FACTOR ASSOCIATED WITH NEUTRAL SPHINGOMYELINASE ACTIVATION (FAN), A MEMBER OF THE BEACH FAMILY OF PROTEINS, REGULATES LYSOSOME SIZE

by

Rishna Shrestha

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STATEMENT OF THESIS APPROVAL

The thesis of **Rishna Shrestha**

has been approved by the following supervisory committee members:

Diane M. Ward, Chair — **05/07/2015** Date Approved

Jerry Kaplan, Member — **05/07/2015** Date Approved

Matthew A. Mulvey, Member — **05/07/2015** Date Approved

and by **Peter E. Jensen**, Chair/Dean of

the Department/College/School of **Pathology**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

Chediak-Higashi Syndrome (CHS) is a rare, autosomal recessive genetic disorder caused by mutations in the lysosomal trafficking regulator gene (*CHS1*) that affects vesicle morphology. The CHS1 gene encodes a protein, Lyst, which belongs to a family of proteins containing a conserved BEACH (BEige And Chediak-Higashi) domain and WD40-repeat domain. The *beige$_j$* mouse has been the best-studied animal model for CHS. Factor associated with neutral sphingomyelinase activation or FAN is the smallest member of the BEACH family. FAN interacts with the Tumor Necrosis Factor Receptor (TNFR1) to activate Neutral SphingoMyelinase2 (NSMase2). The absence of Lyst or FAN gives rise to enlarged lysosomes. Loss of both proteins results in an additive effect, as demonstrated by increased lysosome size in FAN$^{-/-}$ /*beige$_j$* mouse. An additive phenotype suggests that there are at least two pathways to regulate lysosome size. Overexpression of Lyst or FAN results in smaller than normal lysosomes. Overexpression of FAN in the absence of Lyst does not give rise to small lysosomes. NSMases are present on lysosomes and inhibition of NSMases, using GW4869 or 3-O-Methylsphingomyelin, results in enlarged lysosomes. Further, the decrease in lysosome size seen in FAN-overexpressing cells is blocked by GW4869. These results suggest that FAN activation of NSMase at the lysosome is a crucial step in regulating lysosome size.

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CHAPTER 1

INTRODUCTION

Lysosomes

Christian De Duve was the first to describe lysosomes as membrane bound organelles (Appelmans, Wattiaux and De Duve; Duve). Structurally they appear as dense bodies localized in a perinuclear pattern in the cytosol. Lysosomes are conserved throughout eukaryotes. Lysosome size can vary from 100 nm up to several microns, although the steady state size of lysosomes in higher eukaryotes is normally about 200-400 nm. Depending on the cell type, lysosome shape varies from spherical to tubular (Lüllmann-Rauch). Lysosomes have an acidic lumen that is enclosed by a single phospholipid-bilayer. The lysosomal membrane is highly glycosylated, which protects lysosomal membrane proteins and lipids from the acidic proteases and lipases-rich environment. There are many lysosomal membrane proteins and integral membrane proteins such as Lysosome-Associated Membrane Protein (LAMP)-1 and LAMP-2, Lysosome Integral Membrane Protein (LIMP)-2 and CD63, which are among the most abundant (Saftig, Schröder and Blanz). These proteins provide integrity to the lysosomal membrane as well as act with other lysosomal membrane proteins in signaling.

Soluble NSF (N-ethylmaleimide sensitive fusion proteins) Attachment protein Receptors (SNAREs) mediates the fusion of the lysosomes with other cellular structures such as other lysosomes, late endosomes, autophagosomes and the plasma membrane. This fusion allows for the redistribution of substrates among the lysosomes for efficient degradation and also functions to regulate the size of the lysosomes. Lysosomes also contain transporters on their surface that mediate transport of metabolites, ions and soluble substrates into and out of lysosomes (Saftig and Klumperman). Lysosomes have a vacuolar ATPase proton pump that maintains the acidic luminal pH of 4.6-5.0 (Mellman,

Fuchs and Helenius) for proper functioning of acid hydrolases stored in the lysosome lumen (Holt, Gallo and Griffiths).

Functions of Lysosomes

Lysosome functions can be divided into three main types: degradation, secretion and signaling. The endocytic pathway transports extracellular materials intended for degradation to the lysosome, whereas the intracellular materials that need to be degraded are transported to the lysosome by the autophagy pathway (Settembre et al.). Lysosomes contain more than 50 acid hydrolases including proteases, peptidase, phosphatase, nucleases, glycosidases, sulfatases and lipases for the degradation of specific substrates (Luzio et al.; Bainton).

In addition to cellular clearance, lysosomes are also involved in a secretory pathway known as exocytosis in which lysosomes secrete their contents by fusing with the plasma membrane (Chieregatti and Meldolesi; Verhage and Toonen). Some cells store newly synthesized secretory proteins in specialized lysosomal compartments. These organelles are known as secretory lysosomes or Lysosomes-Related Organelles (LROs) that include melanosomes, lytic granules, platelet-dense granules, Major Histocompatibility Complex (MHC) class II Compartments (MIIC), basophilic and azurophilic granules (Dell'Angelica et al.). These secretory lysosomes have cell-specific functions such as pigmentation, host defense, coagulation and both innate and adaptive immunity. The function of LROs varies depending upon the cell type. The size of these organelles is regulated by the rate of fusion of vesicles with the LRO and the rate of LRO fission, and many of the proteins that regulate these processes have been identified

(Dell'Angelica et al.; Perou, Leslie, et al.; Ward, Leslie and Kaplan; Ward et al.; Durchfort et al.).

Most cells contain secretory lysosomes that are involved in the plasma membrane repair. Plasma membrane injury can be attributed to mechanical stress such as in muscles and skin or by pore-forming toxins from pathogens attempting to gain access to host cells (McNeil and Khakee; Gonzalez et al.). Cells must rapidly reseal their plasma membrane or suffer cell death and the lysosomes have been shown to play the critical role in this mechanism of the cell survival.

Lysosomes play an important role in nutrient sensing and in signaling pathways that are important in cell metabolism and growth. Recently, lysosomes have been identified as signaling organelles that can sense nutrient availability and activate a lysosome-to-nucleus signaling pathway that mediates the starvation response and regulates energy metabolism. A master controller of cell and organism growth, the mammalian Target Of Rapamycin Complex1 (mTORC1), is a sensor of nutrient and growth factor levels that exerts its activity on the lysosomal surface. Hence, it controls the balance between biosynthetic and catabolic states. Under conditions such as starvation, infection, or oxidative stress, mTORC1 is inhibited and the cellular autophagy pathway is activated. Autophagy is a catabolic mechanism that involves the degradation and recycling of a cellular component for cell survival. mTORC1 inhibits autophagy in the presence of nutrients (Settembre et al.).

Diseases Associated with Lysosomal Dysfunction

Lysosomes are involved in many cellular functions such as degradation of intracellular and extracellular materials, pigmentation, coagulation, wound repair, bone and tissue remodeling, pathogen defense, cell signaling and cell death (Saftig and Klumperman). Therefore, disturbances in lysosomal functions contribute to many diseases. For example, in the Lysosomal Storage Disease (LSD) Niemann-Pick the lysosomal substrate sphingolipid accumulates in the lysosome resulting in enlargement and lysosome dysfunction and can result in organism death. Other diseases such as Chediak-Higashi Syndrome (CHS), Gray Platelets Syndrome (GPS), Hermansky-Pudlak Syndrome (HPS) and Griscelli's Syndrome also show an increase in the size of lysosomes and/or LROs, which affects normal function (Clark and Griffiths). CHS is the only one of these disorders that affects all LROs and lysosomes. GPS is an autosomal recessive bleeding disorder characterized by large platelets that lack alpha granules (Gunay-Aygun et al.). HPS is an autosomal recessive disease characterized by a partial albinism and bleeding tendencies resulting from the platelet dysfunction. These phenotypes are associated with the defects in the biogenesis of LROs, including melanosomes, platelet dense granules, lamellar bodies of type II alveolar epithelial cells and lytic granules of Cytotoxic T Lymphocytes (CTLs) and natural killer cells (Di Pietro and Dell'Angelica). Many of the genes responsible for HPS have been identified and they range from adaptor molecules to large multi-subunit complexes involved in LRO biogenesis (Clark and Griffiths). Griscelli Syndrome (GS) is a rare autosomal recessive disorder with patients showing characteristic pigment dilution of the skin and distinct silvery-grey hair; with the presence of large clumps of pigment in the hair shafts and an

abnormal accumulation of mature melanosomes in the center of melanocytes with reduced pigmentation of adjacent keratinocytes (Izumi et al.; Clark and Griffiths).

Chediak-Higashi Syndrome (CHS)

Chediak-Higashi syndrome (CHS) is a rare, autosomal recessive multisystem genetic disorder caused by mutations in the lysosomal trafficking regulator gene *CHS1* (Shiflett, Kaplan and Ward). CHS is characterized by severe immune deficiency, hypopigmentation, bleeding tendency, frequent bacterial infection, oculocutaneous albinism, and progressive neurological dysfunction (Spritz). The hallmark characteristic of CHS is the presence of giant cytoplasmic inclusion bodies and granules in all the cells of the body (Figure 1.1). The enlarged granules include lysosomes and LROs (Shiflett, Kaplan and Ward; Introne, Boissy and Gahl; McVey Ward et al.). The $beige_j$ mouse, named after its beige coat color, has been the best-studied CHS model organism. Somatic cell fusions confirmed that the same gene was defective in $beige_j$ mice and CHS patients (Perou, Justice, et al.). The *Lyst* gene is localized on mouse chromosome 13 (Justice et al.) and the human *CHS* gene to position q42 on chromosome 1 (Barrat et al.; Fukai et al.). The *Lyst* gene in mice was identified using positional cloning and in vitro complementation using Yeast Artificial Chromosomes (YACs; Shiflett, Kaplan and Ward; Nagle et al.). The identification of the human *CHS1* gene and CHS-associated mutations were subsequently identified by sequence analysis using the homology to a mouse *Lyst* sequence as a reference (Barbosa et al.).

Lyst Protein

The *Lyst* gene encodes a ubiquitously expressed protein of approximately 3800 amino acids. It is predicted to be a 430-kilodalton (kDa) cytosolic protein involved in lysosome size regulation. The amino acid sequence contains three to four defined domains including: an amino terminus HEAT (Huntington, Elongation factor 3, PR65/A, TOR) and ARM (Armadillo) domain containing α- helical repeats, a possible perilipin domain, a BEACH (Beige And Chediak) domain containing the amino acid sequence WIDL and WD40-repeats at the carboxyl terminus. The HEAT/ARM domains are predicted to be involved in vesicle trafficking (Andrade and Bork) and may function to mediate membrane interactions (Andrade et al.; Peifer, Berg and Reynolds). The perilipin domain may mediate interaction with lipids (Londos et al.). The conserved amino acid sequence WIDL domain (tryptophan (W), isoleucine (I), aspartic acid (D) and leucine (L)) has no known function and the WD40-repeats may serve as protein-protein interaction domains (Shiflett, Kaplan and Ward; Figure 1.2). WD-repeat proteins are involved in a variety of cellular functions such as cell cycle control, autophagy and apoptosis but the most noted function of WD-repeat proteins is vesicle trafficking (Cullinane, Schäffer and Huizing). Together the WIDL and WD40-repeat domains defined a family of protein called BEACH proteins (Shiflett, Kaplan and Ward; Cullinane, Schäffer and Huizing; Kaplan, De Domenico and Ward).

