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CBL and CBL-B Dictate CSF-1R Endocytic Traffic and Signaling in Macrophages

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BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This work is dedicated to my family and friends.

A very deep feeling of thankfulness to my parents, aunt, whose unconditional love and support for me. My husband's thoughtful care, kindness, and the funny faces make me my life more wonderful and beautiful. My baby in my belly makes me more brave.

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TABLE OF CONTENTS

ABBREVIATIONS

AKT	Protein kinase B also known as AKT
ACK1	Cdc42 associated kinase
BMDM	Bone marrow derived macrophages
CSF-1	Colony stimulating factor-1
CSF-1R	Colony stimulating factor-1 receptor
CME	Clathrin mediated endocytosis
CIE	Clathrin independent Endocytosis
DAG	Diacylglycerol
DKO	Cbl and Cbl-b double knock out
EEs	Early endosomes
EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
ESCRT	Endosomal sorting complex required for transport
GEFs	Guanine-nucleotide exchange factors
Grb2	Growth factor receptor bound protein 2
Hrs	Hepatocyte growth factor-regulated tyrosine kinase
IP3	Inositol (1,4,5) trisphosphate
LEs	Late endosomes
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinases

MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MPs	Macropinosomes
MVB	Multi-vesicular bodies
PI3K	Phosphatidylinositol 3 kinase
PIP3	PtdIns(3,4,5)P_3
PLCγ	Phospholipase Cy pathway
PKC	Protein kinase C
PIP2	Phosphatidylinositol 4, 5 bisphosphate (PtdIns(4,5)P2)
PDK1	Pyruvate Dehydrogenase Kinase 1
PDK2	Pyruvate dehydrogenase kinase isoform 2
PTEN	Phosphatase and tensin homolog
PTK	Protein tyrosine kinase
RTK	Receptor tyrosine kinase
RF	RING Finger
SFKs	Src family kinases
SH2	Src homology 2
SH3	Src homology 3
SLAP	Src-like adaptor protein
STAM	Signaling transducing adaptor molecule
STAT	Signal transducers and activator of transcription
SOS	Son of Sevenless
TKB	Tyrosine kinase binding

UBA Ubiquitin Binding Association

ABSTRACT

CBL AND CBL-B DICTATE CSF-1R ENDOCYTIC TRAFFIC AND SIGNALING IN MACROPHAGES

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Macrophage colony stimulating factor receptor (CSF-1R or MCSFR) is a receptor tyrosine kinase essential for the growth and function of macrophages. Understanding the mechanisms that regulate CSF-1R activation and deactivation will provide insights to clinical treatment of macrophage related diseases including chronic inflammation and cancer. Previously, our laboratory showed that CSF-1R undergoes a novel membrane trafficking route that involves macropinocytosis to deactivate CSF-1R signaling. This thesis makes the discovery that the ubiquitin ligases Cbl and Cbl-b cooperate to regulate CSF-1R endocytosis and traffic to macropinosome in macrophages. Macrophages were derived from mice knocked out for Cbl, Cbl-b or the double knock out (DKO). DKO macrophages hyperproliferated, matching the severe myeloproliferative disorder observed in DKO mice. The CSF-1R and associated proteins were not ubiquitinated in DKO macrophages, unlike single knockouts and wild-type cells suggesting redundant functions of Cbl and Cbl-b. Mapping of the CSF-1R traffic demonstrated that CSF-1R internalization was slower in DKO cells, resulting in prolonged CSF-1R signaling at the plasma membrane and prolonged Akt signaling. Interestingly, CSF-1R transport to the lumen of macropinosome was defective in DKO cells, suggesting that altered membrane transport is responsible for the cellular phenotype. Tyrosine phosphorylation was drastically decreased and ERK signaling was lower in DKO macrophages, possibly

resulting from defective CSF-1R signaling on endosome and macropinosome. One of the key ESCRT proteins, HRS, did not associate with the CSF-1R in DKO macrophages indicating Cbl and Cbl-b are required for ESCRT-mediated transport into the lumen of macropinosome. Surprisingly, the CSF-1R was still degraded in DKO cells by an unknown mechanism. RNA sequencing analysis showed that Cbl and Cbl-b work together to regulate approximately 1,300 genes, while Cbl appears to regulate a unique set of approximately 250 genes in macrophages. In conclusion, Cbl and Cbl-b share partially redundant functions regulating CSF-1R signaling, endocytic traffic and cell growth.

Chapter I

INTRODUCTION

1.1 Introduction to the thesis

Macrophages are a type of immune cells that function in both normal and diseased tissues (Mosser and Edwards 2008, Zarif, Taichman et al. 2014). Macrophage Colony Stimulating Factor-1 (CSF-1 or M-CSF), is the main growth factor for macrophages (Bourette and Rohrschneider 2000). CSF-1 mediates macrophage function by binding to its receptor (CSF-1R) on the surface of macrophages to regulate gene expression, controlling macrophage abundance and guiding the function of macrophages in tissues (Pixley and Stanley 2004). CSF-1R belongs to the type III receptor tyrosine kinases (Stanley and Chitu 2014). The CSF-1R dimerizes upon binding to CSF-1, which activates its kinase activity, results in the tyrosine phosphorylation on its cytosolic tails. The phosphorylated tyrosine residues at the cytosolic tails of CSF-1R provide binding sites for the association of downstream signaling proteins, mediating signaling cascades and activation of the transcriptional factors responsible for the growth, differentiation, and proliferation of macrophages (Pixley and Stanley 2004).

Endocytosis and delivery of activated CSF-1R to the lysosome is essential for cells to control growth factor receptor signaling and prevent out-of-control cellular growth (Miaczynska, Pelkmans et al. 2004, Tomas, Futter et al. 2014). Upon binding to CSF-1, the CSF-1R is quickly internalized by endocytosis to intracellular compartments named

as endosomes. As the cytosolic tail of CSF-1R is still accessible to downstream signaling proteins for signaling with CSF-1R on endosomes (Huynh, Kwa et al. 2012), activated CSF-1R will quickly transport to the lysosome that contains degradative enzymes to be cleaved and destroyed and thus deactivated (Lou, Low-Nam et al. 2014).

The balance between receptor signaling and receptor deactivation exerted by receptor traffic is essential for controlling cell growth and function and preventing tumor development (Tomas, Futter et al. 2014). Disrupted growth factor receptor traffic can lead to the increased or prolonged signaling resulting in the hyperactive growth of cells (Tomas, Futter et al. 2014). Growth factor receptor traffic is tightly controlled and regulated by many molecular "brakes" (inhibitory proteins) that stop receptor signaling. Cbl is an E3 ubiquitin ligase that tags CSF-1R and other growth factor receptors with ubiquitin (Miyake, Lupher et al. 1997, Lee, Wang et al. 1999, Mancini, Koch et al. 2002, Rorsman, Tsioumpekou et al. 2016). Cbl-mediated receptor ubiquitination is an essential regulator in receptor traffic to lysosomes to be deactivated (de Melker, van der Horst et al. 2001, Mancini, Koch et al. 2002).

Defects in receptor ubiquitination lead to out-of-control growth factor signaling and can cause cancer (Lipkowitz 2003, Huangfu and Fuchs 2010). Approximately 5% of leukemia is caused by a defect in Cbl's E3 ubiquitin ligase activity. These cases are caused by hyperactive growth of myeloid cells (myoloproliferative disease) (Nadeau, An et al. 2012). Cbl-b is a homologue of Cbl with E3 ubiquitin ligase activity also expressed in myeloid cells (Thien and Langdon 2005). Mice with either Cbl or Cbl-b protein

depletion are normal while with loss of both Cbl and Cbl-b result in severe myeloproliferative disease (Naramura, Nandwani et al. 2010), suggesting that Cbl and Cbl-b redundantly control myeloid cell growth.

Cbl has a mild effect in regulating macrophage proliferation by slowing CSF-1R internalization from the cell surface (Lee, Wang et al. 1999). However, the role of Cbl-b in controlling macrophage growth and CSF-1R traffic and signaling is unknown. We hypothesize that Cbl and Cbl-b share overlapping functions in controlling macrophage growth through regulating CSF-1R traffic and CSF-1R signaling.

1.2 Macrophages, CSF-1, CSF-1R

1.2.1 Macrophages

Macrophages are cells of the innate immune system that differentiate from myeloid lineage progenitors in the bone marrow and peripheral tissues (Weischenfeldt and Porse 2008). Macrophages residing in different tissues are given different names, such as microglial cells in brain, Kuppffer cells in liver, alveolar macrophages in lung, peritoneal macrophages in peritoneum, and Langherhan cells in skin (Epelman, Lavine et al. 2014). Mature macrophages located in different tissues can sense microbes and cell debris and are able to phagocytose (eat) targets. Macrophages are crucial for maintaining tissues in a healthy state by fighting (guarding) against bacterial infections, clearing apoptotic cells. Macrophages are also involved in disease states such as promoting cancer metastasis (Wynn, Chawla et al. 2013).

1.2.2 CSF-1

Colony stimulating factor 1 (CSF-1), also known as macrophage colony-stimulating factor (M-CSF), is the primary growth factor for macrophages(Mouchemore and Pixley 2012). CSF-1 encoded by a single gene that produces three protein isoforms by alternative post-transcriptional splicing and post-transcriptional cleavage: secreted proteoglycan, secreted glycoprotein and cell surface protein (Alterman and Stanley 1994). The active form of CSF-1 circulating in tissues is mainly the proteoglycan disulfide linked. A wide variety of cells produce CSF-1, including fibroblasts, monocytes, activated macrophages, secretory epithelial cells of endometrium, endothelial cells activated by LPS or cytokines, and bone marrow stromal cells. CSF-1 is important for macrophages differentiation, growth and survival and is elevated at sites of inflammation (Stanley and Chitu 2014).

1.2.3 CSF-1R

CSF-1R, also known as CD115 (Cluster of Differentiation 115), is a transmembrane protein of 150-170 kDa encoded by oncogene c-fms (Stanley and Chitu 2014). CSF-1R is a member of the platelet-derived growth factor receptor family of receptor tyrosine kinases. The extracellular domain of the CSF-1R contains five immunoglobulin-like domains that are highly glycosylated. The intracellular tail contains a transmembrane domain, and an intracellular tyrosine kinase domain that is split into two halves by a kinase insert segment (Figure 1.1) (Stanley and Chitu 2014). Although CSF-1R has two known ligands (CSF-1 and IL-34) that complimentary activate CSF-1R (Wei, Lin et al. 2008), CSF-1R null mice have similar phenotype as

MCSF knock out mice (Dai, Ryan et al. 2002), suggesting that CSF-1R appears the only receptor for CSF-1 (Stanley and Chitu 2014). CSF-1R mediates most, if not all, of the biological effects of CSF-1. In the following, CSF-1R activation by CSF-1 binding will be illustrated.

1.2.4 CSF-1R activation and signaling in macrophages

CSF-1R upon CSF-1 binding dimerizes, which activates its tyrosine kinase activity and leads to phosphorylation tyrosine residues of the intracellular domain(Stanley and Chitu 2014). The phosphotyrosine residues provide sites for signaling molecules to bind and thus induce signaling transduction that lead to gene expression change in nucleus, lead to macrophages growth, survival, and differentiation(Figure 1.1) (Mouchemore and Pixley 2012). Here I will summarize the CSF-1R's phosphorylation residues after CSF-1 activation, and the major signaling pathways induced by CSF-1R activation.

1.2.4.1 CSF-1R phosphorylation, adaptors and downstream signaling molecules in response to CSF

CSF-1R has 20 tyrosine residues on the intracellular domain, and 8 of them are phosphorylated when the CSF-1R is activated in response to CSF-1 (Bourette and Rohrschneider 2000). Most of the phosphorylated tyrosine residues provide binding sites for known downstream signaling molecules that mediate CSF-1 response (Table 1.1).

The relevant phosphorylated tyrosine residues to the thesis are Y559, Y697, Y721, Y921 and Y974 in mouse. CSF-1R Y559 is the first tyrosine being phosphorylated in response to CSF-1. Phosphorylated Y559 at the juxatamembrane section interacts with SH2 domains from Src family kinases (SFKs), which phosphorylate associated proteins including PI3K, Cbl and guanine-nucleotide exchange factors(GEFs) for Rho GTPases. Phosphorylation of Y559 is necessary for the activation of SFK/Cbl/CSF-1R ubiquitination pathway while also is required for the other tyrosine residues phosphorylation on CSF-1R (Xiong, Song et al. 2011, Yu, Chen et al. 2012). Phosphorylated Y697 along with a XNX in C-terminal end of the kinase insert segment interacts with Grb2. Grb2 links CSF-1R to the Ras/Extracellular signal-regulated kinase(ERK) by providing a docking site for SOS; Meanwhile Grb2 also recruits Cbl association to CSF-1R through a SH3 domain (Dey, She et al. 2000). Phosphorylation of Y721 of the kinase insert section activates PI3K/Akt or PLCγ2 pathway, these two pathways cooperate to mediate CSF-1differentiation (Bourette, Myles et al. 1997, Kelley, Graham et al. 1999, Chang, Hamilton et al. 2009, Lee 2011). Phosphorylation of Y921 in the C-terminal domain forms a second site for the adaptor protein Grb2. Y921 appears to dominant Y697 in transducing Grb2-dependent growth factor signals (Mancini, Niedenthal et al. 1997). Y974 is phosphorylated in response to CSF-1 and is a binding site for Cbl. Macrophages express Y974F mutant spread poorly, suggesting that Y974 mediated Cbl recruitment is required for both cytoskeletal remodeling and formation of membrane protrusions (Yu, Chen et al. 2008).

The activation of CSF-1R also leads to phosphorylation of many effector proteins important for signaling and traffic (Pixley and Stanley 2004). Table 1.2 lists the identified tyrosine phosphorylation proteins contain adaptors including Cbl, kinases including PLCγ, SFKs, PI3K, some phosphatases, GEFs and transcriptional factors including STAT1, STAT3, STAT5.

In conclusion, phosphorylation of tyrosine residues on CSF-1R result in activation of CSF-1R, those tyrosine residues are essential for recruiting different signaling molecules, resulting in phosphorylation of the effector proteins important for activation of signaling pathways that will be discussed in the following.

1.2.4.2 Signaling pathways following CSF-1R activation by CSF-1

Phosphorylation of effector proteins physically recruited by activated by CSF-1R initiate multiple signaling which fit the classical RTKs signaling transduction pathways (Figure 1.2). Four of them are well characterized: Mitogen-activated protein kinase cascades (MAPKs) pathway, the lipid kinase phosphatidylinositol 3 kinase (PI3K) pathway, Signal Transducers and Activator of Transcription(STAT), and the phospholipase Cγ (PLCγ) pathway (Katz, Amit et al. 2007). These pathways result in further post-translational modification of target proteins, as well as activation of transcriptional factors, and lead to cellular alterations(Katz, Amit et al. 2007).

1.2.4.2.1 The PLCγ pathway

PLCγ binds to phosphotyrosine of activated receptor via its SH2 domain, and is phosphorylated on its tyrosine by activated receptor (Figure 1.3). The phosphotyrosine, along with its translocation to the plasma membrane leads to enzyme activation of PLCγ. Activated PLCγ hydrolyzes phosphatidylinositol 4, 5 bisphosphate (PIP2) to diacylglycerol(DAG) and inositol(1,4,5) trisphosphate (IP3), which leads to Ca^{2+} release to cytosol. DAG and Ca^{2+} activate protein kinase C family proteins, which phosphorylate various effector proteins, Meanwhile, cytosolic Ca^{2+} also activate Ca^{2+}-dependent protein kinases and phosphatases(Katz, Amit et al. 2007). For CSF-1R, PLCγ2 pathway activated by CSF-1R Y721 phosphorylation promotes macrophages differentiation (Junttila, Bourette et al. 2003).

1.2.4.2.2 The PI3K/AKT pathway

The PI3K/AKT pathway is important for macrophage proliferation, differentiation, and survival. PI3K contains two subunits: the p85 regulatory subunit harboring two SH2 domains, and the p110 catalytic subunit. PI3K is activated by binding of its p85 subunit to the phosphorylated tyrosine of RTK. Alternatively, PI3K is recruited to plasma membrane by activated small G protein Ras. Activated PI3K induces PtdIns(3,4,5)P_3 (PIP3) formation at the inner leaflet of the plasma membrane, which recruits various proteins, such as PDK1 and AKT/PKB containing phospholipid binding domains. AKT recruited to plasma membrane is activated by PDK1 and PDK2, which leads to subsequent phosphorylation of proteins, including major effectors of apoptosis, and several transcriptional factors (Jones 2000). Here,

phosphorylated Y559, Y697, Y721 of CSF-1R could contribute to activating AKT pathway (Pixley and Stanley 2004).

1.2.4.2.3 The STAT signaling pathway

Cytoplasm STAT family transcriptional factors may be phosphorylated by RTKs directly, which results in the dimerization of STAT and translocation to nucleus (Katz, Amit et al. 2007). STAT signaling pathway activates transcription of genes involved in cell proliferation. CSF-1R activation is shown to directly activate STAT1, STAT3, STAT5 involved in STAT pathway (Yeung and Stanley 2003).

1.2.4.2.4 The MAPK signaling pathways

The MAPK pathway is a signaling cascade in which the MAPK elements are activated upon tyrosine and threonine phosphorylation catalyzed by dual-specificity kinases (MAPKKs, MEK) at the Thr-Xxx-Tyr motif in the activation loop of the kinase domain. MAPKKs are regulated by serine/threonine phosphorylation within a conserved motif activated by various upstream activators, including kinases and small GTP-binding proteins. The MAPK pathway is shared by four distinct cascades: the extracellular signal –related kinases (Erk1/2), Jun amino-terminal kinases (Jnk2/3), p38-MAPK and ERK5. Among those signaling cascades, growth factors are the major regulators of Erk1/2 signaling cascade (Katz, Amit et al. 2007).

