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Macropinosome Maturation is a Clathrin Dependent Process in Bone Marrow Macrophages

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MACROPINOSOME MATURATION IS A CLATHRIN DEPENDENT PROCESS IN BONE MARROW MACROPHAGES

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

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TABLE OF CONTENTS

ABSTRACT……vii

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION……..……1

MEMBRANE VESICULAR TRAFFICKING……..……2

GROWTH FACTOR SIGNALING IN MAMMALIAN CELLS……..…7

MACROPHAGES……..……8

CLATHRIN……..…….9

REFERENCES……12

CHAPTER 2: ESTABLISHING CRISPR/CAS9 GENETIC DELETION OF CLATHRIN HEAVY CHAIN

INTRODUCTION…….……..…17

MATERIALS AND METHODS……...19

RESULTS……..…….26

DISCUSSION……...….27

REFERENCES…….…….30

CHAPTER 3: CLATHRIN FACILITATES MACROPINOSOME MATURATION IN MACROPHAGES

INTRODUCTION…….……40

MATERIALS AND METHODS…….….43

RESULTS…….….50

DISCUSSION…….……...55

REFERENCES…….….61

CHAPTER 4 CONCLUSIONS……80

ABSTRACT

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Macrophages nonspecifically take up extracellular fluids, solutes and macromolecules by macropinocytosis. Understanding the mechanisms of macropinosome maturation will inform the study of lipid uptake, viral entry, antigen processing and presentation, as well as regulation of cell growth. Colony stimulating factor-1 receptor (CSF-1R) is internalized by small vesicle endocytosis, trafficked to nascent macropinosomes and degraded. These CSF-1R positive macropinosomes mature through a sequence similar to endosomes, progressing from EEA1 and Rab5 to Rab7 positive vesicles before fusing with lysosomes. Here we report the assembly of clathrin on internalized macropinosomes shown both by live-cell microscopy of cells expressing clathrin light chain-yellow fluorescent protein (CLTA-YFP) and by immuno-staining of endogenous clathrin heavy chain (CHC). Partial depletion of the clathrin heavy chain by siRNA prevented macropinosome-lysosome fusion and impaired degradation of the CSF-1R, with only minimal effects on the delivery of the CSF-1R to the macropinosome. Expression of fluorescent protein fusions demonstrates that clathrin assembled on macropinosomes co-localizes with dynamin and possibly the clathrin adaptor, clathrin-assembly lymphoid myeloid leukaemia protein (CALM). These data indicate a novel role for clathrin in mediating macropinosome maturation, cargo trafficking and

macropinosomes-lysosome fusion in macrophages. We hypothesize that clathrin is involved in an outward budding event during the Rab5 to Rab7 stage of macropinosome maturation. Protein and membrane sorting at this time may predispose the macropinosome to further maturation processes such as attachment to motor proteins and lysosome fusion.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Endocytosis is a fundamental process that occurs in cells and is important for supplying extracellular or membrane-bound macromolecules to cells (Popova, Deyev et al. 2013). Internalization of fluid, solutes, macromolecules, plasma membrane materials and foreign particles in cells occurs by folding of the plasma membrane and formation of vesicles through membrane fission events (Huotari and Helenius 2011). There clathrin-dependent and clathrin-independent endocytic pathways (Lim and Gleeson 2011). The clathrin-dependent endocytic pathway is dependent on clathrin coated pits of nearly 100 nm diameter. Adaptor protein 2 (AP2) initiates the formation of clathrin coated pits by binding receptors and initiating clathrin assembly in lattice-like networks (Mousavi, Malerød et al. 2004). One of the well-studied clathrin independent pathways is macropinocytosis and was identified for the first time by Warren Lewis in 1931 (Lim and Gleeson 2011). Macropinocytosis is a non-receptor mediated process that occurs in response to growth factor stimulation like colony stimulation factor (CSF-1), epidermal growth factor (EGF) and platelet derived growth factor (Haigler HT and McKanna JA 1979). Similarly, lysosomes are organelles that act as a center for the breakdown and degradation of macromolecules such as proteins, carbohydrates and lipids into their respective building blocks (Huotari and Helenius 2011). Lysosomes contain more than 60 kinds of hydrolases like lipases, proteases, and glycosidases for the degradation of

macromolecules (Xu and Ren 2015). The lysosome-mediated degradation process is dependent on the nutrient status of cells and cellular signaling (Settembre, Fraldi et al. 2013). Lysosomes receive cargo from extracellular, cell surface or autophagy origins that are destined for degradation (Xu and Ren 2015). Based on preliminary data collected in our lab, I hypothesized that clathrin plays an important role in the degradation of colony stimulation factor (CSF-1) receptors that internalize through the endocytic/macropinocytic pathway and follow the lysosomal degradation pathway. This is a novel function of clathrin and little study has been done to understand this function of clathrin. In this literature review, I will present information that will help readers to understand the process of macropinosome maturation, macropinosome-lysosome fusion and the relevance of these topics to macrophage biology.

MEMBRANE VESICULAR TRAFFICKING

For eukaryotic cells to function properly, regulation of physiochemical properties of membrane-like balance in temperature, pH, relative humidity and nutrient variations is necessary. Living cells maintain this balance by being organized in a compartment system (De Craene, Bertazzi et al. 2017). Cells are organized by membrane-bound organelles and vesicles such as the nucleus, endoplasmic reticulum, endosomes and lysosomes that are important for organizing biochemistries within cells (Costaguta and Payne 2009).

The plasma membrane is the first barrier that separates the cell and its internal content from the external environment and is composed of a phospholipid bilayer made of sterols, glycolipids and proteins (Spector and Yorek 1985). Membranes are mainly composed of five types of phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE),

phosphatidylserine (PS), phosphatidylinositol (PtdIns) and sphingomyelin (SM). Among these phospholipids, phosphoinositides (phosphorylated form of phosphatidylinositol) play an important role in signaling and vesicular transport of lipids and proteins from one compartment to another in eukaryotic cells (Kornberg and McConnell 1971). Phosphoinositides function by recruiting and activating effector protein and facilitating cellular functions like vesicular budding, membrane fusion and cytoskeleton functioning (De Craene, Bertazzi et al. 2017).

In the vesicular trafficking process, vesicles loaded with specific protein form cargo at the donor compartment, and are received by the specific compartment where they are required (Costaguta and Payne 2009). The proteins and mechanisms that facilitate vesicular protein transport are found to be conserved from yeast to humans (Schu 2001).

Macropinocytosis and formation of macropinosomes

Macropinocytosis is a type of endocytosis in which the cell non-selectively internalizes solutes and nutrients in a liquid form (Lim and Gleeson 2011). Macropinocytosis takes place in the presence of growth factors like macrophage colony stimulating factor-1 (CSF-1) and epidermal growth factor (EGF) (Haigler, McKanna et al. 1979). Actin polymerization causes membrane ruffling to form lamellipodia that fold back to fuse with the basal membrane and form large vesicles that are known as macropinosomes (Norbury, Hewlett et al.) Lamellipodia are membrane protrusions located at the edge of cells that help in migration of cells. Macropinosomes are different than other endocytic vesicles and have diameters generally greater than 0.2 μm and can be as large as 5 μm (1994). They can be identified based on size and with fluid-phase markers like dextran, Lucifer yellow, or horseradish peroxidase (Lim and Gleeson 2011).

Physiologically, macropinocytosis has various functions. Macropinocytosis promotes tumor advancement and metastasis (Lim and Gleeson 2011). Neutrophils also depend on macropinocytosis for chemotactic response. Neutrophils kill foreign pathogens by identifying, moving towards and killing them. Cell surface receptors CR1 in neutrophils regulate neutrophil's activities and these receptors are internalized through macropinocytosis (Carpentier, Lew et al. 1991). Another important function of macropinocytosis is to help antigen-presenting cells like macrophages and dendritic cells present antigen to T-cells.

Endosome formation and maturation

The exact mechanism for the formation of early endosomes is not clear; however, the membrane and its volume is known to derive from the fusion of endocytic vesicles. Previously, it was defined as the first site for receiving cargo; however, it also acts as an organelle for sorting cargoes that are received by it (Huotari and Helenius 2011). Early endosomes receive endocytic cargoes from different pathways like caveolin- and ARF6-dependent pathways in addition to clathrin-mediated pathways (Mayor and Pagano 2007). Maturation of early endosomes to late endosomes involves removal of proteins and lipids that enable sorting of cargo and other components to the plasma membrane or the trans-Golgi network. Important membrane proteins that are not recycled are then sent to the lysosome via early endosomes for degradation (Gruenberg and Maxfield 1995).

Indicators of endosome maturation

Regardless of the mechanism of initial formation, newly formed late endosomes undergo a variety of changes. By the time they are ready to fuse with lysosomes, some 10-40 min later they are seen to be transformed from early endosomes and share few

similarities with early endosomes. The maturation process involves formation of additional intraluminal vesicles (ILV), changes in Rab proteins, drop in lumenal pH, change in morphology, shift in choice of partners, gain in lysosomal hydrolases and membrane proteins (Huotari and Helenius 2011). Rab5 is exchanged for Rab7. Intra-luminal vesicles are formed. The luminal pH drops from pH 6.0 to 4.9. The tubular extensions on early endosomes are lost, and matured endosomes acquire a round or oval shape and grow in size. Lysosomal hydrolases and membrane proteins are gained in matured endosome. Endosomes associate with a new set of microtubule dependent motors that help them to move into the peri-nuclear region of the cell. Changes in the ionic environment of lumen include increases in chloride ion concentration and change in calcium, sodium and potassium ion concentration (Huotari and Helenius 2011).

Intra-luminal vesicles and formation of multi-vesicular bodies

Multivesicular bodies (MVB) can be defined as acidic endocytic organelles that have luminal vesicles inside of them. They were first observed under electron microscopy as unique membrane closed structure with intraluminal vesicles (ILVs). They are also known by the name of late endosomes, endocytic carrier vesicles and the pre-vacuolar compartment in yeast (Clague and Urbé 2008). MVBs can be differentiated from other types of organelles with internal membranes like autophagic bodies or multilamellar proteins based on the types of endocytic markers they have. MVBs contain endocytic markers like Rabs, LAMPs and endocytosed tracers such as large dextrans or other fluorescent molecules (Piper and Katzmann 2007). MVBs are recognized as very dynamic and versatile because inward budding and ILV formation depend on growth factor stimulation (White, Bailey et al. 2006). ILVs start to form in early endosomes. The

cytosolic surface of early endosomes has both clathrin and endosomal sorting complex required for transport (ESCRT) machinery that helps to sort ubiquitinated proteins to ILVs (Huotari and Helenius 2011).

ESCRT machinery

Endosomal sorting complex required for transport (ESCRT) machinery is conserved from archae to animals and facilitates inward budding and cargo sorting, the scission of membrane neck and formation of ILV (Piper and Katzmann 2007). Multivesicular bodies are important intermediates that help in trafficking of ubiquitinated receptors and other cargoes that are destined for degradation (Hurley 2008).

Lysosomes

Lysosomes are acidic compartments that are filled with more than 60 different types of hydrolases (Kolter and Sandhoff 2005). The lack of mannose-6-phosphate receptors in lysosome helps to distinguish it from endosome (Luzio, Pryor et al. 2007). They are responsible for the degradation of extracellular particles from endocytosis and intracellular particles from autophagy. The digested products are transported out of lysosomes via vesicular membrane trafficking. Lysosomes contain more than membrane proteins and are well facilitated with machinery to sense nutrient availability that determines number, size and activity of lysosomes. Defects in functions of lysosomes like defects in degradation, export or trafficking cause lysosomal dysfunction and lysosomal storage disorders (Xu and Ren 2015).

Endosome-lysosome fusion

Lysosomes act as the final compartment of the endocytic pathway (Luzio, Gray et al. 2010). It is recognized that delivery of endocytosed macromolecules to the lysosome for

degradation is mediated by direct fusion of lysosomes with late endosomes (Pryor and Luzio 2009). Evidence for the direct fusion of lysosomes with endosomes first came from immuno-electron microscopy and cell-free content mixing assays (Luzio, Pryor et al. 2007). Cell free experiments have also shown that the lysosome is able to fuse more effectively with late endosomes compared to early endosomes (Mullock, Bright et al. 1998)

GROWTH FACTOR SIGNALING IN MAMMALIAN CELLS

In multicellular organisms, cells cells require receptor mediated signaling pathways that are initiated by extracellular growth factors to divide and proliferate (DeBerardinis, Lum et al. 2008). There are several pathways in mammals that play important roles in signaling for growth and proliferation. Growth factor signaling mediated by PI3K/Akt pathway helps to increase glucose uptake and hence glucose uptake increased glycolysis (Ward and Thompson 2012). Likewise, mTOR kinase and the Myc transcription factor regulate the uptake and utilization of amino acids for protein and nucleic acid synthesis (Morrison 2012).

Colony stimulating factor (CSF-1) and its receptor (CSF-1R)

CSF-1 is a hemotopoetic growth factor can promote the growth of pure colonies of macrophages from bone marrow progenitors Stanley ER et al. (1976). CSF-1 is able to act on its target cell by binding to CSF-1R which is a member of the type III protein tyrosine kinase receptor family (Chen, Liu et al. 2008). The CSF-1R gene is located on chromosome 5 (5q 32) in human and chromosome 18 (18 D) in mouse (Stanley and Chitu 2014). CSF-1R expression is low on hematopoietic stem cells and high on monocytes, tissue macrophages, osteoclasts and myeloid dendritic cells (Stanley and Chitu 2014).

CSF-1 and interleukin-34 (IL-34) are the two identified ligands for CSF-1R (Lin, Lee et al. 2008). CSF-1 is seen to be circulating and regulating extracellular fluid throughout the body (Janowska-Wieczorek, Belch et al. 1991) whereas IL-34 is found to be present only in the microenvironment where they are expressed (Tian, Shen et al. 2013).

MACROPHAGES

Macrophages are phagocytic cells that are able to clear about 2x 10^{11} red blood cells every day (Mosser and Edwards 2008). This rapid clearance procedure is necessary for survival of the organism. Macrophages also clear cellular debris and cells that undergo apoptosis independent of immune-cell signaling (Kono and Rock 2008). Macrophages are found to be present in almost all kinds of tissues as they differentiate from circulating blood mononuclear cells and migrate to normal tissue or inflamed tissue (Gordon and Taylor 2005). Macrophages contribute to the innate immune response by responding to immediate endogenous stimuli post injury or infection. They also respond to adaptive immune signals produced by antigen-specific immune cells (Gordon 2007).

Classification of macrophages

In vitro studies have suggested that macrophages can be divided into different groups: myeloid derived suppressor cells, M1 macrophages, M2 macrophages and regulatory macrophages (Benoit, Desnues et al. 2008). M1 macrophages are seen to produce high levels of inflammatory cytokines, oxygen and nitrogen intermediates (Mosser and Edwards 2008). M2 macrophages help in wound healing, tissue organization, phagocytosis and receptors expression (Cybulsky, Cheong et al. 2016). Macrophages also have different names based on the location of tissue where they reside. They are called osteoclasts if present on bone, alveolar macrophages in lungs, microglial

cells in the central nervous system, histiocytes in connective tissue, Kupffer cells in liver and Langerhans cells in skin (Italiani and Boraschi 2014).

Fetal liver and bone marrow derived macrophages

Bone marrow derived macrophages are primary macrophage cells that are isolated from bone marrow and cultured in vitro in the presence of specific growth factors. Specifically CSF-1 promotes differentiation from monocytes into macrophages (Weischenfeldt and Porse 2008).

CLATHRIN

Clathrin is 180 kDa protein and plays an important role in membrane bending and endocytosis. Clathrin protein acts as molecular scaffold and assists vesicular cargo uptake at the cell membrane by assembling into cage-like structures (Kirchhausen, Owen et al. 2014). It assists in vesicle formation, transport and pinching through interaction between its subunits and membrane phospholipids (Robinson 2015).

Structure and function

Clathrin is trimeric in structure with three heavy chains and one light chain associated forming one triskelion (Kirchhausen, Owen et al. 2014).These three legs are kinked and originate from a central vertex; this structure is referred to as a "triskelion." Clathrin heavy chain has three regions: proximal region, distal region and N-terminal domain. The proximal region is close to the vertex and the light chain attaches to it (Royle 2006). Clathrin-mediated endocytosis (CME) is the well-studied function of clathrin. CME is the process in which clathrin coated vesicles internalize the required materials into cells from the surface of the cells. This process is very important for neurotransmission, transduction of signals, plasma membrane activities regulation and

survival of eukaryotic life (McMahon and Boucrot 2011). Besides endocytosis, there are many other important cellular functions of clathrin. Clathrin facilitates cargo sorting at endosomes by acting as endosomal sorting complex required for transport (ESCRT) machinery (McMahon and Boucrot 2011). It also assists in mitosis and protein secretion from trans-Golgi network (McMahon and Boucrot 2011).