Sequencing has shown that Lyst is a highly conserved protein in all eukaryotes (Shiflett, Kaplan and Ward) and some eukaryotes have several family members. Mammals have nine BEACH proteins and the slime mold *Dictyostelium discoidium* has six, *Drosophila melanogaster* and *Arabidopsis thaliana* have five BEACH proteins,

Caenorhabditis elegans has three BEACH proteins and *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* each have one BEACH protein (Shiflett, Kaplan and Ward).

The nine mammalian BEACH (Figure 1.3) proteins include: Lyst, which is proposed to act as a scaffold protein that mediates fusion/fission events of vesicles in the endocytic pathway (Tchernev et al.); Neurobeachin (NBEA), which localizes to the trans-Golgi of mammalian cells and binds to signaling complex Protein Kinase A (PKA) and plays a role in neuronal membrane trafficking (Wang et al.); Neurobeachin-like 1(NBEA1), Neurobeachin-like 2(NBEA2), Lipopolysaccharide-Responsive Beige-like Anchor protein (LRBA), which is predicted to be important for host defense against infections and for cell proliferation; WD and FYVE zinc finger domain (FYVE domains mediate binding to phosphatidylinositol-3-phosphate, which regulates endocytic and autophagic membrane trafficking [Simonsen et al.]) containing protein 3(WDFY3) plays a role in clearance of neurologically related protein complexes; WD and FYVE zinc finger domain containing protein 4 (WDFY4) may have a significant role in autoimmune diseases (Yang et al.); Neutral Sphingomyelinase activation Associated Factor or Factor Associated Neutral sphingomyelinase (NSMAF or FAN), which is implicated in TNFR1-mediated activation of NSMase and WD repeat domain 81 (WDR81), which is a transmembrane protein that when mutated results in recessive syndrome of quadrupedal walking, mental retardation and cerebellar hypoplasia (Gulsuner et al.).

Neutral Sphingomyelinase Activation Associated Factor (FAN or NSMAF)

FAN is the smallest mammalian BEACH protein (948 amino acids) consisting of a short N-terminal segment followed by the BEACH and WD domains (Cullinane, Schäffer and Huizing). FAN has been shown to act in the Tumor Necrosis Factor (TNF) pathway. TNF is a cytokine primarily produced by activated macrophage that signals through two distinct cell surface receptors, 55 kDa (TNF-R55) and 75 kDa (TNF-R75). These receptors transmit signals to the cytoplasm and nucleus, which result in the activation of two major transcription factors, Nuclear Factor κB (NF-κB) and c-Jun. The activation of transcription factors results in the expression of genes important for several biological processes such as cell growth, cell death, development, oncogenesis, inflammation and stress responses (Krönke et al.; Tartaglia and Goeddel; Rothe et al.; Chen and Goeddel). Several studies have shown that both TNF-R55 and TNF-R75 are independently active in signaling the TNF response but the majority of TNF's biological activities are initiated by TNF-R55 (Barbara et al.; Grell et al.; Gehr et al.; Tartaglia, Weber et al.; Espevik et al.; Tartaglia, Rothe, et al.; Wiegmann et al.). FAN interacts with the Neutral Sphingomyelinase activating Domain (NSD) of TNF-R55 upon ligand binding to activate Neutral Sphingomyelinase (NSMase) present at the plasma membrane. NSMase generates the lipid second messenger ceramide from sphingomyelin at the plasma membrane. The generated ceramide is involved in intracellular signaling including secretion of cytokines and growth factors and loss of organelle integrity (Adam-Klages et al.; Clarke and Hannun; Clarke et al.). There are three known NSMases and FAN has been associated with signaling through TNF-R55 in response to TNF

binding to activate NSMase2 (Wu, Clarke and Hannun; Kolesnick and Krönke). In addition to FAN's role in NSMase activation, FAN has been shown to play a role in apoptosis (Ségui et al.; O'Brien et al.), actin reorganization in macrophages (Peppelenbosch et al.) and control of lysosome permeability (Werneburg et al.). FAN has also been shown to have a role in autophagy (Behrends et al.).

FAN-deficient Mouse

A FAN-deficient mouse was generated to understand the role of FAN in activating NSMase. $FAN^{-/-}$ mice show defects in TNF-induced NSMase activation and also show a delay in cutaneous wound repair (Kreder et al.), which is a phenotype also associated with the loss of Lyst. Microscopic analysis shows that $FAN^{-/-}$ lysosomes are slightly enlarged (Figure 1.4), again similar to the loss of Lyst (Möhlig et al.). These phenotypes suggest that FAN plays a role in regulating the lysosome size. This could be a direct effect or FAN's activation of NSMase at other vesicular locations may affect lipid content of those vesicles, membrane curvature, vesicular budding or fusion and thus lysosome size (Cremesti, Goni and Kolesnick).

FAN Binding Partners

In the TNF-receptor pathway, NSMase2 is activated by TNF-R55 in response to TNF. The NSMase activation Domain (NSD) within the TNF-R55 serves as the binding site for FAN. FAN then recruits Receptor for Activated C-Kinase 1 (RACK1) and the nuclear Embryonic Ectoderm Development (EED) protein. In response to TNF binding at

the plasma membrane, EED translocate from the nucleus to the plasma membrane where it binds to nSMase2 and the TNF-R55-FAN-RACK1 complex inducing NSMase activation (Figure 1.5; Philipp et al.).

RACK1 is a WD repeat protein that is highly conserved from *Chlamydomonas* to humans (Neer and Smith). It is a scaffolding protein, which interacts with several signaling pathways involved in the coordination of cell adhesion, movement, and division (Möhlig et al.). It also plays an important role in cell survival and in wound healing (McCahill et al.). RACK1 is a component of the eukaryotic ribosome and is involved in the recruitment of the ribosome to intracellular sites to regulate translation (Nilsson et al.). RACK1 binds to activate PKC (Protein Kinase C) isotypes and translocates to the cellular membranes preventing its premature degradation (Mochly-Rosen and Gordon).

EED, a nuclear WD repeat protein of Polycomb group (Sewalt et al.), plays a role in lymphocyte proliferation, differentiation, organogenesis, and embryonic development. At the plasma membrane EED interacts with integrins, which are noncovalently associated heterodimeric cell surface adhesion molecules that mediate cell-cell, cell-extracellular matrix, and cell-pathogens interaction. It also plays a vital role in leukocyte trafficking and migration, immunological synapse formation, costimulation, and phagocytosis (Witte et al.; Morin-Kensicki et al.; Luo, Carman and Springer).

A proteomic study identified FAN (NSMAF) as an Autophagy related 8 protein (Atg8) -interacting protein, a protein involved in regulating the closure of the autophagosome. That study also showed that small interfering RNA (siRNA) reductions in FAN resulted in a decreased autophagic flux. Those results suggest that FAN acts in regulating lysosome size by affecting autophagy and the reformation of lysosomes after

autophagy although a mechanistic role for FAN in autophagy remains to be elucidated (Behrends et al.).

Aims

The goal of this thesis is to characterize the roles of FAN and Lyst in lysosome homeostasis and lysosome size maintenance.

The first aim is to determine if FAN and Lyst work in the same biochemical pathway to regulate lysosome size. Preliminary experimental data demonstrate that loss of either protein increases lysosome size and overexpression of either FAN or Lyst reduces lysosome size.

The second aim is to identify the pathway and the partners that FAN might be working through to regulate the lysosome homeostasis and size.

The approaches that will be used to address these questions include: the generation of a double knockout FAN$^{-/-}$*; beige$_j$/beige$_j$* mouse to determine if FAN and Lyst are in the same or different pathways for regulating the size of lysosomes; inhibition of NSMase with specific NSMase inhibitor GW4869 to determine if NSMase activity affects lysosome size and if FAN requires NSMase activity to alter lysosome size; overexpression of epitope-tagged FAN to examine if there are changes in lysosome size and identify FAN partners; silencing of FAN binding partners using specific siRNA to determine the role of FAN binding partners in regulation of lysosome size; and lysosome lipidomics to determine if the absence of FAN or Lyst alters cellular and lysosomal lipid contents.

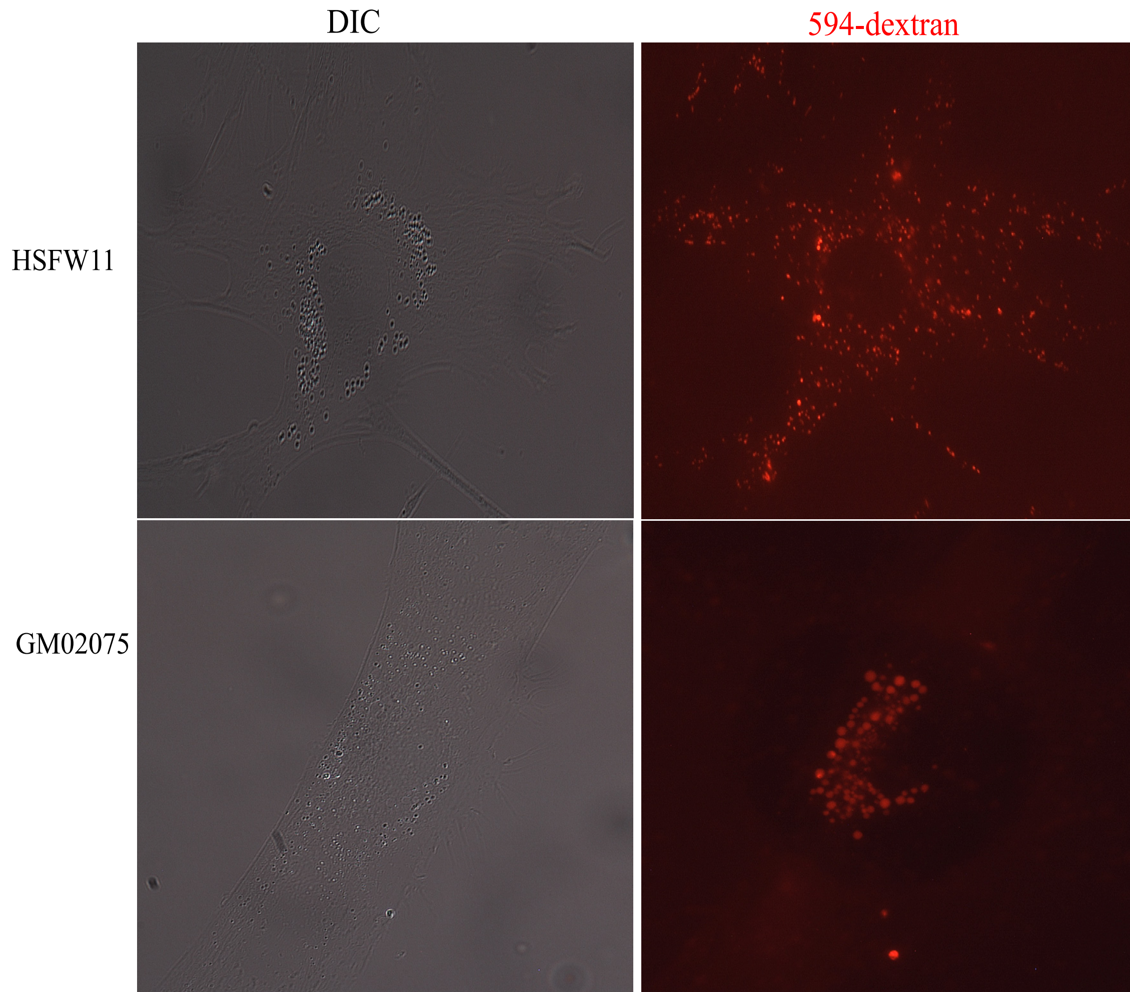

Figure 1.1: Enlarged lysosomes in CHS patient fibroblasts
Human fibroblast from HSFW11 (wild type) and GM02075 (CHS) patients were plated on glass coverslips. Cells were incubated with 5 µg/ml Texas red dextran (mw 10,000 da) at 37°C overnight, followed by a 2-hour chase to allow all dextran to localize to lysosomes. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY).

