mM Glycine for 5 minutes. Cells were washed 2 more times with cold PBS and then lysed in PLC lysis buffer. Biotinylated proteins were isolated by incubating 0.7-1mg of lysate with 30µl of streptavidin-agarose beads overnight at 4°C.

2.2.9 Transfection into HEK293T Cells
HEK293T cells were grown to 70 % confluency in 10 cm plates and then transfected with 2 µg of each plasmid by diluting 3 µl Lipofectamine 2000 (Invitrogen) per microgram of DNA in OPTI-MEM. After 6 hours, cells were washed, replaced with fresh media, and incubated at 37°C for an additional 24 hours before harvesting.

2.2.10 Indirect Intracellular Staining
TF-1 inducible SLAP expression cells were treated with 1 µg/mL doxycycline for 24 hours or left untreated. Cells were harvested, washed twice in PBS, and then resuspended in PBS containing 10 µg/mL Fc Block (eBioscience) at a concentration of 10 x 10⁶ cells/mL. Three million doxycycline treated or untreated cells were fixed in 0.5 mL cold Flow Cytometry Fixation buffer (R&D Systems) for 10 minutes at room temperature, washed in PBS, and permeabilized with 0.9 mL -20 C methanol for 20 minutes at 4⁰C. Cells were washed twice with PBS after fixation and permeabilization and then incubated in 100 µl PBS containing 0.6 µg of primary antibody (anti-SLAP 49.53, Santa Cruz Biotechnology) at room temperature for 30 minutes. Cells were washed again with PBS followed by incubation in 100 µl PBS containing 0.2 µg of secondary antibody (Alexa Fluor 488 Goat Anti-Mouse IgG, Life Technologies) for 20 minutes at room temperature in the dark. Stained cells were analyzed on a FACSCalibur Instrument followed by data analysis using FlowJo software (v 9.4.11, TreeStar).

2.3 Results

2.3.1 SLAP Binds to Tyrosine Phosphorylated Proteins after GM-CSF Stimulation

To understand how SLAP regulates GM-CSFR we wanted to identify proteins that interact with SLAP in a GM-CSF depleted and stimulated state. Since GM-CSF stimulation leads to tyrosine phosphorylation of proteins and new protein interactions, we first examined tyrosine phosphorylated proteins that interacted with SLAP in a GM-CSF dependent manner. We used the TF-1 human erythroleukemia cell line as our system of study since it endogenously expresses GM-CSFR and relies on GM-CSF for proliferation. To examine GM-CSF dependent interactions of SLAP with tyrosine phosphorylated proteins, WT and mutant GST-SLAP fusion proteins were incubated with lysates from TF-1 cells (Fig. 2-1A).
Immuno-blotting of proteins bound to GST SLAP fusions using antibodies against phosphorylated tyrosine residues revealed a number of tyrosine phosphorylated proteins that interacted with SLAP. Notably, we consistently saw proteins migrating at approximately 70 and 120 kDa (pp70, pp120) that bound to SLAP in a GM-CSF dependent manner (Fig. 2-1B). No proteins were bound with GST alone, indicating these interacting proteins are specific to SLAP. The SH3 and SH2 domains, but not the C-terminus of SLAP, were required for pp70 binding as only the ΔC mutant retained binding to pp70 (Fig. 2-1B). pp120 required the SH3 and SH2 domains as well as the C-terminus of SLAP for binding, as mutations in any of these regions resulted in loss of interaction (Fig. 2-1B).

Proteins at approximately 90 and 110 kDa (pp90, pp110) were also observed to interact with SLAP regardless of GM-CSF stimulation, although these interactions were not seen consistently (Fig. 2-1B). Tyrosine phosphorylated pp90 interacts with WT SLAP in both TF-1 starved and stimulated lysate and required the SH2 domain and C-tail of SLAP, since mutations in these regions resulted in loss of binding to pp90 (Fig. 2-1B). The SH3 domain of SLAP appears to be partially required for interaction with pp90 as SH3 mutants show partial loss of binding (Fig. 2-1B). pp110 interacted with WT, ΔC, and SH3 mutant forms of SLAP independent of GM-CSF stimulation, but did not interact with SLAP SH2 mutants (Fig. 2-1B). Taken together, these data indicate that a number of tyrosine phosphorylated proteins interact with SLAP in both a GM-CSF dependent and independent manner, and different proteins require different regions of SLAP to form an interaction.
Figure 2-1. SLAP Interacts with Tyrosine Phosphorylated Proteins after GM-CSF Stimulation.

A. Schematic of WT GST-SLAP proteins and mutants. R111K is an arginine to lysine mutation at amino acid 111 that disrupts binding of the SH2 domain to substrates. P73L is a proline to leucine mutation at amino acid 73 that disrupts binding to the SH3 domain, and ΔC is a truncation at of the C terminus between amino acids 187 and 281. B. WT GST-SLAP and SH3, SH2, and ΔC mutants were mixed with TF-1 lysate with (+) or without (-) GM-CSF stimulation (100 ng/mL). Western blot was performed with antibodies against phospho-tyrosine (pY 4G10). Fast green stain of the membrane shows even loading of GST fusion proteins.
2.3.2 The E3 ligases CBL, RNF126, and RABRING7 Interact with a Distinct Pattern of Tyrosine Phosphorylated Proteins after GM-CSF Stimulation

Since SLAP is an adaptor that can link the E3 ligase CBL to its substrates, we tested whether the SLAP-interacting E3 ligases, CBL, RNF126, and RABRING7 also interacted with similar patterns of tyrosine phosphorylated proteins after GM-CSF stimulation. The TKB domain of CBL and SLAP both interact with tyrosine phosphorylated pp70 and pp120 in GM-CSF stimulated TF-1 lysate (Fig. 2-2A). CBL also interacted with a distinct tyrosine phosphorylated protein of approximately 130 kDa, which co-migrated with phosphorylated βc of GM-CSFR (Fig. 2-2A). However, reprobing the membrane with anti- βc antibodies did not show CBL interaction with βc, despite this interaction having been previously reported (Bunda et al, 2013). This may be a result of an indirect interaction between βc and CBL that can only be detected with the very sensitive 4G10 antibody. As the anti-βc antibodies used are raised against the C-terminus of βc which becomes tyrosine phosphorylated after GM-CSF stimulation, these modified residues may also interfere with antibody binding. Alternatively, this 130kDa band could represent a different tyrosine phosphorylated protein downstream of GM-CSFR.

RNF126 and RABRING7 bound a single tyrosine phosphorylated protein of 130 kDa, distinct from tyrosine phosphorylated proteins that interact with SLAP (Fig. 2-2B). This 130 kDa protein may also represent βc, although probing the membrane with anti- βc antibodies could not confirm this interaction. Since the tyrosine phosphorylated proteins that interact with SLAP and CBL overlap, this may suggest these proteins are working together in GM-CSFR regulation, whereas RNF126 and RABRING7 interact with distinct phosphorylated proteins which may act further down the GM-CSFR regulatory pathway.
Figure 2-2. SLAP, CBL, RNF126 and RABRING7 Interact with a Distinct Pattern of Tyrosine Phosphorylated Proteins after GM-CSF Stimulation.
A. GST-SLAP and GST-CBL TKB fusion proteins were mixed with starved TF-1 lysate with (+) or without (-) 5 minutes of GM-CSF stimulation (100 ng/mL). Bound proteins were separated by SDS-PAGE alongside GM-CSFRβ immunoprecipitations (β IP) and normal rabbit IgG control (IgG). Western blot was performed using anti-phospho-tyrosine antibody (4G10). B. SLAP, RNF-126, and RABRING7 GST fusion proteins were mixed with starved TF-1 lysate with (+) or without (-) 5 minutes of GM-CSF stimulation (100 ng/mL). Western blot was performed using anti-phospho-tyrosine antibody (4G10).
2.3.3 SLAP Interacts with SHP2 and CBL Downstream of the GM-CSFR

To determine the identity of the tyrosine proteins that interacted with SLAP in a GM-CSF dependent manner, GST-SLAP pull-downs were probed with antibodies against candidates known to be involved in GM-CSF signaling. The tyrosine phosphatase SHP2 was identified as a GM-CSF dependent SLAP interacting protein, likely corresponding to pp70 (Fig. 2-3). CBL also interacted with SLAP in a GM-CSF dependent manner, which may correspond to pp120 (Fig. 2-3). SLAP pull-downs were also probed with antibodies against the GM-CSF pathway proteins JAK2, STAT5, LYN, and PI3-Ka, but no interaction with these proteins was detected (data not shown). We also attempted to identify the 90 kDa SLAP associated protein. The adaptor protein GAB2 and tyrosine kinase FES, are both 95 kDa proteins, but we were unable to confirm an interaction with either protein since the available antibodies were raised against GST fusion proteins (data not shown).