Clathrin adaptors

Clathrin adaptors are proteins that help in connecting clathrin scaffolding to plasma membrane components. These components can be phospholipids, transmembrane proteins or both. Until now at least 20 clathrin adaptors have been found and studied. All 20 adaptors are common in structure. They have a compact folded domain and long unstructured domain which helps them to connect to N-terminal domain of clathrin (Edeling, Smith et al. 2006).Vertebrate genomes encode five types of adaptor proteins namely AP1 through AP5 (Kirchhausen, Owen et al. 2014). AP1 and AP2 interact directly with the N-terminal domain of clathrin heavy chain (Popova, Deyev et al. 2013). AP2 is involved in trafficking at the plasma membrane and clathrin-mediated endocytosis whereas AP1 and AP3 are important for intracellular trafficking events. AP4 and AP5 do not have functions in coating vesicles and are the least studied (Robinson and Bonifacino 2001).

SNAREs and membrane fusion

Membrane fusion is an important biological process in which two initially separated lipid bilayers combine to form a single unit of bilayer (Jahn, Lang et al. 2003). Membrane fusion is responsible for many universal events like fertilization, intracellular trafficking, viral infection and many others (Chernomordik and Kozlov 2005). However, two bilayers

oppose each other for spontaneous membrane fusion events (Chernomordik, Melikyan et al. 1987). This repulsive force is the result of hydration repulsion and electrostatic repulsion of equally charged membrane surfaces (Kozlovsky, Chernomordik et al. 2002).The energy that is required to overcome the energy barrier to facilitate membrane fusion is provided by special fusion proteins known as SNARE proteins (Han, Pluhackova et al. 2017).The SNARE proteins consists of a large superfamily that has 60 different membrane proteins and are present in both mammalian and yeast cells (Fasshauer, Sutton et al. 1998).

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CHAPTER 2

ESTABLISHING CRISPR/CAS9 GENOMIC EDITING FOR DELETION OF THE CLATHRIN HEAVY CHAIN

INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (Cas9) were discovered from an adaptive immune mechanism present in many types of bacteria and most of the archaea (Cong, Ran et al. 2013). Recently, technology has been developed to use the CRISPR system to genetically engineer cells and organisms for bioscience and biomedical applications (Cong, Ran et al. 2013). In our lab, we have developed CRISPR/Cas9 to make targeted base pair insertions or deletions (indels) leading to frameshift mutations and the functional knockout of a gene.

In bacteria, the CRISPR system works based on the coordination of different types of Cas endonucleases. The CRISPR system is divided into two types based on differences in components and their action (Makarova, Wolf et al. 2015). Type one requires a number of different effector proteins; whereas, the mostly used type two is simple and involves only one RNA guided endonuclease, Cas9, which is sufficient to cleave foreign genetic material (Shmakov, Abudayyeh et al. 2015).

The CRISPR system works in three steps to carry out an immune response against foreign DNA (Garneau, Dupuis et al. 2010). The first step is the acquisition stage in which fragments of foreign DNA are incorporated into the CRISPR locus of the host's genomic DNA as spacers between CRISPR RNA (crRNA) repeats. In the second step,

Cas proteins are expressed, CRISPR arrays with acquired spacers then transcribe the pre-crRNA. Pre-crRNA is cleaved and matures to crRNAs with the help of Cas proteins and host factors. This fully matured crRNA contains a spacer sequence that targets it to the invading genome (Deltcheva, Chylinski et al. 2011). In the third step, Cas proteins recognize the suitable target with the help of crRNA, cleaves the invading genome and protects the bacterial host cells from infection of invading pathogens (Christin and Beckert 2016). Many CRISPR systems work based on the presence of a sequence specific protospacer adjacent motif (PAM) that is next to crRNA target site in the invading genome. The absence of this PAM sequence in the host genome protects the host genome from self-cleavage (Hsu, Lander et al. 2014). Cas9 is the RNA-guided endonuclease that cleaves the target DNA in the type two CRISPR system and is the most widely used among bacteria species (Jinek, Chylinski et al. 2012). The cleavage of the target genome by Cas9 depends on a duplex of two RNAs : CRISPR RNA and tracr RNA. CrRNA is 20 base pairs and recognizes invading DNA through its base-pairing region. Tracr RNA is unique to type two CRISPR system and hybridizes with crRNA (Jinek, Chylinski et al. 2012), (Cong, Ran et al. 2013), (Jiang, Bikard et al. 2013). These cr and tracr RNA can be fused into a chimeric single guide RNA (sgRNA) to simplify the system. Chimeric single protein, single RNA, Cas9-sgRNA is the most widely used system for genome editing. This Cas9-sgRNA complex binds to genomic DNA that base pairs with sgRNA and is next to a PAM sequence and causes cleavage within the base-pairing region (Jinek, Chylinski et al. 2012).

A retroviral transduction system was used to produce DNA complementary to the inserted guide RNA using reverse transcription process. Retroviral transduction systems

are efficient in transducing genes into primary cells that are difficult to transduce using other kinds of transduction system. This system also produces stable cell lines after transduction (Cepko and Pear 2001).

This chapter presents the information that are relevant to understanding clathrin heavy chain gene knockout in bone marrow derived murine macrophages using CRISPR/Cas9 genomic editing. It presents the reader with methods for CRISPR/Cas9 genome editing including methods for guide RNA selection; molecular biology techniques for cloning the guide RNA gene into the shuttle vector for viral expression; packaging cell line transfection and titer determination, and macrophage transduction.

A retroviral transduction system was used to produce DNA complementary to the inserted guide RNA using reverse transcription process. Retroviral transduction systems are efficient in transducing genes into primary cells that are difficult to transduce using other kinds of transduction system. This system also produces stable cell lines after transduction (Cepko and Pear 2001).

MATERIALS AND METHODS

Cas9/GFP bone marrow macrophage isolation

Transgenic mice with Cas9 expression were used in the study (Jackson Labs, Bar Harbor, ME). Bone marrow macrophages were isolated from femurs of 6-week-old mice. Femurs were separated and bone marrow was flushed using Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium. This newly isolated bone marrow was collected in a petri-dish. Isolated bone marrow was centrifuged at 500 g for 5 min. After

centrifugation, supernatant was discarded and pellet formed was resuspended in fresh warm bone marrow media. Cells were left to grow in 10 cm petridish in incubator at 37 ^{0}C with 5% CO_2 for 2 days. After 24 h of isolation these cells are known as day 1 macrophages. The cells were grown until day 4 and were frozen on day 4 in freezing media (with 10% DMSO) to preserve for a longer time. Cells were counted using hemacytometer. One to two million cells per ml media was added to cryo vials, labelled and frozen at -80^{0}C for 24 h in a freezing container before storage in liquid nitrogen tank. The viability of frozen cells was checked after they were completely frozen. Cas9 is co-expressed with green fluorescent protein (GFP) and the expression of GFP under fluorescence microscope was verified.

Guide RNA design

Clathrin heavy chain gene information and sequence was found in the National Center for Biotechnology Information (NCBI). The gene ID for clathrin heavy chain is (Cltc) 67300. Once the gene and specific target region for editing with CRISPR/Cas9 was identified, the region along with 50-100 upstream and downstream region were copied. These sequences were submitted to Genscript.com, a gRNA design tool website, and six guide RNAs were obtained. Two sets of guide RNA sequences were ordered. For each guide RNA sequence sense and antisense oligos were ordered. Four bases (CACC) were added to the 5' end of the sense oligo or five bases (CACCG) were added for the sequence that did not start with a G nucleotide in order to make the guide RNA expression constructs. Four bases (AAAC) were added to the 5' end of the antisense guide RNA sequence.

The GEcKO library has gRNAs for all genes of mice. The cleavage of the target genome by Cas9 depends on a duplex of two RNAs: CRISPR RNA and tracr RNA. CrRNA is 20 base pair (bp) and recognizes invading DNA through its base-pairing region. Tracr RNA is unique to type two CRISPR system and hybridizes with crRNA (Jinek, Chylinski et al. 2012), (Cong, Ran et al. 2013), (Jiang, Bikard et al. 2013). These cr and tracr RNA can be fused into form a chimeric single guide RNA (sgRNA) to simplify the system. Guide RNAs designed using Genscript and oligos from GECKO library are listed (Table 1).

Selection of plasmid vector and molecular cloning

pIB2 is a retroviral expression vector. Blasticidine gene from pIB2 was deleted and the vector was converted to pIB3. Restriction sites in the plasmid are spots where the original sequence is cleaved using digestion enzymes and guide RNA ends are ligated to. Bpi1/Bbs and Bg1 II are the two restriction sites that are present in pIB3 vector and were cleaved using restriction enzymes. Two sticky ends were created and guide RNAs bind to these ends. pIB3 vector also had mTq2 (turquoise 2) gene inserted as marker. After cloning was completed, vectors were send for sequencing to check the insertion of guide RNA.

Oligos Annealing

Oligos were resuspended in annealing buffer that is made up of 10 mM Tris, 50 mM NaCl, 1mM EDTA and pH 7.5-8 and mixed in equimolar concentration. Two μg of each oligos were mixed in a total volume of 50 μl. Integrated DNA technologies (IDT) oligos were brought to 100 μM and were around 700 ng/μl (3μl per oligo). Fast digest buffer

can be added for annealing. There are two methods to obtain efficient annealing which are explained below. We used method 2 for our cloning.

Method 1:

Mixed oligos were placed in 1.5 mL microfuge tube and the tube were placed in 90-95 ^{o}C hot block for 3-5 minutes. Tube was removed from hot block and allowed to cool at room temperature for around 45 minutes.

Method 2

Mixed oligos were placed in PCR tube. A thermocycler was programmed to start at 95^{o}C for 2 min and PCR tube was placed on it. Then PCR tube was cooled to 25^{o}C for over 45 min. A separate program was set in the thermocycler known as "Anneal". This program reduced temperature in 5^{0}C for minutes each down to 35^{0}C.

Ligation

The annealed cassette was quantified and was around 80 ng/ul and this is the required amount. Mix listed below was used to digest the vector and ligate the cassette. This reaction is known as Golden gate ligation reaction (indicated in table 2).

This reaction was carried in "GG ligate" program set in thermocycler. Vector was then transformed into competent bacteria. Vector only control ligation was also done to check the background (Addgene protocols).

Transformation

Bacterial transformation was done to multiply the plasmids with target guide RNAs. Antibiotic selection kills cells that do not have antibiotic resistance gene in them. After this step, restriction digestion was done again to confirm whether Bpi1 restriction site

was cleaved or not. Missing Bpi1 site confirmed that guide RNAs were inserted at that place. Bpi1/Bgl2 restriction enzyme was used for restriction digestion and Bgl2 restriction enzyme protects host genome against foreign DNA. Either positive or negative result were obtained.

Negative result was confirmed with two bands: One band was for plasmid with length between cBpi1 and Bgl 2 and second band was for remaining length of plasmid.

Positive result was confirmed with the presence of a single band. Then selection was done for all of the plasmid as indicated (Figure 4). Since 50% positive result was expected from cloning, more clones were screened from each construct in separate experiments to increase the chance of positive result.

Miniprep preparation to extract DNA.

Then miniprep was done (Wizard Plus SV Minipreps DNA Purfication Systems) to obtain positive colony plasmids that contain guide RNA. Solution of plasmid was mixed with mili-Q water. DNA was extracted and platE was transfected. Also, sequencing was done to check if the guide RNA sequence were in the identified positive colonies by restriction enzyme digestion.

Making viral supernatant using Platinum E cells

PlantinumE (PlatE) cells are retroviral packaging cell lines that have all proteins such as gag, pol, env required for virus production. PlatEs were transfected to get supernatant containing viruses for infecting and transduction macrophages. Viral supernatant was harvested from the PlatE culture every 2 days.

Protocol used for making Retroviral Supernatant from Platinum E cells

About 1×10^6 PlatinumE cells were plated per well in a six-well tissue culture plate. The number of wells depend on the number of plasmids from which viral supernatant is to be made. (Platinum E cells are cultured under blasticidin and puromycin selection. Do not add antibiotics to cells plated for transfection). Cells were left in the incubator for at least 24 h before the next step. One ug of pIB3-gRNA plasmid construct was added to 100 uL of PBS. Eppendorf tube was capped and the bottom was gently tapped to mix the DNA and PBS solution. Four uL of 1mg/mL solution of PEI was added to the mixture, the tube was closed and tapped to mix the contents. Incubated for 10 minutes at 37 degree Celsius. (PEI has a high positive charge density and it forms positively charged complexes with DNA. Positively charged PEI-DNA complexes easily attach to negatively charged groups on cell membranes and they are eventually taken up easily by the cell). Media on PlatE cells were replaced with fresh DMEM/FBS and put back in the incubator to equilibrate the temperature to 37 degrees Celsius. 10 minutes is usually more than enough so you can do this right after you start incubating your PEI-DNA mixture. Eppendorf tube were removed gently from the incubator. 200uL pipette was used to gently pipette the solution without mixing it. Pipette was adjusted in such a way that only a single drop of solution was ejected at a time. The drops were spread carefully over the entire surface of the well. Do not mix the solution or shake the plate. Cells were incubated and plasmid expression was observed after 48 hours. (pIB3-gRNA constructs had mTq2 (mturquoise2) expressed cells and appeared cyan/blue in the microscope). Viral supernatant was harvested every 48 hours for maximum 2 days and used as soon as possible within 1-2 hours.

Macrophages were then transduced with 1ml of viral supernatant to 2ml of media. Ten uM cyclosporin was used to increase transduction efficiency in macrophages.

Measuring viral titer using NIH 3T3 Cells

NIH 3T3 cells were used to check the viral titer. Viral titer is measured to test the strength of virus. To know the transduction efficiency of retroviral solution, simple transduction experiment was performed using 3T3 cells. About 200,000 3T3 cells per well were plated in a 6 well non-tissue culture treated plate. Cells were incubated for about 24 h. Retroviral supernatant was added to NIH3T3 cell culture media in 1:1, 1:2, 1:4 and 1:8 ratios. Media with viral supernatant was switched around 24 hours post transduction. At day 3, use the BD Accuri was used to count the number of cells expressing mturquoise2 marker to get an idea of transduction efficiency. Alternatively, snap was taken from various fields of view of each well to get an idea of transduction efficiency. Viral titer was observed to be decreased with decrease in concentration of viral supernatant.

Cas9 bone marrow macrophage transduction

Bone marrow macrophages with cas9 gene engineered in it were used for transduction. Green fluorescent protein was used as marker for cas9. Bone marrow macrophages were transduced with viral supernatant harvested as soon as possible within 1-2 hours. Cyclosporin was added to macrophages together with viral supernatant to increase transduction efficiency. The first media change was done within 24 h post transduction. After this media was switched every two days. It took 7 days to knock out the clathrin gene in macrophages.

Optimizing transfection

PlatEs were transfected with viral supernatant and imaged two days post transfection under Cyan fluorescent protein (CFP). Based on counting of cells expressing mturquoise 2, efficiency of transfection was found to be around 30 %. Best day for viral supernatant harvest was 48 hours post transfection and up to two harvest. Estimating viral titre with 3T3 cells.3T3 cells were transduced with viral supernatant to know the strength of virus. Plated 3T3 cells were transfected with 100,50,25 and 12.5% viral sup. Cells with more concentration of viral supernatant expressed more fluorescent protein.

Transduction optimization

Retro-virus were used for transducing macrophages. Viral supernatant used to transduce macrophages were harvested two days post transfection and used within an hour of harvest. Harvested viral supernatant is best when used as soon as possible (within 1 hour after harvest) and the efficiency of viral titer reduces by 100-fold with one freeze/thaw cycle. Images were taken two-day post transduction and cells expressing mtq2 were seen to be around 50% compared to total cells. Cas9 Bone marrow macrophages were plated 24 h before transduction. Cas9 BMM were plated on day 5 (day 1 post thawing) and viral supernatant was harvested 48 hours after transfection and used within 1 hour of harvest. This produced best result with increased knockout efficiency (Cellbiolabs, 2004-2017).

RESULTS

Six guide RNAs were obtained based on the site of target in host gene. Vector was digested and designed guide RNAs were inserted in the plasmid using golden gate reaction. Reagents and volume used in the reaction is listed (Table 3). Cyan fluorescent protein was used as a marker for guide RNAs and final length of the plasmid was 6207 bp (Figure 1). After cloning was completed, vectors were send for sequencing to check the insertion of guide RNA. Negative result was confirmed with two bands: One band was for plasmid with length between cBpi1 and Bgl 2 and second band was for remaining length of plasmid. Positive result was confirmed with the presence of a single band. Then selection was done for all of the plasmid (Figure 2). Since 50% positive result was expected from cloning, more clones were screened from each construct in separate experiments to increase chance of positive result. Then miniprep was done to obtain positive colony plasmids with guide RNA and sequencing was done to check if the guide RNA sequence were in the identified positive colonies by restriction enzyme digestion. PlatEs were transfected to get viral sup and viral supernatant was reported as mtq2 (Figure 3). Strength of viral titer was estimated using 3T3 cells with different concentrations of virus for each type of guide RNAs. Viral titer strength was found to be highest with concentration 1:1 and 1:2 (Figure 4). Finally, macrophages with transduced with the highest concentration of viral supernatant and imaged two-day post transduction (Figure 5). Transduced macrophages were stained with clathrin antibody in day 8, 9 and 10 post transduction and ran under flow cytometer to observe the fluorescence (Figure 6).