The Erk1/2 activation process by activated receptor is schematically shown (Figure 1.4). It is initiated via SH2 domain of Grb2 recruitment to phosphotyrosine residue. SH3 domain of Grb2 interacts with the guanine nucleotide exchange factor, Sos, which is recruited to the vicinity of the plasma membrane, where it promotes the active GTP bound active form of Ras. GTP-bound Ras then binds and activates Raf

(MAPKKK) (Kyriakis, App et al. 1992), which induces the phosphorylation of serine residue in the activation loop of Mek to form Mek1/2 (MAPKK). Mek1/2 then activates Erk1/2 to active form by phosphorylation of adjacent threonine and tyrosine residues, spaced by a glutamic acid at the activation loop (Yoon and Seger 2006). Erk1/2 activation phosphorylates cytoplasmic and cytoskeleton proteins, and also various transcriptional factors including Sp1, E2F, Elk-1 and AP-1 after its translocation to nucleus. Phosphorylated Y697, Y721, Y921 of CSF-1R could all possibly contribute to MAPK pathway (Pixley and Stanley 2004).

In summary, the multiple phosphorylation sites of CSF-1R lead to different signaling pathways that are just the major highway of information flow, and between them, one pathway possibly connects with another. Also, the signaling pathways are regulated temporally and spatially according to the location and stability activated CSF-1R that will be discussed in the following.

1.3. Cellular membrane compartments in macrophages

Cells internalize fluid, macromolecules, plasma membrane components, and particles by invagination of the plasma membrane and form vesicles, endosomes or vacuoles through membrane fission. In mammalian cells, internalized cargos include a wide range of nutrients and their carriers, receptor-ligand complexes, fluid, lipids, membrane and membrane proteins, extracellular-matrix components, cell-debris, bacteria, and virus etc. Internalized cargos are sorted, recycled, stored, or degraded by trafficking through endosomes (Huotari and Helenius 2011). Endosomes are cellular compartments that contain a recycling circuit for plasma membrane components and

their ligands, a degradative system for digestion of macromolecules, and a unidirectional pathway for transporting fluid and selected membrane components to degradative system (Huotari and Helenius 2011).

Cargos after endocytosis follow different routes and are dynamically sorted through different for recycling to the cell surface or target to lysosome for degradation. In mammalian tissue culture cells, endosomes in general are categorized to early endosomes (EEs), Late endosomes (LEs), and lysosomes, and EEs, LEs, and lysosome provide the classical endocytic pathway.

1.3.1 Early endosomes(EEs)

EEs are the compartment formed from primary endocytic vesicles fuse with each other in the cell cytosol and they are the main sorting station in the endocytic pathway (Huotari and Helenius 2011). EEs in cytosol dynamically acquire functional proteins to their membrane surface. One of the proteins complex, Rab5 together with Vps34/p150, a type III phosphoinositol 3-kinase [PI(3)K] along with its product phosphoinositide (PI) PtdIns(3)P define the identity of EEs. EEs are slightly acidic with pH of 6.8 to 5.9 and are relatively small and move through microtubules in cells (Vonderheit and Helenius 2005).

1.3.2 Late endosomes(LEs)

Mature LEs are morphologically round or oval and have a diameter of 250-1000nm (Bayer, Schober et al. 1998). The limiting membrane of LEs contains LAMP1 and the lumen contains acid hydrolases, and the pH of lysosome ranges from 6.0-4.9

(Maxfield and Yamashiro 1987). LEs formed at the cytoplasm peripheral area traffic centripetally toward nuclear area in a unidirectional death pathway where they fuse with each other to form larger endosomes and fuse in a kiss and run transiently and eventually fuse with lysosomes (Luzio, Pryor et al. 2007). LEs contain mannose-6-phosphate receptors, tetraspanins, and SNARES that may escape degradation after fusion with Lysosome. Lysosome Consists of a collection of vacuoles of heterogeneous composition, morphology, and density due to the diversity of cargos, cargo degradation variance and the existence of other feeder pathways of which the classical endosome pathway is a major one (Huotari and Helenius 2011).

1.3.3 Lysosome

Lysosome is an essential location in cell that contains lots of hydrolases for degradation process. Also, lysosome serve as a storage for preserved membrane protected by LAMPs, and other substances resistant to degradation (Huotari and Helenius 2011).

EEs, LEs, Lysosomes are scattered and undergo dynamic and continuous protein exchange, transformation, fusion and fission, provide the classic traffic route for cargos. Of the cellular cargos, receptor tyrosine kinases (RTKs) are special due to that they carry signaling that is important for many cell functions, for instance, CSF-1R as one of RTKs, is able to initiate the signaling important for cell growth and other functions (Figure 1.1, 1.2) (Dey, She et al. 2000, Roepstorff, Grovdal et al. 2008). Different RTKs take different traffic route, and the same RTKs possibly take different

traffic route in different cell types and triggers (Madshus and Stang 2009). Based on this classical endocytic pathways, in the following I will illustrate the possible traffic routes taking by RTKs including endocytosis, endocytic traffic, and degradation.

1.4. Receptor endocytosis, endocytic traffic and degradation

Receptors Tyrosine kinases (RTKs) on the plasma membrane mediate signaling cascades that direct the cell either turnover extracellular stimuli to decrease extracellular stimuli concentration, terminate receptor signaling from plasma membrane by endocytosis. Receptors after endocytosis transport through the endocytic pathway, then are targeted for degradation in lysosome or being recycled back to plasma membrane.

1.4.1 Endocytosis

Endocytosis is an active transport during which cell transport molecules into the cell. In general, there are two endocytosis mechanisms upon receptor activation including Clathrin Mediated Endocytosis (CME), and Clathrin independent Endocytosis (CIE), some of the pathway are constitutive, while others are triggered by specific signals or hijacked by pathogens (Mayor, Parton et al. 2014). In different cells models and different receptors, different endocytosis pathway can be initiated.

1.4.1.1 Clathrin Mediated Endocytosis (CME)

CME is well recognized in the downregulation of transmembrane receptor signaling transduction. CME is induced within minutes of ligand recognition of receptors and

forms a coated pit mediated by a cytosolic protein clathrin on the inner leaflet of the plasma membrane (Figure 1.5). The pit buds into the cell produces small endosomes (~100nm in diameter). CME happens in all cells and the CME process mediates many receptor-ligand complex internalization (Marsh and McMahon 1999). By doing this, the cell not only brings in cells with a small area of plasma membrane, but also membrane proteins and small volume of extracellular fluid.

1.4.1.2 Clathrin independent Endocytosis(CIE)

CIE refers to several endocytosis mechanisms do not use clathrin for bringing material into the cell (Figure 1.5), including small scale endocytosis, macropinocytosis and phagocytosis. They differentiate from each other by their mechanisms, kinetics of the endocytic vesicle formation, associated molecular machinery, and cargo destination (Mayor, Parton et al. 2014). Small scale endocytosis form heterogeneous population of small size vesicles/endosomes (<200nm), while macropinocytosis and phagocytosis internalize large volume of extracellular material and large area of plasma membrane, thus produce larger diameter compartments, namely macropinosomes and phagosomes, respectively. These different CIE pathways may share some of the same molecular machinery, especially those use actin in membrane rearrangement (Bohdanowicz and Grinstein 2013).

1.4.1.2.1 Small scale CIE

Small scale endocytosis can be classified by the requirement of dynamin during the process, thus are divided to dynamin dependent and independent endocytosis (Figure

1.5). The other simple classifier is the presence or absence of a coat on the formed pit (Mayor and Pagano 2007). Among the CIE pathways, the best characterized one is the dynamin-dependent caveolar endocytosis (Mayor, Parton et al. 2014). Recently, a fast-acting tubulovesicular endocytic pathway independent of clathrin and AP2 marked and controlled by a membrane remodeling protein named endophilin (Boucrot, Ferreira et al. 2015).

1.4.1.2.2 Macropinocytosis and phagocytosis

Macropinocytosis happens when receptors response to growth factors, in which, plasma membrane ruffles (effect of actin polymerization) and closes at their distal margins to gulp large volume of extracellular fluid and forms macropinosomes (Figure 1.5) (Swanson 2008). Phagocytosis is initiated by receptor (Fc receptor, recognize antibodies) mediated ingestion of cell particles, result in plasma membrane and the actin surrounding the target particles, forming intracellular compartment named phagosomes (Swanson 2008). Macropinocytosis simply differs from phagocytosis by their ability to form macropinosomes without particles. For macrophages, macropinocytosis and phagocytosis are important for their immune function in clearing antigen.

Due to that macropinosomes are more relevant to the thesis comparing to phagosomes, here I will illustrate a little more about macropinosomes. Macropinosomes are large endocytic vacuoles (0.2-5um) that form during Macropinocytosis, which is an actin-dependent process initiated by plasma membrane rearrangement. Due to large dimension of macropinosome, they can be differentiated from other small endosomes

by labeling with fluorescent labeled dextran (High molecular weight, 40-150kD). Macropinosomes are formed frequently in macrophages and are major compartments of non-selectively uptake of membrane, nutrients, fluid, and antigens (Swanson 2008, Lim and Gleeson 2011). Large size Macropinosomes are induced in response to CSF-1 stimulation in bone marrow derived macrophages (Racoosin and Swanson 1989). Once formed, macropinosomes undergo a maturation process, during which, macropinosomes shrink, acquire, and lose different endocytic protein markers while move toward the lysosome. During the early stage of macropinosome maturation, macropinosomes share some EEs markers including transferrin receptors, EEA1 in bone marrow macrophages. Very quickly (2-4min CSF-1 stimulation), macropinosomes are devoid of transferrin receptors and acquire Rab7, a marker of LEs. Macropinosomes then fuse with the tubular lysosome (Racoosin and Swanson 1993).

In summary, the possible endocytosis mechanism for RTKs are CME, CIE. CME is the major endocytosis mechanism and well-studied for many RTKs (Madshus and Stang 2009). For CSF-1R, it is shown that CSF-1R internalized to small size endosomes and internalization of CSF-1R is attenuated by dynamin inhibitor (Lou, Low-Nam et al. 2014), suggesting that CSF-1R possibly take CME, or small scale CIE pathways instead of large scale CIE. Next, I will discuss the endocytic traffic after RTKs endocytosis.

1.4.2 RTKs endocytic traffic and degradation

After internalization, signaling receptors are transported to endosomes either to be recycled or degraded. The specific motifs and interactions determine receptors to be recycled or to be degraded. Many signaling receptors are targeted to endocytic degradation pathway and are efficiently degraded in late endosomes and lysosomes. Ubiquitination of receptors (will explain more later) after ligand activation is reported to direct receptors to multi-vesicular bodies (MVB) mediated by a set of endosomal sorting complex required for transport (ESCRT) (Welchman, Gordon et al. 2005). The ESCRT sorting complex are collectively referred to ESCRT0, ESCRTI, ESCRTII, ESCRTIII. One of the ESCRT0 component is HRS-STAM (hepatocyte growth factor-regulated tyrosine kinase substrate-signaling transducing adaptor molecule) complex. HRS directly interact with ubiquitinated receptors through ubiquitin binding domain and recruit receptors, while it also interacts and thus recruit ESCRTI complex. These events will lead to the formation of MVB that contain receptors in late endosome or lysosome (Figure 1.7), Consequently, ubiquitinated receptors will be degraded in MVB of late endosome and lysosome and inaccessible for downstream signaling molecules (Miaczynska 2013). Again, EGFR is one of the best studied RTKs that using MVB as a deactivation mechanism in late endosomes. CSF-1R takes macropinosome as an route to be deactivated and is deactivated by intraluminal budding into the lumen of macropinosome (Lou, Low-Nam et al. 2014), suggesting that CSF-1R is most likely being deactivated through a new MVB platform (macropinosome), however, the molecular mechanism is not clear.

In terms of receptor fate, CSF-1R is known to not be recycled and is degraded in lysosome in macrophages (Lee, Wang et al. 1999, Lou, Low-Nam et al. 2014), however, not all receptors will be targeted for degradation in lysosome depending on the threshold of ligand concentration. For example, EGFR stimulated with different concentration of EGF will have different route. Low concentration of EGF will trigger the CME process and recycling of EGFR, while high concentration of EGF confer EGFR to the CIE and the degradation of EGFR in lysosome (Sigismund, Algisi et al. 2013),

In summary, RTKs activated by their ligand endocytosis and traffic to EEs, LEs and are degraded in lysosome or recycled back to plasma membrane, different from this classical route, CSF-1R undergoes a novel membrane trafficking route that involves macropinosome most likely through ESCRT machinery mediated MVB process to degrade CSF-1R in lysosome (Guilbert and Stanley 1986, Lou, Low-Nam et al. 2014). From here, I will illustrate how the different RTKs endocytic traffic outcome will affect the signaling of RTKs.

1.4.3 Receptor tyrosine kinases (RTKs) endocytic traffic and the regulation on Receptor signaling

Many signaling pathways of RTKs from ligand activation, including MAPK pathway as well as PI3K pathway is initiated from the plasma membrane (Brankatschk, Wichert et al. 2012, Sousa, Lax et al. 2012), RTKs endocytosis is thought to initiate termination of the signaling cascade (Roepstorff, Grovdal et al. 2008). However, it is

shown that endocytosis of activated RTKs continues signaling on intracellular endosomes and the endocytic traffic of RTK may facilitate the propagation of a subset of signaling pathways initiated at plasma membrane, or it may allow RTK activate pathways that are different from those activated from the plasma membrane through signaling proteins specifically located to endosomes (Vieira, Lamaze et al. 1996, Joffre, Barrow et al. 2011, Huynh, Kwa et al. 2012).

1.4.3.1 Endocytic traffic Regulators of RTKs signaling

1.4.3.1.1 Grb2

Grb2 plays opposing roles in regulating RTKs signaling. Grb2 is recruited to activated RTKs and facilitate RTKs signaling by activating MAPK pathway, and it also mediates RTKs downregulation in a Cbl mediated ubiquitination pathway.

1.4.3.1.2 Cbl proteins

Cbl is an E3 ubiquitin ligase typically involved in ubiquitination of RTKs and effector proteins during the RTK signaling activation process. Ubiquitination of proteins are targeted for degradation. Structurally, Cbl contains a tyrosine kinase binding domain (TKB), a Ring finger domain (RF), a proline domain (PR) (Figure 1.6).

The Cbl proteins are highly conserved gene family, and the name derives from the retroviral onco-protein v-Cbl, which is a dominant mutant dominant its cellular homolog Cbl, promoting development of B cell leukemia in mice. Mammalian

genome encodes three Cbl proteins: Cbl, Cbl-b, and Cbl-c. Cbl and Cbl-b are expressed in a wide range of tissues, Cbl-c is restricted to epithelia.

The Cbl family RING finger ubiquitin ligases (E3) are recruited to many RTKs upon RTK activation and regulate RTK turnover. Of the E3 ubiquitin ligases, Cbl and Cbl-b are enriched in hematopoietic stem cells. it contains tyrosine kinase binding domain (four-helix bundle(4H), a calcium-binding EF hand and a modified SH2 domain), RING finger domain(RF), C-terminal proline rich domain(P) and Ubiquitin Binding Association domain(UBA).

The TKB domain named due to its ability to bind to phosphotyrosine residues in multiple protein tyrosine kinases (PTK) (Meng, Sawasdikosol et al. 1999). TKB recognizes most of PTKs through the consensus sequence (N/D)XpY(S/T)XXP(Meng, Sawasdikosol et al. 1999). TKB domain is capable of multiple interactions with its binding partners. TKB domain mediate binding of Cbl to other proteins besides PTKs with varies binding mode. For example, proteins like Src-like adaptor protein (SLAP) and tubulin bind Cbl through the other domain independent of SH2 domain (Tang, Sawasdikosol et al. 1999). The L domain and RING finger domain are very conserved in Cbl family.

The RF domain of Cbl mediates the E3 ubiquitin ligase activity of Cbl, it is separated from TKB domain by the L domain. TKB conferring substrate specificity and RING bring in an E2 ubiquitin-conjugating enzyme to mediate ubiquitination and degradation of activated PTKs. The L domain is also critical for the E3 activity of Cbl. Phosphorylation of Tyr-371 and Tyr-368 at L domain was shown to involve in EGFR signaling regulation (Levkowitz, Waterman et al. 1999). Tyr mutation at these

residues constitutively activate E3 activity (Kassenbrock and Anderson 2004), Tyr-371 phosphorylation is essential for Cbl-dependent ubiquitination of Src in vitro (Yokouchi, Kondo et al. 2001). Moreover, Tyr-371 phosphorylation involves in the interaction of Cbl with PI-3 kinase (PI3K) and possibly interact with Crk in adipocytes to positively regulate glucose transport (Miura, Sajan et al. 2003, Miura, Sajan et al. 2004).

The P domain contains a number of SH3 binding motifs for signaling adaptors. The C-terminal part of Cbl proteins contains major sites of tyrosine for phosphorylation (Tyr-674, Tyr-700, Tyr-731, Tyr774), which provide binding sites for SH2 domain containing proteins(Liu, Kimura et al. 2002, Steen, Kuster et al. 2002, Grossmann, Kolibaba et al. 2004). Besides, the LZ domain in C-terminal mediates dimerization of multiple proteins thus facilitate protein-protein interaction and phosphorylation (Bartkiewicz, Houghton et al. 1999), and the UBA domain binds to ubiquitin. UBA domain is not essential for E3 ubiquitin ligase of Cbl and its role is less clear (Levkowitz, Klapper et al. 1996).

1.4.3.1.3 Ubiquitination of RTKs

Protein ubiquitination is a post-translational and reversible modification in which Ub, a 76-amino-acid polypeptide, is covalently attached to the ε-amino group of lysine residues in target proteins by E3 ubiquitin ligases(Madshus and Stang 2009). It is shown that in EGFR example that protein can be concurrently monoubiquitinated (one Ub on one lysine), multiubiquitylated (several monoubiquitylated lysine resides on one protein), and polyubiquitynated (several Ub molecules on one lysine in one

protein through each of the seven lysine residues within Ub itself) (Peng, Schwartz et al. 2003).

Among the RTKs, EGFR has been extensively studied for the function of ubiquitination in endocytosis. CSF-1R and EGFR both belong to RTKs and share very similar structure, here I will use EGFR as example to illustrate the function of ubiquitination in RTKs endocytosis and thus to infer to CSF-1R endocytosis. Ubiquitination of EGFR targets both EGFR internalization and degradation. Cbl mediated ubiquitination of EGFR is recognized by ubiquitin-binding proteins of the CME protein machinery and thus facilitates recruitment of activated EGFR to clathrin coated pits and promotes CME (Madshus and Stang 2009, Bertelsen, Sak et al. 2011). However, EGFR mutant with 15 lysine residues lacking at the tyrosine kinase domain of EGFR deplete EGFR ubiquitination but still internalizes (Huang, Goh et al. 2007). Thus the contribution ubiquitination to RTK internalization is cell type and physiological condition dependent.