2.3.4 CBL and SHP2 Interact with Each Other and Bind to SLAP with Similar Kinetics

Since SHP2 and CBL have previously been identified as interacting proteins (Tanaka et al, 2008), we investigated whether they interact in TF-1 cells. TF-1 cells over-expressing HA tagged CBL were depleted and stimulated with GM-CSF, followed by immunoprecipitation of CBL using anti-HA antibodies. Co-immunoprecipitation of CBL with SHP2 was observed in TF-1 cells in a GM-CSF dependent manner (Fig. 2-4A). Additionally, the interactions of SLAP with SHP2 and CBL have similar kinetics over a GM-CSF time course, where optimal binding is seen at 2 minutes post GM-CSF stimulation and decreases towards 10 minutes post stimulation (Fig. 2-4B). Taken together, this may indicate that SLAP, CBL, and SHP2 are all present in the same complex after GM-CSF stimulation or that SLAP is directly binding to one
Figure 2.3. SLAP Interacts with SHP2 and CBL in a GM-CSF Dependent Manner.
WT GST-SLAP was incubated with starved TF-1 lysate with (+) or without (-) 100 ng/mL of GM-CSF stimulation. Western blot was performed with antibodies against SHP2 and CBL. Fast green stain of the membrane shows even loading of GST fusion proteins.
Figure 2.4 CBL and SHP2 Interact with Each Other and Bind to SLAP with Similar Kinetics.

A. IPs with HA antibodies were performed with starved HA-CBL over-expressing TF-1 lysate with (+) or without (-) GM-CSF Stimulation. Normal mouse IgG was mixed with TF-1 lysate as a control. Western blot was performed to assess CBL binding to SHP2. B. WT GST-SLAP was mixed with lysate from TF-1 cells that were starved then stimulated with 100 ng/mL GM-CSF for 2, 5, 10, and 20 minutes. Western blot was performed using CBL and SHP2 antibodies for GST pull-downs and lysates were probed using phospho-CBL and phospho-SHP antibodies. Fast green stain of the membrane shows even loading of GST fusion proteins.
of CBL or SHP2, while the protein that is not binding directly to SLAP may be pulled down indirectly.

2.3.5 Mapping of the Regions Important for SLAP/SHP2 Interaction

As the interaction between SLAP and SHP2 is novel, we wanted to map which regions of SLAP and SHP2 were important for binding. To address this, GST fusion proteins of SLAP or SLAP mutants that abolished interactions between the SH2 or SH3 domains, as well as a ΔC truncation mutant were used. SLAP SH3 and SH2 mutants no longer interacted with SHP2 in stimulated TF-1 lysate, while the interaction between SLAP ΔC and SHP2 was unaffected (Fig. 2-5A). This suggests that the SH3 and SH2 domains of SLAP are important for SHP2 interaction, while the C-tail of SLAP is dispensable. Although the SH3 and SH2 domains of SLAP and SLAP2 share high sequence identity (36 % and 59 % respectively), SLAP2 was unable to pull-down SHP2 from GM-CSF stimulated TF-1 lysate (Fig. 2-5B). This shows specificity of the SH3 and SH2 domains of SLAP for binding to SHP2.

Since the SH2 domain was important for SHP2 binding to SLAP, and this interaction was GM-CSF dependent, we proposed that the tyrosine residues 542 and 580 which become phosphorylated upon GM-CSF stimulation, may be important for this interaction. This was tested using 293T cells transiently co-transfected with SLAP and either WT or SHP2 mutants with one or both of the tyrosine residues mutated to phenylalanine (Fig. 2-6A). SLAP co-immunoprecipitated with both WT and SHP2 mutants, indicating that these tyrosine residues are not necessary for this interaction (Fig.2-6B).
Figure 2-5. SLAP SH3 and SH2 Domains are Necessary for Binding to SHP2
A. WT GST-SLAP and SH3, SH2, and ΔC mutants were mixed with starved TF-1 lysate with (+) or without (-) 100 ng/mL GM-CSF stimulation. Western blot with anti-SHP2 antibodies was performed to assess SLAP binding requirements to SHP2. B. WT GST-SLAP and GST-SLAP2 were mixed with starved TF-1 lysate with (+) or without (-) 100 ng/mL GM-CSF stimulation. Western blot with anti-SHP2 antibodies. Fast green stain of the membrane shows even loading of GST fusion proteins.
Figure 2-6. SHP2 Tyrosine Phosphorylated Residues 542 and 580 are Not Required for Binding to SLAP
A. Schematic of SHP2 protein showing SH2 domains, phosphatase domain, tyrosine phosphorylated residues, and proline rich region (PPR). B. FLAG-SHP2 and SHP2 tyrosine mutants were transfected into 293T cells with (+) or without (-) SLAP-MYC/HIS. IPs were performed with MYC antibodies followed by Western blot probing for FLAG.
2.3.6 SLAP Expression in TF-1 Cells

The GM-CSF dependent interaction between SLAP and CBL supports the hypothesis that SLAP regulates GM-CSFR in a CBL dependent manner, but how these proteins affect GM-CSFR trafficking remains unclear. To test the effects of SLAP and CBL on GM-CSFR regulation, we chose to use a modified TF-1 cell system that over-expresses both SLAP and CBL. We first examined endogenous SLAP expression in TF-1 cells at both the RNA and protein levels. Similar to what is reported in Jurkat cells (Sosinowski et al., 2000), in TF-1 cells SLAP was found to be expressed at the RNA level, (Fig. 2-7A) but SLAP protein could not be detected (data not shown).

Since transfection efficiency of the TF-1 cell line is low and as a negative regulator of GM-CSFR, SLAP may negatively regulate cell proliferation, we decided to make a stable TF-1 cell line with inducible expression of SLAP. The pLVX Tet-ON 3G (EF1α version) system was used to make doxycycline inducible TF-1 cell lines. This system involved the transfection of two vectors. The first vector, pLVX Tet-ON, constitutively expresses a trans-activator protein, which upon addition of doxycycline, can interact with the minimal promoter of the second vector, pLVX TRE3G-SLAP myc/his, leading to initiation of SLAP expression. Positive cells were selected and maintained in G418 and puromycin, following sequential electroporation with pLVX Tet-ON and pLVX TRE3G-SLAP myc/his. Another TF-1 cell line which constitutively over-expresses HA tagged CBL was used to generate cells that inducibly over-express SLAP. To test the expression of SLAP in these stable cell lines, cells were treated with doxycycline and lysates from both lines were probed with SLAP antibodies. SLAP protein expression was detected in both TF-1 and TF-1 HA-CBL cell lines only when cells were treated with doxycycline, indicating that this system is tightly controlled (Fig. 2-7B).
Since there were difficulties choosing single clones using limiting dilution methods, both of the inducible SLAP expression cell lines used in further studies were made up of a pool of clones. Therefore, to measure the proportion of cells expressing SLAP protein, indirect intracellular flow cytometry was performed in the TF-1 inducible SLAP expression line. This analysis showed that approximately 30% of cells expressed SLAP upon addition of doxycycline (Fig. 2-7C). Despite the limitations due to the fact that SLAP is only expressed in a percentage of TF-1 cells, we used these cells to study the effects of SLAP over-expression on GM-CSFR down-regulation.