Figure 1.2: The Lyst protein
A schematic representation of the Lyst protein containing ARM/HEAT repeats, a WIDL domain and a WD40-repeat domain. The Lyst protein contains 3801 amino acids. Together the WIDL and WD40-repeat domains defined a family of proteins called BEACH proteins.

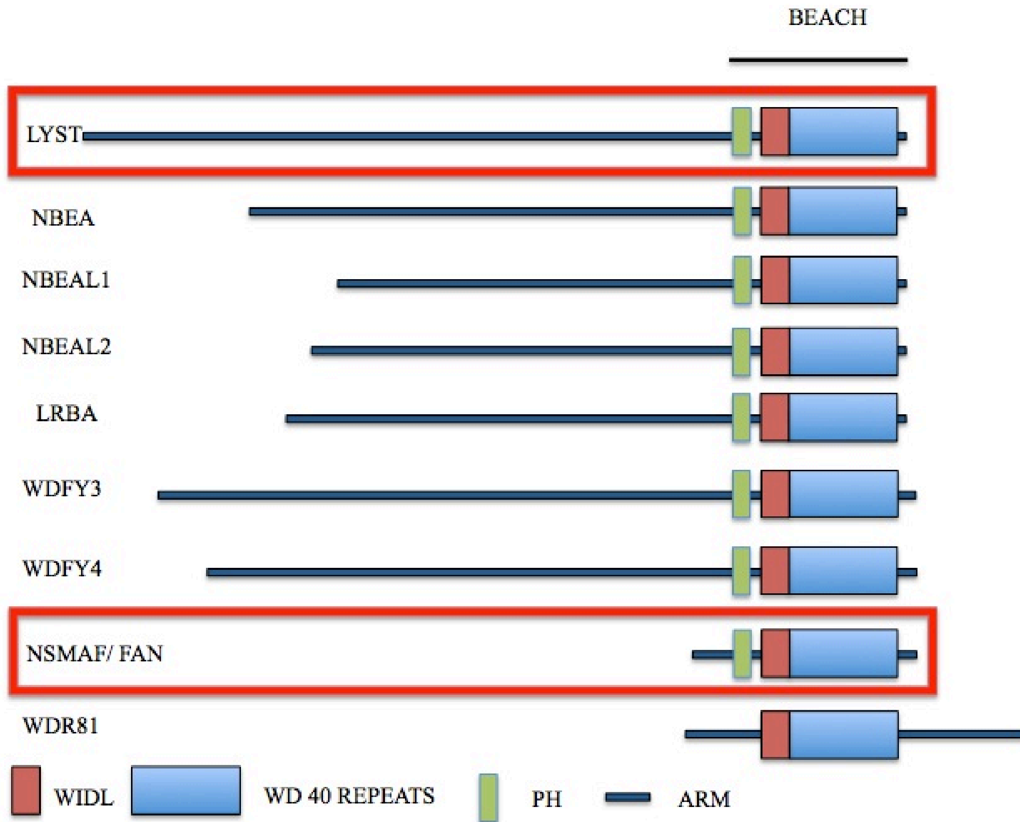

Figure 1.3: Mammalian BEACH family of proteins
A schematic representation of mammalian proteins belonging to the BEACH family.

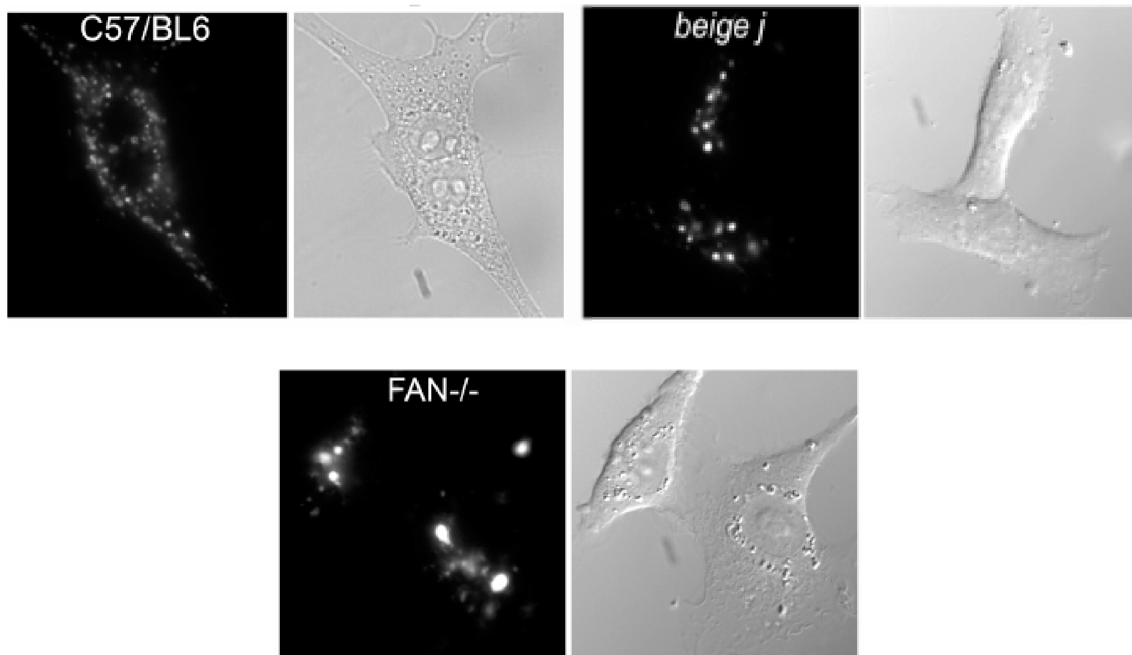

Figure 1.4: Enlarged lysosomes in *beige$_j$* and FAN$^{-/-}$ mouse fibroblasts
Fibroblasts from C57BL/6, *beige$_j$* and FAN$^{-/-}$ mice were plated on glass coverslips. Cells were incubated with 5 µg/ml Texas red dextran (mw 10,000 da) at 37°C overnight, followed by a 2-hour chase to allow all dextran to localize to lysosomes. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY).

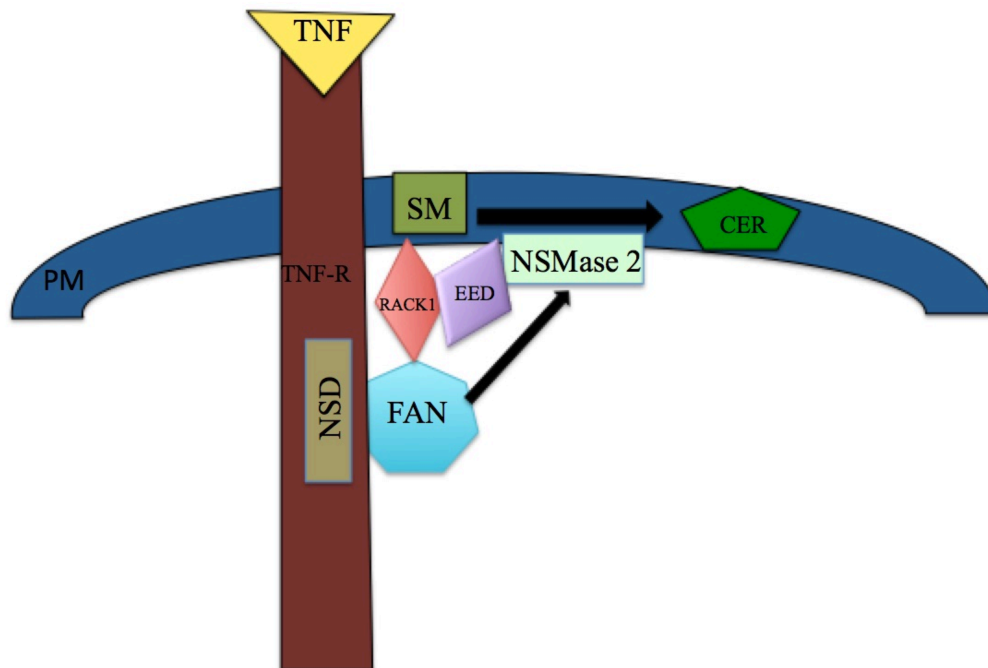

Figure 1.5: FAN binding partners
FAN binds to the NSD of TNFR1 in response to TNF binding. FAN binding recruits RACK1 and EED to induce NSMase activation. Adapted from (Philipp et al.). (PM, Plasma Membrane; TNF-R, Tumor Necrosis Factor Receptor; FAN, Factor Associated with Neutral Sphingomyelinase Activation; NSMase2, Neutral Sphingomyelinase 2; SM, Sphingomyelin; Cer, Ceramide).

CHAPTER 2

MATERIALS AND METHODS

Cells and Media

Bone marrow cells were isolated from femurs of 6 to 8 week-old C57BL/6, *beige*$_j$ mice, or FAN$^{-/-}$ mice and seeded on 10-cm petri dishes. Macrophages were cultured in RPMI medium (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 20 µM b-mercaptoethanol supplemented with 20% Fetal Bovine Serum (FBS) and 30% L-cell conditioned medium. Fibroblasts from mouse C57BL/6, *beige*$_j$, FAN$^{-/-}$ or FAN$^{-/-}$*beige*$_j$, mouse NIH 3T3 fibroblast, HeLa, HEK293 and *fro/fro* fibroblast (obtained from Dr. Yusuf Hannun Medical University of South Carolina, Charleston, South Carolina) were maintained in DMEM (*Dulbecco's Modified Eagle's medium*) with 10% FBS and penicillin/streptomycin.

Plasmids

FAN was cloned into pCMVTag2B (Agilent Technology, Santa Clara, CA) or pEGFP-C1 to generate an amino-terminally epitope-tagged FAN. Plasmid containing NSMase2 was a generous gift from Dr. Yusuf Hannun (Medical University of South Carolina, Charleston, South Carolina). Plasmids pcDNA 3.1(-) Myc-His A RACK1 and pFLAG CMV EED were generous gifts from Dr. Dieter Adam (Universität Kiel, Kiel, Germany).

Transfections

Cells were plated onto tissue culture plates and allowed to grow for 24 to 48 hours to 50-80% confluence. The cells were transfected with various constructs using Amaxa

nucleofector technology (Lonza, Walkersville, MD) according to manufacturer's directions.