1.4.4 RTKs traffic and cancer

Disrupted endocytic trafficking of RTKs is shown to play important role in roles in oncogenesis. Traffic defects lead to the wrong subcellular location and poor downregulation of EGFR are associated with enhanced signaling and may contributes to cancer development (Roepstorff, Grovdal et al. 2008).

Again, EGFR as one of the RTKs are best studied in its traffic and the finding in EGFR study can be used to guide CSF-1R research. Several oncogenes have been proposed to affect EGFR trafficking. One RhoGTPase GEFs named Vav2 has been

shown to enhance ERK, AKT signaling of EGFR by delaying EGFR internalization. Another oncogene, Cdc42 associated kinase (ACK1) interacts with ubiquitinated EGFR and facilitate the degradation of EGFR (Mahajan and Mahajan 2010). Trafficking of EGFR regulates tumorigenesis via interacting with the tumor suppressors PTEN and SPRY2 (sprouty homolog 2). Reduced SPRY2 expression causes hyper-activation of PI3K/AKT signaling and thus increased cell proliferation and invasion in prostate cancer (Gao, Patel et al. 2012). In other side, increased EGFR internalization and sustained EGFR signaling on early endosome also enhance EFGR endosome specific signaling (Winograd-Katz and Levitzki 2006).

In summary, RTKs signaling is regulated by endocytosis, and endocytic traffic due to the spatial and temporal availability of different signaling molecules on different cellular location (plasma membrane, EEs, LEs, lysosomes, macropinosomes) (Brankatschk, Wichert et al. 2012, Huynh, Kwa et al. 2012, Miaczynska 2013). Same RTK taking different traffic routes possibly have different signaling outcomes (Francavilla, Papetti et al. 2016). Next, I will illustrate in detail the novel traffic route of the CSF-1R, the possible signaling outcomes if this unique route is disrupted, and what can be investigated based on this characterized traffic route.

1.5. A novel mechanism of CSF-1R deactivation through macropinosome in bone marrow macrophages

A novel model for CSF-1R endocytosis and downregulation was reported by our lab in 2014. The CSF-1R upon binding to CSF-1 is endocytosed to small size endosomes,

and macropinocytosis triggered by CSF-1 induce nascent macropinosomes in the absence of CSF-1R. Interestingly, CSF-1R on small size endosomes transport to the lumen of macropinosome to be deactivated (Lou, Low-Nam et al. 2014) and is degraded after transport to macropinosome (Figure 1.8). Macropinosomes generated from macropinocytosis contain fluid nutrient and are usually used by cells for supporting growth or antigen clearance (Lim, Teasdale et al. 2012, Commisso, Davidson et al. 2013). However, being a platform for RTK subcellular locating, and downregulation is first reported. This traffic route is different from the classical endocytic route for other RTKs: RTKs endocytosis, appearing on EEs, then transport to LEs through intraluminal vesicle budding to be degraded in lysosome or being sorted back to plasma membrane (Sorkin and Goh 2009). In the CSF-1R traffic model, macropinosomes could possibly be a platform for specific signaling pathway of CSF-1R to prorogate, or for specific signaling pathway to be deactivated. Disrupting the tightly controlled traffic pathway could possibly leads to those "macropinosome-based" signaling pathways deregulation and cause cellular phenotype. The unique CSF-1R traffic route provide us basis and model to investigate: 1. What molecular machinery control and regulate this unique route? 2. What is the function of macropinosomes for CSF-1R signaling? 3. How is the CSF-1R transported to macropinosomes? Answering those questions is very important for the understanding macrophages function guided by CSF-1R traffic and signaling, and thus provide fundamental biology for translating research finding to clinical treatment of macrophage related diseases including chronic inflammatory, autoimmune diseases and leukemia.

FIGURES

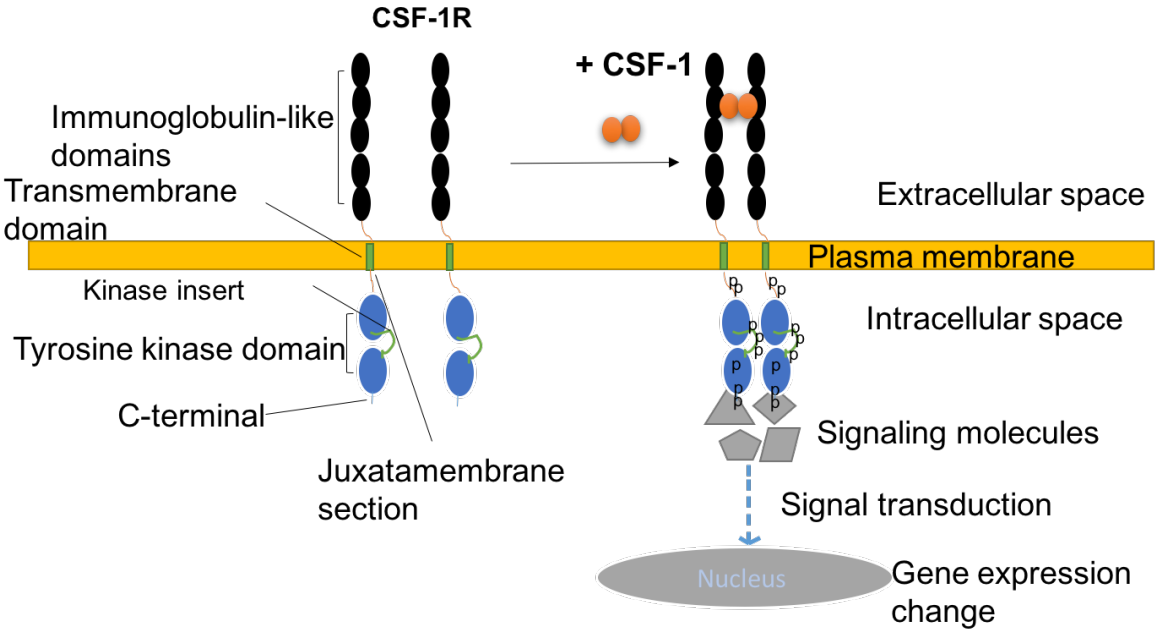


Figure 1.1 Structure diagram of CSF-1R and activation by CSF-1.

CSF-1R on cell surface dimerize upon binding to CSF-1, which activates CSF-1R kinase activity, result in tyrosine phosphorylation on intracellular kinase domains, lead to signaling transduction pathways that activate gene expression.

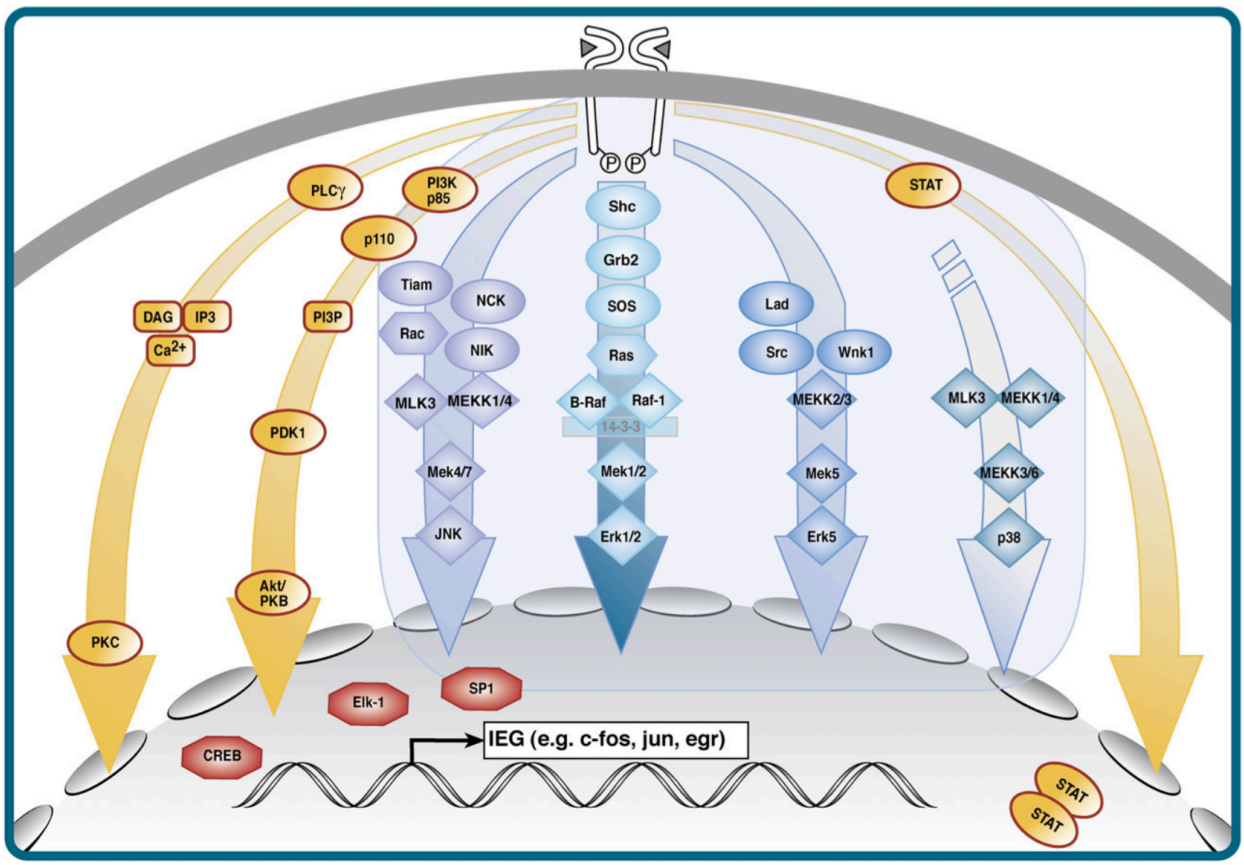


Figure 1.2 Signaling pathways initiated by activated receptor tyrosine kinases.

Ligand binding leads to RTKs activated pathways: PLCγ, PI3K/AKT pathway, MAPK pathway(including JNK, Erk1/2, Erk5, p38 pathways), STAT pathway. (Katz, Amit et al. 2007).

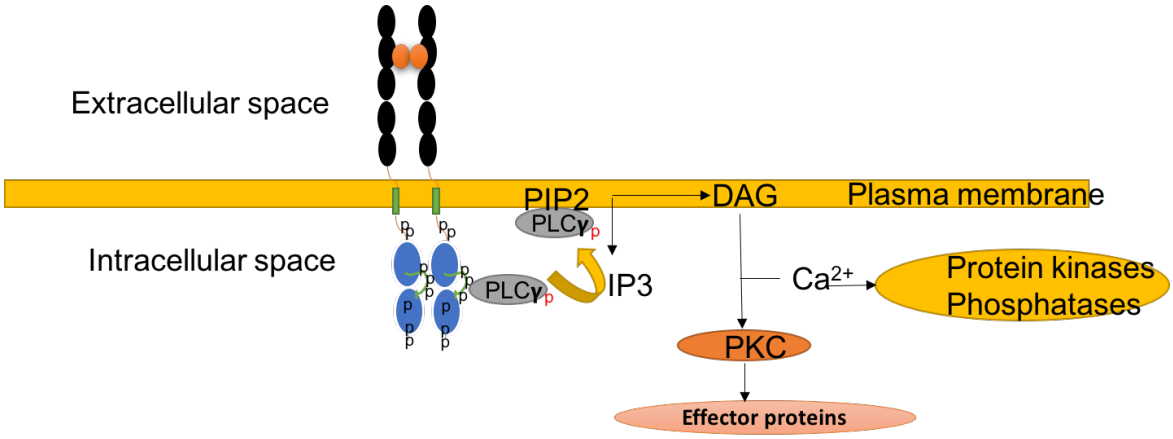


Figure 1.3 Schematic diagram of PLCγ signaling pathway.

PLCγ binds to phosphotyrosine of activated receptor via its SH2 domain, and is phosphorylated on its tyrosine by activated receptor. The phosphotyrosine, along with its translocation to the plasma membrane leads to enzyme activation of PLCγ. Activated PLCγ hydrolyzes phosphatidylinositol 4, 5 bisphosphate (PIP2) to diacylglycerol(DAG) and inositol(1,4,5) trisphosphate (IP3), which leads to Ca^{2+} release to cotyosol. DAG and Ca^{2+} activate protein kinase C family proteins, which phosphorylate various effector proteins, Meanwhile, cytosolic Ca^{2+} also activate Ca^{2+}-dependent protein kinases and phosphatases.

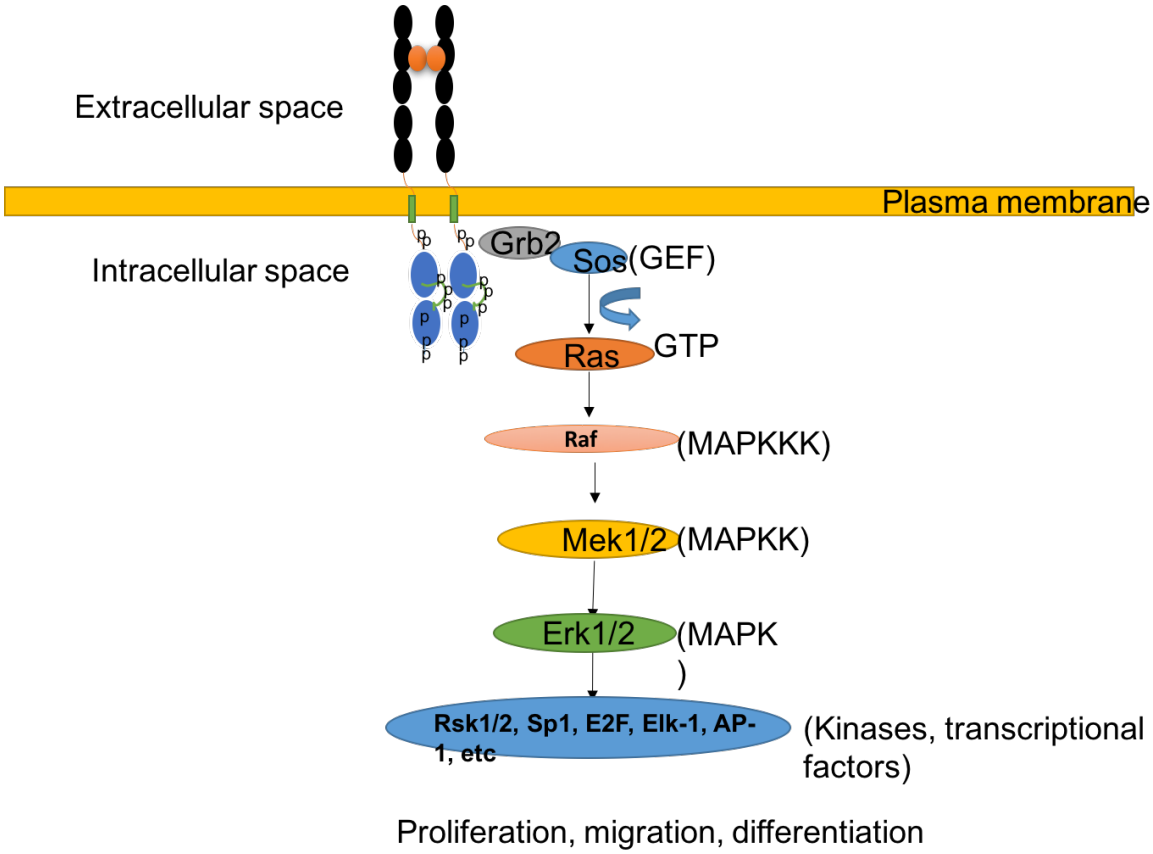


Figure 1.4 Schematic diagram of ERK signaling pathway.

SH2 domain of Grb2 recruitment to phosphotyrosine residue. SH3 domain of Grb2 interacts with the guanine nucleotide exchange factor, Sos, which is recruited to the vicinity of the plasma membrane, where it promotes the active GTP bound active form of Ras. GTP-bound Ras then binds and activates Raf (MAPKKK), which induces the phosphorylation of serine residue in the activation loop of Mek to form Mek1/2(MAPKK). Mek1/2 then activates Erk1/2 to active form by phosphorylation of adjacent threonine and tyrosine residues, spaced by a glutamic acid at the activation loop. Erk1/2 activation phosphorylates cytoplasmic and cytoskeleton proteins, and also various transcriptional factors including Sp1, E2F, Elk-1 and AP-1 after its translocation to nucleus.

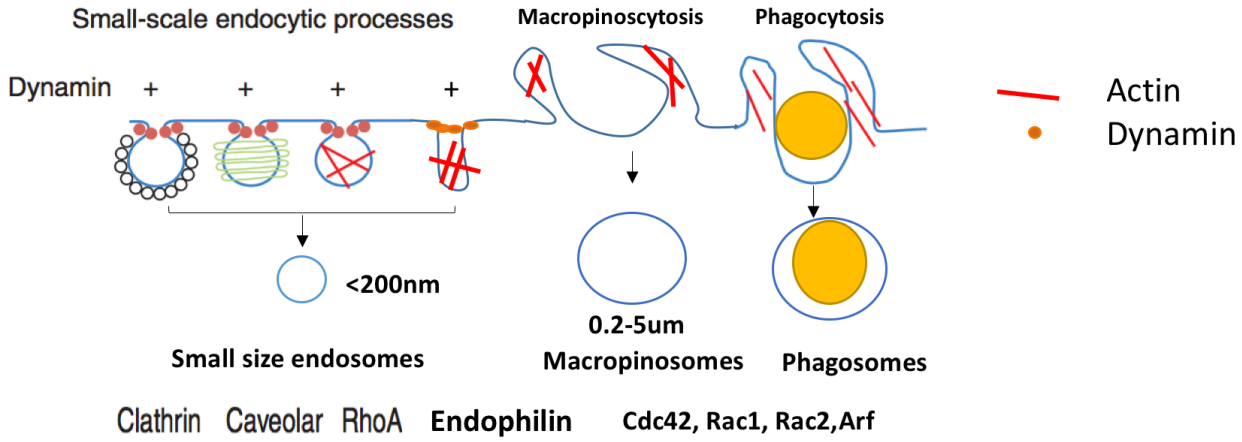


Figure 1.5 Different mechanisms of endocytosis.