2.3.7 SLAP and CBL Over-Expression Alters Surface Levels of βc after GM-CSF Stimulation

SLAP inducible TF-1 and TF-1 HA-CBL cell lines were used to test the effect of SLAP and CBL over-expression on GM-CSFR endocytic trafficking by measuring changes in cell surface levels of βc following GM-CSF stimulation. Previously published data from the McGlade lab showed that in SLAP knock-out dendritic cells, βc remained at the cell surface over 10 minutes of GM-CSF stimulation, while in WT cells a robust decrease in surface levels of βc was observed (Liontos et al., 2011). Therefore, we predicted that over-expression of SLAP might enhance down-regulation and decrease surface levels of βc. To test this, starved TF-1 and TF-1 HA-CBL cells were treated with doxycycline or left untreated, followed by stimulation with GM-CSF for 2, 5, and 10 minutes. After stimulation, cell surface proteins were biotinylated, cells were lysed, and streptavidin beads were used to isolate biotinylated surface proteins. Figure 2-8A shows that over-expression of SLAP alone does not appear to change the levels of βc on the cell surface following GM-CSF stimulation. Quantification of the band intensity from 2 independent experiments at both the 0 and 10 minute time points indicate that the percentage
Figure 2-7. SLAP expression in TF-1 cells and generation of inducible SLAP expression TF-1 cell lines.

A. RNA was isolated from TF-1 and Jurkat cells and reverse transcribed to make cDNA. SLAP and β-actin specific primers were used to detect SLAP and β-actin gene expression by PCR amplification. Samples containing no cDNA template were used as a control (NT). B. TF-1 and TF-1 HA-CBL inducible SLAP expression cell lines were treated with doxycycline (+) or left untreated (-) for 24 hours. Cell lysates were probed with SLAP, CBL, and HA-CBL antibodies. C. TF-1 inducible SLAP expression cell lines were treated with doxycycline (+dox) or left untreated (-dox) for 24 hours. Cells were washed, fixed, permeabilized, and stained indirectly for SLAP using Alexa Fluor 488 conjugated secondary antibodies. Gates were formed around SLAP negative (left gate) and SLAP positive (right gate) populations with percentage of total cells displayed inside the gates.
of βc remaining at the cell surface after 10 minutes is 37 % in control cells and 29 % in SLAP over-expressing cells (Fig. 2-8B). In TF-1 HA-CBL expressing cells, over-expression of SLAP decreases the percentage of βc remaining on the cell surface after 10 minutes from 38 % in control cells to 5% (Fig. 2-8B). Over-expression of HA-CBL alone did not appear to alter βc surface levels compared to WT TF-1 cells, as the percentage of βc remaining at the surface after 10 minutes of stimulation was 38 % and 37 % respectively. From these data, we conclude that SLAP and CBL over-expression together, but not alone, can alter GM-CSFR trafficking as seen through a decrease in βc surface levels.

2.4 Discussion

GM-CSF is a cytokine which stimulates proliferation, differentiation, and survival of myeloid cells through interaction with GM-CSFR and initiation of JAK/STAT, RAS/MAPK, and PI3-K/AKT signaling pathways. Mechanisms which terminate GM-CSF signal activation are important for regulating normal cell function, as hyperactive signaling downstream of GM-CSFR can lead to myeloproliferative diseases and leukemia. One mechanism used by other cytokine and growth factor receptors to terminate signaling events is by the internalization and degradation of the receptor. Although the details of this process in GM-CSF signaling are poorly understood, the adaptor protein SLAP has been shown to be involved in down-regulation of GM-CSFR. To gain insight into the mechanism by which SLAP regulates GM-CSFR, we sought out proteins that interacted with SLAP downstream of GM-CSFR and identified CBL and SHP2 as GM-CSF dependent SLAP interacting proteins. We also found that over-expression of CBL and SLAP together can alter GM-CSFR trafficking, providing support for the hypothesis that SLAP functions to down-regulate GM-CSFR in a CBL dependent manner.
Figure 2-8. SLAP and CBL Over-Expression Alters Surface Levels of βc after GM-CSF Stimulation.

A. TF-1 and TF-1 HA-CBL cells with (+SLAP) and without (-SLAP) induction of SLAP expression, were stimulated with 10 ng/mL GM-CSF for the indicated times and surface proteins were labelled with EZ-link-sulfo-NHS-LC-Biotin. After cell lysis, biotinylated proteins were pulled down using streptavidin beads. Western blot using βc was used to evaluate surface levels of the receptor. B. Bar graphs show the average percentage of βc at the cell surface at 10 minutes post-stimulation compared to the 0 minute control (TF-1 cells n = 2, TF-1 HA-CBL cells n = 3). Error bars display standard error of the mean. *p = 0.001
Although the mechanism by which SLAP regulates GM-CSFR and downstream signaling has not been elucidated, both SLAP and SLAP2 can regulate several other hematopoietic receptors by functioning as adaptor proteins that links CBL to receptors, thus mediating their ubiquitylation and degradation. In GM-CSFR signaling, the role of CBL has not yet been defined, but CBL mutations which disrupt its E3 ubiquitin ligase function are seen in some JMML patients leading to hypersensitivity to GM-CSF and overactive RAS signaling downstream of GM-CSFR. This provides evidence that CBL may function as a negative regulator of GM-CSFR and/or downstream signaling. In this study, we observed that SLAP interacts with CBL in TF-1 cells only after GM-CSF stimulation (Fig 2-3), whereas SLAP2 interacted with CBL independent of GM-CSF stimulation (data not shown). This suggests these two proteins may have separate functions downstream of GM-CSFR. SLAP likely plays a non-redundant role in GM-CSFR regulation since in dendritic cells, only Slap, but not Slap2 deficiency leads to deregulation of GM-CSFR and downstream signaling (unpublished data). Additionally, the process of receptor mediated endocytosis and degradation occurs after cytokine or growth factor stimulation, therefore the GM-CSF-dependent interaction between SLAP and CBL fits a model in which SLAP regulates GM-CSFR following GM-CSF stimulation through the recruitment of CBL to the receptor. To further support this model, Slap has also been shown to interact with GMRα in a GM-CSF independent manner providing a route by which SLAP may link CBL to GM-CSFR (Liontos et al., 2011), although in this study we could not consistently see this interaction in TF-1 cells. Similarly, although the interaction between βc and CBL has been reported in one study, following GM-CSF stimulation (Bunda et al., 2013a), we were unable to confirm this interaction, possibly due to its transient and/or indirect nature.
Additional evidence to support the model that SLAP is regulating GM-CSFR in a CBL-dependent manner is the finding that over-expression of SLAP and CBL together, but not separately, led to a decrease in surface levels of βc after GM-CSF stimulation. This in levels of βc at the cell surface could be a result of an increase in receptor degradation, an increase in receptor internalization, or a decrease in receptor recycling. Other studies examining degradation of GM-CSFR in TF-1 cells observed a decrease in total βc levels at 30 minutes post-stimulation (Bunda et al., 2013b; Martinez-moczygemba and Huston, 2001), suggesting that the decrease in surface levels of βc we observe after 10 minutes of stimulation is not likely due to an increase in receptor degradation. CBL has previously been implicated in RTK internalization and degradation in the lysosome but not directly in the role of receptor recycling, therefore, although a decrease in receptor recycling cannot be ruled out, it is less likely that this is the cause of altered βc surface levels in SLAP and CBL overexpressed TF-1 cells. Altered βc internalization in TF-1 cells over-expressing SLAP and CBL may explain the decrease in surface levels of βc. Supporting this idea, another study looking at internalization of GM-CSF in WEHI 3BD+ cells found that approximately 60% of GM-CSF was internalized after 15 minutes of stimulation (Walker and Burgess, 1987), which is similar to what we observed in our biotinylation experiments where there is a loss of 62% of βc at the cell surface following 10 minutes of stimulation.