RNA Interference

Cells were treated with Non Specific (NS), EED, RACK1, NSMase1 and NSMase3 specific oligonucleotides (Dharmacon SiGenome SiRNA SMARTpool, Dharmacon RNA Technologies, Layfette, CO) using Oligofectamine Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. About 150,000 cells were treated with siRNA in Optimem reduced-serum medium (Invitrogen, Carlsbad, CA) for 6 hours at 37°C before serum-replete medium was added for overnight growth. Media was changed and cells were allowed to grow in DMEM with 10% FBS for 48 or 72 hours before analysis were completed.

mRNA Analysis by RT-PCR

To isolate total RNA, cells were harvested as previously described following Qiagen RNeasy kit protocol. For the analysis, RT-PCR (Reverse Transcription-Polymerase Reaction) was performed using specific primers and quantification was done using software Gel Doc XR+. Primers used: NSMase 1 (forward primer 5' CGTGCTTTACAAGGCAGTTTCTGG 3' and reverse primer 5' CTCTCTGCTGGTCCGTGGGTA 3'), NSMase 3 (forward primer 5'GTACCAGATCATCAATGGGCTG 3' and reverse primer 5'CTGTCCTGCAAATCTGTGGTTG 3'), EED (forward primer

5’AGACGAGAATGATGACGCTGTTCAG 3’and reverse primer 5’CAAGGTAACTCTGTTGCTTCCTAC 3’) and RACK1 (forward primer 5’GGGACAAGCTGGTCAAGGTA 3’ and reverse primer 5’GGGATCCATCTGGAGAGACA 3’). The RT- PCR was performed with an initial synthesis of cDNA at 60°C for 30 minutes followed by denaturation of DNA at 94°C for 2 minutes, then 40 cycles of 94°C for 30 seconds and 55°C for 30 seconds and 68°C for 45 seconds, followed by 68°C for 5 minutes.

Microscopy

Cells were plated onto 1 mm x 18mm glass coverslips in the respective growth medium, incubated at 37ºC with 5 µg/ml Alexa 488- or Alexa 594-dextran (mw 10,000) (Invitrogen, Carlsbad, CA) overnight followed by a 2-hours chase to allow all dextran to localize to lysosomes. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA lens using Optronics Picture Frame software (Olympus Inc., Melville, NY). For immunofluorescence analysis, cells plated on glass coverslips were fixed in 3.7% formaldehyde (Sigma-Aldrich, St. Louis, MO) in Phosphate Buffered Saline (PBS) for 20 minutes at room temperature. Cells were washed in PBS, permeabilized with 0.01% Saponin/PBS/1% Bovine Serum Albumin (BSA) and incubated with 1:100 rat anti-Lamp1, 1:100 rabbit anti-myc, 1:250 rabbit anti-flag followed by 1:750 Alexa 488 or Alexa 594 conjugated goat anti-rat IgG, Alexa 594 goat anti-mouse IgG, Alexa 488 conjugated goat anti-rabbit IgG. Quantifications of lysosome size were done on epifluorescent images using greater than 20 fields per cell type with 2-5 cells per field. Measurements of lysosome diameter were performed using NIH Image J software

(Durchfort et al.).

Neutral Sphingomyelinase Inhibition

NSMase effect on lysosome size regulation was studied by treating cells with NSMase inhibitors GW4869 (Cayman Chemicals, Ann Arbor, MI) or 3-O-Methylsphingomyelin at a concentration of 5 µM for 24-hours or for different interval of time, and lysosome size was measured by live cell microscopy as previously described (Nina Durchfort et al.).

Neutral Sphingomyelinase Activity Assay (^{14}C Assay)

Wild type C57BL/6 and *fro/fro* fibroblasts were plated onto 100 mm tissues culture plates and allowed to grow for 24 to 48 hours to 50-80% confluence. The cells were lysed in lysis buffer plus 2X protease inhibitor cocktail (Roche Applied Science, Boulder, CO), sonicated and centrifuged at 500 x g at 4'C for 10 minutes and the supernatant was transferred to a new eppendorf. Protein determinations were performed and samples (150-300 µg/reaction) in 100 µl of lysis buffer were prepared. The reaction was started by adding 100 µl of reaction mixture (200 µM Sphingomylein, 100 µM phosphatidylserine, 200 mM Tris, pH 7.4, 10 mM $MgCl_2$ 0.2 % Triton X100, 5mM Dithiothreitol (DTT), Roche EDTA- free tablet) containing ^{14}C-sphingomyelin to the cell lysate, mixed by vortexing and then incubated at 37°C for 120 minutes. The reaction was stop by adding 1.5 ml chloroform/methanol (2:1), 0.4 ml of water and vortexed for 15 seconds. The mixture was centrifuged at 2000 x g for 5 minutes at room temperature, the

upper aqueous phase transferred to scintillation vials containing 10 ml of scintillation fluid and radioactivity was measured.

Lipidomic Analysis

Bone marrow-derived macrophages were cultured from femurs of C57BL/6, *beige$_j$*, and FAN$^{-/-}$ mice. Cells were washed in ice-cold PBS, scraped and centrifuged at 200 x g at 4'C for 5 minutes. Lipidomic analysis was performed using an Agilent 1290 Infinity LC system coupled to an Agilent 6520 Quadrupole Accurate-Mass Q-TOF LC-MS instrument equipped with an Acquity UPLC CSH C18 (100 x 2.1 mm; 1.7 um) column. The gradient starts at 15% mobile phase B then increases to 30% B over 4 minutes, it then increases to 52% B from 4-5 minutes, then increases to 82% B from 5-22 minutes, then increases to 95% B from 22-23 minutes, then increases to 99% B from 23-27 minutes. From 27-38 minutes it is held at 99% B, then decreases to 15% B from 38-38.2 minutes and is held there from 38.2-44 minutes. Flow is 0.3 mL/min throughout. Mobile phase A consists of ACN: water (60:40 v/v) in 10mM ammonium formate and 0.1% formic acid, and mobile phase B consists of IPA: water (90:10 v/v) in 10mM ammonium formate and 0.1% formic acid. The column temperature was set to 65°C. Acquisition was performed in positive and negative ESI mode acquiring from *m/z* 100-1700. The mass spectrometer was operated using the following parameters while in positive mode: dry gas temperature, 350°C; dry gas flow, 11.1 L/min; nebulizer pressure, 24 psig; Vcap, 5000V; fragmentor, 250 V; skimmer 74.4 V; sample injection, 3 µL. The mass spectrometer was operated using the following parameters while in negative mode: dry gas temperature, 260°C; dry gas flow, 8.6 L/min; nebulizer pressure, 21 psig; Vcap,

5000V; fragmentor, 100 V; skimmer 75 V; sample injection, 5 μL. Tandem mass spectrometry was conducted using the same LC gradient and at a collision energies of 20V.

CHAPTER 3

RESULTS

Generation of FAN$^{-/-}$/*beige$_j$* Mouse

FAN$^{-/-}$ mice were crossed with *beige$_j$* mice and bred to homozygosity (FAN$^{-/-}$*; beige$_j$/beige$_j$)*. The absence of Lyst results in a coat color defect but the absence of FAN was not appreciated to alter the C57BL/6 color. The loss of both Lyst and FAN is additive in affecting coat color (Figure 3.1) and at the cellular level the loss of both FAN and Lyst results in an additive effect on altering lysosomes size (Figure 3.2). These results provide evidence that FAN and Lyst act in different pathways to alter lysosome size.

Inhibition of NSMase Activity

FAN has been shown to interact with TNFR1 to activate NSMase2 at the plasma membrane. To determine if the level of NSMase affects steady state lysosome size, cells were incubated with the specific NSMase inhibitor GW4869 or 3-O-Methylsphingomyelin and the lysosome morphology was examined by fluorescence live-cell microscopy. Microscopic analyses showed that inhibition with GW4869 (Figure 3.3) or 3-O-Methylsphingomyelin gave rise to enlarged lysosomes (Figure 3.4). These results demonstrate that NSMase activity is important for regulating lysosome size.

Overexpression of NSMase2

To determine if NSMase2 levels are limiting in regulating lysosome size, NSMase2-GFP under a CMV promoter was transfected into HeLa cells and the size of lysosomes assessed using epifluorescence microscopy. No changes in lysosome size were

observed when NSMase2-GFP was overexpressed in the cell (Figure 3.5). Previous studies demonstrated no change in lysosome size when NSMase2-GFP was overexpressed in either FAN$^{-/-}$, or *beige$_j$* fibroblasts (Durchfort). This result demonstrates that increased levels of NSMase2 do not affect lysosome size. We did note that a fraction of NSMase2-GFP could be found coincident with lysosomes. This could be an effect of overexpression or this may indicate that NSMase2 has a function at lysosomes.

Overexpression of FAN

Previous studies demonstrated that overexpression of Lyst gave rise to small lysosomes localized to the periphery of cells (Perou, Leslie, et al.). To determine if overexpression of FAN would have a similar affect a construct containing epitope-tagged (GFP) mouse FAN expressed under the control of the CMV promoter was generated and transfected into C57BL/6, FAN$^{-/-}$, or *beige$_j$* fibroblasts. Transfected cells were incubated with 5 µg/ml Texas red dextran overnight, chased in growth media for 2 hours and lysosome morphology assessed using epifluorescence microscopy. Overexpression of GFP-FAN reduced lysosome size in wild type and FAN$^{-/-}$ cells but not in *beige$_j$* cells (Figure 3.6). The reduction in lysosome size seen in FAN-overexpressing cells was prevented by the addition of the NSMase inhibitor GW4869 (Figure 3.7).

The characterization of FAN as an activator of NSMase identified NSMase2 as the activated NSMase (Philipp et al.). To determine if FAN is acting through NSMase2 to alter lysosome morphology we obtained fibroblasts from the *fro/fro* mouse, which harbors a deletion mutation, that blocks expression of NSMase2 (Khavandgar et al.) and examined lysosome morphology and whether or not FAN overexpression altered

lysosome morphology in the absence of NSMase2. No changes in lysosome size, distribution, or morphology were observed in *fro/fro* cells compared to wild type C57BL/6 fibroblasts. Additionally, the reduction in the lysosome size seen when FAN was overexpressed was still observed in *fro/fro* cells (Figure 3.8). We confirmed that the *fro/fro* mouse fibroblast showed less neutral sphingomyelinase activity compared to the wild type C57BL/6 cells (Figure 3.9). Together, these results demonstrate that like Lyst, FAN overexpression reduces lysosome size and that the absence of NSMase2 does not affect lysosome size nor the reduction in lysosome size seen when FAN is overexpressed.

RNA Interference of NSMase1 and NSMase3

To determine if FAN is working through other NSMases, RNAi was used to try to reduce the levels of NSMase1 and NSMase3. No alteration in lysosome morphology was observed in NSMase1 and NSMase3 RNAi-treated cells (data not shown). Analysis of mRNA levels was done by RT-PCR. The result showed a 58% reduction of NSMase1 mRNA, however, no reduction of NSMase3 mRNA level was observed (Figure 3.10). These results suggest that reduced level of NSMase1 does not alter lysosome morphology; however, a 58% reduction in NSMase1 mRNA may not be sufficient to produce an effect. Further studies are needed to reduce NSMase1 and NSMase3 levels and then analyze the lysosome phenotype in absence of FAN in order to determine if either of these NSMases plays a role in regulating lysosome size.