Best day for viral supernatant harvest was 48 hours post transfection. Up to two harvest of viral supernatant provided optimum result. Transduction efficiency of Cas9 bone

marrow macrophages was around 50%. Day of viral sup and bone marrow macrophage determined the transduction efficiency. Right day for cas9 BMM plating was 24 hours before transduction time. We thawed day4 cas9 bone marrow macrophages from liquid nitrogen tank 24 h before transduction with viral supernatant.

DISCUSSION

The CRISPR/Cas9 system for genomic editing is a complex technique that requires a lot of skill and expertise. Due to the viral transduction, it was time consuming as there are many steps involved in the process. Each step required enough skills to make it work. Another difficult aspect of the process was in manipulating macrophages. Macrophages are tricky to work with, as being cells of the innate immune system they have mechanisms to sense and get rid of any foreign genetic materials they encounter. Hence, we faced trouble for some significant amount of time to knockout clathrin gene using CRISPR/Cas9 in macrophages. However, the end result was successful. We were able to knock out clathrin heavy chain gene using this CRISPR/Cas9 system even though the knockout efficiency was quite low compared to our expectation.

CRISPR/Cas9 system has helped cells to take advantage of non-homologous end joining (NHEJ) DNA repair mechanism in the absence of DNA template after DNA double-strand break (Hsu et al., 2014). Its use in insertion, deletion and modification of gene has been helpful in understanding the specific functions of individual gene. This system could also be used in drug development, therapeutics for many genetic disorders along with correction of harmful mutations (Tebas et al., 2014). Difficulty in manipulation of eukaryotic genes, non-specificity and error prone non homologous end joining technique

used for DNA double strand breaks are some of the challenges of CRISPR (Hsu et al., 2014).

The selection of the clathrin heavy chain gene as a target was problematic because clathrin is also involved in cell division and cells are able to survive without it only until they divide. It also took a week to deplete cellular protein levels following viral transduction due to the time it took for reverse transcription of the gRNA into the host genome, expression of the gRNA, DNA cutting, mRNA depletion and protein depletion. We were using primary bone marrow macrophages which senesce after three weeks in culture, and therefore not able to create a stable knockout cell line. We had to knockout genes each time before the experiments. Timing between transduction with viral supernatant and the experiment also played an essential role in the successful knockout of the gene.

Based on experimental observation, the efficiency of gene knockout with this technique was very low. CRISPR/Cas9 technique is useful in knocking out surface protein compared to intracellular protein. Only a good antibody is able to detect intracellular proteins. Still, it has wide functions and is a powerful tool for cell biology. Alt-R CRISPR-Cas9 system is new approach to existing CRISPR/Cas9. In this system, gRNA oligos are transfected directly into macrophages containing the Cas9 enzyme. This new system is easier because it eliminates viral transduction, which has many steps and is time consuming.

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(https://www.cellbiolabs.com/faq/viral-expression-faq/retroviral-expression-packaging)

(http://www.addgene.org/protocols/annealed-oligo-cloning/)

Table 1. List of guide RNAs from Genscript and oligos from GecKo library

gRNA (name)	**gRNA target sequence**
Cltc CRISPR guide RNA 1	GATCGTCATTCTAGCCTCGC
Cltc CRISPR guide RNA 2	TCATATCAATGATTACCACC
Cltc CRISPR guide RNA 3	TGATGATCTGGCATCCTGCG
Cltc CRISPR guide RNA 4	TTATTTGCAGCAAAATCGTG
Cltc CRISPR guide RNA 5	CAGTTACATATCATTGAAGT
Cltc CRISPR guide RNA 6	CTAGCTGCATGCCCTTCAAT

Table 2. List of oligos from GecKO library

Cltc, MGLibA_10908	GATCGTCATTCTAGCCTCGC
Cltc, MGLibA_10909	TCATATCAATGATTACCACC
Cltc, MGLibA_10910	TGATGATCTGGCATCCTGCG

Note: The oligos downloaded from the GeCKOv2 library are the same as the oligos generated from the Genscript website (see first three oligos from Genscript).

Table 3. Reagents with their volume used for golden gate reaction

S.N	Reagents	Amount
1	Water	Up to 20 µL
2	10x FD buffer	2 µL
3	25 mM ATP	1 µL
4	pIB3 30	100 ng
5	Annealed cassette	100-500 ng
6	BbsI	0.5 µL
7	T4 DNA ligase	1 µL

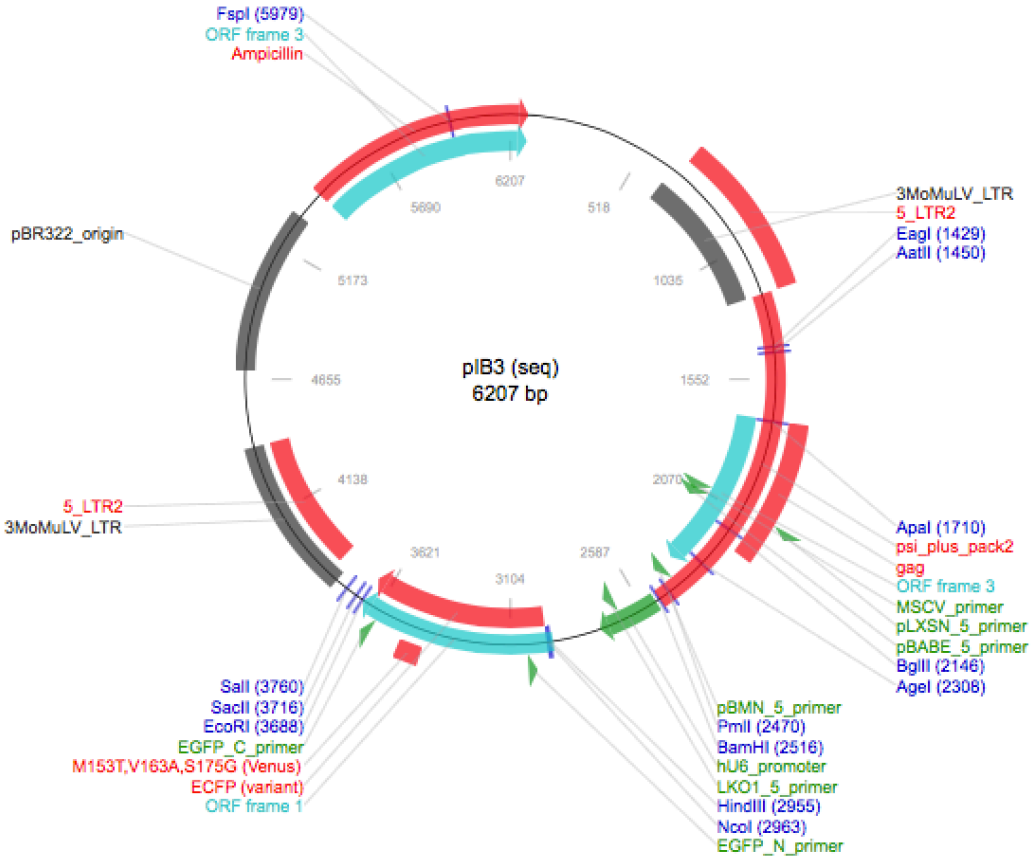


Figure 1. pIB3-mTq2 plasmid vector (Addgene, 2013). CFP marker was inserted in the piB2 plasmid and converted to piB3. It is 6207 base pair long with Bg1II restriction site.

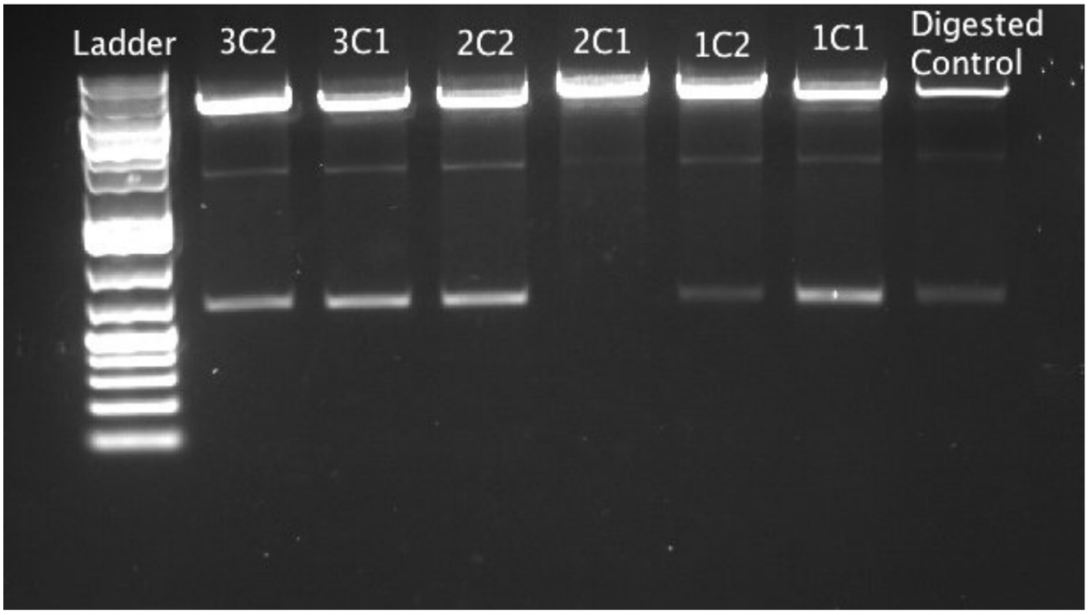


Figure 2. Cloning result for guide RNAs. Two colonies were selected from each guide RNA construct and cultured in Lysogeny Broth (LB) and supplemented with ampicillin for 6 h. A total of 200 ng DNA was digested from each colony with Bg1 II and Bpi1 enzymes. Clone 1 of gRNA2 shows positive result and all other were tested negative.

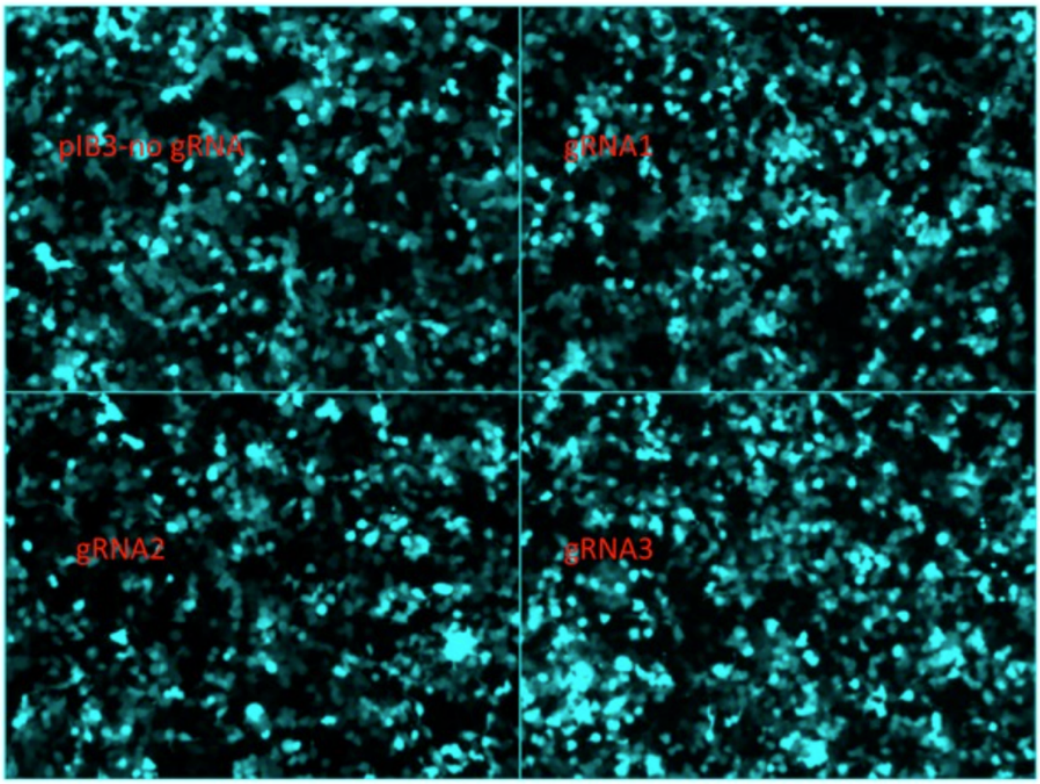


Figure 3: **PlatinumE cells were transfected with 1ug DNA of each construct (gRNAs1-3 and pIB3-control).** Images captured 4 days post transfection show strong expression of turquoise 2, which is the marker for expression of gRNA or control plasmid

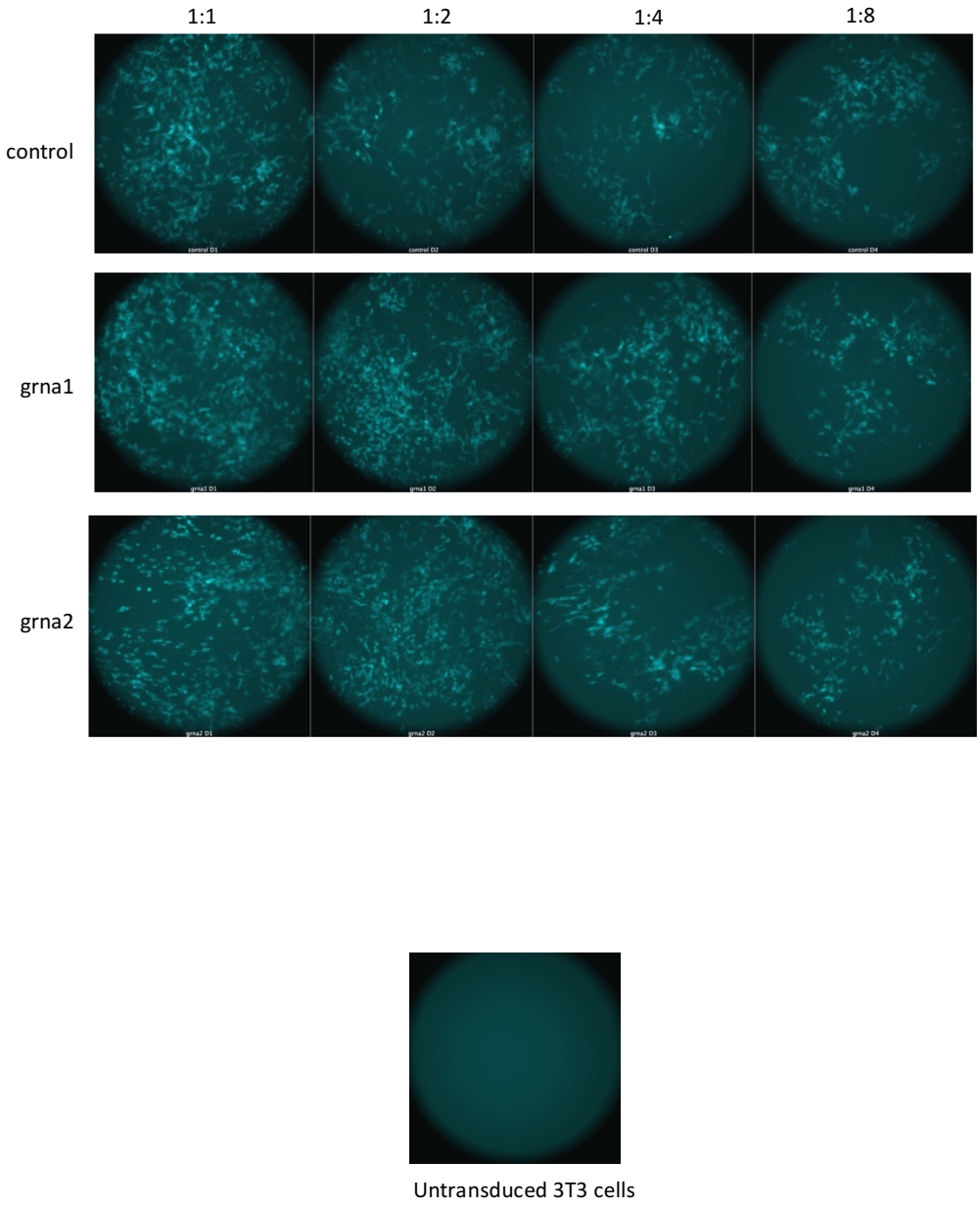


Figure 4: **Estimation of viral titer using 3T3 cells with different concentrations of virus in each type of guide RNAs**. Concentration of 1:1 and 1:2 is seen to express more CFP compared to others in all three guide RNAs.