It is possible that SLAP and CBL could be negatively regulating GM-CSFR and downstream signaling by increasing the rate of receptor internalization and therefore altering the kinetics of receptor degradation. In regulation of EGFR signaling, CBL has been shown to promote internalization of the receptor by clathrin-dependent endocytosis via interactions with adaptor proteins such as Grb2 (Jiang et al., 2003). Internalization of EGFR into endosomes changes the spatial localization of the receptor complex, which ultimately leads to signal
termination (Schmidt and Dikic, 2005). The role of endocytosis in initiating and terminating signaling from βc has been studied in TF-1 cells stimulated with IL-5, where endocytosis is required to transmit signals as well as for the proteasomal degradation of the cytoplasmic region of βc, which terminates signaling (Lei and Martinez-Moczygemba, 2008). Therefore, if regulation of GM-CSF signaling through βc functions similarly to IL-5 signaling, SLAP and CBL may regulate GM-CSFR and downstream signaling by altering the kinetics of signal activation and proteasomal degradation of GM-CSFR.

In addition to confirming the interaction of SLAP with CBL, a novel GM-CSF dependent interaction between SHP2 and SLAP was identified. Interestingly, SLAP binds both SHP2 and CBL with similar kinetics and both SHP2 and CBL interact with each other in a GM-CSF dependent manner, suggesting that these proteins may form a complex (Fig 2-9). The idea that CBL and SHP2 may form a complex with SLAP is supported by studies that have mapped the CBL/SHP2 interaction in different cell types. In Hela cells and fibroblasts which do not express SLAP, only the SH2 domains of SHP2 and the tyrosine residues in the C-terminus of CBL were required for interaction (Chernock et al., 2001; Tanaka et al., 2008), whereas in T-cells which do express SLAP, the C-terminus of SHP2 was required for CBL binding (Hoff and Brunner-Weinzierl, 2007). In the mapping data from our study, we found that the SH2 and SH3 domains of SLAP were required for binding to SHP2 (Fig 2-5A). Although the phosphorylated tyrosine residues in the C-tail of SHP2 were not required for binding to SLAP, the proline rich region of SHP2 may be important for SLAP interaction with its SH3 domain. With our results and those from previous mapping studies, a speculative model can be made in which SLAP interacts with the proline rich region of SHP2 via the SH3 domain, and the TKB domain of CBL via the C-tail, thus linking the SHP2 SH2 domains to the phosphorylated tyrosine residues on the C-terminal region of CBL (Fig. 2-9). As SHP2 promotes signal activation of the RAS
pathway downstream of GM-CSFR, SLAP may play a role in the negative regulation of GM-CSF signaling by recruiting CBL to SHP2, which then allows CBL to ubiquitylate and target SHP2 for degradation. Alternatively, the interactions of SLAP with CBL and SHP2 serve different purposes, resulting in a balance between positive and negative regulatory signals.

In conclusion, this study provides support for the model that SLAP regulates GM-CSFR in a CBL-dependent manner, although further investigation is required to validate this model. It has also revealed a novel interaction between SLAP and SHP2, which may contribute to the regulation of GM-CSFR by SLAP.
Figure 2-9 Model of SLAP/CBL/SHP2 Complex Formation

Mapping of CBL/SLAP, CBL/SHP2, and SLAP/SHP2 interactions led to a model where SLAP, CBL, and SHP2 interact together in a complex via different domains. The C-tail of SLAP and N-terminus of CBL are necessary for CBL/SLAP interaction (1). The C-terminus of CBL and N-terminal SH2 domains of SHP2 are necessary for CBL/SHP2 interactions (2). The SH3 and SH2 domains of SLAP are necessary for interactions with SHP2. Although the region on SHP2 that is required for SLAP binding has not been identified, we propose the proline rich region (PRR) on SHP2 can interact with the SH3 domain of SLAP, as Y542 and Y580 were not important for SLAP/SHP2 interaction (3).
Chapter 3
The Role of SLAP in GM-CSF Induced Proliferation, and Dendritic Cell Development

3.1 Introduction

GM-CSF stimulates the proliferation and survival of myeloid progenitor cells and the production and activation of mature granulocytes and macrophages. For this reason GM-CSF has been administered to patients undergoing chemotherapy to restore their myeloid cell population. GM-CSF is also important for stimulating dendritic cell (DC) differentiation in vitro. Within the last decade, vaccines which involve the activation of DCs using GM-CSF have become a promising immunotherapy to treat cancer. It is therefore important to understand how dendritic cells mature to develop effective vaccines. We have recently identified Slap as an important protein in GM-CSF-stimulated DC development, as loss of Slap expression in bone marrow derived dendritic cells (BM-DC) results in impaired DC maturation. To better understand how SLAP regulates DC maturation we performed microarray experiments to evaluate the gene expression in SLAP knock-out (KO) BM-DC. We also examined the effect of SLAP on other GM-CSF-induced cellular processes. In this study we identified differentially expressed genes in SLAP KO BM-DC that may be important in DC maturation and also found that the role of SLAP in regulation of GM-CSF signaling is specific to differentiation in DC.

3.2 Materials and Methods

3.2.1 Mice

Generation of Slap -/- BALB/c mice has been previously described (Sosinowski et al., 2001). All experiments were performed on mice between 6 and 10 weeks old. WT Balb/c control mice
were ordered from the Toronto Centre for Phenogenomics (TCP) and were age-matched for individual experiments. For experiments with littermate controls, Slap KO Balb/c mice were crossed with WT Balb/c mice obtained from TCP to produce Slap -/- Balb/c heterozygous mice. These heterozygous mice were bred and genotyping was performed to identify Slap -/- and Slap +/- for use in qPCR experiments. Mice were bred and maintained under TCP guidelines and all procedures were approved by the Hospital for Sick Children Animal Care and Use Committee.

3.2.2 Colony Formation Assay
Bone marrow cells were obtained from the femurs and tibias of WT and Slap KO mice and prepared as single-cell suspensions. Erythrocytes were removed by lysis in an ammonium chloride buffer and the remaining cells were mixed with MethoCult M3234 methylcellulose medium (Stem Cell Technologies) at 3 × 10^4 cells/mL in the presence of increasing concentrations of GM-CSF. One millilitre of MethoCult mixture was plated on 35 mm dishes in duplicate and incubated at 37 °C. After 8 days, Granulocyte-Monocyte colony forming units (CFU-GM) were counted. CFU-GM were defined as clusters of 40 or more cells consisting of granulocytes, monocytes-macrophages, or both.

3.2.3 Cell Proliferation Assay
TF-1 and TF-1 HA CBL inducible SLAP cell lines were cytokine depleted at a concentration of 0.1 x 10^6 cells/mL in complete RPMI + 10 % FBS. After 24 hours, total viable cell number was measured by trypan blue exclusion using the ViCell Viability Counter (Beckman Coulter). Cells were then stimulated with 0.5 and 2.5 ng/mL GM-CSF and treated with 1 µg/mL doxycycline to induce SLAP expression or were left untreated. After incubation for 48 hours at 37° C, total cell number was measured again and normalized to the cell count at 0 hours to compare changes in cell number between different conditions.
3.2.4 Generation of BM-DC

BM-DC were generated following the protocol previously used in our lab (Liontos et al., 2011). Briefly, bone marrow was harvested from the femurs and tibias of WT and Slap -/- mice. Red blood cells were lysed in ammonium chloride lysis buffer and remaining bone marrow cells were plated in 6-well dishes at a concentration of 1 x 10^6 cells/mL in complete RPMI containing 10% FBS, 50mM 2-mercaptoethanol, 1% penicillin (100U/ml), 1% streptomycin (100U/ml), 10ng/ml GM-CSF and 5ng/ml IL-4 as indicated. On days 3 and 6, half of the media was removed and replaced with fresh media supplemented with additional GM-CSF and IL-4 to maintain cytokine concentrations at 10ng/mL and 5ng/mL respectively. Cells were harvested after 9 days for experimental use.