Overexpression and RNA Interference of FAN Binding Partners

FAN has been shown to interact with RACK1 to activate NSMase (Möhlig et al.). This activation is dependent upon RACK1 binding to the polycomb protein EED (Philipp et al.). To determine if FAN binding partners RACK1 or EED play a role in regulating the lysosome size, epitope-tagged RACK1 or EED was overexpressed and lysosome size assessed. No changes in lysosome size or morphology were observed with either RACK1 (Figure 3.11) or EED overexpression (Figure 3.12). We did note that a fraction of RACK1-myc could be found coincident with lysosomes. This could be an effect of overexpression or this may indicate that RACK1 has a function at lysosomes.

RNA interference was used to knockdown RACK1 or EED levels in cells. mRNA analyses by RT-PCR showed a 57.5% reduction of EED mRNA and 37.79% reduction in RACK1 mRNA (Figures 3.13, 3.14). Further experiments are needed to improve the RNAi efficiency and once better "silencing" is obtained lysosome size will be assessed to determine if there is a role for RACK1 or EED in maintaining lysosome size.

Lipidomic Analysis

As changes in cellular lipid content can alter lysosome size (Sbrissa et al.; Mukherjee and Maxfield), we postulated that changes in lysosome size could be attributed to alterations in the lipid composition and that the absence of Lyst or FAN may results in changes in lipid profiles. We isolated bone marrow-derived macrophages of C57BL/6, *beige*$_j$ and FAN$^{-/-}$ mice. Cells were then subject to lipidomic analyses using tandem LC-MS. Little difference in lipids was observed between *beige*$_j$ and C57BL/6 cells but significant changes were observed when comparing C57BL/6 to FAN$^{-/-}$ (Table

3.1). Principle Components Analysis (PCA) between C57BL/6 and FAN$^{-/-}$ lipids showed greater than 20 altered lipids (Figure 3.15). Further analysis showed that 15 different lipid were significantly changed in the absence of FAN (Figure 3.16). The identities of some of these lipids are known and suggest that the levels of free fatty acids and possible ceramides were significantly decreased in the absence of FAN. These results show that the absence of FAN alters cellular lipids. Further experiments will focus on the lysosome lipid profiles.

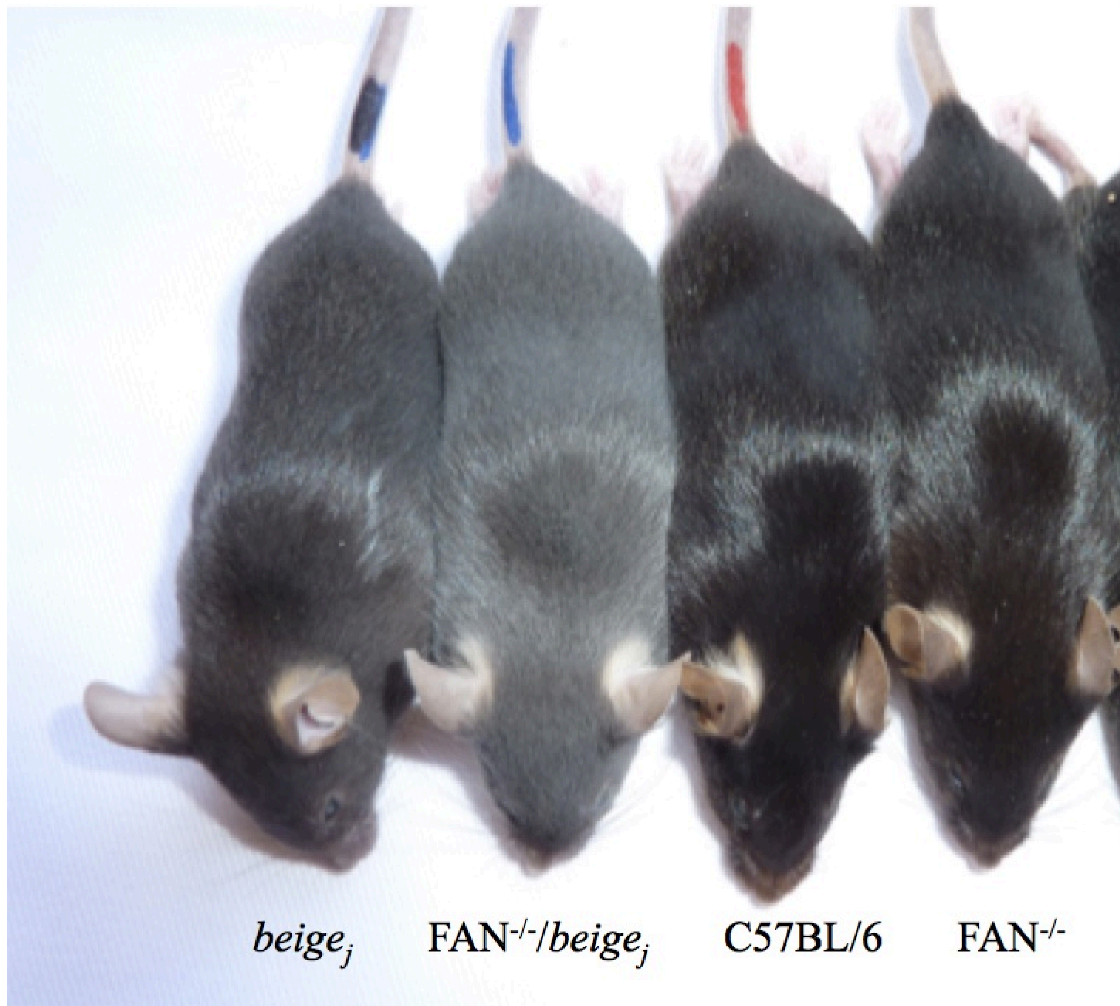

Figure 3.1: Coat color difference between C57BL/6, *beige*$_j$, FAN$^{-/-}$, FAN$^{-/-}$/ *beige*$_j$ mice

The *beige*$_j$ mouse has a lighter coat color compared to C57BL/6. The absence of FAN does not show an alteration in coat color, whereas the *beige*$_j$ coat color defect was exaggerated in the FAN$^{-/-}$/ *beige*$_j$ mouse.

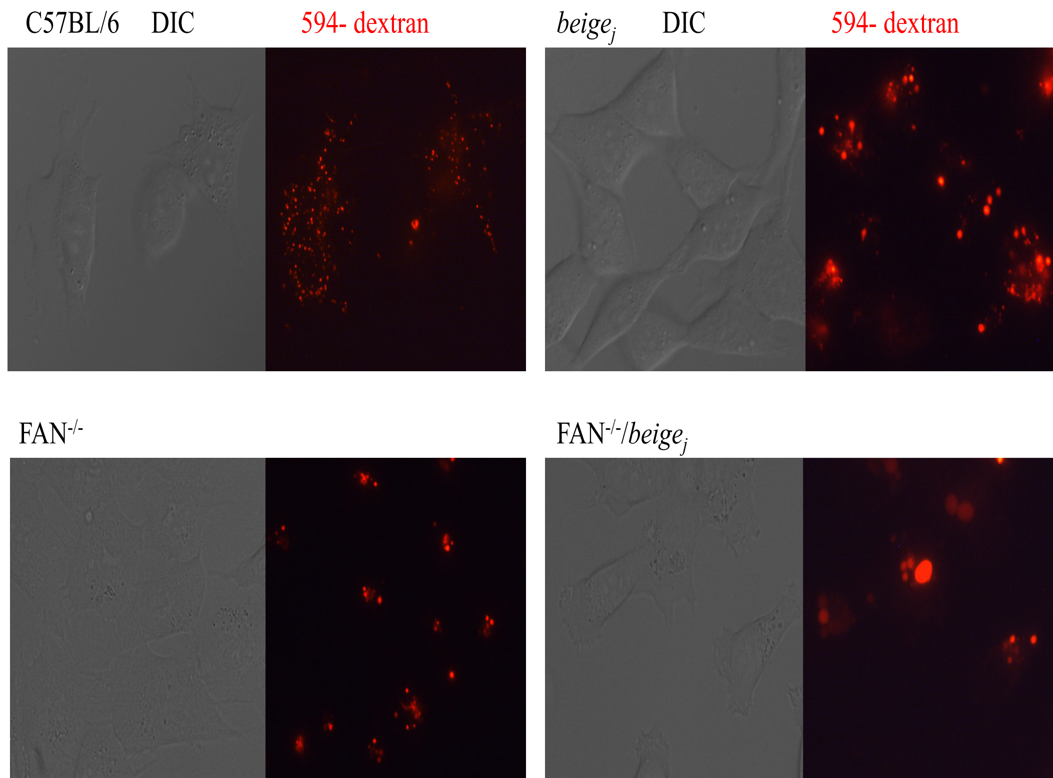

Figure 3.2: The absence of FAN and Lyst results in enlarged lysosomes
Fibroblast from C57BL/6, *beige*$_j$, FAN$^{-/-}$, FAN$^{-/-}$/ *beige*$_j$ mice were plated on glass coverslips and incubated with 5 µg/ml Texas red dextran (mw 10,000 da) at 37°C overnight, followed by a 2-hour chase to allow all dextran to localize to lysosomes. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY).