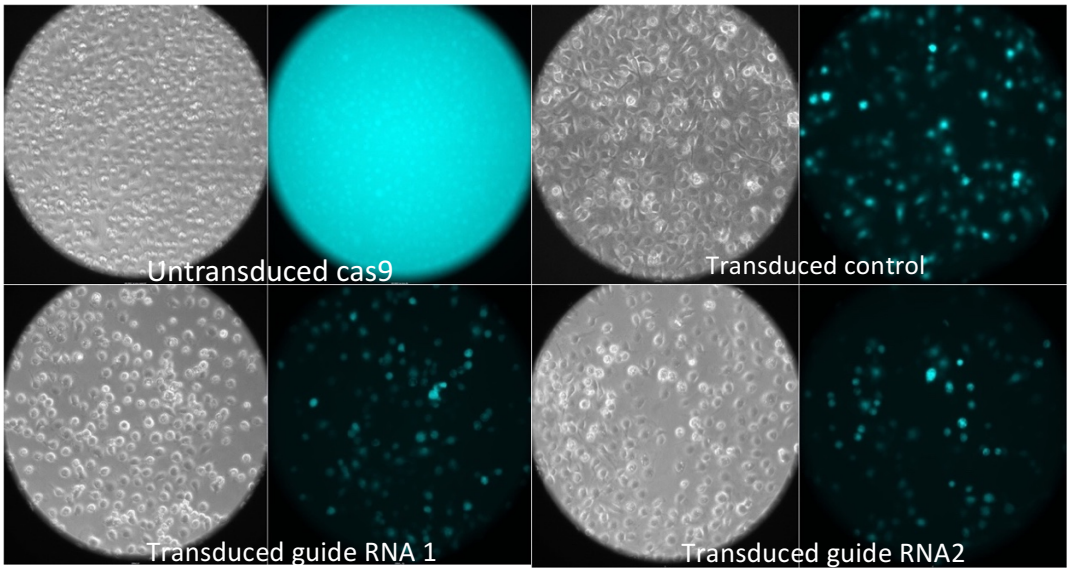
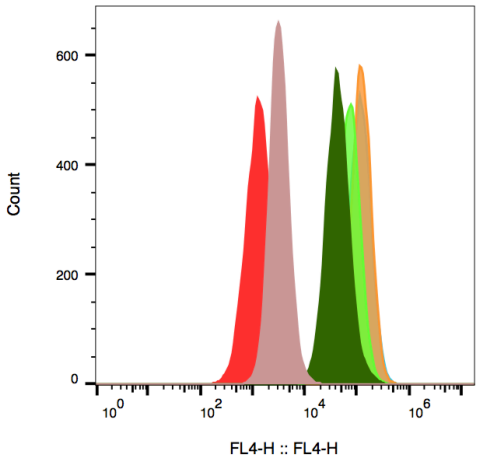


Figure 5: Estimating transduction efficiency of Cas9 Bone marrow macrophages. Transduction efficiency was found to be 50% based on counting. Transduced cells with control grna, grna1 and grna2 is seen to have Cyan fluorescent protein (CFP) expressed whereas transduced do not express.

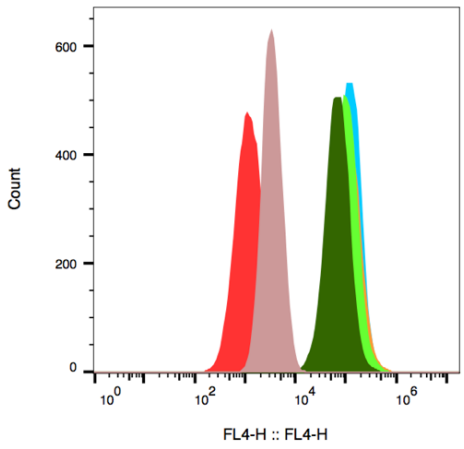
(Note: Contrast and exposure time were different for transduced and untransduced cells. This was done to show the presence of cells in untransduced images).

Day 8 macrophages post transduction



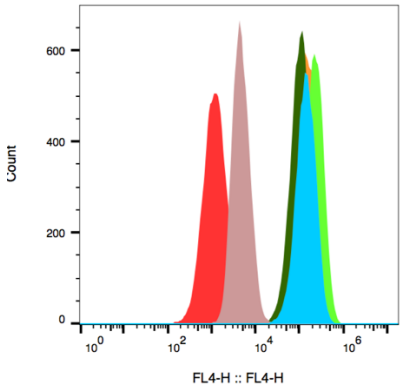
	Sample Name	Subset Name	Count
	C01 day8,sec only,control.fcs	Bone marrow macrophages(bmm)	10869
	B04 day 8,stained,grna3.fcs	Bone marrow macrophages(bmm)	10998
	B03 day 8,stained grna2.fcs	Bone marrow macrophages(bmm)	10082
	B02 day8,stained grna1.fcs	Bone marrow macrophages(bmm)	10292
	B01 day 8,stained,control.fcs	Bone marrow macrophages(bmm)	10235
	A01 Day 8,unstained control.fcs	Bone marrow macrophages(bmm)	10748

Day 9 macrophages post



	Sample Name	Subset Name	Count
	C05 day9,sec only,control.fcs	Bone marrow macrophages(bmm)	10570
	B08 day 9,stained,grna3.fcs	Bone marrow macrophages(bmm)	10550
	B07 day9,stained,grna2.fcs	Bone marrow macrophages(bmm)	10092
	B06 day9,stained,grna1.fcs	Bone marrow macrophages(bmm)	8329
	B05 day9,stained,control.fcs	Bone marrow macrophages(bmm)	10288
	A05 day9,unstained control.fcs	Bone marrow macrophages(bmm)	10846

Day 10 macrophages post transduction



	Sample Name	Subset Name	Count
	B09 day 10,stained,control.fcs	Bone marrow macrophages(bmm)	10356
	C09 day 10,sec only,control.fcs	Bone marrow macrophages(bmm)	10799
	B12 day 10,stained,grna3.fcs	Bone marrow macrophages(bmm)	11464
	B11 day10,stained,grna2.fcs	Bone marrow macrophages(bmm)	10963
	B10 day 10,stained,grna1.fcs	Bone marrow macrophages(bmm)	10867
	A09 day10,unstained,control.fcs	Bone marrow macrophages(bmm)	11432

Figure 6: Knockout cells stained for clathrin under flow cytometry. Knockout cells with guide RNA1, 2 and 3 were stained for clathrin on Day 8, 9 and 10 post transduction. Secondary only and unstained control were also used beside clathrin

CHAPTER 3

CLATHRIN FACILITATES MACROPINOSOME MATURATION IN MACROPHAGES

INTRODUCTION

Macropinocytosis is the non-selective and clathrin-independent uptake of nutrients and solutes from the extracellular space (Swanson and Watts 1995). This cellular process was first discovered by Warren Lewis in 1931 in rat macrophages (Jones 2007). Actin polymerization causes membrane ruffling from the plasma membrane. Subsequently, the lamellipodia fuse back with plasma membrane giving rise to large endocytic organelles known as macropinosomes (Lim and Gleeson 2011). Phagocytes such as macrophages and dendritic cells undergo high macropinocytic activity even in absence of growth factors (Swanson and Watts 1995). However, many other cell types require growth factors like colony stimulation factor-1 (CSF-1), epidermal growth factor (EGF) or mitogens like phorbol 13-myristate 12-acetate (PMA) to form macropinosomes (Kasahara, Nakayama et al. 2007).

Colony stimulation factor-1 receptor is a receptor tyrosine kinase in the platelet derived growth factor (PDGF) family. Like other members of this family, CSF-1R has extracellular, transmembrane and intracellular domains. CSF-1 receptor is present on the surface of the plasma membrane (Figure 1). When ligand CSF-1 binds to the receptor, receptor dimerization and phosphorylation occur leading to downstream activation of Akt and ERK as well as actin polymerization. Actin polymerization causes membrane ruffle formation and macropinosomes. Receptors internalized through endosomes and are subsequently trafficked to macropinosomes prior to lysosomal degradation as indicated

(Figure 1). Mechanisms are unknown for endosome-macropinosome heterotypic fusion, macropinosome maturation and macropinosome-lysosome fusion. Presumably, these events lead to the degradation of activated receptors, which is important for growth control. Hence, our work is focused on understanding the underlying mechanism involved in macropinosome maturation.

Little is known about macropinosome maturation, but some evidence suggests that following their formation, macropinosomes mature in a similar sequence as other endocytic compartments (Racoosin and Swanson 1992). Soon after internalization, macropinosomes gain EEA1 and Rab5 similar to "early endosomes" before converting to Rab7-positive compartments similar to "late endosomes" and finally fusing with lysosomes (Racoosin and Swanson, 1993; Hewlett et al., 1994)

The goal of this project was to elucidate the mechanisms of macropinosome maturation based on the existing knowledge about endosome maturation. Early endosomes act as cargo sorting organelles. Signaling receptors such as epidermal growth factor receptor (EGFR) that traffic to the lysosome for degradation have cytosolic domains for sorting machinery recruitment (Jovic, Sharma et al. 2010). Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) binds to mono-ubiquitinated cargo through its ubiquitin interacting motif and sort them for degradation. Hrs also has clathrin box domain present in C-terminal domain that binds clathrin. Both flat clathrin lattices and Hrs were observed by electron microscopy on portions of early endosome membranes. These clathrin were morphologically different than clathrin present in cell membrane (Raiborg, Bache et al. 2002). Hrs also has a phosphatidylinositol 3-phosphate-binding FYVE domain that plays important role for endosomal targeting (Bache, Raiborg et al. 2003). Hrs forms novel

sorting micro domains composed of both ubiquitin and clathrin and may function as an endosomal clathrin adaptor. Through recruitment of clathrin, Eps15 and other proteins in the restricted domain of early endosomes, Hrs promotes maturation of early endosomes to late endosomes (Raiborg, Grønvold Bache et al. 2001).

From our data, we observed clathrin assemblies around macropinosomes with CSF-1 receptors, those macropinosomes merged with other macropinosomes like organelles faster and degradation of the receptor was quicker. However, in the absence of clathrin, fusion between macropinosomes was quite slow and defects in receptor degradation was observed. Hence, we hypothesized that clathrin could be facilitating the fusion of macropinosomes and hence degradation of CSF-1 receptors. Clathrin could also be sorting cargoes to prevent fusion of macropinosomes. However, this function of clathrin is the least studied one. Due to the bigger size of macropinosomes, they are easier to study and findings can also be related to endosomes. Preliminary data show the assembly of clathrin along with Hrs in endosomes. The morphology of these clathrin were different than the ones present in membrane. The function of clathrin was identified by using cargoes like transferrin receptors. The ubiquitinated transferrin receptor bound to domains with Hrs and clathrin later degraded, whereas transferrin receptor unbound to clathrin and Hrs recycled back (Raiborg, Bache et al. 2002). This finding also provided us with hint to look into the role of clathrin in trafficking of CSF-1 receptor and maturation of macropinosomes.

MATERIALS AND METHODS

Media preparation and reagents

Bone marrow media (BMM) was used for the growth and differentiation of macrophages. BMM was prepared by mixing 20% premium heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), 30% of L-cell (Lou, Low-Nam et al. 2014)with Penicillin and Streptomycin (Pen Strep) (30-002-Cl, Corning, Manassas, VA), and 2-mercaptoethanol (ThermoFisher Scientific, Waltham, MA) in Dulbecco's modification of Eagles Media containing 4.5 g/L glucose, L-glutamine and sodium pyruvate (DMEM) (from Corning, Manassas, VA). All these reagents were mixed together and filtered using 0.22 um pore size PVDF Durapore membrane (EMD Millipore corporation, Darmstadt, Germany).

Other important media used was starvation media which is also known as 10% FBS in DMEM. It was used to starve macrophages of growth factor, colony stimulation factor. This media was prepared by mixing 500 ml of Dulbecco's modification of Eagles Media containing 4.5 g/L glucose, L-glutamine and sodium pyruvate (DMEM) (from Corning, Manassas, VA), 10% of heat inactivated fetal bovine serum (FBS) (from Atlanta Biologicals, Flowery Branch, GA) and 1% of Penicillin and Streptomycin (Pen Strep) (30-002-Cl, Corning, Manassas, VA).

Isolation of bone marrow macrophages

Femurs were harvested from adult C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) (Swanson 1989). Bone marrow was flushed with the help of 0.5 inch, 26-gauge needle and 5 ml sterile Luer Lock syringe using phosphate buffered saline (DPBS)

without calcium or magnesium. This newly isolated bone marrow was collected in a petri-dish. Isolated bone marrow in DPBS –Ca –Mg was centrifuged at 300 g for 5 min. After centrifugation supernatant was discarded and pellet formed at the bottom was resuspended in fresh warm bone marrow media. This media with cells was incubated in 10 cm petridish at 37 ^{0}C with 5% CO_2 for 2 days. Macrophages are expected to adhere to a non tissue-culture treated culture dish by 4 days of culture. On day 4, media is replaced with fresh medium, and other types of cells which are non-adherent get discarded leaving a homogenous primary macrophage culture.

After 24 h of isolation these cells are known as Day 1 macrophages. The cells were grown till day 4 and prepared for freezing on day 4. Ten percent DMSO with respect to total media was added to cells to preserve them. Cells were counted using hemacytometer. One million cells per ml media was added to cryo vials, labelled and frozen at -80^{0}C for 24 h in a freezing container before moving it to liquid nitrogen tank. Cells were moved to nitrogen tank post 24 h. Viability of frozen cells were checked after they are completely frozen.

Fetal liver macrophages isolation and culture

Fetal liver macrophages were isolated from livers of neonatal C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). To isolate fetal liver macrophages, livers were isolated from mouse fetuses from 15-19 gestational days. All laws and policies were followed in accordance to South Dakota State University Institutional Animal Use and Care committee. Liver tissue was dissociated mechanically using sterile forceps. Tissues were passed through 1 ml pipette tip and single cell suspension was created. Cells were plated at the density of 8x10^{6} cells per 10 cm non-tissue culture treated cells. Bone

marrow media was used for growth and differentiation of macrophages. Cells were cultured for at least 8 weeks before conducting any experiment. (reference FLM paper)

Transduction with fluorescent fusion protein constructs (clathrin-YFP, dynamin)

Fetal liver macrophages were starved of CSF-1 for 18 h prior to experiments to up-regulate CSF-1R on the surface of the cells. Cells were incubated with 200 ng/ul CSF-1 for the indicated time points prior to fixation (for IF) or lysis (for western blot). The Dynamin-2 (DNM-2) mCherry constructs were created using Clonetech plasmids obtained from the Hoppe laboratory. Overlap extension PCR and conventional restriction enzyme cloning methods were used to create retroviral pIB2 plasmids containing the DNM-2 Wildtype (WT) and DNM-2 K44A genes. Clathrin light chain (CLC) turquoise-2 FLMs were created using a similar retroviral transduction approach before sorting via Fluorescence-activated cell sorting (FACS) on a flow cytometer. Human embryonic kidney (HEK) 293 cells were used as a packaging cell line for plasmids. They were co-transfected with the DNM-2 mcherry retroviral pIB2 plasmid., a pCL-Eco packaging plasmid and a pCMV-VSV-G pseudotyping plasmid. The viral supernatant produced by the HEK 293 cells was then added to the sorted CLC-Tq2 to produced a stable, doubly transfected DNM-2 mCherry/CLC-Tq2 FLM cell line.

Immunofluorescent staining

Immunofluorescent staining was done to visualize and quantify knockout phenotypes, CSF-1R, EEA1, Rab5, Rab7, Clathrin, CALM. Cells were plated for IF on both small and bigger coverslips. 4% PFA was used for 10 min to fix the cells and 100 % ice-cold methanol was used for 1 min to permeabilize the cells. The permeabilization step is done to create holes in the cells so that antibodies can have easy access to the protein of our

target. Some experiments need harsh detergents like 0.1% triton-x and some need mild detergent like saponin. Harsh detergents permeabilize even the organelles along with cell membranes whereas mild detergents permeabilize only membranes. Blocking step follows permeabilization step. Blocking is done for at least 1 h with 2.5% bovine serum albumin powder (ThermoFisher Scientific, Waltham, MA) mixed with 1X phosphate buffer saline (PBS). The blocking step helps in binding non-specific proteins present in cells beside our target proteins with antibodies present in bovine serum albumin. Cells were washed 3 times with 1x PBS after every step. Primary antibodies were added to cells based on the dilution provided by the datasheet for overnight at 4^0C. Antibodies have higher affinity at 4^0C. Secondary antibody is specific to host of primary antibody and is added for an hour in 1:500 dilution with PBS. Nuclear staining with DAPI (ThermoFisher Scientific, Waltham, MA) is done for 5-20 min. Coverslips are then washer 3X with PBS and mounted on glass slides with Flouromount-G (Southern Biotech, Birmingham, AL). After fluoromount-G dries coverslips are sealed in glass slide with a drop of clear nailpolish. Now coverslips are ready for imaging under fluorescent microscopy.

Table 1. Primary antibodies used for immunofluorescence experiments.