3.2.5 Microarray and Analysis

RNA was isolated from BM-DC generated from 3 wild-type and 3 Slap -/- mice between 6 and 10 weeks of age using Trizol Reagent (Gibco) and isopropanol precipitation (BM-DC generation was performed by Dr. Larissa Liontos). RNA was reverse transcribed to fluorescently labelled cDNA by The Centre for Applied Genomics (Toronto) using the Ambion WT Expression Kit (RT) and Affymetrix GeneChip WT Terminal Labeling Kit. cDNA samples were hybridized to an Affymetrix Mouse Gene 1.0 ST chip, and CEL files were imported into Partek Genomics Suite for data analysis. Data was processed by quantile normalization, RMA background correction and log2 transformation. T-tests were performed between WT and SLAP KO groups to determine fold-change with corresponding p-values. A list of differentially expressed genes was made by selecting genes with an absolute fold-change greater than 1.3 and a p-value less than 0.05.
3.2.6 Quantitative PCR
RNA was isolated from day 9 BM-DC using Qiagen RNeasy kit as per manufacturer’s protocol. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen) as per manufacturer’s protocol. qPCR assays were performed by Dr. Donna Berry on an Applied Biosystems StepOnePlus Real-Time PCR System using the SYBR Select Master Mix (Life Technologies). GAPDH was used as a house-keeping gene to normalize cDNA levels. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
</table>
| Zhx2       | Fwd: 5’CTCTGTGTTGCCCCAGTTAGC
Rev: 5’GGGTCTTTGCAAAAGTGCTC |
| Arhgap19   | Fwd: 5’TGCAATGACTCTCCCCTTC
Rev: 5’CCAATTcGCTGAAACACCTCT |
| Arl2bp     | Fwd: 5’AGAGCAGTGGCTGGAGCGTA
Rev: 5’CGTGAATGTGAGCAGCATGT |
| Tcfe3      | Fwd: 5’CCATTGGGTCCAGCTCAGAA
Rev: 5’GCAGTGCCTCCTGGAAAGATAG |
| Gapdh      | Fwd:5’CTCCCACTCTTCCACCTTGC
Rev:5’CCACCACCCCTGTTGCTGTAG |

3.3 Results

3.3.1 SLAP Does Not Regulate GM-CSF Induced Cell Proliferation
As Slap has been implicated in the regulation of GM-CSF and IL-4 stimulated DC development, we wanted to investigate whether SLAP plays a role in other GM-CSF induced cellular processes. For this reason, we examined the effect of SLAP deficiency or over-expression on GM-CSF induced proliferation in mouse BM and in human TF-1 cells.
Granulocyte-Macrophage colony forming assays were performed using BM from WT and Slap KO mice to measure proliferation of early myeloid progenitors stimulated with GM-CSF. In BM cells cultured in methocellulose media supplemented with GM-CSF, granulocyte-macrophage progenitor cells (also called CFU-GM for Colony Forming Units-Granulocyte, Monocyte) proliferate and differentiate into either granulocyte or monocyte/macrophage colonies. To measure the proliferation of CFU-GM, the number of colonies formed after 8 days of culture was examined. Data from these experiments are presented in Figure 3-1 by comparing total colonies formed, as well as by comparing the percentage of maximum colony formation in WT and Slap KO BM. The average colony count from three experiments with BM cells growing in 0.1 and 1 ng/mL of GM-CSF show that there is not a significant difference in total colonies formed between WT and KO samples (Fig. 3-1A). Similarly there was no significant difference in the percent of maximum colony formation at 0.01 and 0.1 ng/mL GM-CSF in Slap KO and WT BM (Fig. 3-1B). From these data we conclude that Slap deficiency does not alter the proliferation of CFU-GM at different concentrations of GM-CSF, suggesting that Slap is not essential for regulating proliferation of CFU-GM.

Although Slap did not appear to be essential for regulating GM-CSF induced proliferation of CFU-GM, we tested whether over-expression of SLAP altered proliferation of TF-1 cells using the TF-1 inducible SLAP expression cell line described in Chapter 2. Since we found that over-expression of SLAP and CBL together but not separately led to changes in GM-CSFR trafficking (Fig. 2-8), we also used the TF-1 HA-CBL inducible SLAP expression cell line to examine whether these two proteins together would lead to a change in GM-CSF induced cell proliferation. TF-1 and TF-1 HA CBL cells were cytokine depleted for 24 hours, SLAP
Figure 3 – 1. Slap Deficiency Does Not alter CFU-GM Proliferation
A. Cells extracted from bone marrow of WT and Slap KO mice were grown in methocult supplemented with 0.1, and 1 ng/mL of GM-CSF for 8 days. CFU-GM counts are expressed as a total colony number. Data are an average of three independent experiments. B. Cells extracted from bone marrow of WT and Slap KO mice were grown in methocult supplemented with 0.01, 0.1, and 1 ng/mL of GM-CSF for 8 days. CFU-GM counts are expressed as a percent of the maximum colony number at 1 ng/mL GM-CSF. Data are an average of two independent experiments.
expression was induced with doxycycline, and cells were stimulated with different concentrations of GM-CSF for 48 hours. Trypan blue exclusion was used to quantify the total viable cells at 48 hours to estimate relative changes in cell proliferation. In TF-1 cells over-expressing SLAP and CBL alone or together, we observed a trend of decreased total cell number in TF-1 cells cultured with 2.5 ng/mL GM-CSF, although these changes were not statistically significant (Fig. 3-2). These data suggest that CBL and SLAP over-expression does not affect TF-1 cell proliferation, although it is possible that using a cell system with a higher percentage of cells expressing SLAP would allow us to detect a significant effect. Supplementary assays would also need to be performed to differentiate between effects on survival or proliferation.

3.3.2 Slap Deficient Mouse BM-DC have Different Gene Expression Patterns Compared to WT BM-DC

SLAP does not appear to play a clear role in GM-CSF induced cell proliferation in either mouse myeloid progenitor cells or in TF-1 cells, leading us to propose that SLAP may play a role exclusively in GM-CSF induced differentiation. In Slap deficient BM-DC, a block in maturation was observed, but the mechanism underlying this phenotype is not understood. We postulated that loss of Slap may impair DC maturation through deregulation of GM-CSFR signaling which alters DC gene expression. To assess whether Slap KO DC had altered gene expression compared to WT DC, microarray analysis of gene expression from 3 WT and 3 Slap KO BM-DC samples was performed using the Affymetrix Mouse Gene 1.0 ST Chip. To globally visualize the gene expression data, Principal Components Analysis (PCA) was employed as it is a method of simplifying multidimensional data without sacrificing variance information. The first three components were analyzed for each sample and could explain 68.3% of the variance observed. This analysis was displayed on a three-dimensional plot showing
Figure 3 – 2. SLAP and CBL Over-Expression do Not Alter TF-1 Cell Proliferation. TF-1 and TF-1 HA-CBL cells were cytokine starved for 24 hours followed by the addition of doxycycline to induce SLAP expression. Cells were then incubated with 0.5 or 2.5 ng/mL of GM-CSF for 48 hours. Proliferation was measured by normalizing the cell count at 48 hours compared to the cell count at 0 hours. Relative proliferation was determined by comparing the normalized cell count in each condition to control cells which do not over-express CBL or SLAP upon addition of doxycycline. Data are an average of three independent experiments and error bars represent standard error of the mean.
that WT and Slap KO DC samples can be clustered into distinct groups, although there is a large variance between samples in the Slap KO group (Fig. 3-3). Overall the PCA analysis suggests that some of the variance in gene expression between samples can be explained by the presence of Slap gene expression; however other unidentified variables are also contributing to this variance (Fig. 3-3).