Small-scale endocytic process include Clathrin mediated endocytosis(CME), and Clathrin independent endocytosis(CIE, including Caveolar, RhoA, endophilin assocated endocytosis) produces small size endosomes that are typically small than 200 nm, they require a GTPase protein Daynamin assist in membrane scission. Macropinocytosis produces macropinosomes that has large size of 0.2-5um, phagocytosis is Fc receptor mediated antigen uptake and produces the phagosomes. Reproduced from (Mayor, Parton et al. 2014)

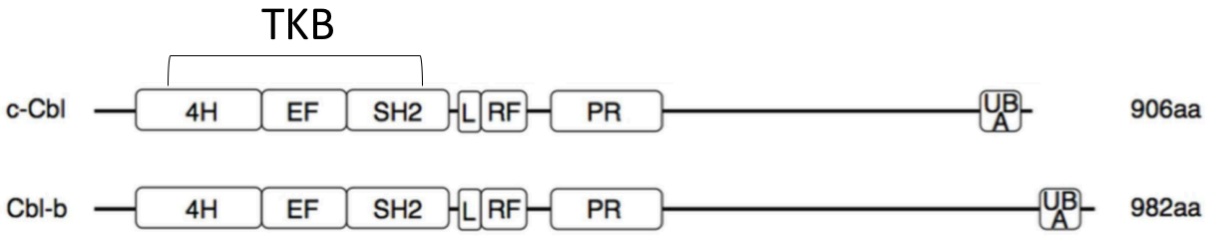


Figure 1.6 Functional domains of Cbl family proteins.

The tyrosine kinase binding domain(TKB) contains a 4-helical bundle(4H), EF-hand domain, and SH2 domain. A conserved linker region(L) spate the TKB from a RING finger (RF) domain that binds to E2 enzymes. The proline-rich domain(PR) interacts with SH3 domains of proteins in endocytosis and signaling. Cbl and Cbl-b contain an ubiquitin associated domain(UBA) that engage in ubiquitin binding and dimerization. Modified from (Lutz-Nicoladoni, Wolf et al. 2015)

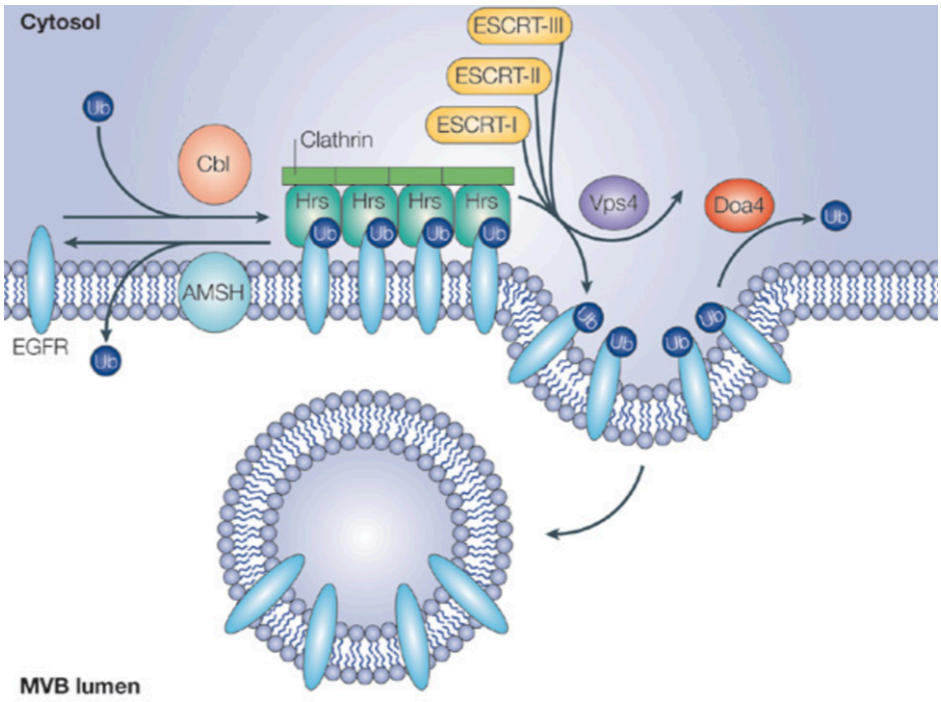


Figure 1.7 Cbl mediated EGFR ubiquitination target EGFR sort to intraluminal vesicles of MVB on endosome.

On the membrane of an endosome, Cbl mediated ubiquitination of EGFR are recognized by Hrs, then ubiquitinated EGFR is passed to ESCRT I, II, III that all have the ubiquitination binding domain, intraluminal budding mediated by ESCRT I, II, III, and Vps4, to transport ubiquitinated EFGR to the lumen of endosome, meanwhile EGFR is deubiquitinated by Doa4, an enzyme that can remove ubiquitin, this process results in formation of multi-vesicular bodies(MVB) (Welchman, Gordon et al. 2005).

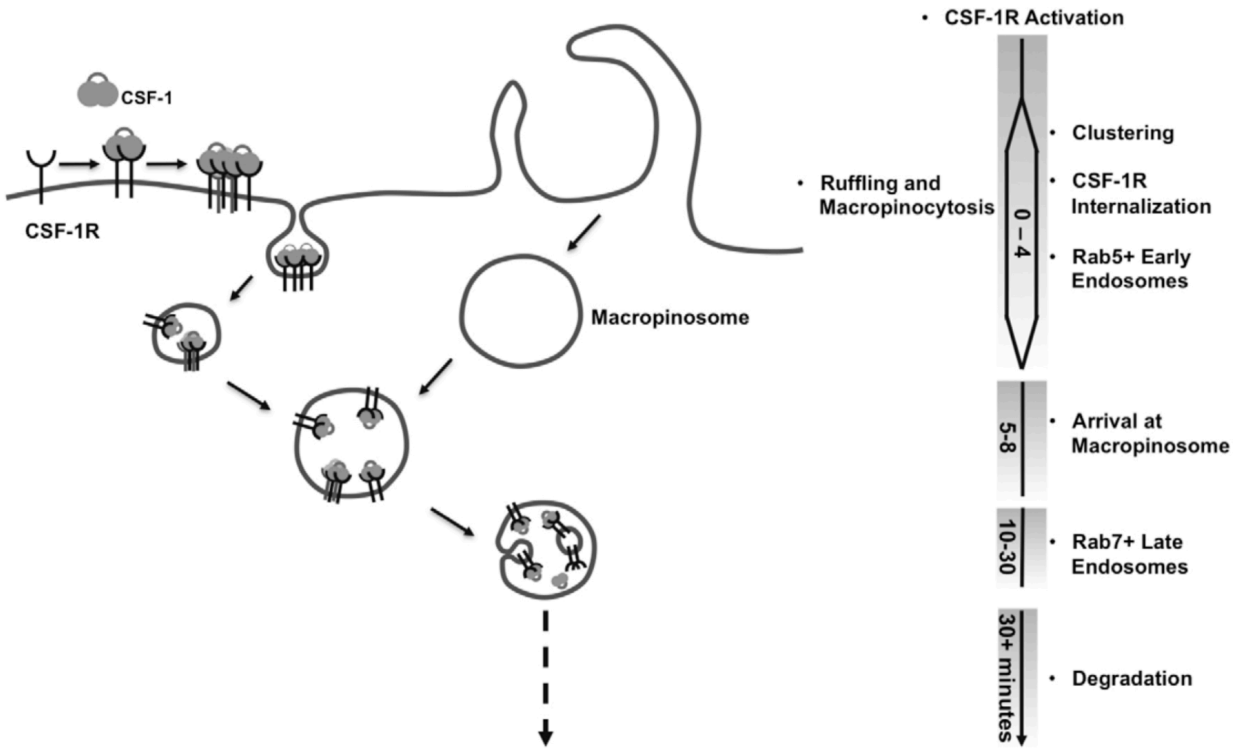


Figure 1.8 CSF-1R degradation mechanism.

Upon CSF-1 binding, CSF-1R dimerize, and endocytosis to small vesicles, meanwhile, macropinocytosis is triggered and macropinosomes form without CSF-1R present, CSF-1R is transport from endosomes to nascent macropinosomes and is degraded (Lou, Low-Nam et al. 2014).

TABLES

Table 1.1 Tyrosine phosphorylation sites on CSF-1R, adaptors that are recruited, signaling transduction pathway, and the function in mouse macrophages. Reproduced from (Pixley and Stanley 2004)

Phosphorylation sites	Adaptors	Downstream Signaling	Function
Y544	p55	unknown	Prolifertaion
Y559	SFKs	PI3K-Cbl-RhoGTPase-WASP	Cell adhesion, spreading, phagocytosis, motility
Y697	Grb2	1.Mona-Shc-Gab2-Gab3 2.Sos-Ras/Raf-1-ERK 3. Cbl-degradation	Differentiation, proliferation
Y706	N/A	STAT1	Proliferation
Y721	PI3K PLCγ	1.SHIP1-Gab3 2.PKCφ -PKare 3.ROS-ERK	Differentiation, oncogenic signaling
Y807	N/A	Activation loop, adaptors not reported	Prolifearation
Y921	Grb2	1.Shc-Gab2-Gab3 2.Cbl	Proliferation Attenuate CSF-1R signaling
Y974	Cbl	Ubiquitination	Attenuate CSF-1R signaling

Table 1.2 Tyrosine phosphorylated proteins in response to CSF-1. Reproduced from (Pixley and Stanley 2004).

Protein	Full name/Function
SFK[b,c]	Src family kinases
Grb2[c]	Adaptor
Mona[b,c]	Monocyte adaptor; adaptor
Socs1[b,c]	Suppressor of cytokine signaling-1; adaptor
PLCγ[b,c]	Phospholipase C-γ
P85PI3K[b,c]	Regulatory subunit of PI3K
Cbl[b,c]	Casita B lineage; ubiquitin ligase, adaptor
FMIP[b,c]	FMS-interacting protein; unknown fucntion
PP2A[b]	Protein phosphatase 2A
Pyk2[b]	Proline-rich and Ca^{+2} activated tyrosin kinase
MAYP[b]	Macrophage actin associated and tyrosine phosphorylated protein
Iba1[b]	Actin bundling
Gab2[b]	Grb2 associated binder-2, adaptor
Gab3[b]	Grb2 associated binder-3, adaptor
SHIP1[b]	SH2 domain containing polyinositol phosphatase-1
SHP2[b]	SH2 domain containing phosphatase-2 phosphatase-2
PKC φ[b]	Protein kinase C- φ
STAT1,3,5[b]	Signal transducers and activators of transcription 1,3,5, transcriptional factors
Vav[b]	Rho family guanine nucleotide exchange factor

[b] Tyrosine phosphorylated in response to CSF-1
[c] Directly associate with CSF-1R

Chapter II

ABSTRACT

Endocytosis and intracellular traffic regulates and shapes signaling from growth factor receptors. Here, we demonstrate that Cbl and Cbl-b have overlapping function in regulating macrophage growth by regulating the endocytosis and endocytic traffic of the CSF-1R. Analysis of bone marrow derived macrophages from $Cbl^{-/-}$, $Cbl\text{-}b^{-/-}$ or $Cbl^{-/-}/Cbl\text{-}b^{-/-}$ (double knockout, DKO) mice revealed that Cbl and Cbl-b redundantly control membrane transport and signaling of the CSF-1R. DKO macrophages, but not single knockouts demonstrated accelerated growth, mirroring the myeloprolifotirve disease of the mice. In DKO we observed a loss of ubiquitination of the CSF-1R and associated proteins following exposure of cells to CSF-1. The loss of ubiquitination correlated with slowed CSF-1R internalization and elevated AKT signaling in DKO macrophages. Previously we defined a novel endocytic pathway in macrophages in which the CSF-1R is internalized by small vesicle endocytosis and then transported to the lumen of newly formed macropinosomes where it is subsequently degraded. This pathway was fully functional in WT and single Cbl knockouts, but not in DKO macrophages, indicating that rapid transport to the macropinosome requires Cbl or Cbl-b function even though they are dispensable for CSF-1R degradation. RNA seq analysis show that the altered traffic in DKO cells correlated with altered gene expression and that a small number of genes were regulated uniquely by Cbl and no genes were uniquely regulated by Cbl-b.

INTRODUCTION

Cbl and Cbl-b are E3 ubiquitin ligases that regulate the endocytic traffic and signaling of receptor tyrosine kinases (RTK) and immunoreceptors including EGFR, PDGFR, c-Kit,

CSF-1R, TCR and FcγR (Matsuo, Hazeki et al. 1996, Erdreich-Epstein, Liu et al. 1999, Schmidt and Dikic 2005, Marois, Vaillancourt et al. 2011, Voisinne, Garcia-Blesa et al. 2016). Upon activation, Cbl or Cbl-b are recruited to phosphotyrosine residues on the cytoplasmic tail of RTKs by the adaptor Grb2 or through direct interaction mediated by their tyrosine kinase binding domain (Miyake, Lupher et al. 1997, Ettenberg, Keane et al. 1999, Pennock and Wang 2008). Cbl and Cbl-b mediated ubiquitination of RTKs controls the subcellular location and degradation of receptors thereby regulating RTKs signaling. In addition to ubiquitin ligase activity, Cbl can act as an adaptor/scaffold protein by associating with a variety of SH2 and SH3 containing singling proteins including Crk, Fyn, Lck, PI3K, and Shc (Miyake, Lupher et al. 1997).

The CSF-1R is an RTK that mediates most of the effects of CSF-1 in promoting differentiation, growth and immune functions of macrophages. Although rare, aberrant CSF-1R activity can contribute to the pathogenesis of human cancer and as a model RTK understanding its traffic and signaling will provide insight into its two closest and high frequency oncogenes c-Kit and Flt3 (Ridge, Worwood et al. 1990, Sapi 2004). Recently we demonstrated that in macrophages, the endocytosis and degradation of the CSF-1R is mediated by a novel pathway that involves small-vesicle endocytosis followed by subsequent traffic to macropinosome that are form in response CSF-1R signaling (Lou, Low-Nam et al. 2014). Although advances have been made toward CSF-1R endocytic traffic and signaling regulation (Lee, Wang et al. 1999, Huynh, Kwa et al. 2012, Lou, Low-Nam et al. 2014), the understanding of CSF-1R endocytic traffic and signaling remains to incomplete.

Cbl gene mutations have been identified in more than 10% patients with juvenile myelomonocytic leukemia, which is a type of myeloproliferative disease with excessive proliferation of myeloid cells (Loh, Sakai et al. 2009, Makishima, Cazzolli et al. 2009, Sanada, Suzuki et al. 2009). Mice lacking Cbl or Cbl-b in the hematopoietic compartment are non-lethal, while mice carrying the double knock out for Cbl and Cbl-b develop severe myeloproliferative disease and do not typically live past average life-span of 65 days (Naramura, Nandwani et al. 2010). The myeloproliferative disease suggests that Cbl and Cbl-b function redundantly in myeloid cells.

Here we have analyzed macrophages produced from WT, Cbl knock out, Cbl-b knock out and Cbl/Cbl-b double knock out(DKO) mice. Cbl and Cbl-b are found to regulate CSF-1R internalization and transport to macropinosome thus CSF-1R signaling possibly through their redundant E3 ubiquitin ligase function, but not CSF-1R degradation.

MATERIALS and METHODS

Reagents

Dulbecco's Modified Eagle Medium (#SH30022, GE Healthcare Life Sciences), fetal bovine serum (#SH30088, GE Healthcare Life Sciences) and L-cell supernatant were used for cell culture. Recombinant CSF-1 (#:574806, Biolegend) was used for CSF-1 stimulation time course experiment. Alamar blue reagent (#DAL1025, ThermoFisher Scientific) was used for growth assay of macrophages. CSF-1R antibody (#SC692, Santa Cruz Biotechnology), CSF-1R antibody(AFS98, eBioscience), p-ERK antibody (#9101,

Cell Signaling Technology), pAKT antibody (#4691, Cell Signaling Technology), p-Tyrosine antibody (#P-Tyr-100, Cell Signaling Technology), and pCSF-1R(Y721) antibody (#49C10, Cell Signaling Technology) were used for western blot or immunofluorescence, goat anti-Rat Dylight 594 and Goat anti-Rabbit Dylight 488 conjugated secondary antibodies (ThermoFisher Scientific) were used for detecting primary antibodies. Texas-Red Dextran(70 kD) (#D1864, ThermoFisher Scientific) was used to label macropinosomes. GeneJET RNA Purification Kit (#K0732, ThermoFisher Scientific) and Turbofree DNA free Kit was from (# AM1907, ThermoFisher Scientific) were used to purify total RNA for RNA seq. ToxinSensorTM Gel Clot Endotoxin Assay Kit (L00351, GenScript) was used to check medium contamination from LPS.

Generation of WT, Cbl$^{-/-}$, Cbl-b$^{-/-}$, and DKO bone marrow derived macrophages(BMDM)

WT, Cbl$^{-/-}$, Cbl-b$^{-/-}$, and DKO bone marrow derived macrophages were differentiated with CSF-1 containing medium from bone marrow isolated from the bone of WT mice, Cbl knock out, Cbl-b knock out , Cbl and Cbl-b double knock out(DKO) in the hematopoietic compartment of the mice(Naramura, Nandwani et al. 2010).

Growth assay of BMDM

Bone marrow cells were isolated from bone and plated on 10 cm non-tissue treated dishes or 6 well plates in DMEM+10% FBS supplemented with CSF-1 (100ng/mL). For growth assay between Day5-25, alamar blue assay was applied. Day 5 macrophages were plated on 96 well plates in CSF-1 (100ng/mL) DMEM+10% FBS in 6 replicates with starting cell number of about 1,000, cells were re-plated after reaching 90% confluence. Alamar

blue dye was applied to measure cell number every other day from Day 5 to Day 25. Alamar blue dye reaction with cells was done in 37 ^{o}C CO_2 incubator for 40 min. Microplate Reader (BioTek Synergy 2) was used with excitation of 550 nm and emission at 585 nm to measured reaction products and calculate cell number, data was normalized to number at Day 5. For growth assay from Day 81 to Day 94, macrophages plated on 6 well plates were counted for at least 10 field of view with triplicate wells, and the cell number was recorded and plotted against days of cell age.