Primary Ab against	Species	Product code	company	Type
Mouse CD115(CSF-1R)	Rat	AFS98	eBioscience	Monoclonal
Clathrin heavy chain	Mouse	MA1-065	Thermoscientific	Monoclonal
Clathrin heavy chain	Rabbit	D3C6	Cell signaling	Monoclonal
Rab5	Rabbit	C8B1	Cell signaling	Monoclonal
Rab7	Rabbit	D95F2	Cell signaling	Monoclonal
PICALM	Rabbit	Bs-11665R	BIOSS	Polyclonal
Eea1	Rabbit	C45B10	Cell signaling	Monoclonal

Imaging

Cells in coverslips were imaged with fluorescent microscopes. An EVOS FL imaging system in lab was used for fixed cells. It has filter cubes for GFP, Texas red, Cy5 and DAPI (ThermoFisher Scientific, Waltham, MA). A confocal microscope with Andromeda spinning disk was used for experiments with live cells. For red fluorescent protein, 561nm laser with Zet 442/514/561x excitation wheel and 605/64 emission wheel was used. For green fluorescent protein, 445 laser with Zet 442/514/561x excitation

wheel and 537/26 emission wheel was used.

Flow cytometry

Flow cytometry experiments were done to confirm clathrin knockout. Bone marrow macrophages were grown in petridishes. They were fixed with 4% PFA, permeabilized with 0.1% saponin and blocked with 2.5% BSA. Primary and secondary antibody staining was done at the end. Cells were in suspension state in centrifuge tubes when all the steps were carried out.

Live cell microscopy

An Andromeda spinning disk confocal microscope was used to images live cells. Dextran uptake assays, co-localization assays were carried for detection of CALM and Clathrin, clathrin and macropinosomes co-localization and macropinosome lysosome fusions. Cells are plated in 25 mm glass coverslips at the density of $3x10^5$ depending on the day of experiment. Cell chamber with live cell imaging buffer (LCIB) is used and coverslips were placed on it to take movie or images. In some case cells are pre-labelled with fluorescent dextran and images later. Oregon green dextran and Texas red dextran are used for labelling macropinosomes and lysosomes.

Transmission electron microscopy

Transmission electron microscopy samples were fixed and sent to the South Dakota School of Mines and Technology for imaging. Clathrin heavy chain gene was knockout from bone marrow derived macrophages using CRISPR/Cas 9 technique. A total of 500,000 clathrin knockout cells were plated on 6 cm dish. They were starved of CSF-1 overnight. They were stimulated with CSF-1 for 0, 5, 15 and 30 minute. Cells were

removed from the dish, moved to microfuge tubes, centrifuged at 500 g for 2 min. They were resuspended in fixative. Fixative used was 2% Glutaraldehyde from Sigma Aldrich (50 wt. % in H_20), (glutaraldehyde was diluted using 4% PFA to make 2% Glutaraldehyde). Microfuge tube were wrapped with parafilm and shipped with ice on styroform box. Received samples were fixed with glutaraldehyde, paraformaldehyde and osmium tetroxide to preserve them in natural state. Dehydration step follows fixation as water is incompatible with high vacuum condition in TEM column. Organic solvents like Ethanol and acetone in increasing concentration are used for dehydration step. Ultra-microtomy is used for sectioning of cells and are used for imaging under microscope.

Image Analysis

Images were analyzed using ImageJ(32-bit) (National Institutes of Health, Bethesda MD) software.

RESULTS

Clathrin localizes around macropinosomes in CSF-1 stimulated macrophages

Bone marrow macrophages transfected with a gene for a clathrin light chain-yellow fluorescent protein (CLTA-YFP) fusion were starved of CSF-1 overnight and then exposed to CSF-1 and imaged by live-cell microscopy. Clathrin assembled around macropinosomes at different time points as indicated (Figure 2A). A clathrin assembly is seen around macropinosomes from 6-11 min post CSF-1 addition and correlates with CSF-1 arrival at the macropinosome. To confirm, the clathrin was assembled around macropinosomes, cells were stimulated with CSF-1 and observed for uptake of Texas red

dextran. This 70 KDa dextran sugar molecule can only be taken up by only macropinocytosis due to size exclusion from other routes of entry such as endocytosis. The merged image shows the presence of clathrin assemblies around dextran positive vesicles. Immunofluorescence experiments confirmed the presence of endogenous clathrin on macropinosomes and provide localization for its co-localization with CSF-1R (Figure 2C). These results suggest that the observed clathrin assembly on macropinosomes is not a result of clathrin overexpression.

The fate of the macropinosome and its cargo is different in the absence of clathrin

Clathrin heavy chain (CHC) in bone marrow derived macrophages were knocked down using siRNA and scrambled siRNA smart pool was used as control. Cells were starved of CSF-1 overnight and stimulated the following day with 100 ng/ml CSF-1 for 5,15, 30 and 45 minutes (Figure 3).

In CHC knock down cells, CSF-1 receptors were not degraded even at 45 min whereas majority of CSF-1R were degraded by 30 min post stimulation in control cells (Figure 3). However, we couldnot distinguish the compartment type in CHC KD cells (as early endosome or late endosome) where undegraded receptor were stuck. In control cells, result was opposite to knock down cells. Receptor degradation started at 30 min and ended by 45 min. Hence, we observed the difference in pattern of receptor degradation in control and knockdown cells in presence and absence of Clathrin. This suggests that clathrin could be playing role in CSF-1R degradation. However, the actual mechanism or process behind the receptor degradation by clathrin is not well understood. One possibility could be the involvement of clathrin in macropinosome maturation.

Clathrin knockdown and control cell lysosomes were pre-labeled with Texas red dextran and macropinosomes formed during stimulation with CSF-1were labelled with Lucifer yellow. In control cells, 30-min post stimulation with CSF-1, Lucifer yellow was seen to be co-localized with Texas red indicating macropinosomes had fused with lysosomes. Fusion between macropinosomes and lysosomes was disturbed in clathrin knockdown cells. To confirm clathrin knockdown cells had normal lysosomes cells were stained using Lamp1 antibody (Figure 4). The staining pattern looks similar for both control and knocked down cells. Hence, clathrin is necessary for the fusion of macropinosomes and lysosomes but the lysosome is seen to be unaffected in the process.

Clathrin, EEAl and CSF-1R are co-localized in CLTA-YFP transfected Fetal Liver macrophages (FLMs).

Fetal liver macrophages (FLMs) were transfected with clathrin light chain construct to overexpress fusion protein yellow fluorescent protein. They were starved overnight of CSF-1 and stimulated following day for 3, 6 and 9 min. Merged images at some spot show the co-localization between clathrin, CSF-1R and EEA1 (Figure 5). This data shows that the normal trafficking route of CSF-1R is through early endosomes. Clathrin also co-localized with both EEA1 and CSF-1R.

Colony stimulation factor -1 receptor gets trapped in Rab7 positive compartments in clathrin depleted macrophages.

Wild type and clathrin knockdown macrophages were starved overnight, stimulated with CSF-1 for 15 and 45 min. They were stained with CSF-1R and Rab7 antibodies. CSF-1 receptors in wild type macrophages were degraded at 45 min and were Rab7 negative; whereas, CSF-1R in CHC KD macrophages were un-degraded even at 45

min post CSF-1 stimulation and were Rab7 positive as indicated (Figure 6). This can be an important observation to understand the mechanism of macropinosome-lysosome fusion. This result shows that CSF-1R gets stuck to the Rab7 compartment and are prevented from maturing and fusing with lysosomes.

CSF-1R is seen to be present in early endosomal antigen 1 (EEA1), Rab5 and CALM positive compartments in CHC heavy chain knockout cells

Bone marrow derived macrophages were used on day 7 post retro-viral transduction to perform experiments as indicated (Figure 7). Clathrin heavy chain gene was knocked out using guide RNA constructs and CRISPR/Cas9 technique. Macrophages were stained for endogenous CSF-1R and CALM at 30 min, CSF-1R and EEA1 at 30 min and CSF-1R and Rab5 at 15 min post CSF-1 stimulation. Clathrin assembly lymphoid myeloid leukemia protein (CALM) is a clathrin adaptor. During endocytosis, when a specific molecule binds to a target receptor in plasma membrane, clathrin and other molecules like AP2, epsin and CALM transports to plasma membrane from cytoplasm and facilitated CME process (Rai, Tanaka et al. 2014). In clathrin KO cells we were also able to see a ring like compartment with CSF-1R stuck in it as observed in figure 3 and 4. These compartments were also seen to be CALM positive. Endogenous CSF-1R and Eea1 were stained with Antibody for CSF-1R and Eea1 as indicated (Figure 7B). Knockout cells with expected phenotypes were seen to be EEA1 positive. Endogenous staining for both CSF-1R and Rab5 is shown as indicated (Figure 7C). Rab5 is a protein from the Rab family that regulates activities of early endosomes (Stein, Dong et al. 2003). Knockout cells with ring like compartments were also seen positive for

Rab5. Hence, it can be concluded that knockout cells with un-degraded receptors are trafficked through early endosomes.

Clathrin and Dynamin2 K44A both co-localize around macropinosomes, after addition of CSF-1.

FLMs transfected with clathrin light chain fusion yellow fluorescent protein were transfected with dynamin2-mCherry constructs. GTPase dead Dynamin 2 K44A mutants were also created. Macropinosomes were labelled with Alexafluor 647 dextran of 10,000 molecular weight and stimulated with CSF-1. Wildtype cells and mutant were imaged under confocal microscope. Colocalization between clathrin and dynamin was observed at 12 min post stimulation in wild type and 8 min post CSF-1 stimulation in mutant. Wildtype and mutant cells showed co-localization around macropinosomes in merged images as indicated (Figure 8A). Quantified result from figure A for control and mutants is shown in a line graph as indicated in (Figure 8B). The line graph shows co-localization between clathrin and dynamin in both cells types.

The clathrin adaptor CALM and clathrin light chain localize around macropinosomes in clathrin light chain transfected FLMs

FLMs transfected with clathrin light chain yellow fluorescent fusion protein (YFP) were transfected again with CALM (mCherry) constructs. The goal was to see if CALM functions together with clathrin to facilitate macropinosome-lysosome fusion. These CLC-YFP FLMs were stimulated with CSF-1 for 2 min and imaged under 2 channels in confocal microscope. Localization between CALM and clathrin was observed visually as indicated (Figure 9) and later quantified using image j software. Quantified data also shows the assembly of CALM with clathrin at some spots. Hence, presence of

clathrin adaptor CALM together with clathrin might indicate a clathrin-mediating sorting event on the membrane.

HRS localizes to CSF-1R positive macropinosomes in CHC KD and control cells.

Bone marrow derived macrophages were transfected with either clathrin heavy chain or scrambled siRNA (control). BMM were starved of CSF-1 overnight, prior to the experiment. Cells were stimulated with CSF-1 for 10 min and fixed and immunostained for HRS and CSF-1R as indicated (Figure 10). CSF-1R positive macropinosomes were Hrs positive in both control and clathrin knocked down cells.

Unstimulated bone marrow derived macrophages under electron microscopy

Day 7 wild macrophages were imaged under transmission electron microscopy. Clathrin assembly was observed around the organelles presumably macropinosomes/endosomes that are highlighted as indicated (Figure 11).

Clathrin heavy chain knockout using CRISPR/Cas9 technique also shows similar result in CSF-1R receptor degradation pattern as with siRNA technique.

Clathrin heavy chain gene was knocked out in bone marrow macrophages with CRISPR/Cas9 technique using two different guide RNAs. Endogenous CALM and CSF-1R were stained using respective antibodies. Unique ring like structure with CSF-1 receptors are seen in macrophages with guide both guide RNAs whereas control shows opposite result as indicated (Figure 12). Ring like structure persists even at 45 min time point. This result is in accordance to the figure 3A where receptors are not degraded in the absence of clathrin.

DISCUSSION

Here we show that clathrin facilitates the maturation of macropinosomes and subsequent fusion with lysosomes in bone marrow derived murine macrophages. Colony stimulating factor-1 receptor (CSF-1R) was used as cargo to study the process of macropinosome maturation. Clathrin assemblies were observed around macropinosomes following CSF-1 stimulation. To understand the function of clathrin, clathrin heavy chain protein was knocked down or knocked out. In the absence of clathrin, degradation CSF-1R was delayed. In cells without clathrin, the size of macropinosomes became larger than control cells. This could be due to increased homo and heterotypic fusion, reduced membrane recycling, or decreased intraluminal budding. Macropinosomes in clathrin knockdown cells were Rab5 and Rab7 positive endocytic compartments indicating that these vesicles may be stuck in an intermediate phase between early and late endosomes. Endosome maturation requires conversion of Rab5 to Rab7, and Rab proteins participate in vesicle transport processes in its GTP bound form. To activate Rab5, Rabex5, a guanine exchange factor (GEF) is recruited to early endosomes. Removal of Rab5 and its replacement by Rab7 is necessary to form late endosomes and transport cargoes to lysosomes. Removal of Rab5 requires involvement of GTPase activating protein (GAP) to activate Rab5's hydrolysis activity (Huotari and Helenius 2011). Hence, in our macropinosomes that fail to mature, Rab5 may have lost its ability to recruit GAP activity and hence no hydrolysis activity occurs. Results showed that clathrin plays a role in maturation of macropinosome but more needs to be known about the exact mechanism.

Macropinocytosis is a type of endocytosis that facilitates non-receptor mediated uptake of nutrients, antigens and solute molecules. It is an actin dependent process, and

starts from cell membrane ruffles, which give rise to the large endocytic vesicles known as macropinosomes (Lim and Gleeson 2011). Macropinocytosis has a prime importance in both amoeba and mammalian cellular feeding processes. The phagotrophic cells ingest nutrients via macropinocytosis, accumulate them in endosomes and fuse their content to lysosomes for degradation (Bloomfield and Kay 2016). Extracellular polypeptides are taken up by macropinosomes, digested in the endocytic pathway and provided to cells as essential and non-essential amino acids (Commisso, Davidson et al. 2013). Macropinosomes can also be utilized as organelles for delivering therapeutic cargoes to the cells as they uptake and transport required materials in bulk (Gilleron, Querbes et al. 2013). For example, lipoprotein vehicles to target hepatocytes have been developed recently and they enter the cells through macropinosomes (Dong, Love et al. 2014). Understanding mechanisms of macropinosome cargo sorting, macropinosome maturation and membrane trafficking will help to understand the key mechanisms behind several diseases like cancers that are result of deregulated signaling and trafficking. Many viruses and bacteria also use macropinocytosis as a medium to enter cells (Bloomfield and Kay 2016). Better understanding of macropinocytosis and membrane trafficking can also provide knowledge and ideas to design new therapeutic approaches to manage and cure existing diseases.

Macropinocytosis mostly occurs in response to growth factor stimulation. However, there are antigen presenting cell types like dendritic cells, macrophages and B cells that are capable of constitutive macropinocytosis (Norbury, Hewlett et al. 1995, Norbury, Chambers et al. 1997). Macropinosomes are very different from other types of endocytic vesicles as they lack distinct coat membrane. They are heterogeneous in size

(more than 0.2 um in diameter) and supply cells with large amount of nutrients, macromolecules and membranes (Lim and Gleeson 2011).

Macropinosomes undergo maturation processes following their formation. Macropinosomes mature through both homotypic and heterotypic fusion with endosomes and fuse with lysosomes for degradation (Dolat and Spiliotis 2016). EEA1 functions as a tethering molecule and helps in both homo and heterotypic fusion of early endosomes (Raiborg, Grønvold Bache et al. 2001). With time they begin to decrease in size, gain or lose different endocytic protein and move centripetally to fuse with lysosomes (Racoosin EL, Swanson JA. 1993). Macropinosomes formed after 1 min of treatment with CSF-1 in bone marrow macrophages were stained as positive for transferrin receptors and are known as early macropinosomes. After 2-4 min, the same macropinosomes lacked transferrin receptors and had acquired Rab7 proteins. Rab7 protein is known as marker for late endosomes and these Rab7 positive macropinosomes are known as late macropinosomes (Lim and Gleeson 2011). Rab7 and lysosomal glycoprotein coincided at even later time points and hence macropinosomes fuse with tubular lysosomal structures (1993). In HEK293 cells stimulated with epidermal growth factor, macropinosomes mature in a similar fashion as in macrophages. The only difference is the absence of sortin nexin 5 (SNX5) as tubules from macropinosomes in these epithelial cells. This reduces surface area of macropinosome and helps acquire endosome maturation marker Rab7 (Kerr, Lindsay et al. 2006). However, the case is different with macropinosomes formed in EGF-treated A431 cells (epidermoid carcinoma human cell lines). They neither acquire late endosome marker Rab7 nor fuse with lysosomes (1994) . Macropinosomes formed 5 min post treatment with EGF are positive for transferrin receptors and faintly

positive for EEA1 (1989) (Hamasaki, Araki et al. 2004). EEA1 helps in homotypic fusion between macropinosomes (Araki, Hamasaki et al. 2006). At the end, macropinosomes fuse back with plasma membrane and release their content to outside of the cell (Hewlett et al., 1994 ; West MA et al., 1989).