Differentially expressed genes between WT and Slap KO samples were identified by statistical analysis comparing the mean intensity values of Slap KO and WT DC samples for individual genes. A list of 71 differentially expressed genes was produced by selecting genes with an absolute fold-change greater than 1.3 and a p-value less than 0.05. Of these differentially expressed genes, 27 were down-regulated in Slap KO BM-DC and 44 were up-regulated. The fold-change values ranged from -3.17 to +5.54, with 90% of the genes being differentially expressed by an absolute fold change less than 2. These small changes in gene expression could be a result of the large variance between samples that cannot be explained by Slap expression. Therefore it is difficult to predict whether fold-change values below 2 are biologically significant. Nonetheless, differentially expressed genes were grouped into 7 categories based on known or predicted gene attributes (Fig. 3-4). These include: interferon inducible genes, membrane proteins, monocyte surface proteins, transcription factors, cytoskeletal organization/protein trafficking, macromolecule modifying enzymes, and miscellaneous genes. Organization of these differentially expressed genes into categories reveals that genes that are induced by interferons and that code for membrane proteins or monocyte surface markers are frequently up-regulated in Slap KO BM-DC. In contrast, the majority of differentially expressed genes identified in this study that are involved in cytoskeletal regulation and transcription are down-regulated in Slap KO DC. Differential
Figure 3 – 3. Exploratory Gene Expression Analysis from WT and Slap Knock-out BM-DC Samples. RNA isolated from BM-DC of 3 WT and 3 Slap KO mice was reverse transcribed and hybridized to an Affymetrix Mouse Gene 1.0 ST chip. Principal Components Analysis showing the correlation of gene expression from 3 WT (blue spheres) and 3 Slap KO (red spheres) arrays. Samples plotted closely together have similar intensity values across the whole probeset. The hatched circles separate the samples into distinct groups that correlate to each other (red grouping Slap KO, blue grouping WT).
<table>
<thead>
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<th>Interferon Inducible Genes</th>
<th>Transcription</th>
<th>Miscellaneous</th>
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<tr>
<td>I830012016Rik</td>
<td>Zhx2</td>
<td>Pisd-ps1</td>
</tr>
<tr>
<td>2010002M12Rik</td>
<td>Trps1</td>
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<td>Oas1g</td>
<td>Epas1</td>
<td>Gm13154</td>
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<td>Hist2h3c2</td>
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<td>Oasl2</td>
<td>Tdrd3</td>
<td>Gm10101</td>
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<td>Iifi202b</td>
<td>Mfap1a</td>
<td>Prl2c5</td>
</tr>
<tr>
<td>Oas3</td>
<td></td>
<td>Gm12253</td>
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<td>Slfn5</td>
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<table>
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<td>Slf1</td>
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<tr>
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<td>Grem1</td>
</tr>
<tr>
<td>Igkv5-45</td>
<td>Gsdmc2</td>
</tr>
<tr>
<td>Tmem71</td>
<td>Arl2bp</td>
</tr>
<tr>
<td>Ms4a4a</td>
<td>Ap1m2</td>
</tr>
<tr>
<td>Raet1d</td>
<td>Arhgap19</td>
</tr>
<tr>
<td>Ms4a6d</td>
<td></td>
</tr>
<tr>
<td>Il13ra1</td>
<td></td>
</tr>
<tr>
<td>S1pr5</td>
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</tr>
<tr>
<td>Gpr82</td>
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<tr>
<th>Monocyte Surface Proteins</th>
<th>Macromolecule Modifying Enzymes</th>
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<tr>
<td>Cd300e</td>
<td>Has2</td>
</tr>
<tr>
<td>Cd300ld</td>
<td>St3gal1</td>
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<tr>
<td>Fcgr1</td>
<td>Gm5683</td>
</tr>
<tr>
<td>Cd209f</td>
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</table>

Figure 3-4. Differentially Expressed Genes in Slap KO BM-DC
A list of differentially expressed genes in Slap KO and WT BM-DC was made by selecting genes with an absolute fold-change greater than 1.3 and a p-value less than 0.05. Genes are divided into 7 categories based on similar attributes. Fold-change (Slap KO vs WT) is presented beside each gene symbol. Red and black correspond to up-regulated and down-regulated genes respectively.
expression of genes with these attributes are also observed in other studies that analyzed transcriptional changes in DCs during development, although a limited number of genes identified in this study have also been identified in these reports (Ancuta et al., 2009; Granucci et al., 2001; de la Fuente et al., 2009; Le Naour et al., 2001). Il13ra1, Mtss1, and Tcfe3 which are all differentially expressed genes identified in this study are also differentially expressed in specific subsets of human peripheral blood monocytes and UV treated human monocyte-derived DC, suggesting these genes may be good candidates to study further (Ancuta et al., 2009; de la Fuente et al., 2009). Genes that were separated into to the miscellaneous and macromolecule modifying enzymes categories had similar proportions of up-regulated and down-regulated genes and did not have an apparent connection to DC development. This implies that these genes are not likely to be good candidates to explain how Slap deficiency leads to impaired DC maturation.

### 3.3.3 Differential Expression of Zhx2, Tcfe3, Arghap19, and Arl2bp in Slap KO BM-DC can be Validated by qPCR

From the list of differentially expressed genes obtained from microarray analysis, 4 genes were selected to be validated by qPCR. Zhx2, Arghap19, and Arl2bp were selected based on their functions in cytoskeletal regulation and transcription, as well as for their absolute fold-change which is greater than 2. Although the transcription factor Tcfe3 was differentially expressed only by a factor of 1.36, it was chosen based on its gene function and previous reports of its differential expression in dendritic cells (Ancuta et al., 2009; de la Fuente et al., 2009). Differential expression of all 4 genes was confirmed by qPCR in agreement with the microarray data, although there were differences in the magnitude of fold-change observed (Table 3.1). Absolute fold-change detected for the differentially expressed genes Zhx2, Arl2bp, and Tcfe3 were all minimally increased in qPCR data by values between 1 and 2.8 (Table 3.1).
Conversely, the fold-change value for the differentially expressed gene Arhgap19 was increased by an absolute value of 49.8 in qPCR data compared to microarray analysis. This large discrepancy in fold-change is not unusual in qPCR data and could be due to differences in RNA quality, reverse transcription methods, and normalization methods between microarray and qPCR experiments (Morey et al., 2006).

3.3.4 Zhx2 is Differentially Expressed in the Balb/Cj Mouse Strain and Human Cell Lines

As Zhx2 displayed the highest fold-change in gene expression between Slap KO and WT BM-DC and a high correlation was observed in qPCR data, we chose to explore Zhx2 function in more detail. Zhx2 is a transcriptional repressor that is differentially expressed in both cortical neural progenitor cells and erythroid cells during differentiation, where a decrease in gene expression is associated with increased cell differentiation (De Andrade et al., 2010; Wu et al., 2009). In the Balb/cJ mouse strain, a retroviral insertion was detected in a Zhx2 gene intron resulting in decreased mRNA transcript levels and is responsible for the increased level of α-fetoprotein in these adult mice (Perincheri et al., 2008). To ensure that mouse strain was not giving rise to the differential expression observed, we measured Zhx2 gene expression levels in BM-DC derived from WT and Slap KO littermates by qPCR. These data confirmed that Zhx2 is up-regulated in Slap KO BM-DC compared to the WT littermate control (Fold-change of +28, data not shown). To determine whether changes in Zhx2 gene expression would translate to increased expression at the protein level, WT and Slap KO BM-DC lysates were immune-blotted with antibodies against mouse Zhx2. Zhx2 protein expression could not be detected in either sample (data not shown) however, we were able to detect human Zhx2 protein using antibodies specific for human Zhx2 and observed abundant Zhx2 protein expression in the
human lymphoblastic cell lines SUPB15 and CMLT1 as well as lower levels of protein expression in HEK293 and Jurkat cells line (Fig. 3-5).

3.4 Discussion

Signaling through GM-CSFR in different cellular contexts can lead to multiple cellular fates, but the mechanism by which these diverse responses are controlled is not well understood. Here we show that GM-CSF-induced proliferation and differentiation appear to be differentially regulated by the adaptor protein SLAP, as change in SLAP expression did not alter GM-CSF-induced cell proliferation, despite playing a role in GM-CSF-induced DC differentiation. The effect of SLAP on cell proliferation downstream of GM-CSFR was examined using two approaches; through the evaluation of CFU-GM in bone marrow from Slap KO mice and by measuring changes in TF-1 cell proliferation when SLAP is over-expressed.