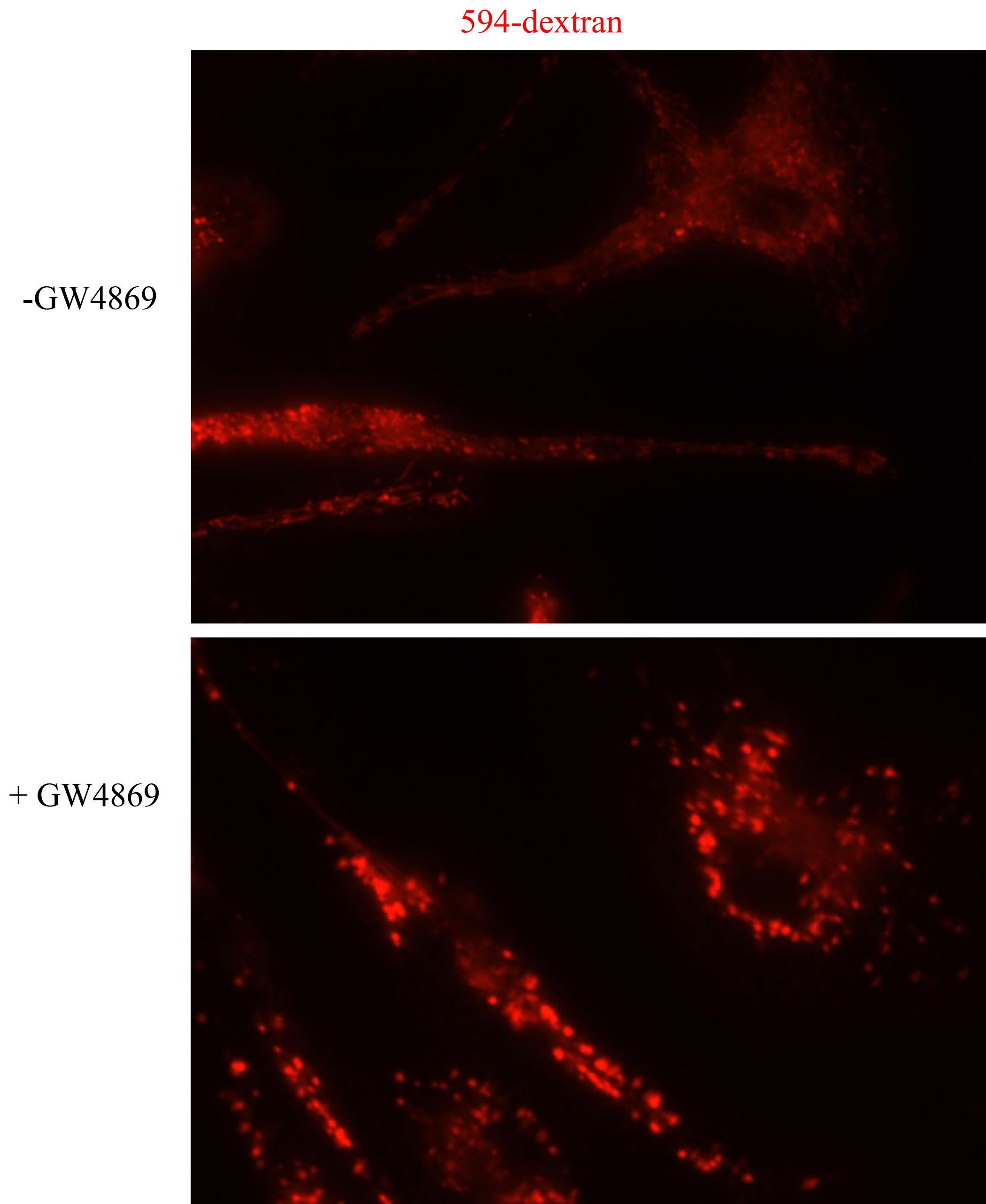

Figure 3.3: NSMase inhibition induces an increase in lysosome size
C57BL/6 cells were plated on glass coverslips and incubated with 5 μg/ml Texas red dextran (mw 10,000 da) at 37°C overnight, followed by a 2-hour chase to allow all dextran to localize to lysosomes. Five μM NSMase inhibitor GW4869 was added and incubated at 37°C overnight. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY).

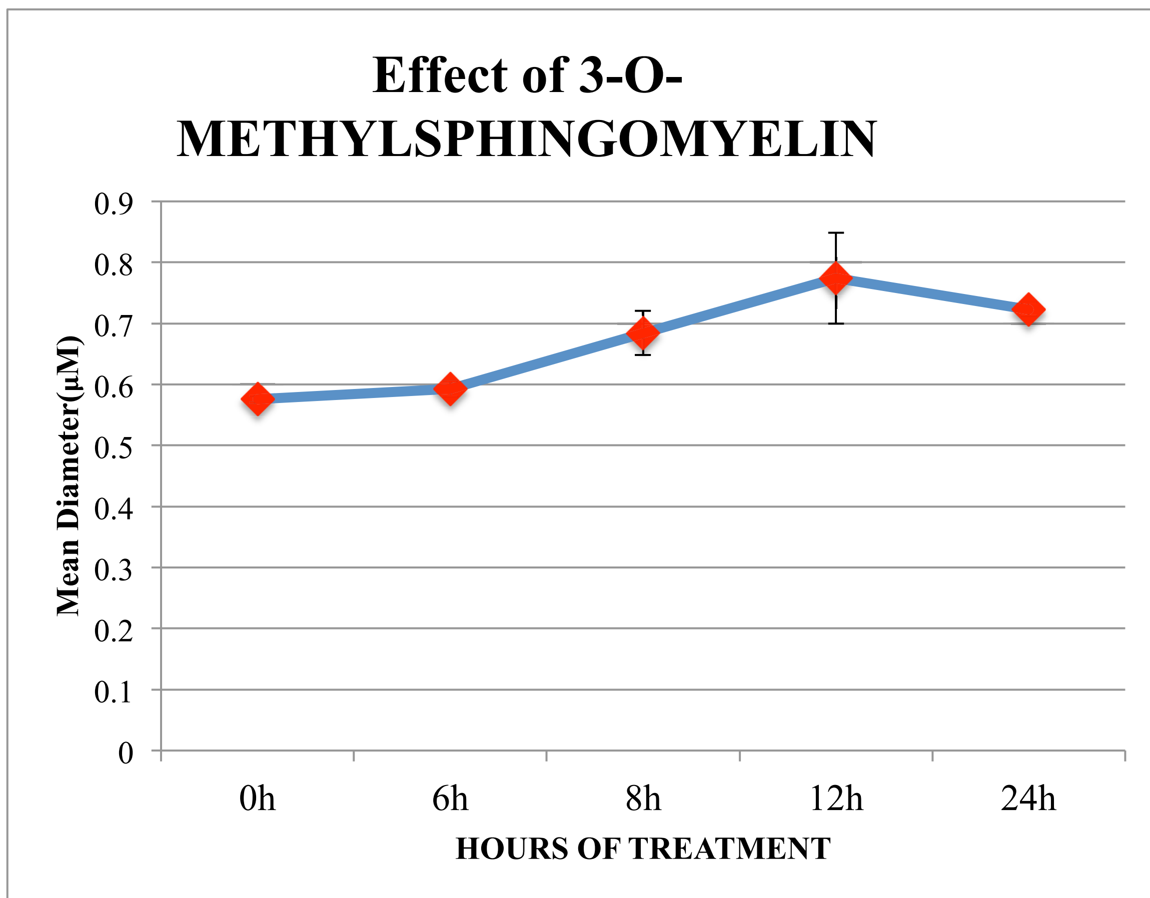

Figure 3.4: Time-dependent effect of NSMase inhibitor 3-O-Methylsphingomyelin on lysosome size

C57BL/6 cells were plated on glass coverslips and incubated with 5 µg/ml Texas red dextran (mw 10,000 da) at 37°C overnight, followed by a 2-hour chase to allow all dextran to localize to lysosomes. Cells were then incubated with 5 µM NSMase inhibitor 3-O-Methylsphingomyelin at 37°C for different time interval and images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY). Measurement of lysosome size was done on epifluorescent images using NIH Image J software.

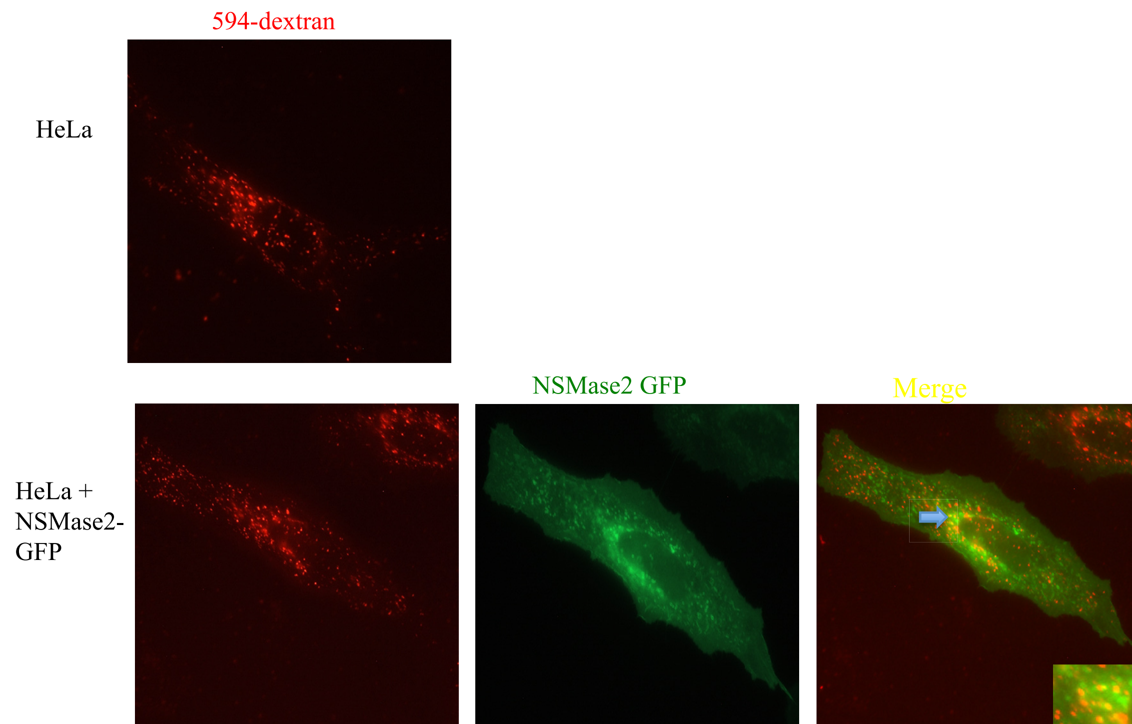

Figure 3.5: NSMase2 overexpression does not alter lysosome size
HeLa cells were transfected with the plasmid pCMV NSMase2-GFP. Cells were plated on glass coverslips and incubated with 5 µg/ml Texas red dextran (mw 10,000 da) at 37°C overnight, followed by a 2-hour chase to allow all dextran to localize to lysosomes. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY). The arrow denotes the area blown up as an insert showing colocalization of NSMase2 on Texas red dextran loaded lysosomes.