Rab GTPases belong to the Ras superfamily and are key regulators of endocytosis. They regulate each step of endocytic traffic like selection of cargo, identification of target membrane for fusion and vesicle budding (Somsel Rodman and Wandinger-Ness 2000, Smythe 2002) . Among 60 total Rab proteins, 13 are involved in the endocytic pathway and even smaller number have been well studied (Stein, Dong et al. 2003). Rab5 and Rab15 are involved in the early endosomal pathway. Rab5 sorts cargo, facilitates cytoskeletal movement and helps in homotypic fusion of early endosomes (Zerial and McBride 2001) whereas Rab15 functions oppositely of Rab5. It prevents movement of cargo to Rab5 and promotes perinuclear recycling (Zuk and Elferink 2000). Cargoes that reach early endosomes follow two paths. Either cargoes are sorted for recycling back to plasma membrane or they are sorted for degradation in lysosome. In the recycling pathway two more Rabs: Rab4 and Rab11 are involved. The Rab4 domain in early endosomes accepts sorted cargo and promotes faster delivery of them to plasma membrane (van der Sluijs, Hull et al. 1992) . Similarly, Rab11 is located in perinuclear recycling endosomes and facilitates transport of cargo sorted for recycling to plasma membrane (Calhoun and Goldenring 1996). Cargoes that follow the degradation pathway depend on Rab7 for transport from early to late endosome (1995). Rab7 could also be involved in transport of cargo from late endosome to lysosome with the help of Rab interacting lysosomal protein (RILP), one of its effector (Cantalupo,

Alifano et al. 2001). RILP is a small GTPase found in cytosol that binds Rab7-GTP at its C-terminus end and can be recruited to late endosome and lysosomal compartments (Cantalupo, Alifano et al. 2001). Lysosome-associated membrane protein (Lamp1) is an important membrane protein in lysosome that has luminal and cytoplasmic domain. Luminal domain is highly glycosylated and cytoplasmic domain is short with only 10-11 amino acids. Late endosomes and lysosomes contain high amount of Lamp1 in inactive state. Lamp1 is also present in some amount in plasma membrane and early endosomes (Cook, Row et al. 2004).

The hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) helps in cellular trafficking and signal transduction. Hrs has a phosphatidyl 3-phosphate binding FYVE domain that targets endosomes (Raiborg, Grønvold Bache et al. 2001). FYVE domains are membrane targeting domains that are specific to phosphatidylinositol 3-phosphate and are found in proteins that have role in trafficking pathways. FYVE is named after the the first letter of four proteins they were discovered in: Fab1, YOTB, Vac1 and EEA1 (Wywial and Singh 2010). Hrs and EEA1 are found at distinct regions of early endosomes and therefore suggest that they have different functions. Clathrin and Hrs had strong co-localization at early endosome membranes. The C-terminus of Hrs has a clathrin box motif that is functional and interacts with the beta propeller domain of clathrin heavy chain. Hence, Hrs could be functioning as endosomal clathrin adaptor and also helping in maturation of early to late endosomes (Raiborg, Grønvold Bache et al. 2001). Based on our data, the clathrin assembly is observed around macropinosomes with CSF-1R in bone marrow derived macrophages.

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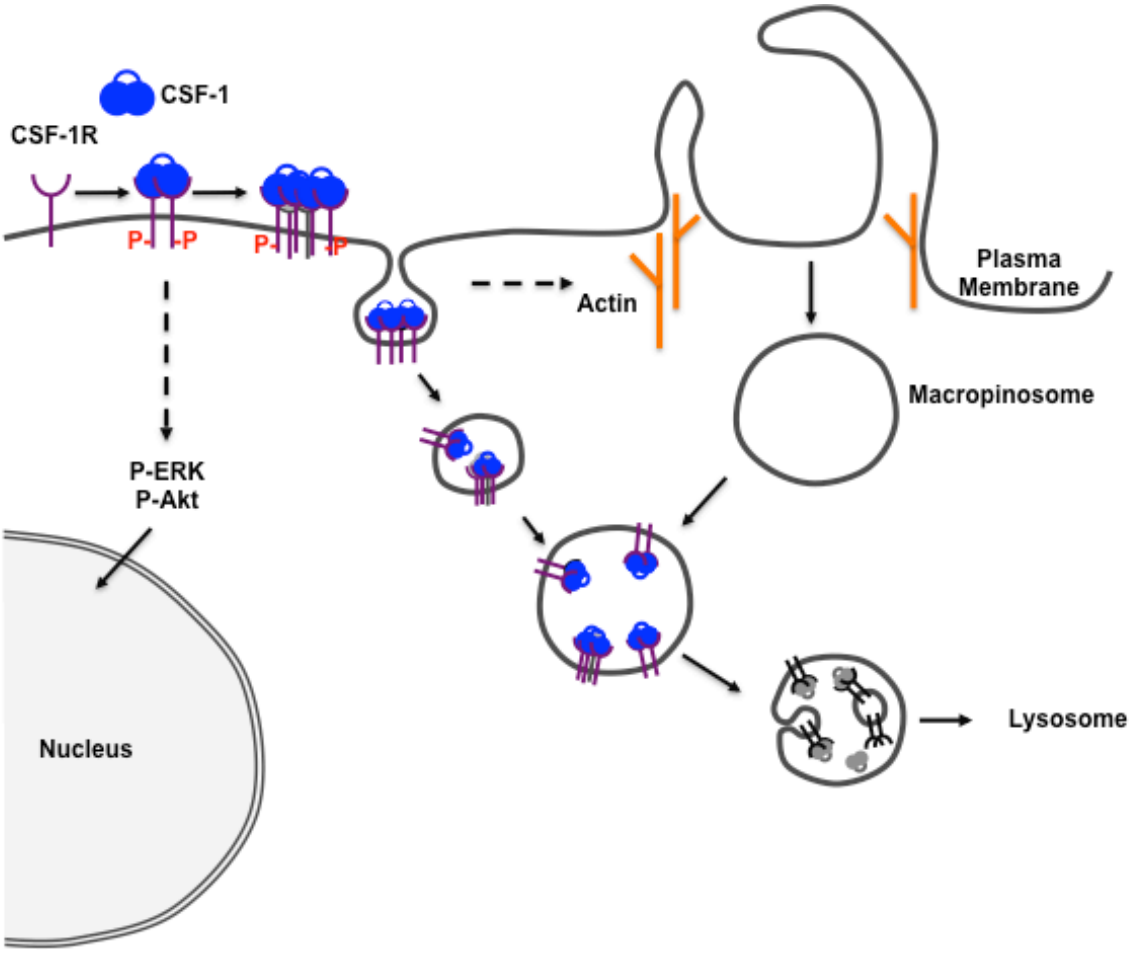

Figure 1. Upon receptor activation, CSF-1R is internalized by small vesicle endocytosis, traffics to macropinosomes and then is degraded.

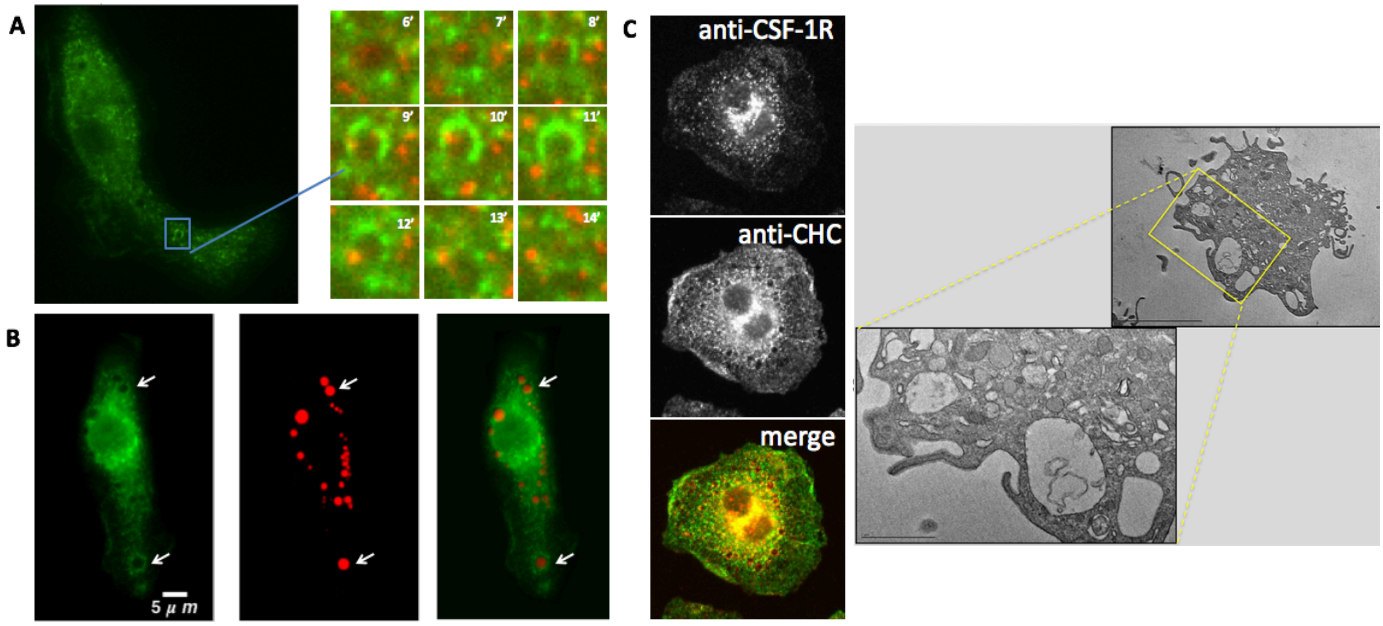

Figure 2. Clathrin light chain-YFP localizes to macropinosomes in BMM following CSF-1 stimulation. A) Bone marrow macrophages were transfected with clathrin light chain-YFP, exposed to 100 ng/ml CSF-1 and observed by confocal microscopy. CLTA-YFP (green) co-localized with fluorescent CSF-1 (red). Times indicated are minutes post CSF-1 stimulation. B.) CLTA-YFP macrophages were co-exposed to CSF-1 (unlabeled) and 70 kD Texas Red Dextran. Texas Red Dextran positive macropinosomes were surrounded by clathrin assemblies. C.) Endogenous clathrin (green) co-localizes with CSF-1R (red) on macropinosomes following CSF-1 stimulation. D) TEM image with Clathrin ring around macropinosome.

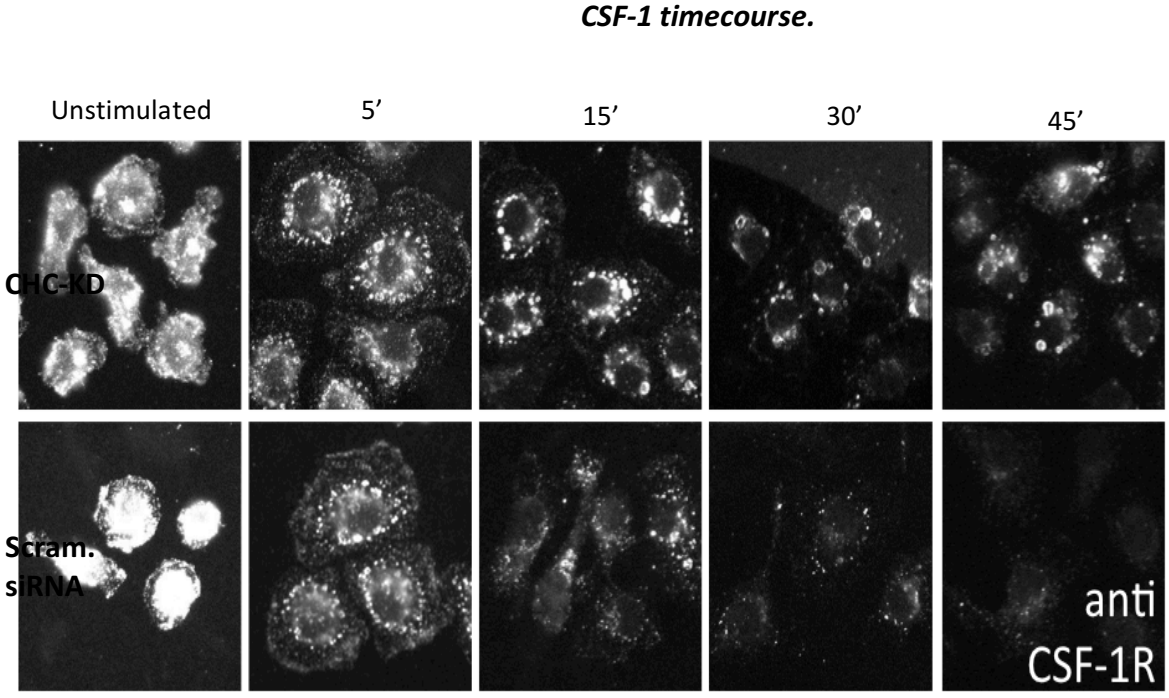

Figure 3. Clathrin heavy chain knockdown prolongs CSF-1R residency on macropinosomes and appears to delay CSF-1R degradation. Clathrin heavy chain was depleted by transfection with siRNA (smart pool) against clathrin heavy chain. Control cells were transfected with scrambled siRNA. Cells were starved of CSF-1 overnight, then stimulated with 100ng/ml CSF-1, fixed with 4% paraformaldehyde at the indicated times. CSF-1R was detected by immunofluorescence staining and imaging under epi fluorescence microscopy.

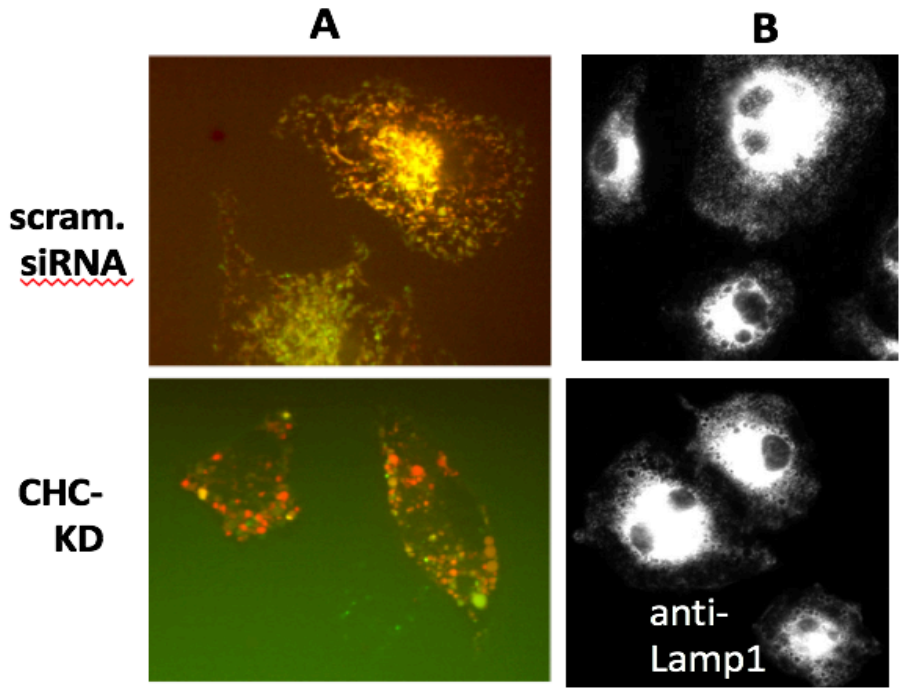

Figure 4. Macropinosome-lysosome fusion is dependent on clathrin in bone-marrow derived macrophages. A) Lysosomes were labeled with Texas Red dextran (70 kD), and macropinosomes were labeled with Lucifer Yellow. After 30 min, macropinosome contents were delivered to lysosome in control but not CHC-KD cells.These are live cells imaged under confocal microscopy. B) Lamp 1 stained macrophages were imaged under epi-fluorescence microscopy. Lamp1 immunostaining indicates intact reticular structure of lysosome in control and CHC-KD macrophages.