The CFU-GM assay is commonly used in the diagnosis of JMML, as bone marrow cells from these patients have increased colony formation at low concentrations of GM-CSF, indicating a hypersensitivity to this cytokine (Freedman et al., 1996). JMML is caused by mutations in SHP2, c-CBL, RAS, and NF-1, leading to the deregulation of RAS/MAPK signaling downstream of GM-CSFR (De Vries et al., 2010). Since SLAP interacts with both CBL and SHP2 in a GM-CSF dependent manner (Chapter 2) and SLAP deficient DC also show deregulation of signaling downstream of RAS, it was somewhat surprising that Slap deficient BM cells did not show hypersensitivity to GM-CSF as well. One explanation for this is that SLAP may play a redundant role in regulating GM-CSF induced proliferation. Therefore, when SLAP expression is lost, other mechanisms may be used to regulate proliferation of myeloid
Table 3-1. Validation of Differentially Expressed Genes in Slap KO BM-DC by qPCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold-Change</th>
<th>Microarray</th>
<th>qPCR</th>
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<tbody>
<tr>
<td>Zhx2</td>
<td>+5.5</td>
<td>+6.5</td>
<td></td>
</tr>
<tr>
<td>Tcfe3</td>
<td>-1.4</td>
<td>-3.5</td>
<td></td>
</tr>
<tr>
<td>Arhgap19</td>
<td>-3.2</td>
<td>-53</td>
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</tr>
<tr>
<td>Arl2bp</td>
<td>-2.4</td>
<td>-5.2</td>
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</tr>
</tbody>
</table>

Microarray and qPCR data showing fold change (Slap KO vs WT) of ZHX2, Tfe3, Arhgap19, and Arl2bp expression

Figure 3-5  ZHX2 protein expression in lymphoblastic cell lines. Lysates form lymphoblastic cell lines SUPB15, Jurkat, and CMLT1 were probed with anti-human ZHX2 antibodies to measure protein expression. Lysate from the human embryonic kidney cell line HEK293 was also probed as a control.
cells. Indeed, in CFU-GM assays using BM cells from Cbl KO mice, hypersensitivity to GM-CSF was also not observed, but a double knock-out of Cbl and Cbl-b resulted in a hypersensitivity to GM-CSF and other cytokines (Naramura and Nandwani, 2010). This implies that CBL and CBL-b have redundant roles in regulation of GM-CSF induced cell proliferation. Since SLAP interacts with CBL to regulate hematopoietic receptors but has not been reported to interact with CBL-b, it may be interesting to examine GM-CSF influenced colony formation in BM cells lacking both Slap and Cbl-b expression.

We also did not observe an effect of SLAP over-expression on proliferation of TF-1 cells stimulated with GM-CSF (Fig. 3-2), which further supports the idea that SLAP plays a role specifically in the regulation of GM-CSF-induced differentiation and not proliferation. Alternatively, the involvement of SLAP in regulating GM-CSF-induced cellular processes may instead be specific to cell contexts rather than cell processes. Examining the effect of SLAP on GM-CSF-induced cellular processes in other primary cells and cell lines may help to distinguish how SLAP regulates GM-CSFR signaling.

As we have previously observed that loss of Slap expression impairs DC maturation but the mechanism of how this occurs is unclear, we performed microarray analysis to evaluate changes in gene expression between Slap KO and WT DC. Although we did not see a large number of differentially expressed genes, we did identify several gene expression changes that may help explain the phenotype observed. We identified several genes involved in cytoskeletal organization that were differentially expressed in Slap KO DC. Two of these genes are Arhgap19 and Arl2bp, which are both down-regulated in Slap KO BM-DC. Arhgap19 is hematopoietic-specific GTPase activating protein (GAP) that is involved in regulation of cell shape in lymphocytes during early mitosis through the inactivation of RhoA (David et al.,
Arl2bp regulates cytoskeleton rearrangements in some cell types by also regulating activity of RhoA through binding to the RhoA GAP Arl (Taniuchi et al., 2011). As DC undergo profound cytoskeletal rearrangements during maturation, the deregulation of cytoskeletal organization genes we see in Slap KO BM-DC could either be a product of impaired maturation, or could contribute to the cause. Other studies looking at transcriptional changes during DC maturation also found changes in transcription of cytoskeletal organization genes, although these genes do not coincide with those identified in our study (Ancuta et al., 2009; Le Naour et al., 2001).

Differential expression of several proteins involved in transcription were also identified in our microarray study and of particular interest are the transcription factors Tcfe3 and the transcriptional repressor Zhx2. The transcription factor Tcfe3 is part of the family of MiTF/TFE transcription factors along with TFEB, TFEC, and MiTF. It is important in promoting differentiation of mast cells, osteoclasts, B-cells and T-cells (Martina et al., 2014; Yagil et al., 2012), making it an interesting target as potential regulator of GM-CSF induced DC maturation. Tcfe3 is down-regulated in BM-DC after UV treatment, a cell stressor which leads to impaired DC maturation. In this study SLAP was also down-regulated in DC after UV treatment, which suggests that SLAP could be regulating transcription of Tcfe3 which is in turn important for DC maturation.

Zhx2, as described earlier, is differentially regulated during erythroid and neural progenitor differentiation. It also interacts with the transcription factor NF-YA, inhibiting its ability to activate transcription. NF-YA forms a complex with NF-YB and NF-YC on the Y-box of the MHCII promoter, and together with the RFX and CREB complexes binds the trans-activator CIITA to initiate transcription of MHCII genes (Garvie and Boss, 2008). Therefore
up-regulation of Zhx2 in Slap KO BM-DC may inhibit the function of NF-YA to initiate transcription of MHCII, providing one possible explanation for the reduced surface levels of MHCII in Slap KO mice (Fig. 3-6).

Although these experiments did not expose the mechanism by which Slap regulates GM-CSF-induced maturation in DCs, they do provide evidence that Slap deficient DCs have altered gene expression. Further investigation into the function of these gene products in DC may help to understand why loss of SLAP leads to a block in DC maturation.
Figure 3-6 Model of Zhx2 Regulation of MHCII Expression
The transcription factor NF-YA interacts with the Y box upstream of the MHCII and forms a complex with NF-YB to initiate MHCII expression. Interaction of Zhx2 with NF-YA may inhibit the binding of NF-YA to the promoter of MHCII, leading to inhibition of MHCII transcription.
Chapter 4 – Summary and Future Directions

4.1 Summary

The objective of this thesis was to investigate the mechanism by which SLAP regulates GM-CSFR and GM-CSF-induced DC differentiation. To understand how SLAP regulates GM-CSFR we searched for GM-CSF-dependent SLAP interacting proteins in TF-1 cells. A number of tyrosine phosphorylated proteins interacted with SLAP after GM-CSF stimulation. We identified SHP2 and CBL as GM-CSF dependent SLAP interacting proteins. The interaction of SLAP with CBL in a GM-CSF dependent manner supports a model where SLAP couples CBL to GM-CSFR after GM-CSF stimulation, allowing CBL to ubiquitylate and target the receptor to the lysosome for degradation. The interaction between SLAP and SHP2 is novel, therefore the consequence of this interaction is not yet understood. Over-expression of SLAP and CBL together, but not separately led to a decrease in βc surface levels after 10 minutes of GM-CSF stimulation. This indicates that SLAP and CBL may work together to alter GM-CSFR endocytic trafficking, which also supports the idea that SLAP down-regulates GM-CSFR in a CBL dependent manner. Additional studies must be performed to provide further support for this working hypothesis, some of which are described below.

Although Slap regulates GM-CSF induced DC differentiation, it does not appear to play a role in GM-CSF induced proliferation in either TF-1 cells or CFU-GM progenitors. This suggests that SLAP may function specifically in regulation of differentiation downstream of GM-CSFR. We proposed that SLAP may regulate GM-CSF induced DC maturation through regulation of signaling downstream of GM-CSFR, which ultimately controls expression of
genes involved in this process. We did observe differential expression of several genes in Slap KO BM-DC compared to WT. Of particular interest are transcription factors Zhx2 and Tcfe3, which play a role in cell differentiation. Further investigations into the function of these gene products in DC development may help reveal how loss of SLAP expression leads to a block in DC maturation.