Immunofluorescence

Intracellular targets in cells were fixed with 4% (PFA), and permeabilized with 0.1% Triton x-100 at room temperature for p-ERK or fixed and permeabilized with cold methanol at -20 ^{o}C for CSF-1R, phosphorylated Tyrosine, Hrs. Surface CSF-1R in cells was fixed with 2% PFA and not permeabilized (Lou, Low-Nam et al. 2014). Following that, samples were blocked with 2.5% BSA in PBS. Primary antibody was added to incubate with sample for 1h at room temperature or overnight at 4^{o}C, secondary antibody was then added followed by secondary antibody incubation for 1h at room temperature.

Western blot

Macrophages were lysed by M-Per solution at different times following CSF-1 stimulation, lysate was centrifuged for 10 min at 12,000 rpm at 4^{o}C. The total protein was determined by BCA (#P123221, ThermoFisher Scientific) and 20 ug total protein was loaded on a 8% SDS-PAGE gel, at 150V for 1 hour. Protein Transfer to nitrocellulose membrane was achieved at 300 amps for 30 min. The membrane was

blocked in 5% BSA in PBST or TBST solution at room temperature for 30 min, then primary antibody was incubated overnight at 4 ^{o}C followed by secondary antibody at room temperature for 1h.

Microscopy and data analysis

Immunofluorescence images were acquired on a Leica CTR4000 inverted microscope equipped with an QICAM 12-bit color camera via Micromanager software and 60X oil lens. Exposure times were optimized according to sample brightness and held constant for all samples within a staining group. imaging parameters were kept constant for each experimental condition. Cell Nucleus was masked with HCS (#H10325, ThermoFisher Scientific), Pholloidin-dylight647 (#A22287, ThermoFisher Scientific) that can labeling actin cytoskeleton was used to mask cells. CellProfiler (Broad Institute) was used to measure intensity from individual cells over the region defined by the phalloidin stain and HSC.

Macropinosomes were labled by incubating cells in Texas red-Dextran (70 kD) and CSF-1. Sequential immunostaining of the CSF-1R was done on either a custom-built Till iMic microscope (FEI) using 60 X water objective (Lou, Low-Nam et al. 2014), or on a High content microscope (Imagexpresspro XLS, Molecular Devices) using 60X air objective.

RNA Sequencing by Illumina

RNA-seq was performed using indexed libraries prepared using the Illumina Truseq kit, and samples were sequenced by RNA-seq and 150-nt single-end reads were obtained.

Reads were filtered based on quality control set up and mapped to Mouse genome . Reads were mapped to mouse genome using RNA-seq pipeline in CLCBio (QIAGEN) and counts were normalized to calculate expression level as RPKM. Raw total exon counts were used to identify significantly differential expressed genes in macrophages across different genotypes using R/Bioconductor's Limma package (Ritchie, Phipson et al. 2015) with cutoff values of fold change>1.5, false discovery rate calculations were calculated based on the Benjamini-Hochberg method with a cut off of 0.05.

RESULTS

Growth phenotype in DKO macrophages

Given that mice that were null for Cbl and Cbl-b in the myeloid compartment developed severe proliferative disease, we sought to determine the growth phenotype of macrophages from these and single knockout mice (Naramura, Nandwani et al. 2010). The initial growth rate of DKO macrophages was similar to WT and Cbl $^{-/-}$, Cbl-b$^{-/-}$ before 15 days. At 15 days, BMDM generally senesce, which was observed for WT and Cbl $^{-/-}$ and Cbl-b$^{-/-}$ BMDM, however, DKO BMDM continued to proliferate (Figure 2.1A). To determine if DKO macrophages immortalize, we replated the cells to the same density at 81 days and measured growth, and DKO BMDM continue proliferation during day 81 to day 94 while WT BMDM already become senescent (Figure 2.1B). Thus, Cbl and Cbl-b work together to control the early stem cell growth and have an important role in tempering CSF-1 signaling for senescence.

Cbl and Cbl-b redundantly ubiquitinate the CSF-1R and its signaling complex response to CSF-1

We speculated that the growth defect observed in DKO BMDM was a result of the loss of ubiquitination of the CSF-1R. Indeed, pull down of the CSF-1R followed by ubiquitin blot indicated that a complete loss of ubiquitination in DKO BMDM relative to WT, Cbl$^{-/-}$ and Cbl-b$^{-/-}$ BMDM (Figure 2.2). These results indicate that Cbl and Cbl-b have overlapping ubiquitination activity in macrophages with Cbl, showing a slightly stronger role in ubiquitination.

Cbl and Cbl-b regulate CSF-1R internalization, phosphorylation and AKT signaling

Given the parallel between DKO macrophage growth phenotype and myeloproliferative disease observed in the parent mice, and a complete loss of CSF-1R and CSF-1R signaling complex ubiquitination, we sought to determine Cbl and Cbl-b's contributions to endocytosis and initial receptor activation. By staining the CSF-1R remaining on the cell surface following CSF-1 stimulation, we were able to record the rate of CSF-1R endocytosis (Figure 2.3A). Internalization of the CSF-1R was slowed in DKO relative to WT (Figure 2.3A). Quantification of these data showed that the CSF-1R had internalization of a single exponential fit with a half-life of 126s (~2min, Figure 2.3B) in WT BMDM. In DKO BMDM a double exponential was needed to fit the data and the corresponding average half-life was 18 min, corresponding to a fast 182s (~3min) and slow 1,980s (~30 min) components. Increased surface CSF-1R level in DKO BMDM was noticed from the quantification graph (Figure 2.3B).

Since CSF-1R internalization was slowed in DKO macrophages, we examined the activation of CSF-1R to evaluate if its phosphorylation was slowed by loss of adaptor

function from Cbl and Cbl-b (Miyake, Lupher et al. 1997, Nadeau, An et al. 2015). Western blot of CSF-1R Y721 showed that the CSF-1R in WT, $Cbl^{-/-}$ and $Cbl\text{-}b^{-/-}$ ran as a smear shifted towered higher molecular weight, but this shift was not present in the DKO sample (Figure 2.4), consistent with Cbl and Cbl-b mediated ubiquitination (Figure 2.2). The upshift in CSF-1R molecular weight was somewhat less pronounced for Cbl, suggesting that Cbl plays a somewhat pronounced role in CSF-1R ubiquitination over Cbl-b. Quantification of CSF-1R phosphorylation indicated that the CSF-1R remained in a phosphorylated state longer in DKO and to a lesser degree in $Cbl^{-/-}$ (Figure 2.4). Together these data indicate that Cbl and Cbl-b have little effect on initial CSF-1R phosphorylation, but that they have an additive effect in mediating the deactivation of the CSF-1R.

Based the slow internalization rate of CSF-1R in DKO macrophages, we hypothesized that p-AKT signaling, which is mainly activated on cell surface (Katz, Amit et al. 2007), would enhanced in DKO macrophages. In WT, $Cbl^{-/-}$ and $Cbl\text{-}b^{-/-}$, p-AKT(S473) was activated by 7min, then deactivated quickly at 15min following CSF-1 stimulation (Figure 2.5). DKO cells however showed prolonged p-AKT(S473) consistent with an extended residence of phospho CSF-1R at the cell surface.

Cbl and Cbl-b facilitate global tyrosine phosphorylation downstream of the CSF-1R

Given the pronounced elevation in AKT signaling, we considered the possibility that Cbl and Cbl-b simply act as attenuators of CSF-1R signaling and that without them there would be a global potentiation of kinase activity. Surprisingly, immunostaining for phosphotyrosine at early time after CSF-1 stimulation indicated tyrosine phosphorylation

in WT BMDM was much more pronounced than in DKO BMDM (Figure 2.6), indicating that Cbl and Cbl-b were needed for rapid and robust downstream kinase cascades. Additional, we noticed that tyrosine phosphorylation in WT BMDM was localized to intracellular punctate structures reminiscent of early endosomes and macropinosomes.

Cbl and Cbl-b redundantly regulate CSF-1R intracellular transport to macropinosome

Previously, we discovered a novel trafficking route for the CSF-1R that involved its endocytosis by small vesicle carriers that were then transported to nascent macropinosomes where the CSF-1R would eventually enter the macropinosome lumen (Lou, Low-Nam et al. 2014). Given the strong defect in kinase cascades, we speculated that Cbl and Cbl-b were required for the transport of the CSF-1R through this pathway. Using a dual staining procedure (Lou, Low-Nam et al. 2014) we imaged macropinosomes by the uptake of with Texas-red dextran, then cells were fixed and permeabilized with 4%PFA and Triton X-100, and stained with CSF-1R (Figure 2.7). Indeed, we observed that that in WT BMDM, the CSF-1R was efficiently transported to a subset of macropinosomes and accumulated within the macropinosome lumen (Figure 2.7A). In the case of DKO BMDM however, the CSF-1R showed only trace quantities of the CSF-1R on the limiting membrane of the majority macropinosomes suggesting that it 'rode in' on newly formed macropinosomes, but was not trafficked to the lumen. Indeed, approximately 40% of the Texas-red labeled macropinosomes lumen contained CSF-1R puncta in WT BMDM whereas macropinosomes in the DKO did not contain CSF-1R

(Figure 2.7 B). We conclude that Cbl and Cbl-b were required for efficient transport of the CSF-1R to macropinosome and mediated the accumulation of the CSF-1R in the lumen of macropinosome.

CSF-1R degradation with an ubiquitin independent mechanism in DKO macrophages

Previously we speculated that transport of the CSF-1R to the macropinosome lumen was the predominant mechanism for its degradation(Lou, Low-Nam et al. 2014) From the immunostaining of the CSF-1R it was clear that it was degraded even in the DKO BMDM. Immunostaining of the CSF-1R in macrophages across different genotypes showed that trafficking of the CSF-1R was distinct in DKO (Figure 2.8) BMDM. Specifically, WT and single KO BMDM rapidly transported the CSF-1R to newly formed macropinosomes (observed as large ring-like objects (Figure 2.8 and Figure 2.9). However, DKO BMDM trafficked the CSF-1R through dispersed vesicles and only occasionally seemed to have CSF-1R associated with macropinosomes (Figure 2.8). Note that there were trace quantities of the CSF-1R on macropinosome-like structures even prior to the addition of CSF-1 (Figure 2.8). Surprisingly, despite the significant difference in trafficking, the CSF-1R was degraded at approximately the same rate in BMDM across different genotypes (Figure 2.9). Given that CSF-1R was degraded in DKO BMDM, we consider whether CSF-1R was degraded in proteasome, another degradation system in cells. From CSF-1R staining from samples treated with proteasome inhibitor (Bortezomib), it was clear that CSF-1R was still degraded in DKO BMDM (Figure 2.10). Thus, Cbl and Cbl-b were required for rapid and organized

transport to the macropinosome but an unknown or cryptic pathway exists for CSF-1R degradation independent of transport to the macropinosome.

Hrs and CSF-1R association is regulated by Cbl and Cbl-b

The defect of CSF-1R transport to macropinosomes in DKO macrophages lead us to ask if there is a defect in intraluminal budding process mediated by ESCRT machinery. It is not known whether ESCRT machinery assembles on macropinosomes but, we hypothesized that ESCRT machinery facilitates CSF-1R transport to macropinosomes in WT macrophages and that ESCRT may act at different locations in DKO BMDM. Immunostaining of showed that Hrs and CSF-1R co-localized on large macropinosomal structures in WT, $Cbl^{-/-}$, and $Cbl\text{-}b^{-/-}$ but not DKO BMDM (Figure 2.11). In fact, little or no Hrs was observed to co-localize with the CSF-1R in DKO cells at all indicating that the receptor was degraded by an unknown pathway.

Given the defects in kinase cascades in DKO macrophages, we sought to determine if ERK signaling, which is a well-known signal downstream of the CSF-1R was affected (Yoon and Seger 2006, Katz, Amit et al. 2007, Huynh, Kwa et al. 2012, Stanley and Chitu 2014). Immunostaining of pERK was examined by immunofluorescence in WT and DKO macrophages with a time course of 30 min CSF-1 stimulation (Figure 2.12) showed CSF-1R ERK activation was quickly activated at 5 min CSF-1 stimulation, and then reduced start from 7 min stimulation and was completely deactivated at 30min stimulation in both WT and DKO macrophages. By quantifying the p-ERK signal, pERK was shown slightly downregulated in DKO macrophages.

Cbl and Cbl-b regulate gene expression downstream of the CSF-1R

We applied RNA Seq. to determine how Cbl and Cbl-b regulation of CSF-1R and signaling and trafficking affect gene expression that involved in cell proliferation and growth. mRNA levels were compared in macrophages across different genotypes cultured at steady state in the presence of CSF-1 (Figure 2.14A). The overall impact on gene expression was mild, with only ~ 1,500 differentially regulated genes with fold changes greater than 1.5, indicating the overall transcriptional program was intact. Interestingly, the expression of approximately ~1,300 genes was dependent on both Cbl and Cbl-b indicating that their overlapping function in regulating the CSF-1R. We did find a small subset of approximately 250 genes that appeared to be regulated by Cbl alone. Of the upregulated genes in DKO macrophages, their molecular function of gene ontology is enriched in growth factor activity (Figure 2.14B), their cellular component is enriched in extracellular space (Figure 2.14C) and their pathway is related to cell proliferation and growth, cell motility, and immune response (Figure 2.14D).

DISCUSSION

Cbl family E3 ubiquitin ligases are important negative regulators of receptor tyrosine kinase signaling pathways (Thien and Langdon 2001, Schmidt and Dikic 2005). Cbl knock out in mice only have mild myeloproliferative disease and is non-lethal, while Cbl and Cbl-b knock out mice develop lethal myeloproliferative disease, suggesting lack of Cbl-b additively contribute to myeloproliferative disease (Naramura, Nandwani et al. 2010). We demonstrate here that (I) Cbl and Cbl-b have overlapping function in regulating macrophage growth, (II) ubiquitination of CSF-1R and associated signaling complex, (III) promoting CSF-1R internalization thereby regulating AKT signaling (IV)

and governing rapid transport of the CSF-1R to macropinosome where it associated with Hrs. Thus Cbl, Cbl-b and ubiquitination act at multiple steps to control the endocytosis, membrane traffic and signal transduction of the CSF-1R.

The dominant function of Cbl with Cbl-b's additive role in controlling macrophage growth, and ubiquitination and endocytosis the CSF-1R

We noticed that Cbl mutants had stronger phenotypes for ubiquitination of the CSF-1R and gene expression. Previous work has shown that macrophages lacking Cbl have a mild growth advantage, mild attenuation of CSF-1R ubiquitination and CSF-1R endocytosis (Lee, Wang et al. 1999). Our findings reproduce some of these phenotypes, however when compared with the phenotype of DKO macrophages, we conclude Cbl or Cbl-b is sufficient for controlling macrophage growth consistent with the myeloprolifotirve phenotype observed in the mutant mice. Loss of both Cbl and Cbl-b resulted in a strong defect in ubiquitination, slowed CSF-1R internalization and dramatically altered transport of the CSF-1R into the lumen of macropinosome. Thus, while Cbl has more pronounced role in regulating the CSF-1R, either Cbl or Cbl-b are sufficient for targeting associating the CSF-1R with Hrs and targeting it to the macropinosome.

We were not surprised to find that p-AKT is hyper activated due to the higher amount of remaining activated CSF-1R(Y721) with slower internalization from the surface due to loss of both Cbl and Cbl-b (Katz, Amit et al. 2007). This is consistent with CSF-1R Y721's function in activation of AKT's signaling pathway on plasma membrane reported (Lee, Wang et al. 1999). In conclusion, the overlapping function of Cbl and Cbl-b in CSF-1R ubiquitination is important for CSF-1R endocytosis and the p-AKT signaling.

Cbl and Cbl-b mediate CSF-1R transport to the lumen of macropinosome

The CSF-1R is transported to the lumen of macropinosome in macrophages where the CSF-1R is packaged into the lumen and degraded (Lou, Low-Nam et al. 2014). Neither Cbl or Cbl-b knockout altered this phenotype, however, DKO cells failed to accumulate the CSF-1R within macropinosomes and failed to associate the CSF-1R with Hrs (Figure 2.7, Figure 2.8, Figure 2.9). Thus Cbl and Cbl-b mediated ubiquitination mediates recognition of ubiquitinated CSF-1R and its transport into the lumen of macropinosome. The CSF-1R transport into the macropinosome lumen likely attenuates CSF-1R signaling, given that the CSF-1R continues to signal after endocytosis (Huynh, Kwa et al. 2012). Thus, the evidence above suggests that Cbl and Cbl-b directs the CSF-1R to the macropinosome which acts as an important platform for CSF-1R signaling and termination.

Cbl and Cbl-b may be important adaptors for CSF-1R full activation thus for tyrosine phosphorylation signaling and p-ERK signaling

Our finding indicate that global phosphorylated tyrosine is drastically reduced and p-ERK signaling pathway. Cbl protein was previously shown to mediate full activation of CSF-1R (Stanley and Chitu 2014). Among 8 tyrosine residues on the intracellular domain of CSF-1R, different tyrosine phosphorylation leads to different signaling pathways (Pixley and Stanley 2004). For instance, Y559, Y697 phosphorylation recruit Cbl that ubiquitinates CSF-1R. Y559 recruitment of Cbl via SFKs mediated ubiquitination of CSF-1R is important for other tyrosine residues phosphorylation of CSF-1R, while Y697 recruitment of Cbl via Grb2 is important for ERK signaling pathway activation and tyrosine phosphorylation (Pixley and Stanley 2004, Stanley and Chitu 2014). Thus our

finding that Cbl and Cbl-b loss caused decreased tyrosine phosphorylation and slightly downregulated ERK signaling suggest that Cbl and Cbl-b redundantly act as adaptors for CSF-1R full activation thus for tyrosine phosphorylation and p-ERK signaling.

In summary, we find that Cbl together with the additive function of Cbl-b mediates CSF-1R ubiquitination, endocytosis, transport to macropinosome, but not degradation of CSF-1. Loss of Cbl and Cbl-b result in deregulated CSF-1R signaling on cell surface and a failure to transport the CSF-1R to the lumen of macropinosome. Furthermore, macropinosomes may be an important CSF-1R signaling platform and is possibly an organelle that could be targeted for developing clinical therapy for oncogenic Cbl family mutations.