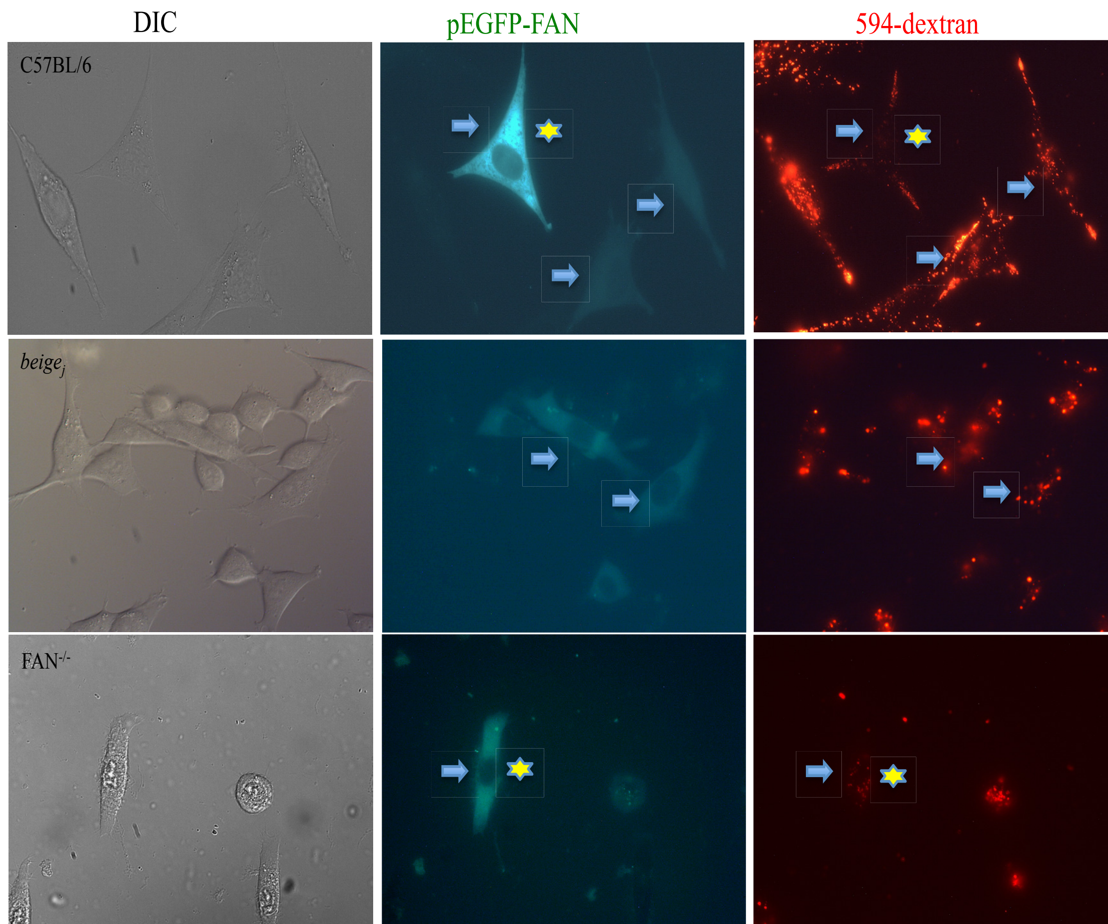

Figure 3.6: FAN overexpression does not reduce lysosome size in the absence of Lyst C57BL/6, FAN$^{-/-}$, *beige*$_j$ cells were transfected with the plasmid pEGFP-FAN. Cells were plated on glass coverslips and incubated with 5 µg/ml Texas red dextran (mw 10,000 da) at 37°C overnight, followed by a 2-hour chase to allow all dextran to localize to lysosomes. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY). Arrows denote GFP-FAN overexpressing cells and stars denote reduction in size when GFP-FAN is overexpressed.

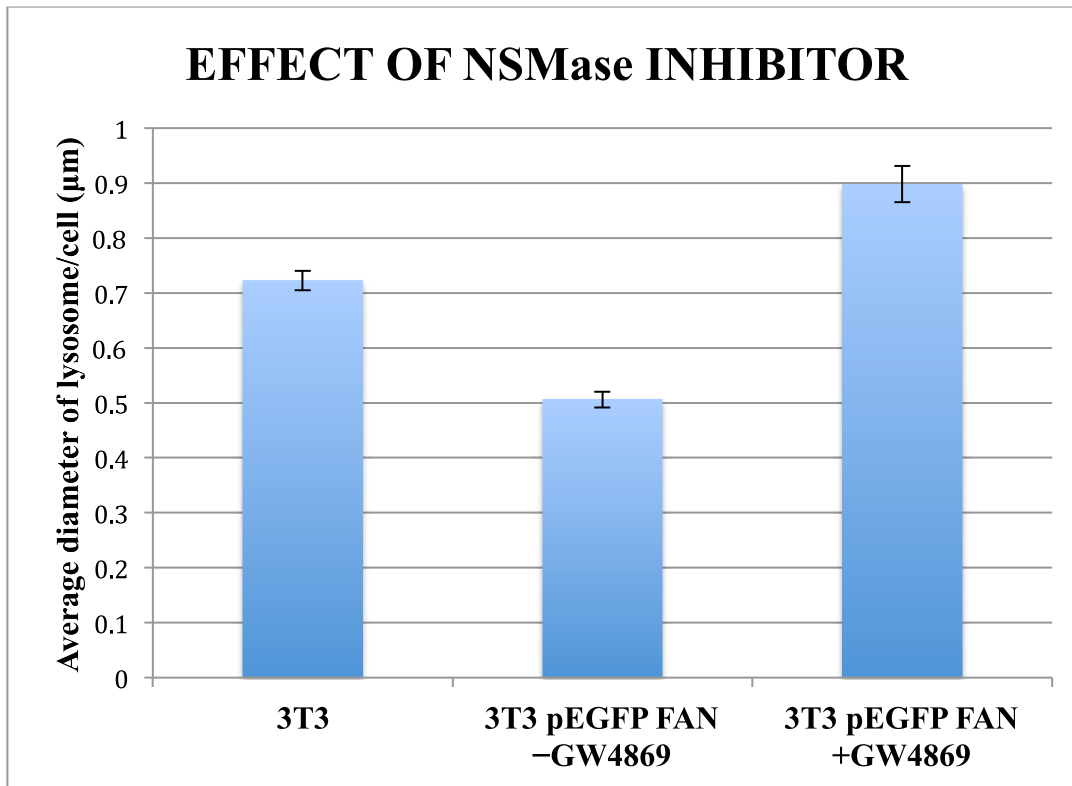

Figure 3.7: Inhibition of NSMase blocks the reduction in lysosome size in cells overexpressing FAN

NIH3T3 fibroblasts were transfected with the plasmid pEGFP-FAN. Cells were plated on glass coverslips and incubated with 5 µg/ml Texas red dextran (mw 10,000 da) followed by a 2-hour chase to allow all dextran to localize to lysosomes. Cells were then incubated with 5 µM NSMase inhibitor GW4869 at 37°C overnight. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA lens using Optronics Picture Frame software (Olympus Inc., Melville, NY). Measurement of lysosome size was done on epifluorescent images using NIH Image J software.

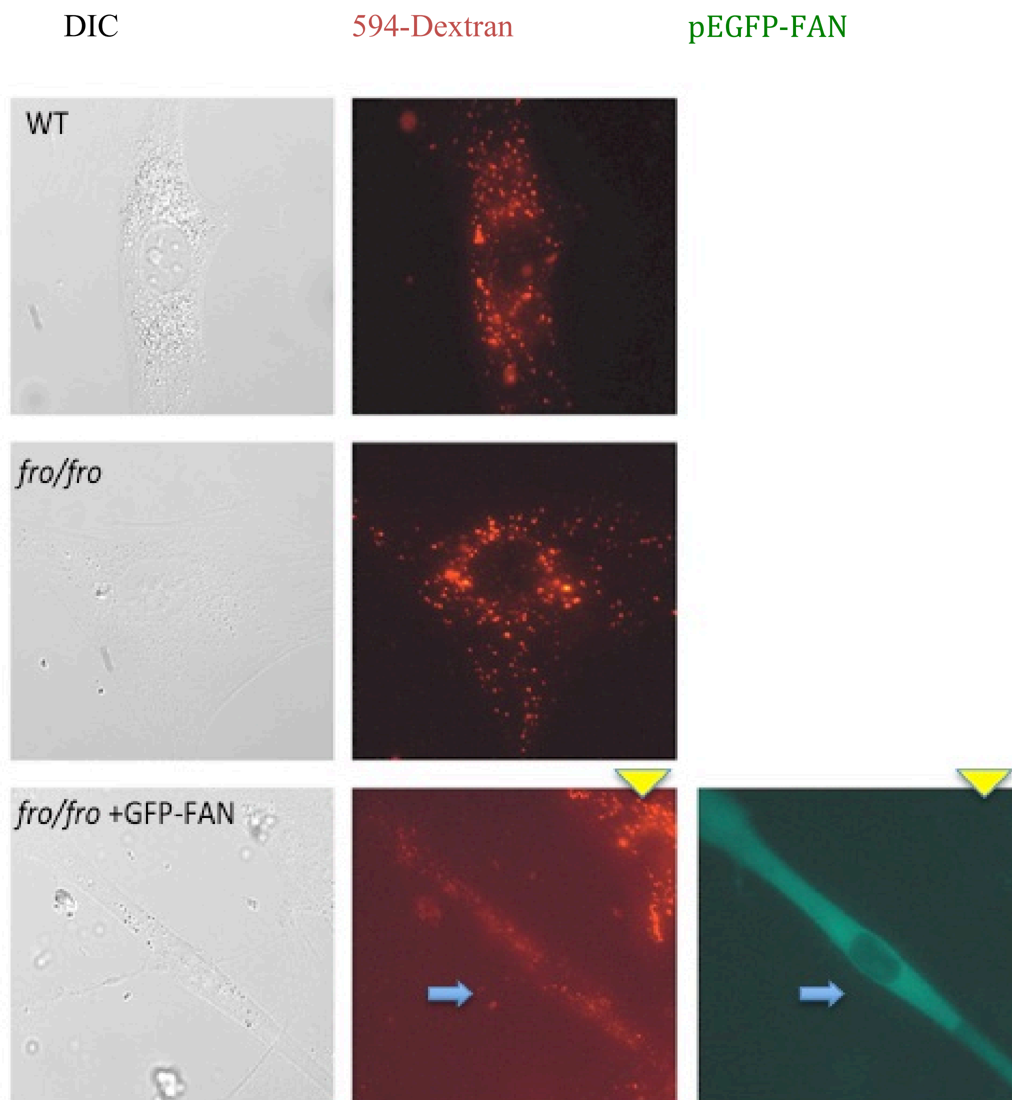

Figure 3.8: The absence of NSMase2 does not affect lysosome size or the FAN-mediated reduction in lysosome size
Fibroblast WT, *fro/fro* (NSMase2 deficient cell), *fro/fro* (NSMase2 deficient cell) transfected with pEGFP-FAN were plated on glass coverslips and incubated with 5 μg/ml Texas red dextran (mw 10,000 da) followed by a 2-hour chase to allow all dextran to localize to lysosomes. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY). Arrows denote GFP-FAN overexpressing cells with small lysosomes and the arrowheads denote an untransfected cell in the same culture with larger lysosomes.

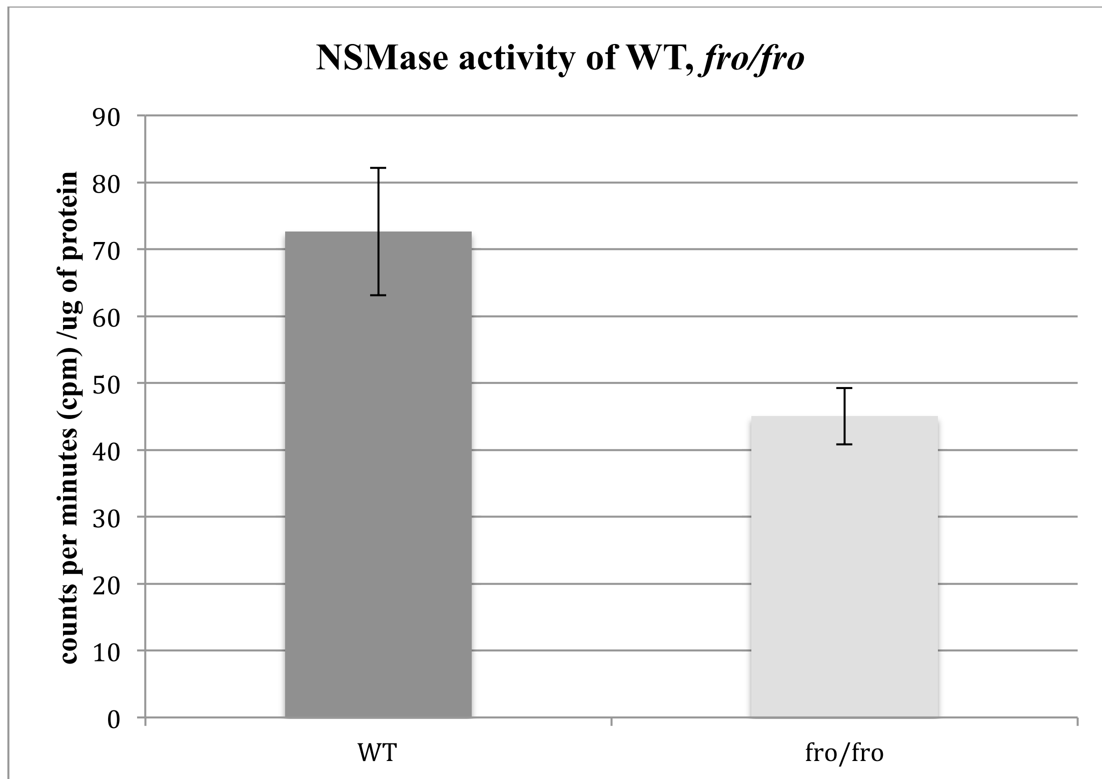

Figure 3.9: ***fro/fro*** **cells exhibit decreased NSMase activity** ***in vitro***
Neutral sphingomyelinase activity was measured in WT and *fro/fro* cells according to the ^{14}C-sphingomyelin protocol described in the Materials and Methods chapter.