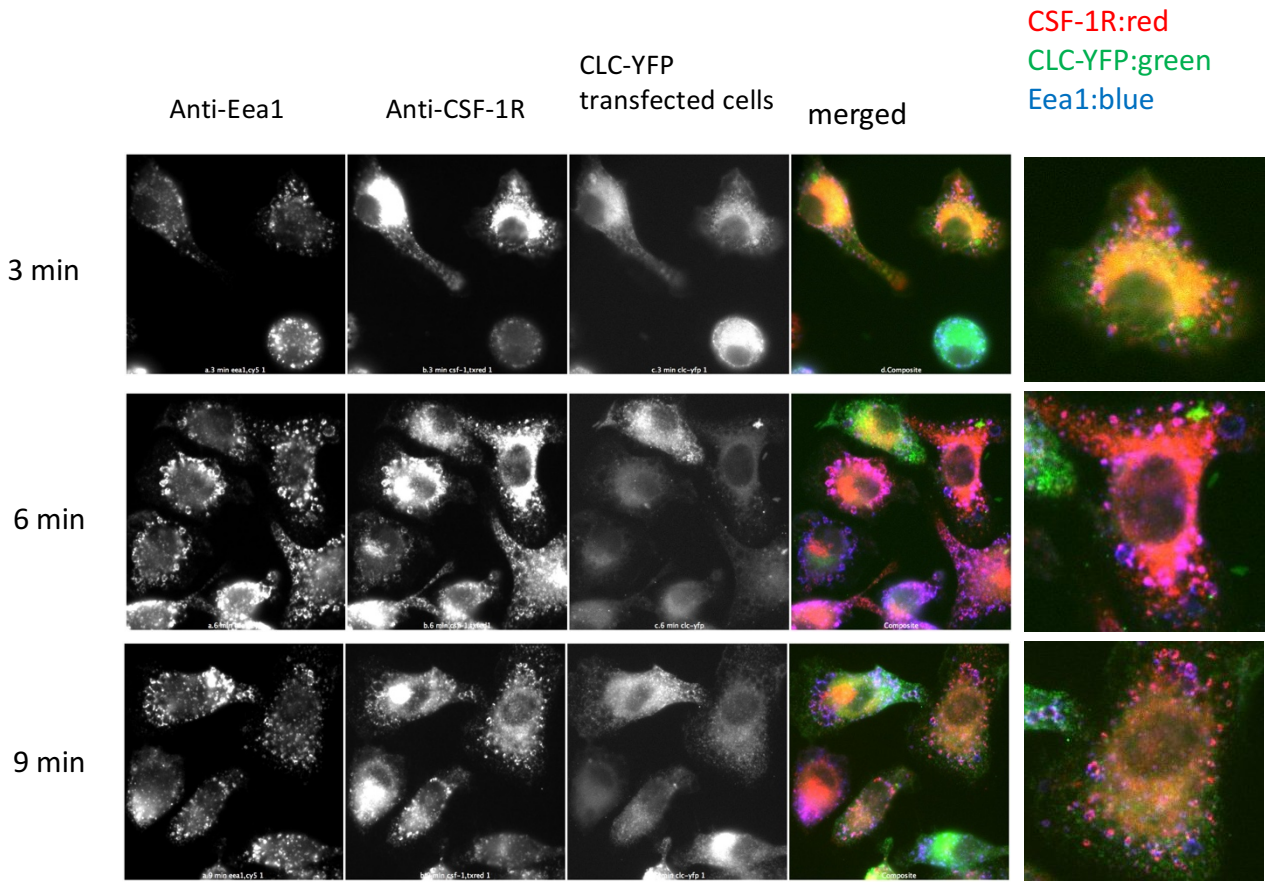

Figure 5. Clathrin, EEAl and CSF-1R co-localize in CLTA-YPF transfected Fetal Liver macrophages (FLMs). FLMs transfected with fluorescent fusion protein containing clathrin light chain fused to yellow fluorescent protein were starved overnight of CSF-1 and stimulated with 100 ng/mlCSF-1 and fixed with 4% paraformaldehyde at indicated times. CSF-1R and EEA1 were detected by immunofluorescence staining and imaged under epifluorescence microscopy.

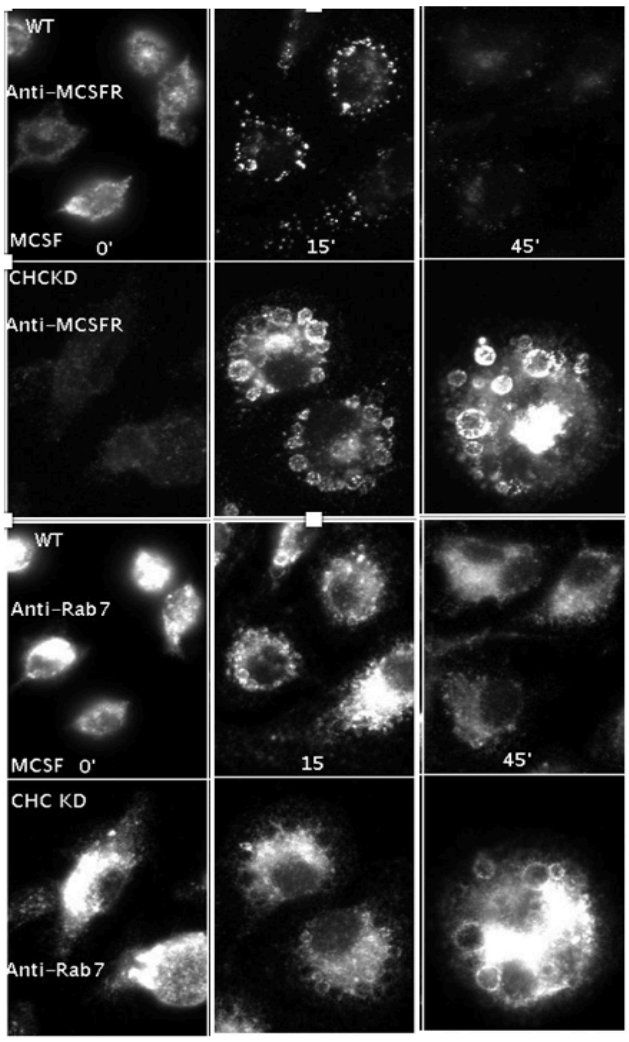

Figure 6. CSF-1R degradation is dependent on clathrin. A siRNA knockdown of clathrin prevents the maturation of macropinosomes and also interferes with the normal degradation of CSF-1R. These macrophages were imaged under epifluorescence microscopy.

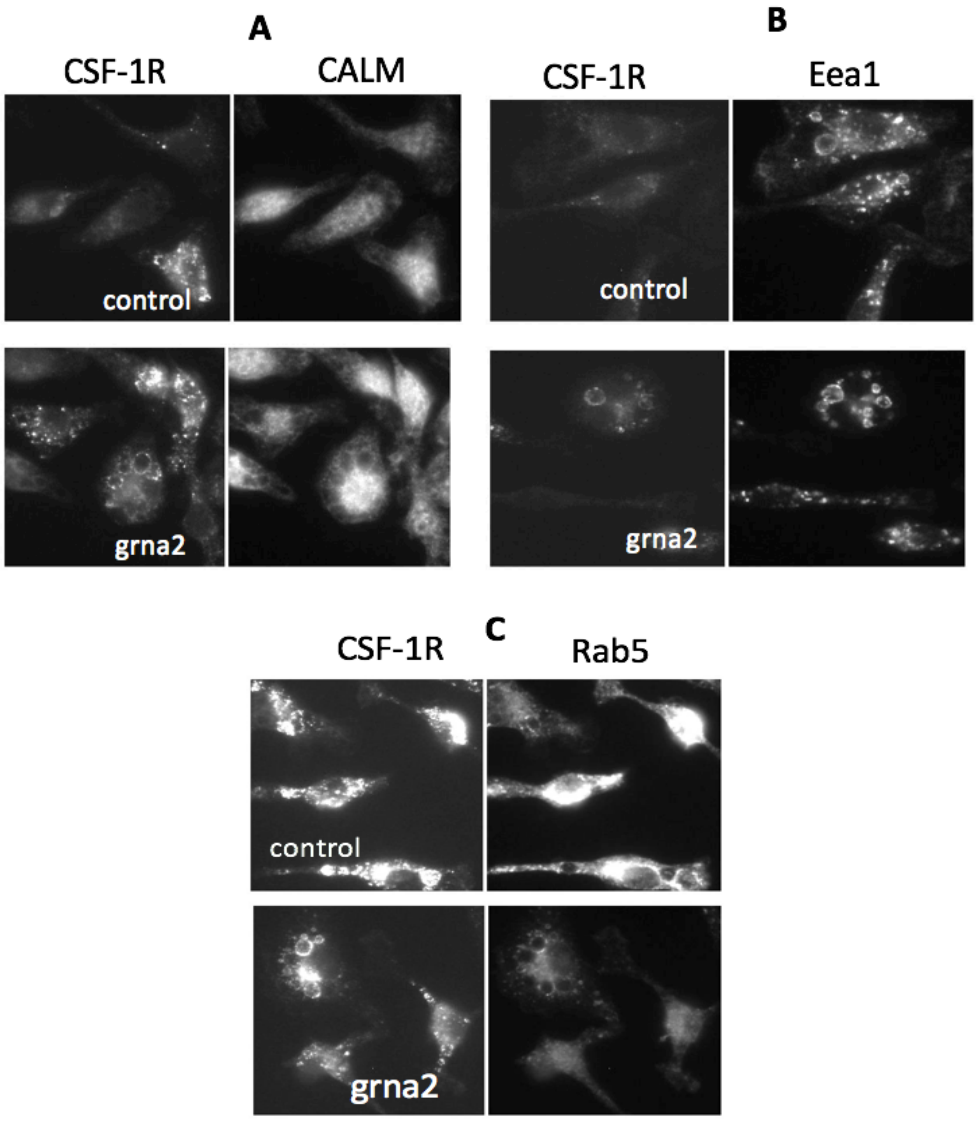

Figure 7. Immunostaining of endogenous CALM, CSF-1R, EEA1 and Rab5 in bone marrow derived macrophages. A) CSF-1R and CALM immunofluorescence 30 min following CSF-1 stimulation B) CSF-1R and Eea1 IF 30 min following CSF-1 stimulation C) CSF-1R and Rab5 15 min following CSF-1 stimulation. All of the cells were imaged with epifluorescence microscopy.

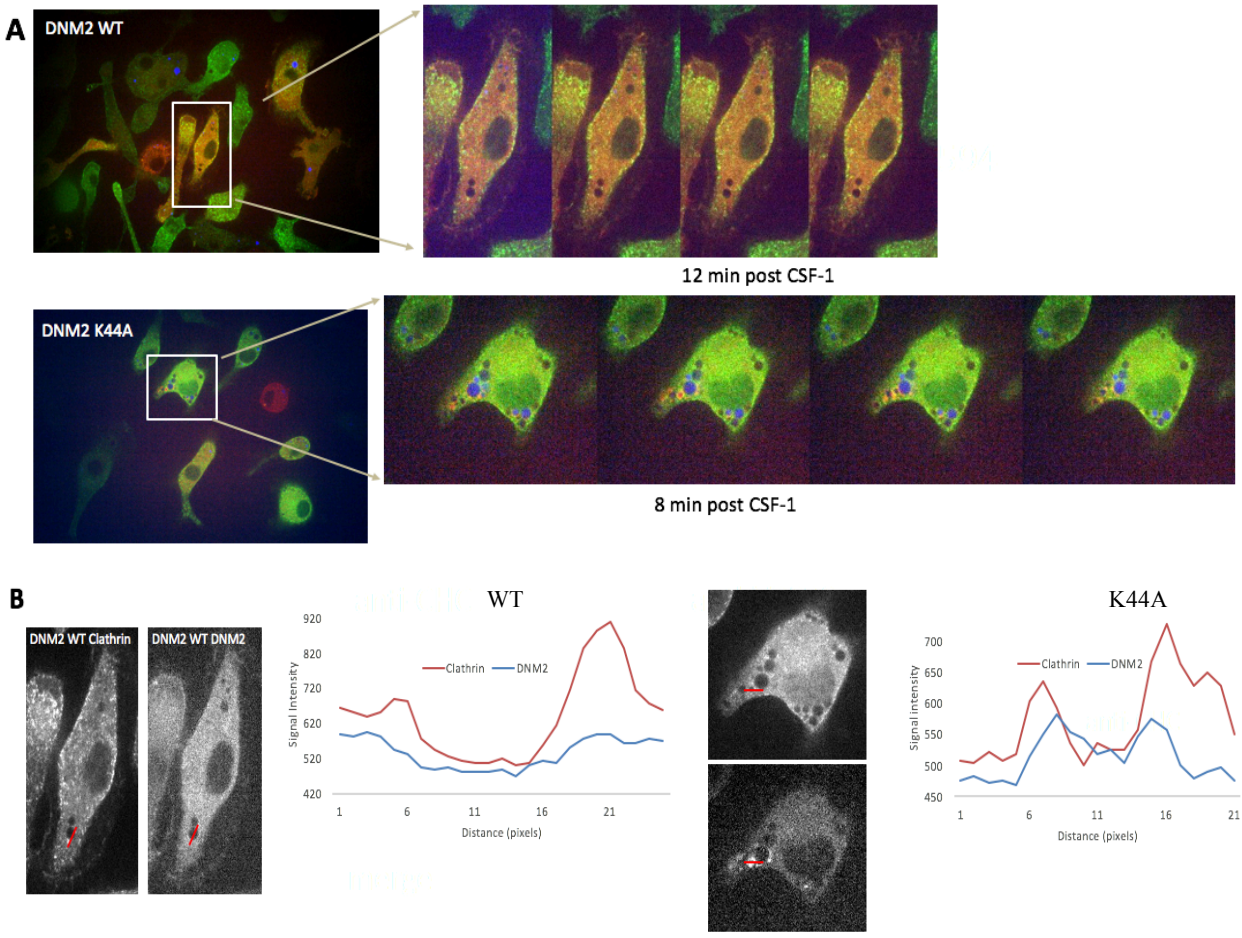

Figure 8. Clathrin and Dynamin2 K44A both co-localize around macropinosomes, after addition of CSF-1. Dynamin K44A is a dominant-negative mutant. It has the ability to polymerize, but limited capacity to bind and hydrolize GTP. A) Doubly transduced cells were imaged following CSF-1 stimulation. Clathrin = green; dynamin2 = red; macropinosomes = blue. B) Pixel intensity was measured along the line in both images and graphed to measure co-localization. These were live cells imaged under confocal microscopy.

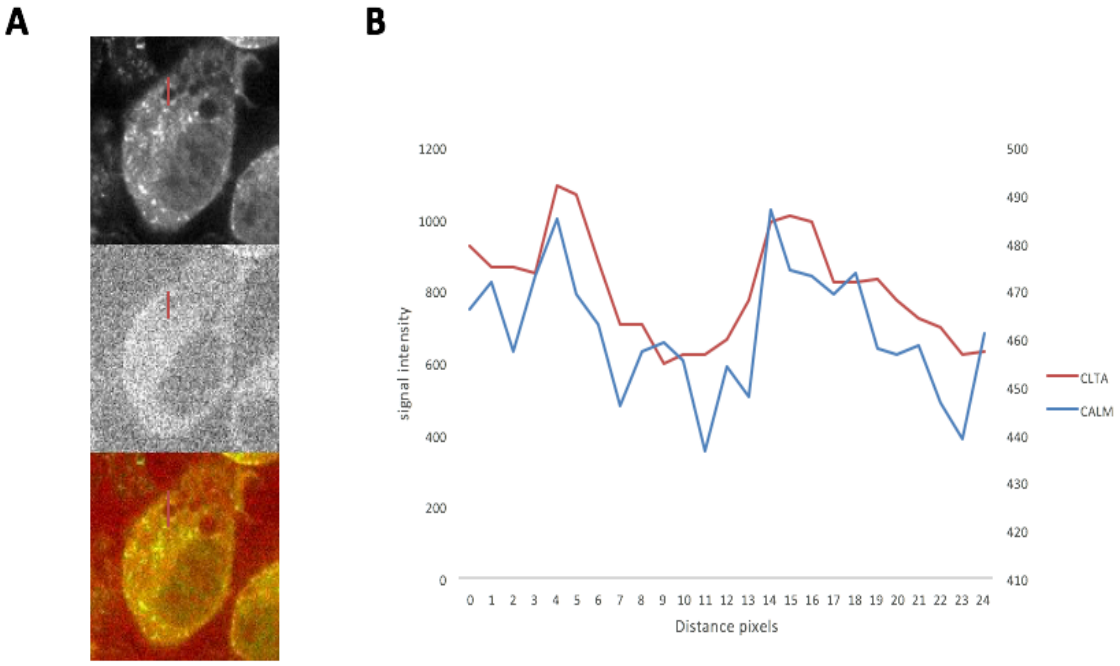

Figure 9. The clathrin adaptor CALM and clathrin light chain localize around macropinosomes in clathrin light chain-YFP CALM-mCherry doubly transfected FLMs. A) FLMs were transfected with clathrin light chain-YFP (yellow) and clathrin adaptor CALM-mCherry (red), exposed to 100 ng/ml CSF-1 and observed by live cell microscopy. B) Pixel intensity was measured along the line in both images and graphed to visualize co-localization. These were live cells imaged under confocal microscopy.