FIGURES

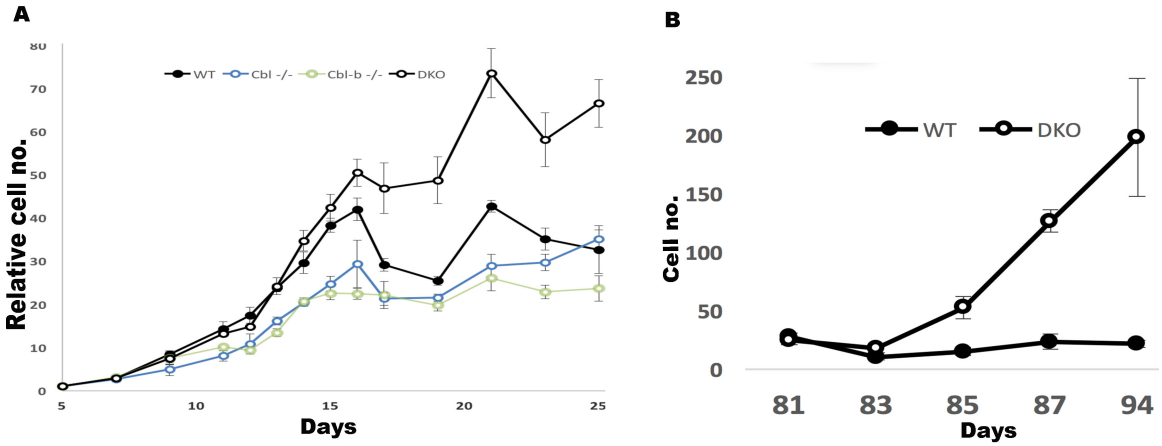


Figure 2.1 Depletion of both Cbl and Cbl-b in macrophages results in immortalization of macrophages. **A.** Growth comparison following replating at Day5 by the alamar blue assay, n=6, error is standard deviation. **B.** Growth measurement during 81 and 94 days of macrophages. N=3, error is standard deviation.

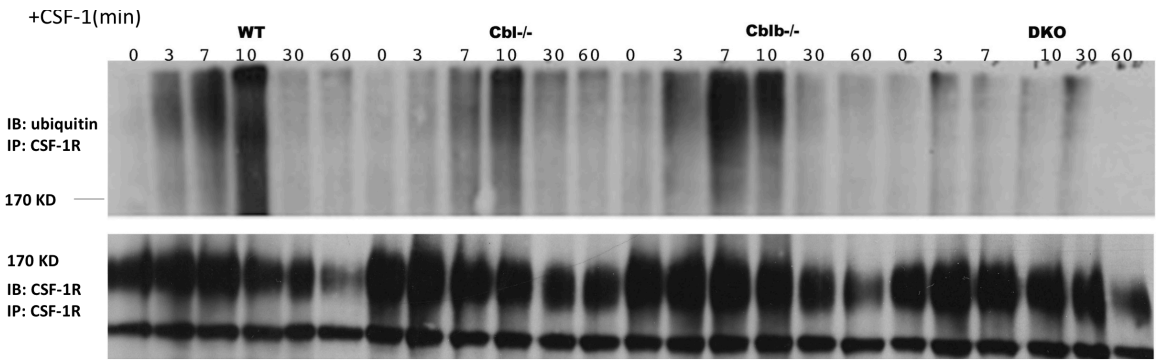
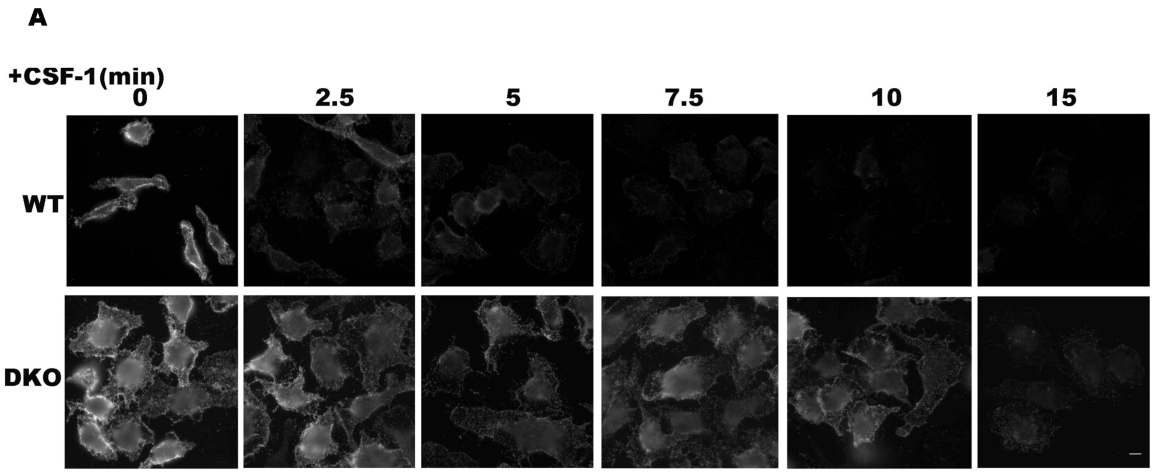


Figure 2.2 Ubiquitination of the CSF-1R and downstream signaling complex by Cbl and Cbl-b.

BMDMs were stimulated with CSF-1 for the times indicated and the CSF-1R was immunoprecipitated, and ubiquitin and the CSF-1R were immunoblotted. The CSF-1R blot showed that two forms of CSF-1R in cells: the mature CSF-1R (fully glycosylated form) with a molecular weight of ~ 165 kD, and the immature CSF-1R pool with a molecular weight of ~130 kD. The ubiquitin blot (top panel) showed extensive ubiquitination at 10 min in WT, $Cbl^{-/-}$ and Cbl-b but not in DKO BMDM. This blot is representative of three trials.


A
+CSF-1(min)
0
2.5
5
7.5
10
15
WT
DKO

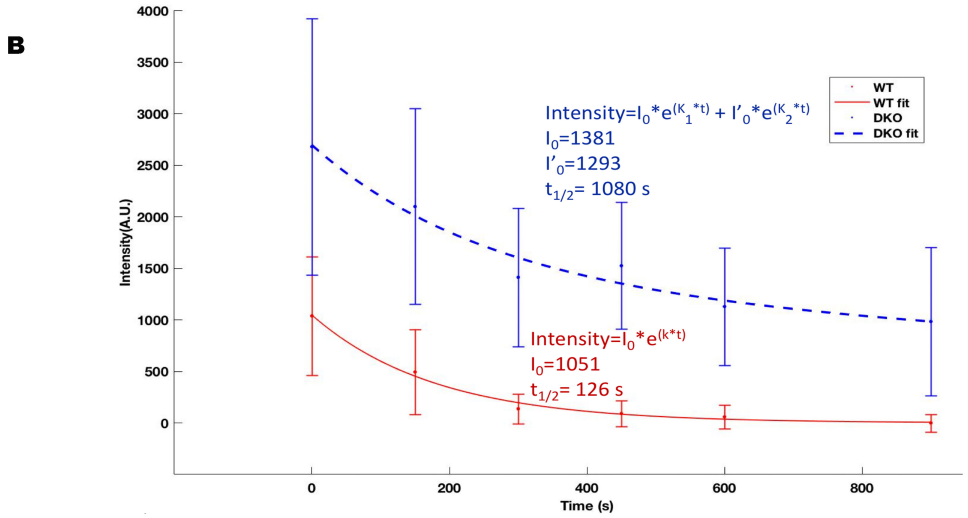

B
Intensity(A.U.)
Time (s)
WT
WT fit
DKO
DKO fit
Intensity=I0*e(K1*t) + I'0*e(K2*t)
I0=1381
I'0=1293
t1/2= 1080 s
Intensity=I0*e(k*t)
I0=1051
t1/2= 126 s

Figure 2.3 CSF-1R internalization was delayed in DKO macrophages.

A. Cell surface CSF-1R in WT and DKO was compared following CSF-1 exposure. CSF-1R in WT quickly internalized at 2.5min as shown in the data with a decreased fluorescent signal, and internalized at 10min. CSF-1R in DKO internalized with a much slower speed with much higher fluorescent than WT at the same time point. Scale bar equals 5 um. **B.** The intensity of surface CSF-1R vs CSF-1 stimulation time point was plotted and the data was applied to exponential fit. Before CSF-1 stimulation(0min), surface CSF-1R in DKO macrophages was about twice as much as CSF-1R in WT macrophages. CSF-1R had a half-life of 126s in WT macrophages, while CSF-1R in DKO quickly internalized in the beginning, then about half of the CSF-1R were internalized with slow rate. The half-life of CSF-1R was 1080 s in DKO macrophages. n=100 cells, scale bars equal 5 um, error bars = standard deviation.

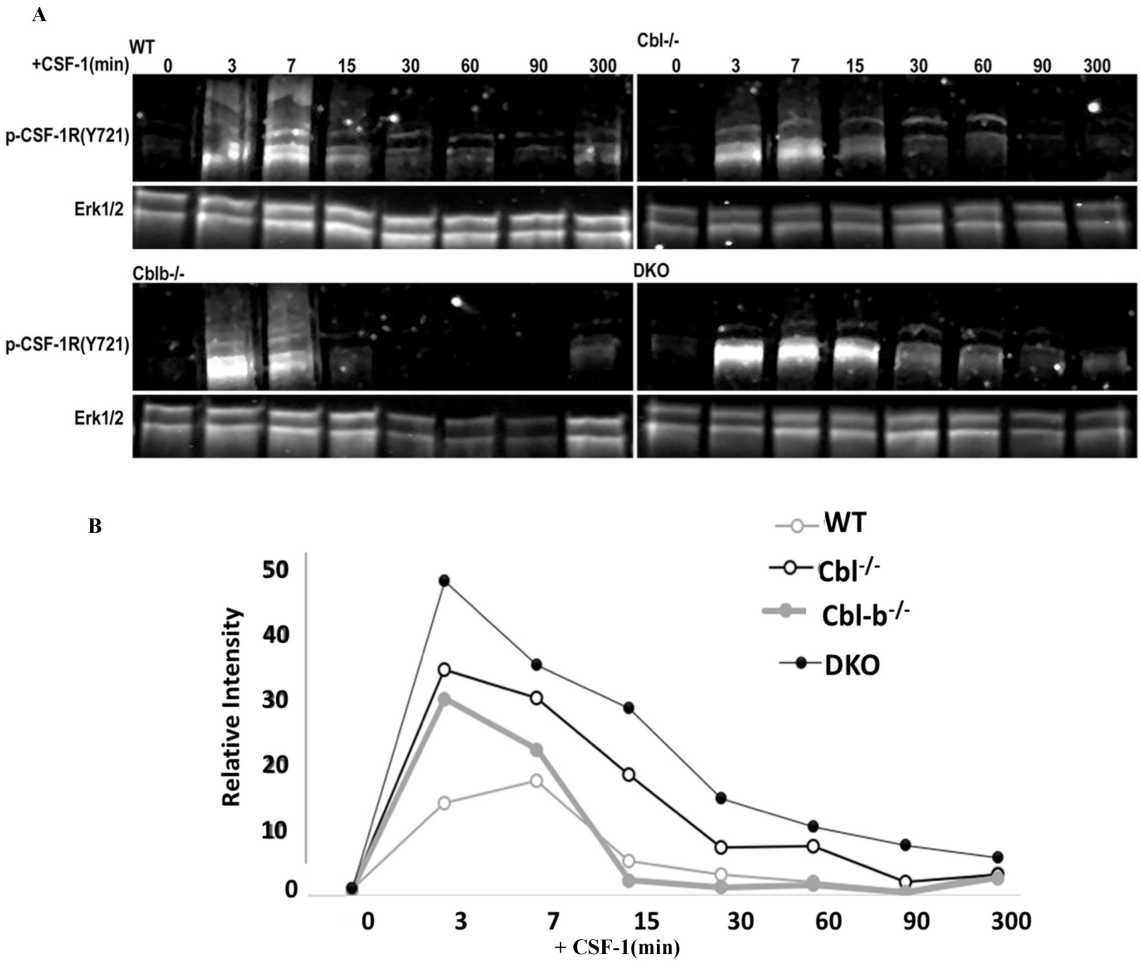


Figure 2.4 CSF-1R phosphorylation was prolonged in DKO and Cbl and Cbl-b had an additive effect in attenuated CSF-1R phosphorylation.

A. CSF-1R Y721 phosphorylation of CSF-1R was examined by western blot. Phosphorylated CSF-1R in WT, Cbl-b$^{-/-}$ and to a lesser extent Cbl$^{-/-}$ ran as smear at 3 and 7min CSF-1 stimulation. Phosphorylated CSF-1R ran as single molecular weight and in DKO macrophages. **B.** The quantification of the signal in 100 cell in each condition shown in A indicated that CSF-1R phosphorylation showed the highest in DKO and but was also deactivated very quickly.

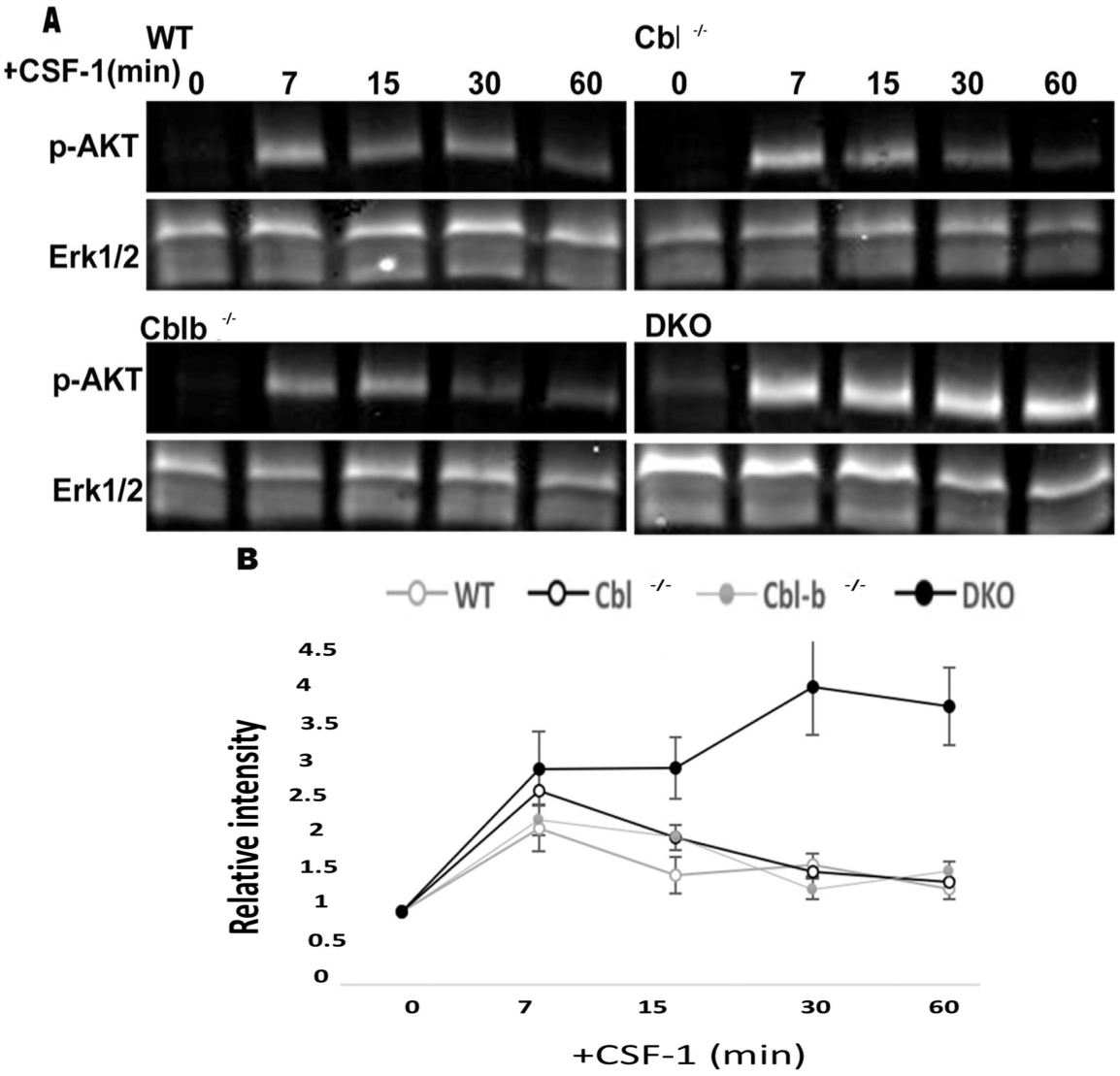


Figure 2.5 AKT signaling pathway was hyper activated in DKO macrophages.

A. AKT S473 phosphorylation was measured in macrophages across different genotypes with indicated time period of CSF-1 stimulation by western blot. AKT phosphorylation duration was longer and was not quickly deactivated in DKO macrophages comparing to WT, $Cbl^{-/-}$, Cbl-$b^{-/-}$ macrophages. **B.** The quantification of A panel, error is standard deviation, image was representative of 2 experiments.

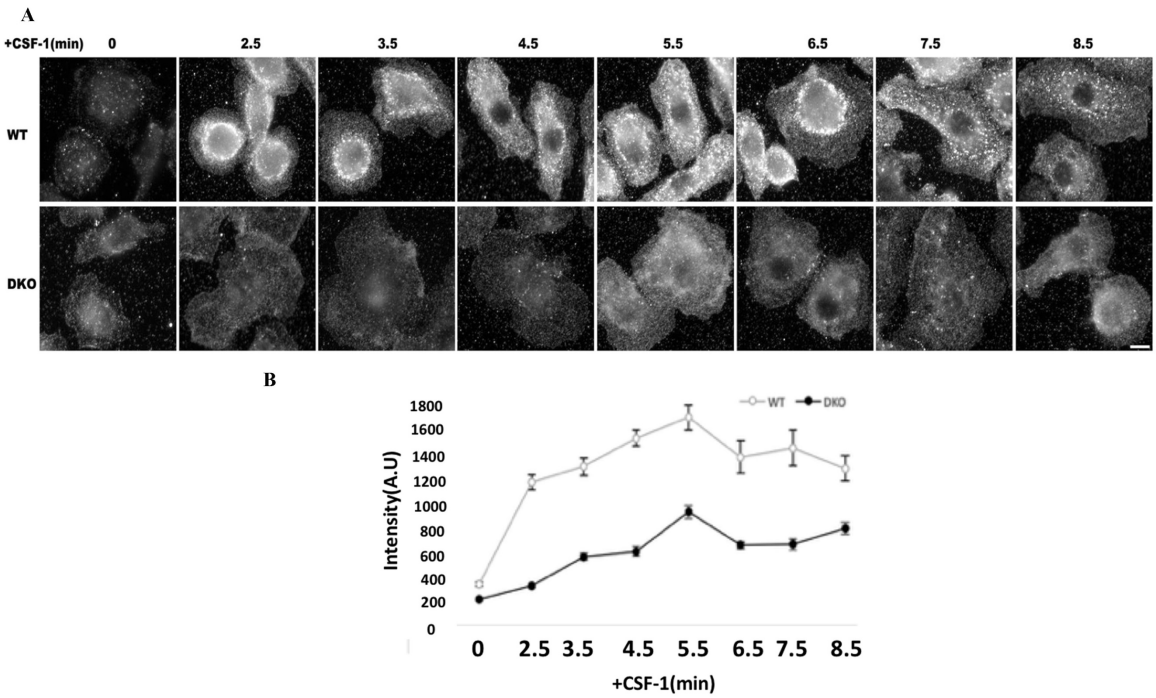


Figure 2.6 p-Tyrosine signaling defect in DKO macrophages.