4.2 Regulation of GM-CSFR and Downstream Signaling by SLAP and CBL

In this study, we observed that over-expression of SLAP and CBL together, but not separately, decreased the surface levels of βc in TF-1 cells after GM-CSF stimulation, although the mechanism by which this occurs is still unclear. Future experiments are needed to confirm whether SLAP regulates GM-CSFR in a CBL-dependent manner. To validate this model, GM-CSFR surface levels can be measured in an HA-CBL expressing TF-1 cell line that inducibly expresses a C-terminal truncated form of SLAP. As the C-tail of SLAP is required for binding to CBL, failure of this SLAP mutant to decrease surface levels of βc would indicate that SLAP regulates GM-CSFR in a CBL-dependent manner.

As SLAP interacts with GMRα in BM-DC (Liontos et al., 2011) we propose this could be the route by which SLAP can link CBL to GM-CSFR. Since SLAP2 did not interact with GMRα in BM-DC, it would be interesting to examine whether over-expression of SLAP2 and CBL together would have an effect on GM-CSFR surface levels. It would also be beneficial to map the regions of SLAP which are important for binding to GMRα, as βc levels could be measured in TF-1 cells over-expressing CBL and a mutant form of SLAP that does not interact with GMRα, to determine whether the interaction between GMRα and SLAP is needed to decrease surface levels of βc.
We predicted that the decrease in surface levels of βc observed in SLAP and CBL over-expressing TF-1 cells was a result of increased receptor internalization. After IL-5 stimulation, βc is internalized by lipid raft and clathrin dependent mechanisms (Lei and Martinez-Moczygemba, 2008). To test this prediction, the mechanism of GM-CSFR internalization could be determined by analyzing the levels of βc when either lipid raft or clathrin dependent endocytosis methods are blocked.

Since deregulation of GM-CSFR in Slap KO BM-DC resulted in enhanced and prolonged activation of Erk and Akt, it would also be of interest to examine whether SLAP and CBL over-expression would affect the activation of downstream signaling molecules. Generation of a cell system with a higher percentage of cells expressing SLAP would eliminate the potential diluting effects of non-expressing cells.

4.3 SLAP Interactions with Proteins Downstream of GM-CSFR

In this study we identified CBL and SHP2 as GM-CSF dependent SLAP interacting proteins and also observed that SLAP interacts with a number of other tyrosine phosphorylated proteins after GM-CSF stimulation. To better understand the mechanisms by which SLAP regulates GM-CSFR and downstream signaling, it would be useful to identify additional GM-CSF dependent SLAP interacting proteins. Mass spectrometry techniques could be employed to identify other tyrosine phosphorylated, unmodified or alternately modified proteins that interact with SLAP in TF-1 cells. This method could also aid in identification of the unknown tyrosine phosphorylated proteins that interact with SLAP, particularly the highly phosphorylated 90 kDa protein (Fig. 2-1).

The observed interaction between SLAP with SHP2 is novel, therefore the consequences of this interaction remain to be elucidated. As both SHP2 and CBL bind to SLAP after GM-CSF
stimulation with similar kinetics and they also interact with each other (Fig. 2-4), we predicted that these proteins may function in a complex together. As SHP2 functions as a protein tyrosine phosphatase and CBL can function as an E3 ubiquitin ligase, future experiments could test whether SHP2 or CBL mediate protein modifications directly on SLAP or each other. A recent study found that SHP2 can regulate CBL activity through dephosphorylation of the CBL inhibitor SPRY2 and that this process requires the adaptor protein ITSN1 (Okur et al., 2014). It is possible that SLAP could be functioning in a similar manner, whereby it acts as a scaffold for multiple proteins with enzymatic activity. Therefore, identification of additional SLAP interacting proteins by mass spectrometry may also be helpful in revealing the function of the SLAP/SHP2/CBL interaction.

### 4.4 Validation of Zhx2 and Tcfe3 as Regulators of DC Development

To understand how SLAP deficiency results in a block in DC maturation, we performed microarray analysis to identify differentially expressed genes in Slap KO BM-DC. From this investigation the transcriptional repressor Zhx2 was observed to be up-regulated and the transcription factor Tcfe3 was down-regulated in Slap KO BM-DC (Fig. 3-4). As Zhx2 and Tcfe3 have both been implicated in the regulation of cell differentiation, these genes would be good candidates for further investigation (De Andrade et al., 2010; Martina et al., 2014; Wu et al., 2009; Yagil et al., 2012). First, the differential expression of these genes should be validated at the protein level, which would provide support for the idea that these gene products play a role in DC maturation. Rescue experiments could also be performed in Slap KO BM-DC to examine whether Zhx2 or Tcfe3 are important in regulating DC development. This would involve infecting Slap KO BM at different time-points during DC differentiation with either shRNA against Zhx2 or a vector that will over-express Tcfe3. Surface levels of MHCII, CD86,
and CD80 could then be measured to evaluate DC maturation. Similarly, WT BM could be infected with shRNA against Tcfe3 or a vector that will over-express Zhx2, at different time-points during differentiation to evaluate whether changes in expression of these genes was sufficient to mimic the impaired DC maturation seen in Slap KO BM-DC.

4.5 The Role of SLAP in Regulation of BM-DC Maturation

Although we observed differential gene expression in Slap KO BM-DC and have some candidate genes to investigate, the mechanism by which SLAP deficiency leads to impaired DC maturation is still unclear. Expression of Slap mRNA is increased between Day 3 and Day 9 of BM-DC development (unpublished data) suggesting that regulation of GM-CSFR and downstream signaling by SLAP may be crucial for normal DC development during these time-points. This means that although microarray analysis of gene expression in WT and Slap KO BM-DC was performed at Day 10 during differentiation, analysis of differential gene expression may be more informative at earlier time points. Additionally, comparing gene expression between WT and Slap KO cells at early time-points during DC differentiation may reveal differentially expressed genes that are the cause of impaired DC maturation rather than an effect of immature DC.

Evaluation of global changes in protein levels in Slap KO and WT DC may also aid in understanding how SLAP regulates DC maturation, as changes in gene expression do not always correlate with protein levels and activity. This type of analysis has previously been performed by another group, which compares protein levels at different stages in DC maturation using 2D gel electrophoresis followed by mass spectrometry (Le Naour et al., 2001). Mass spectrometry methods such as SILAC (stable isotope labeling by amino acids in cell culture) can
quantitatively measure changes in protein levels (Ong, 2002), therefore, would be an effective method in measuring protein level changes between SLAP KO and WT BM-DC.

4.6 SLAP Function in Disease

Since SLAP regulates the stability of several hematopoietic receptors, which can negatively regulate downstream signaling, SLAP may play a role in the progression of immunological diseases and cancer. To date there are no known mutations of the SLAP gene that contribute to disease. Changes in SLAP expression has been implicated in autoimmune arthritis as SLAP deficiency has a protective effect on the development of zymogen induced arthritis in mice containing an inactivating point mutation in the ZAP-70 gene (Peterson et al., 2011). Furthermore, in CD4+ thymocytes of rheumatoid arthritis patients, SLAP protein levels are increased by approximately 2-fold compared to healthy donors (Érsek et al., 2012). A recent study reported that SLAP has tumour suppressor functions in colorectal cancer by promoting the degradation of the EPHA2 receptor (Naudin et al., 2014). In this study, SLAP was found to be highly expressed in healthy epithelial intestine, whereas SLAP expression was reduced in colorectal cancer. Since SLAP is implicated in the negative regulation of GM-CSFR and downstream signaling, the involvement of SLAP in the initiation and progression of JMML could be examined. JMML patients have deregulation of GM-CSF signaling, and both CBL and SHP2, which interact with SLAP in a GM-CSF dependent manner, are mutated in JMML patients (De Vries et al., 2010). Slap KO mice do not show a JMML phenotype or hypersensitivity to GM-CSF, but this may be due to a redundant role of Cbl-b in regulating GM-CSFR levels. Future experiments which cross Slap KO and Cbl-b KO mice could be performed to test whether these mice develop a myeloproliferative disease such as JMML.
Chapter 5 - References