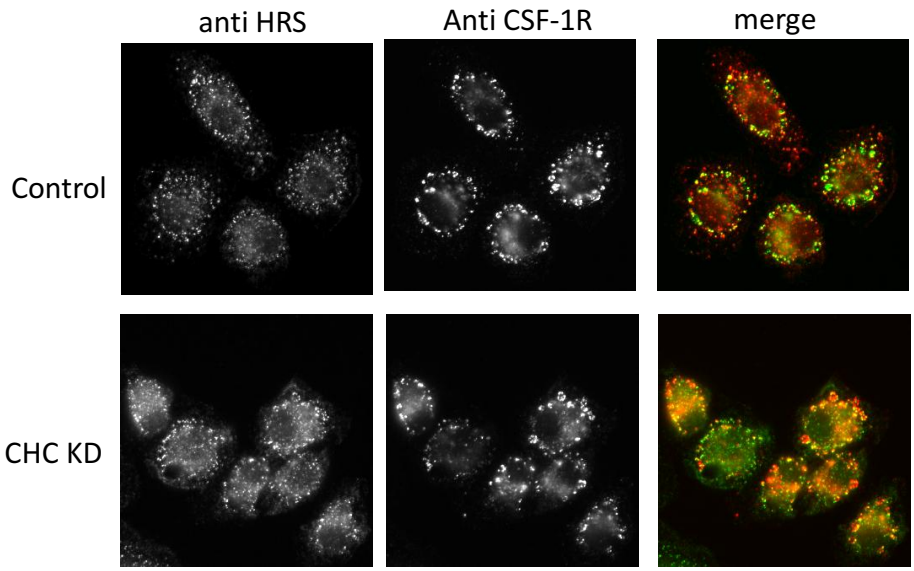

Figure 10. HRS localizes to CSF-1R positive macropinosomes in CHC KD and control cells. BMM were transfected with CHC or scrambled siRNA (control) . Prior to the experiment, BMM were starved of CSF-1 overnight. Cells were exposed to CSF-1 for 10 min and fixed and immunostained for HRS and CSF-1R. Cells were imaged under epifluorescence microscopy.

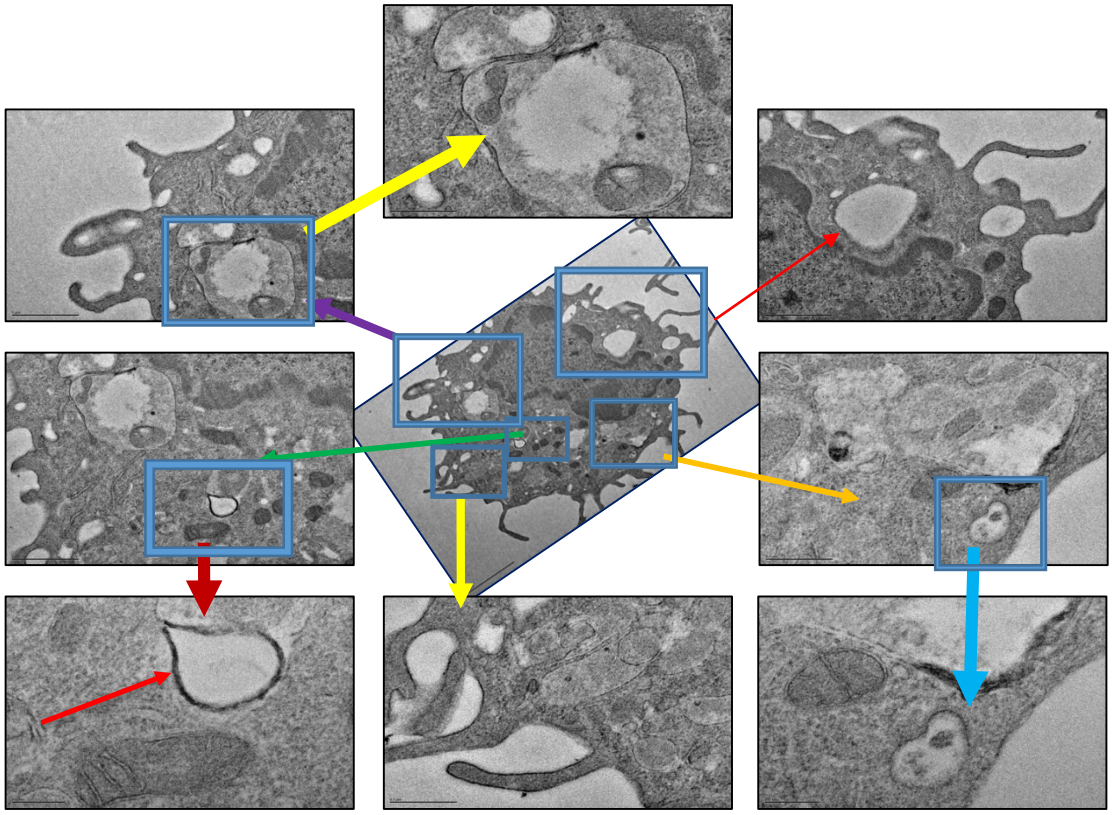

Figure 11: Unstimulated bone marrow derived macrophages under electron microscopy showing a variety of vesicles. Lower yellow arrow refers to ruffles, blue arrow refers to multi-vesicular body, both red arrows refer to vesicle.

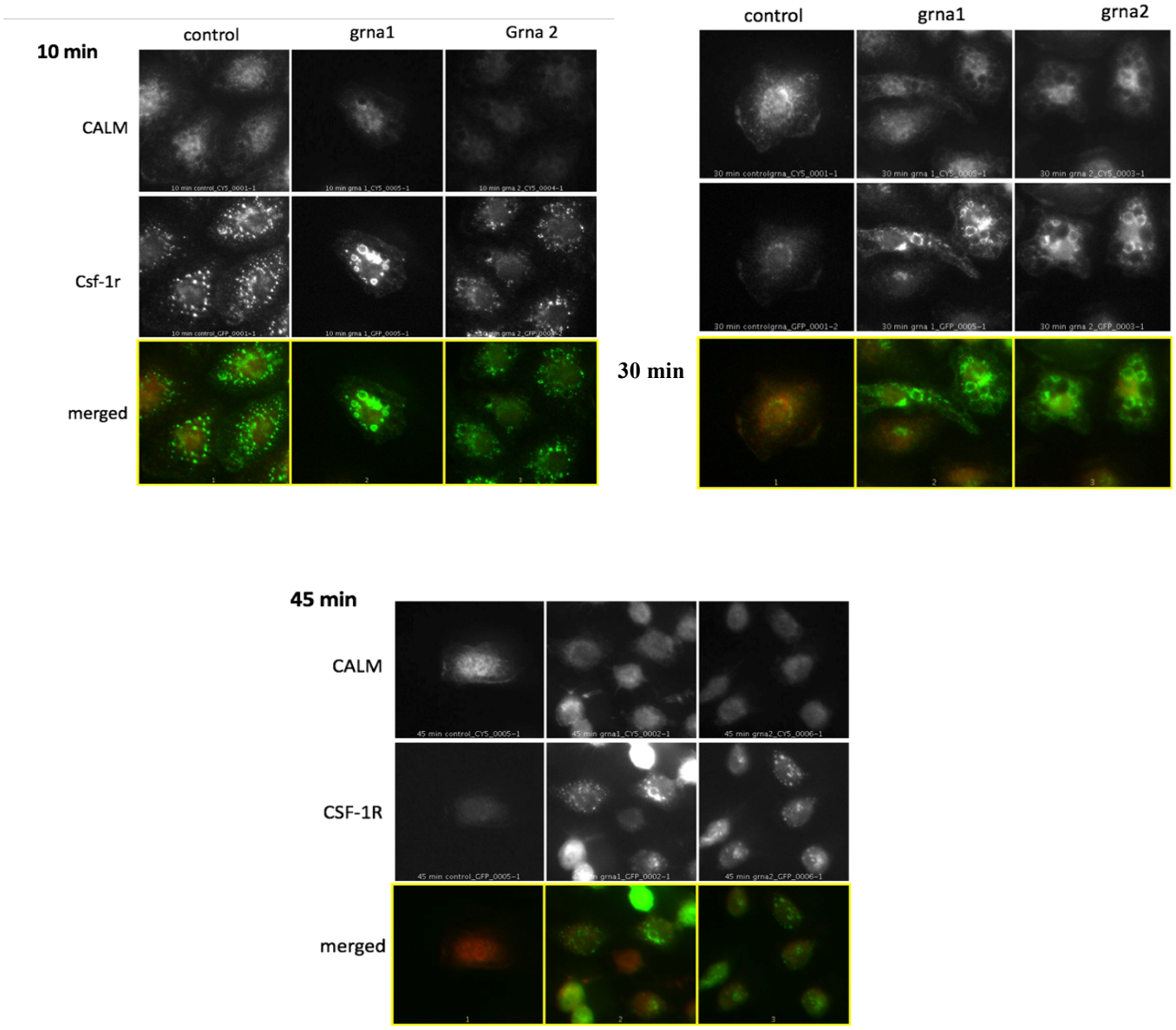

Figure 12. Clathrin heavy chain knockout using CRISPR/Cas9 technique produce same result as siRNA silencing technique. A) macrophages were stimulated for 10 min, B) 30 min and C) 45 min with 100 ng/ml CSF-1. All of them were fixed with 4% PFA for 10 min followed by permeabilization with 100% methanol for 1 min. Cells were imaged under epifluorescence microscopy.

CHAPTER 4

CONCLUSION

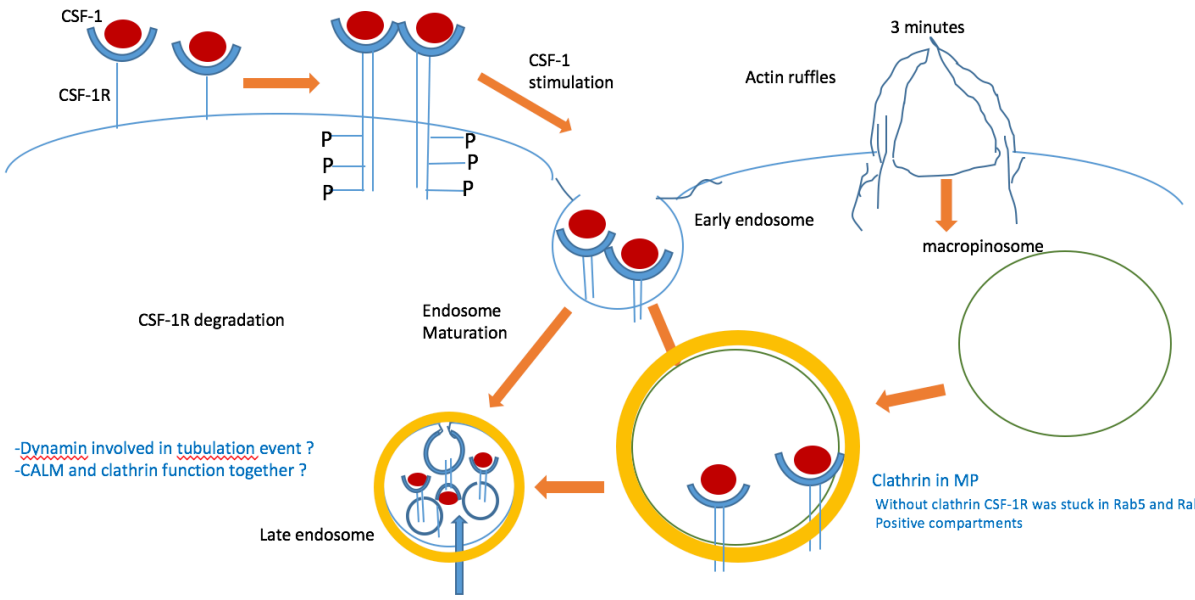

Figure 1: Mechanisms of macropinosome maturation. CSF-1R was used as cargo to understand maturation process of macropinosome. Clathrin assembly was observed around macropinosome with CSF-1R and in the absence of clathrin, macropinosomes were Rab5 and Rab7 positive. Finally, macropinosomes matured to late endosome with the involvement of dynamin and CALM.

The goal of this project was to understand the novel function of clathrin in macropinosomes maturation. Based on preliminary data from our lab, clathrin assembly was observed around macropinosomes in CSF-1 stimulated macrophages. Using clathrin light chain yellow fluorescent protein, the presence of clathrin was observed by live-cell microscopy around macropinosomes at early time points (from 5-15 min post CSF-1

stimulation). To ensure that this assembly and localization was not an anomaly due to clathrin overexpression, endogenous clathrin was immunostained in macrophages and observed in fixed cells. There have been no previous reports of clathrin assembled on macropinosomes. To investigate the function of clathrin in this novel setting, we knocked down clathrin heavy chain protein using siRNA and also deleted the clathrin heavy chaing gene using CRISPR/Cas9. In the absence of clathrin, the CSF-1 receptor was trapped in a large vesicular compartment which were presumed to be macropinosomes/late endosome. This trapping of this normally degraded cargo suggests that these vesicles were unable to mature and fuse with lysosomes in the absence of clathrin. However, the knockdown or knockout of clathrin did not affect the ability of CSF-1R to be endocytosed or for small endosome to fuse with macropinosomes.

Signaling events seemed to be going on in those macropinocytic/early endosomal/ late endosomal compartments which could be important for the regulation of different processes in cells. Based on receptor system and cells, endosomes are capable of fulfilling at least three distinct signaling functions. Endosomes make sure that signal transduction prolongs post ligand binding and receptor internalization. Secondly, they act as scaffolding protein by combining different signaling molecules and hence generate new signals. Thirdly, because of their cytoskeleton dependent movement, they carry signaling molecules to various required places within cell (Sadowski, Pilecka et al. 2009). Signaling from early endosome is important for Rac mediated cell migration as Rab5 in early endosomes activate Rac to generate lamellipodia and peripheral ruffles. (Palamidessi, Frittoli et al. 2008) (Lanzetti, Palamidessi et al. 2004). Similarly, delivery of extracellular solutes by macropinosomes to endolysosomes activate mechanistic target

of rapamycin complex-1 (mTORC1) by amino acids. MTORC1 is a complex cytosolic protein that regulates cell growth and is activated by growth factor receptor signaling and increased amino acids level inside endolysosome (Yoshida, Pacitto et al. 2015) . Even though late endosomes may be seen as organelles for signal deactivation, they still contain activated receptors and downstream effectors (Oksvold, Skarpen et al. 2001). One example of signaling from late endosome is PDZ-GEF1, a guanine nucleotide exchange factor for Rap1, a small GTPase that helps in signal transduction. PDZ-GEF1 interacts with receptor TrkA at late endosomes and activate Rap1 and ERK signaling that is necessary for neurite outgrowth (Hisata, Sakisaka et al. 2007).

There are many unanswered questions that could be helpful in understanding the mechanism of macropinosome-lysosome fusion in the future such as how does Rab7 facilitates macropinosome-lysosome fusion, what is preventing the proper shrinkage of macropinosome and how is CALM helping clathrin. Dynamin was localized with clathrin in bone marrow derived macrophages and could be helping in tabulation of membrane as a result of sorting event (Figure 8, chapter 3).

Some of the potential roles for clathrin could be cargo sorting. Clathrin could be collaborating with Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) in early endosome to sort cargo. Presence of PI3K binding FYVE domain and clathrin binding clathrin box motif in Hrs shows its possibility in endosomal targeting and intracellular trafficking (Raiborg, Grønvold Bache et al. 2001). Novel clathrin binding domain was found in C-terminal 69 residues of Hrs and this raised the possibility of Hrs as an endosomal clathrin adaptor. Through recruitment of clathrin, Eps and other proteins to early endosomes, Hrs also functions in regulation of trafficking and maturation of early

endosomes to late endosomes. Baby hamster kidney cells were transfected with Hrs constructs to observe function of Hrs. Adaptor proteins like AP1, AP2 and AP3 that connect directly with clathrin through a clathrin box sequence were absent on clathrin and Hrs positive early endosomes. However the coated vesicle GTPase, dynamin was observed in early endosomes that were recruited by Hrs (Raiborg, Grønvold Bache et al. 2001). Presence of dynamin in early endosome shows the possibility of dynamin involvement in cargo sorting too.

Clathrin could also be promoting outward membrane tubulation and inward membrane budding. Both outward tubulation and inward membrane budding could cause reduction in size of macropinosome due to the loss of membrane in the maturation process. Maturation of endosomes involve acidification of organelles, microtubule dependent movement and perinuclear localization leading to degradation of cargo. Degradation of cargo occurs through maturation of endosome by fission of tubular endosomes. (Mesaki, Tanabe et al. 2011).

Another possibility could be the involvement of clathrin in facilitating attachment of macropinsomes to microtubules via dynamin. Dynamin was found to be associated with microtubule during its isolation. Dynamin facilitated microtubule bundle by forming regularly spaced cross bridges between them (Shpetner and Vallee). Microtubule transport helps in endosomal movement with the help of motor proteins dynein and kinesin in bidirectional manner (Huotari and Helenius 2011). When microtubules were disrupted late endosomes and lysosomes were dispersed and the maturation of endosome was delayed (Bayer, Schober et al. 1998) (Vonderheit and Helenius 2005).We also observed the presence of dynamin and clathrin around macropinosome (Figure 8, Chapter

3). Here, dynamin could be facilitating the attachment of macropinosomes to microtubule. In the absence of clathrin, cargo was seen to be undegraded. Macropinosome could not fuse with lysosomes and got larger possibly due to presence of excess membrane. We have shown that clathrin is required for degradation of CSF-1 receptors, but future experiments are needed to determine the exact function of clathrin on maturing macropinosomes.

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