A. immunostaining of Tyrosine phosphorylation within 8.5 min CSF-1 stimulation in WT and DKO macrophages. **B.** Quantification of signal from A. 30 cells in each condition were used to quantify the signal, error is standard error of mean. images were representative of 2 experiments. Scale bar, 5 um.

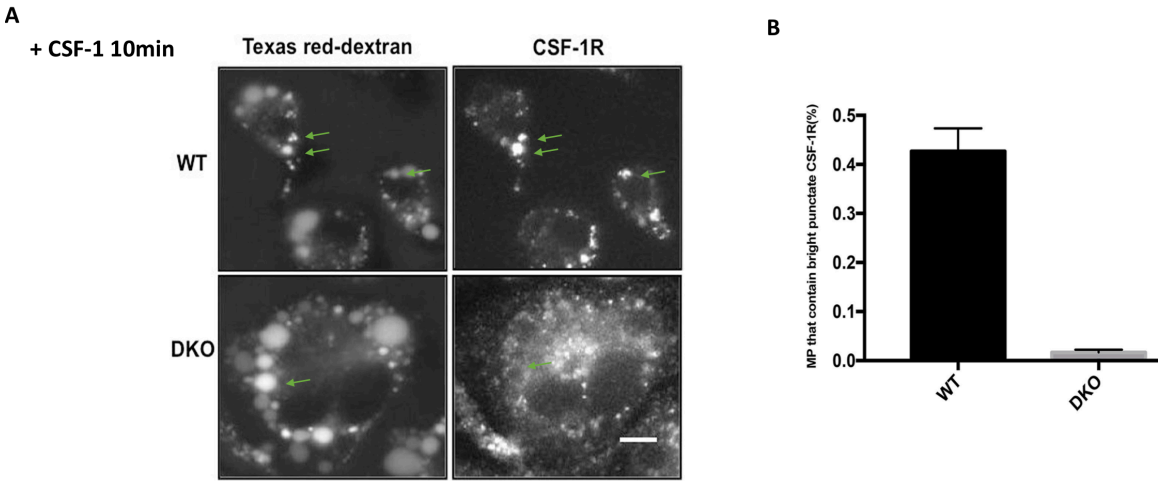


Figure 2.7 Defect of CSF-1R transport to macropinosome in DKO macrophages.

A. Macropinosomes labeling and sequential immunostaining of CSF-1R. Macropinosomes were labeled by the fluid phase marker Texas red-dextran at 10min CSF-1 stimulation then cells were permeabilized and Texas-red dextran leaking out enabled CSF-1R immunofluorescence. CSF-1R located in the lumen of macropinosome in WT macrophages and, while CSF-1R was absent in the macropinosome lumen, only with small amount on the limiting membrane of macropinosome. **B.** Quantification of macropinosome that contain CS-1R. The percentage of macropinosome that contain CSF-1R punctate in its lumen was 40% in WT, while close to 0 in DKO. Error is standard deviation. Scale bar, 5 um.

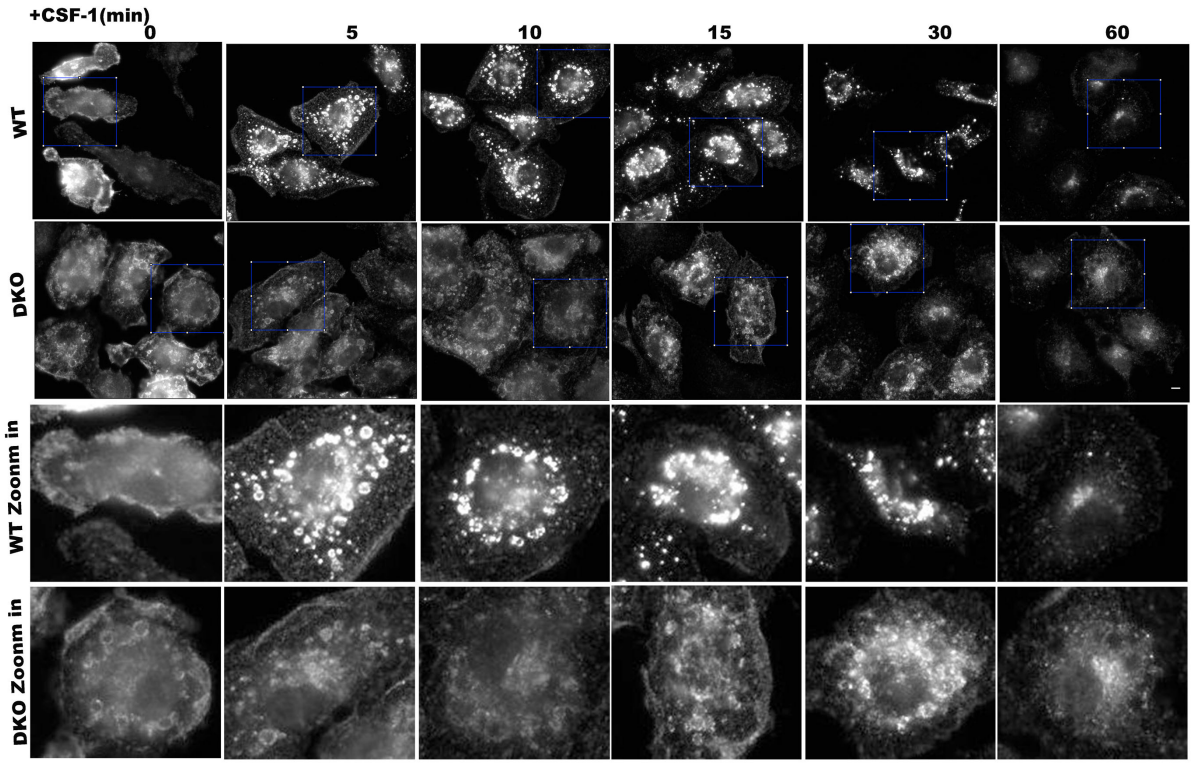


Figure 2.8 CSF-1R degradation in DKO macrophages with an unknown mechanism.

CSF-1R immunostaining in WT and DKO macrophages at CSF-1 stimulation for different time as indicated. CSF-1R internalized and transported to bright donut like structures from 5-10 min, after that, CSF-1R trafficked to the center of the cells where lysosome degrades CSF-1R at about 60 min in WT macrophages. CSF-1R signal did not accumulate as bright donut like structures in DKO macrophages and only few CSF-1R appeared on the limiting membrane of macropinosome, while at about 30min CSF-1R appeared in the center of the cells too and was degraded.

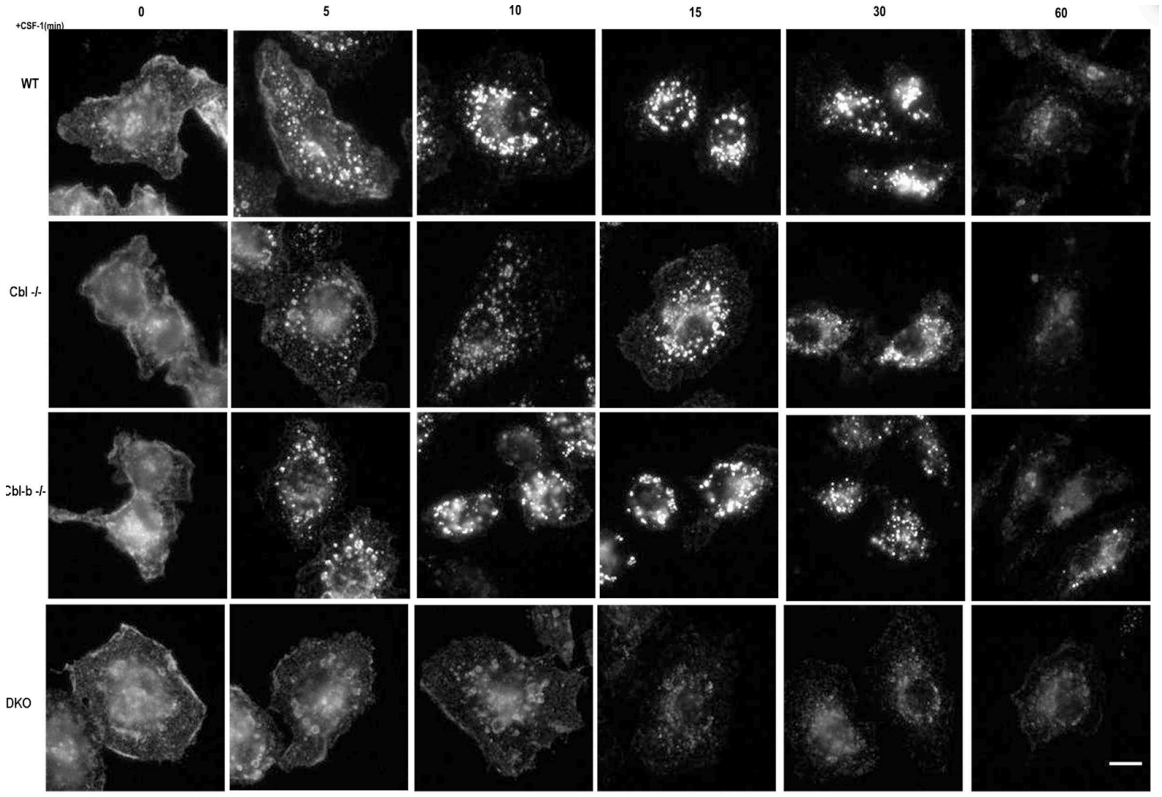


Figure 2.9 CSF-1R endocytic traffic similarly in WT, Cbl$^{-/-}$, Cbl-b$^{-/-}$ but without transport to macropinosome, though CSF-1R was degraded with an unknown mechanism in DKO macrophages.

CSF-1R immunostaining in macrophages across different genotypes at indicated CSF-1 stimulation time period. CSF-1R internalized and transported to bright donut like structures from 5-10 min, after that, CSF-1R traffic to the center of the cells where CSF-1R was degraded in lysosome at about 60 min in WT, Cbl$^{-/-}$, Cbl-b$^{-/-}$ macrophages. CSF-1R signal did not accumulate as bright donut like structures in DKO macrophages and only few CSF-1R appeared on the limiting membrane of macropinosome, while at about 30min CSF-1R appeared in the center of the cells too and was degraded. Scale bar, 5 um.

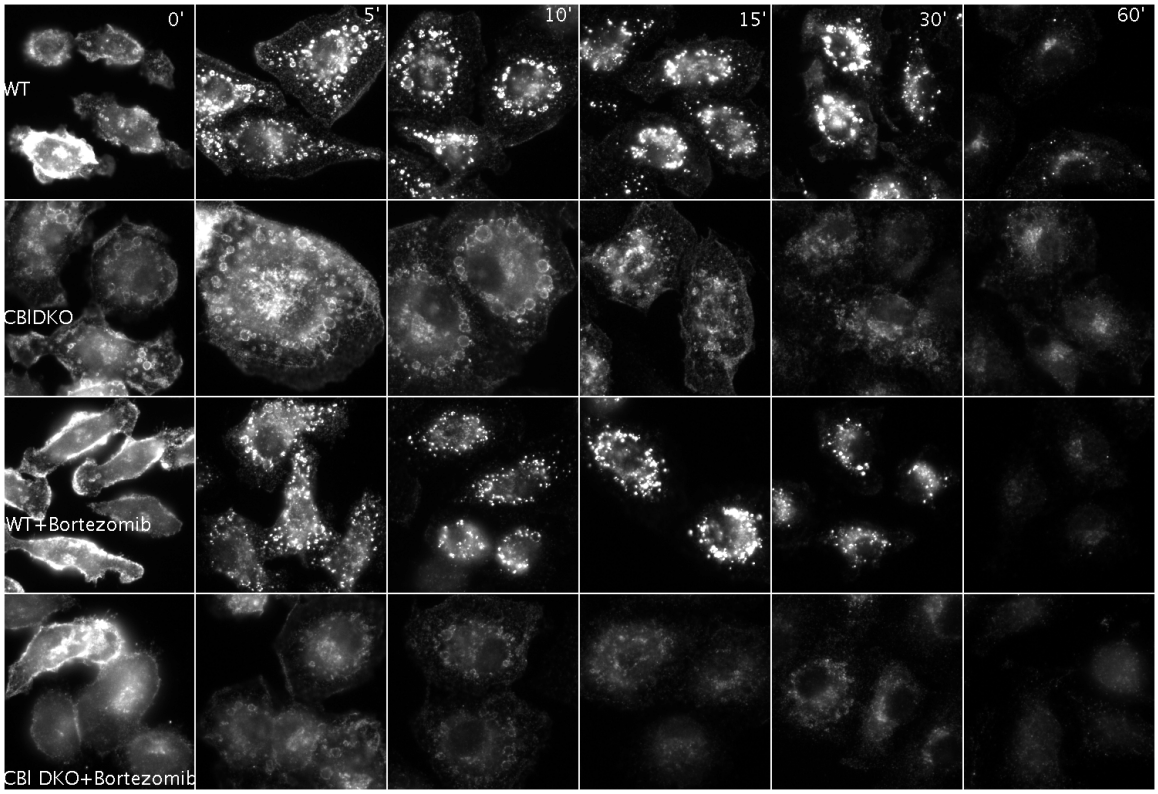


Figure 2.10 The unknown CSF-1R degradation mechanism in DKO macrophages was not a proteasome dependent pathway.

CSF-1R immunostaining in macrophages with or without proteasome inhibitor(Bortezomib) at CSF-1 stimulation time as indicated. CSF-1R traffic and degradation in samples treated with Bortezomib was similar to samples without Bortezomib.

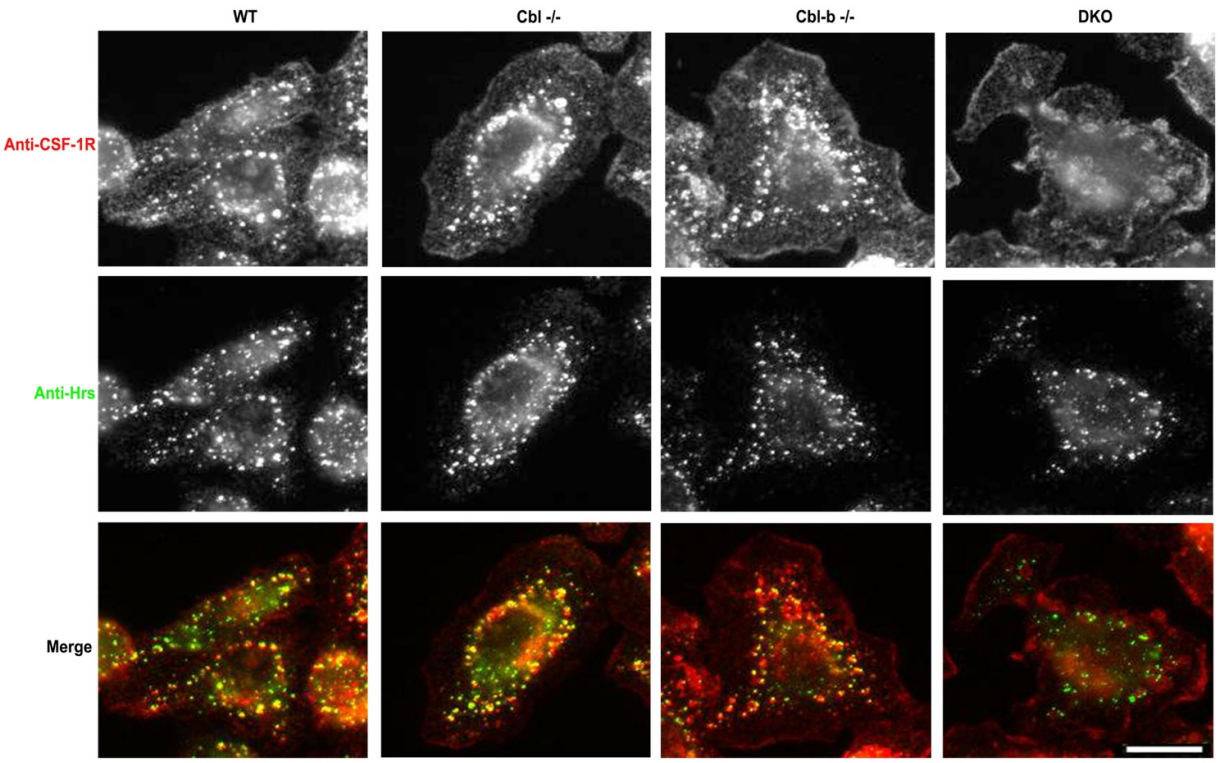


Figure 2.11 Hrs and CSF-1R association is regulated by Cbl and Cbl-b.

CSF-1R and Hrs co-staining in macrophages across different genotypes. CSF-1R stain and Hrs staining in same cells stimulated by CSF-1 for 5min in WT, $Cbl^{-/-}$, and $Cbl\text{-}b^{-/-}$ macrophages. The merge panel showed that CSF-1R and colocalized in WT, $Cbl^{-/-}$, and $Cbl\text{-}b^{-/-}$ macrophages, while CSF-1R in DKO did not colocalize. Scale bar, 5 um

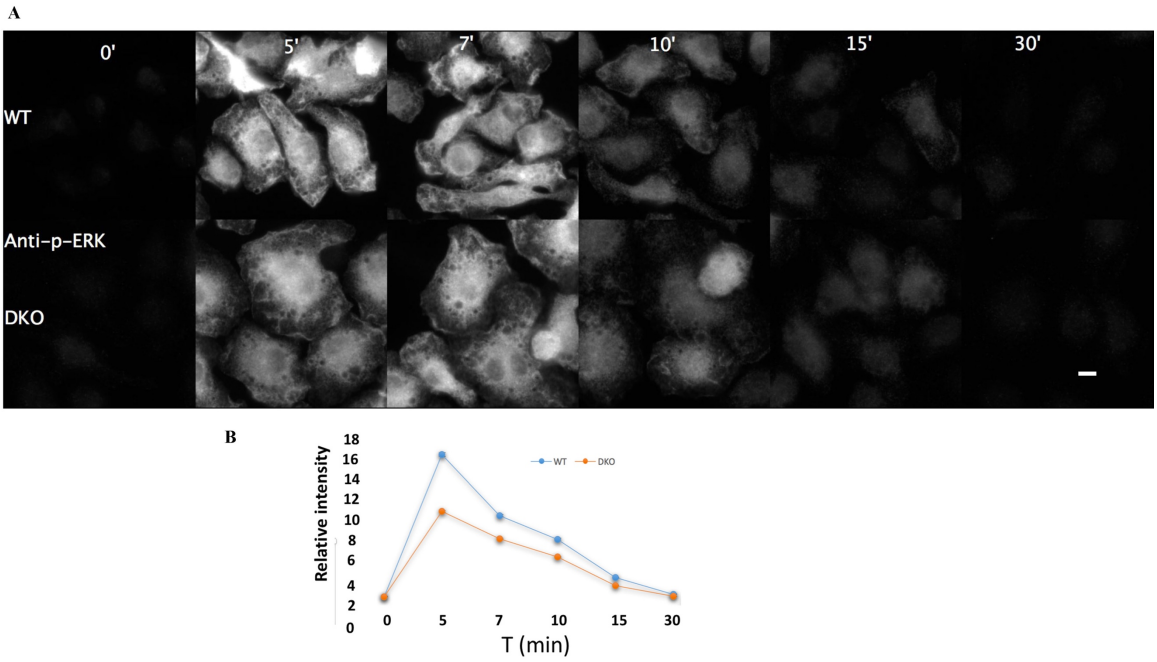


Figure 2.12 ERK signaling pathway is slightly downregulated in DKO macrophages.

A. p-ERK was examined by immunofluorescence in WT and DKO macrophages within 30min CSF-1 stimulation. p-ERK signal quickly increases at 5 min CSF-1 stimulation and slightly dropped at 7min CSF-1 stimulation, and reduced at 10minutes and disappears at 30min in both WT and DKO macrophages. **B.** The quantification of p-ERK signal in 30 cells in each CSF-1 stimulation time point of WT and DKO macrophages. p-ERK signaling was slightly reduced at 5 and 7 min CSF-1 stimulation. Scale bar, 5 um.

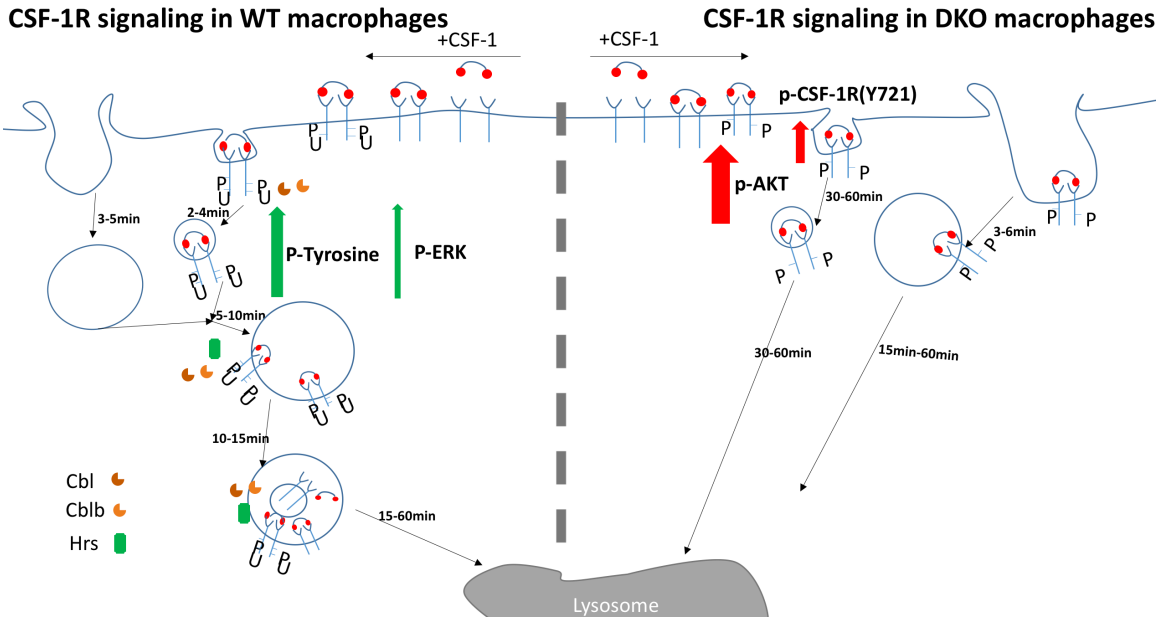


Figure 2.13 Cbl and Cbl-b regulate CSF-1R endocytic traffic and signaling.

CSF-1R fast internalization was redundantly regulated by Cbl and Cbl-b, CSF-1R internalization was reduced by loss of Cbl and Cbl-b (DKO macrophages) and the activation of CSF-1R and the duration and amplitude of AKT signaling pathway indicated by p-AKT level was increased. CSF-1R transport to macropinosome is redundantly regulated by Cbl and Cbl-b, and this defect in DKO macrophages possibly leads to decreased global tyrosine phosphorylation of CSFf-1R and ERK signaling pathway. Eventually, CSF-1R was degraded in DKO macrophages with an unknown mechanism.

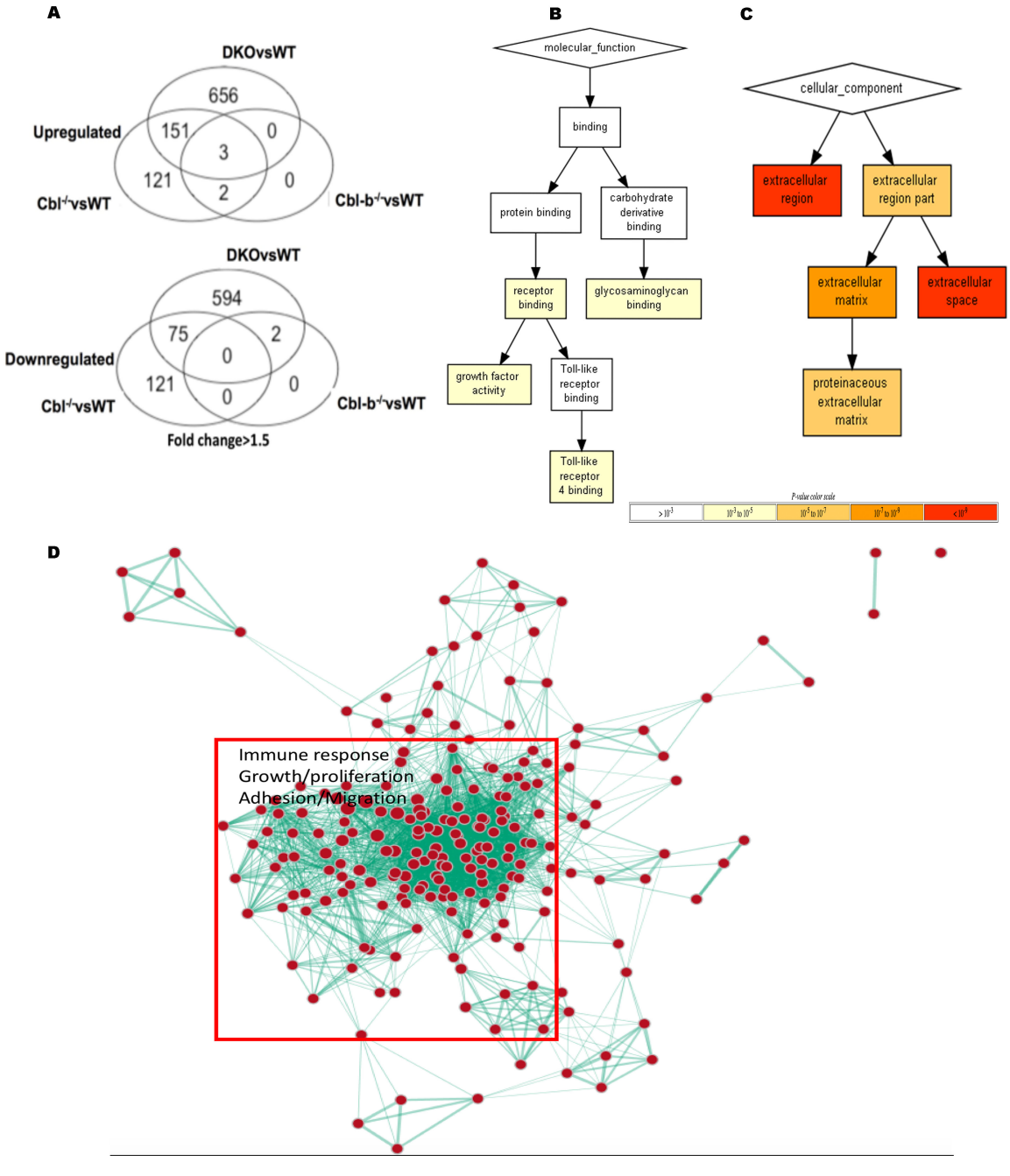

A
DKOvsWT
656
Upregulated
151
0
3
121
0
2
Cbl-/-vsWT
Cbl-b-/-vsWT
DKOvsWT
594
Downregulated
75
2
0
121
0
0
Cbl-/-vsWT
Cbl-b-/-vsWT
Fold change>1.5
B
molecular_function
binding
protein binding
carbohydrate derivative binding
receptor binding
glycosaminoglycan binding
growth factor activity
Toll-like receptor binding
Toll-like receptor 4 binding
C
cellular_component
extracellular region
extracellular region part
extracellular matrix
extracellular space
proteinaceous extracellular matrix
P-value color scale
D
Immune response
Growth/proliferation
Adhesion/Migration

Figure 2.14 Cbl and Cbl-b redundantly regulate the majority of the differential expressed genes.

A. Intersection showed the overlapped differential expressed genes that are either upregulated or downregulated in $Cbl^{-/-}$, $Cbl\text{-}b^{-/-}$, DKO macrophages. Loss of both Cbl and Cbl-b caused the most gene expression change, upregulated genes in DKO macrophages **B.** Molecular function of upregulated genes by GOrilla in DKO macrophages are enriched in growth factor activity **C.** Cellular component of upregulated genes by GOrilla in DKO macrophages are enriched in extracellular space **D.** Network analysis of DKO upregulated genes are involved in cell proliferation and growth, cell motility, and immune response.

Chapter III

CONCLUSION

In this study, we characterized the function of Cbl and Cbl-b in CSF-1R endocytic traffic and signaling. Specifically, we discovered that Cbl and Cbl-b redundantly ubiquitinate the CSF-1R in macrophages subsequently regulating CSF-1R internalization and transport to macropinosome. This work demonstrated that Cbl and Cbl-b are key regulators in this newly discovered pathway CSF-1R degradation (Lou, Low-Nam et al. 2014) and that their ubiquitination of the CSF-1R enables recognition by Hrs and transport into the macropinosome lumen, ostensibly via the ESCRT. Surprisingly, CSF-1R degradation does not require Cbl and Cbl-b mediated ubiquitination, suggesting the existence of an uncharacterized ubiquitin-independent degradation mechanism for the CSF-1R in macrophages. The control of Cbl and Cbl-b on CSF-1R traffic is important for normal CSF-1R signaling. CSF-1R p-AKT signaling amplitude and duration is increased correlated longer half-life of CSF-1R on cell surface, while global Tyrosine phosphorylation based signaling and p-ERK signaling are slightly decreased in simultaneous Cbl and Cbl-b depletion macrophages possibly due to Cbl and Cbl-b's adaptor function for CSF-1R endocytic traffic to endosome and macropinosome.

Redundant and non-redundant function of Cbl and Cbl-b in receptor traffic and signaling

We have shown the overlapping function of Cbl and Cbl-b in ubiquitination and regulating receptor traffic and signaling, but also some dominant function of Cbl over Cbl-b in CSF-1R in protein ubiquitination and regulation of gene expression. Distinct

functions of Cbl and Cbl-b has been reported in regulating EGFR (Ettenberg, Keane et al. 1999, Ettenberg, Rubinstein et al. 1999, Davies, Ettenberg et al. 2004, de Melker, van der Horst et al. 2004). For example, Cbl-b overexpression is more potent than Cbl in inhibiting EGF mediated growth via PI3K-AKT signaling (Ettenberg, Keane et al. 1999). Another study shows that Cbl-b UBA domain binds ubiquitinated proteins, and overexpression Cbl-b UBA domain blocks EGFR degradation, possibly acting as an dominant negative mutants in protein ubiquitination(Davies, Ettenberg et al. 2004). Furthermore, Cbl is shown to interact with EGFR at an early stage of trafficking, while Cbl-b association happens later after Cbl dissociate from EGFR(Pennock and Wang 2008). Together, Cbl and Cbl-b have redundant function as well as non-redundant function in regulating CSF-1R traffic and signaling.

Effects of receptor ubiquitination on receptor phosphorylation

Y721 is one of the major phosphorylation sites during CSF-1R activation, Y721 phosphorylation of CSF-1R is not prevented in DKO macrophages. The specific lysine residues ubiquitinated are not known in CSF-1R, while 6 residues have been mapped on the kinase insert segment of EGFR, and EGFR mutant with ubiquitination defect does not affect receptor kinase activity in EGFR and their tyrosine phosphorylation triggered by activation is similar to wild type EGFR. (Huang, Kirkpatrick et al. 2006). Based on the slightly higher CSF-1R Y721 phosphorylation extent in DKO macrophages, the decaying of Y721 phosphorylation in CSF-1 stimulation kinetics in DKO macrophages is very similar to the one in WT macrophages, which suggest that ubiquitination does not regulate the stability of activated CSF-1R.

Possible ubiquitination mechanism and other cellular proteins ubiquitinated by Cbl and Cbl-b in response to CSF-1R activation

The ubiquitination immunoblot (Figure 2.2) from immunoprecipitated CSF-1R tells us ubiquitinated proteins are a mixture of proteins with different molecular weight, and most of the proteins have higher molecular weight than matured CSF-1R (~170 kD). Limited ubiquitinated proteins are known to associate with activated CSF-1R trafficking, by Combining protein ubiquitination in response to the activated EGFR identified by proteomics with the specific CSF-1R traffic route. Due to the significant size upshift of the ubiquitinated proteins, those proteins are possibly poly ubiquitinatinated. In summary, further proteomics experiments are required to map the interactome of CSF-1R activation, and identify ubiquitinated proteins coupled with CSF-1R traffic and signaling.

The multifunctional signal of ubiquitin and ubiquitin like proteins

Ubiquitination of proteins is first recognized as a targeting signal to degradative pathway(Ciechanover 2005); it has the non-degradative function specifically in signaling transduction(Zaaroor-Regev, de Bie et al. 2010), enzymatic activation(Nguyen, Munoz-Garcia et al. 2011), endocytosis and trafficking(Sorkin and Goh 2009), DNA repair and transcription(Welchman, Gordon et al. 2005, Weake and Workman 2008), which possibly leads to the broad level impact of gene expression regulated by loss of ubiquitination mediated by Cbl and Cbl-b.

Teasing out Cbl protein adaptor function and ubiquitin ligase function in regulating receptor traffic and signaling

Whether Cbl proteins or ubiquitination of proteins regulates CSF-1R internalization and transport to macropinosome is unknown. For receptor endocytosis, a EGFR mutant, weakly ubiquitinated due to replacement of 6 lysine residues on the kinase domain, undergoes normal internalization(Huang, Kirkpatrick et al. 2006, Huang, Goh et al. 2007). Another study shows that Cbl associates with EGFR before EGFR entering clathrin mediated endocytosis pit, and couple with EGFR during the endocytic route, suggesting that the important function of Cbl and ubiquitination in receptor endocytosis (de Melker, van der Horst et al. 2001). For receptor endocytic traffic, our findings indicate that Cbl and Cbl-b mediate CSF-1R transport to macropinosome in an Hrs dependent mechanism.

In future, we will combine established macrophage cell lines dependent on CSF-1R for proliferation and CRSPR to generate DKO macrophages. By complementing these DKO macrophages with truncated Cbl protein or Cbl protein with point mutation,

We will furtherly investigate the mechanism by which Cbl regulates CSF-1R traffic and signaling.

Macropinosome may be an important signaling platform for global tyrosine phosphorylation and p-ERK signaling pathway

Slow CSF-1R internalization results in higher AKT signaling, suggests that CSF-1R activating AKT signaling mainly is initiated on plasma membrane. However, the tyrosine phosphorylation based signaling including STAT, many protein kinases, phosphatases and some of proteins involved in ERK signaling require the normal endocytosis of CSF-1R, and are activated along the endocytic traffic to macropinosome. In conclusion,

macropinosomes are important signaling platform for the propagation of global tyrosine phosphorylation and p-ERK signaling.

Cellular alternative degradation mechanisms

We find that CSF-1R is degraded in DKO macrophages in the absence of protein ubiquitination. Other degradation mechanism except from ubiquitination directed lysosome degradation exist to compromise CSF-1R ubiquitination degradation defect. Endoplasmic reticulum (ER)- associated degradation (ERAD) pathway and intramembrane proteolysis are additional mechanisms are used to drive regulated protein turnover (Yao, Works et al. 2005, Ruggiano, Foresti et al. 2014). ERAD target misfolded proteins of the ER for ubiquitination and degradation by proteasome, and Intramembrane proteolysis dislocate and cleave full-length proteins by proteases in cytosol in an irreversible way and turnover membrane proteins in proteasome(Hachmeister, Bobowski et al. 2013). We attempt to block proteasome function by drug inhibition to test those two possibility, but CSF-1R is still degraded in DKO macrophages. Thus, CSF-1R degradation in lysosome is more likely but the mechanism is still puzzling to us.

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