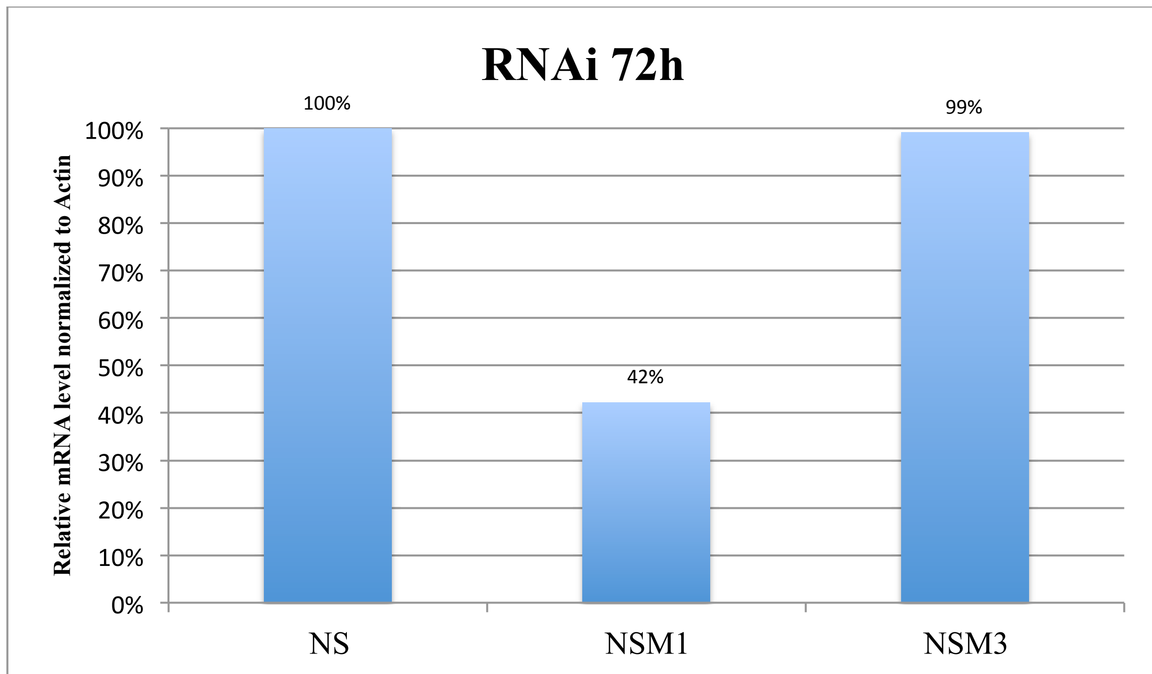

Figure 3.10: mRNA analysis of NSMase1 and NSMAse3 by RT-PCR
HeLa cells were treated with Non Specific (NS), NSMase1 or NSMase3 specific oligonucleotides for 72 hours, RNA isolated and analyzed by RT-PCR. mRNA was normalized to actin. Quantification was done with Gel Doc XR+ software (n=1).

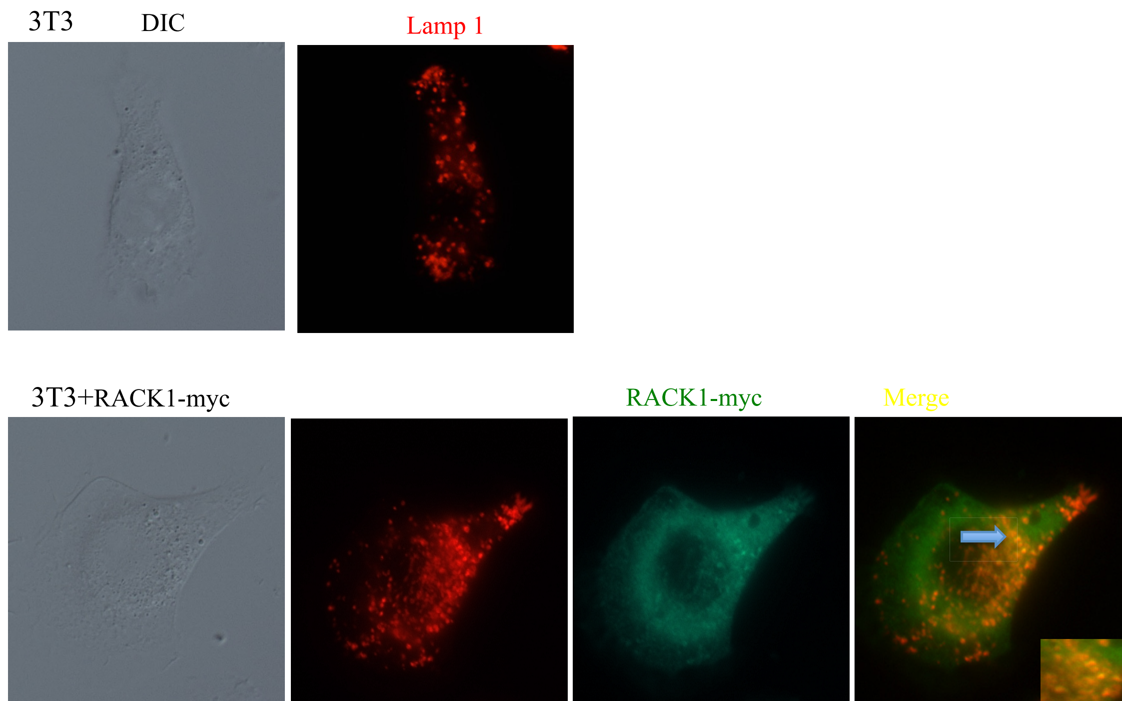

Figure 3.11: RACK1 overexpression does not alter lysosome size
NIH3T3 fibroblast was transfected with the plasmid pCMV RACK1-myc and grown on glass coverslips overnight followed by fixation in formaldehyde. Lamp-1 and RACK1-myc were localized by immunofluorescence microscopy using a rat anti-Lamp-1 antibody and rabbit anti-myc followed by Alexa 594 conjugated goat anti-rat IgG and Alexa 488 conjugated goat anti-rabbit IgG. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY). The arrow denotes the "blown-up" area showing colocalization of RACK1-myc on Lamp-1 positive lysosomes.

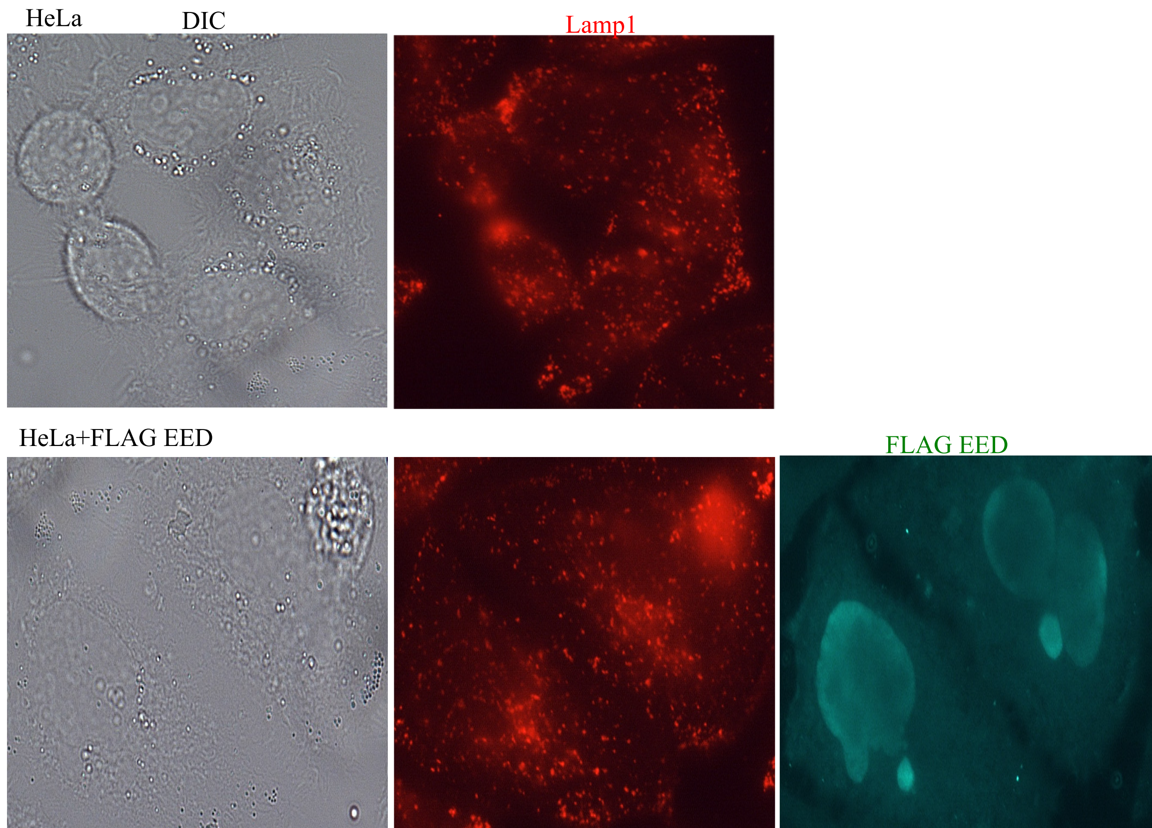

Figure 3.12: EED overexpression does not affect lysosome size
HeLa cells were transfected with the plasmid pCMV FLAG-EED. Cells were plated on glass coverslips and fixed in formaldehyde. Lamp-1 and FLAG-EED were localized by immunofluorescence microscopy using a mouse anti-Lamp-1 antibody and rabbit anti-flag followed by Alexa 594 conjugated goat anti-mouse IgG and Alexa 488 conjugated goat anti-rabbit IgG. Images were captured on an Olympus BX51 microscope with a 60X, 1.4NA objective using Optronics Picture Frame software (Olympus Inc., Melville, NY).

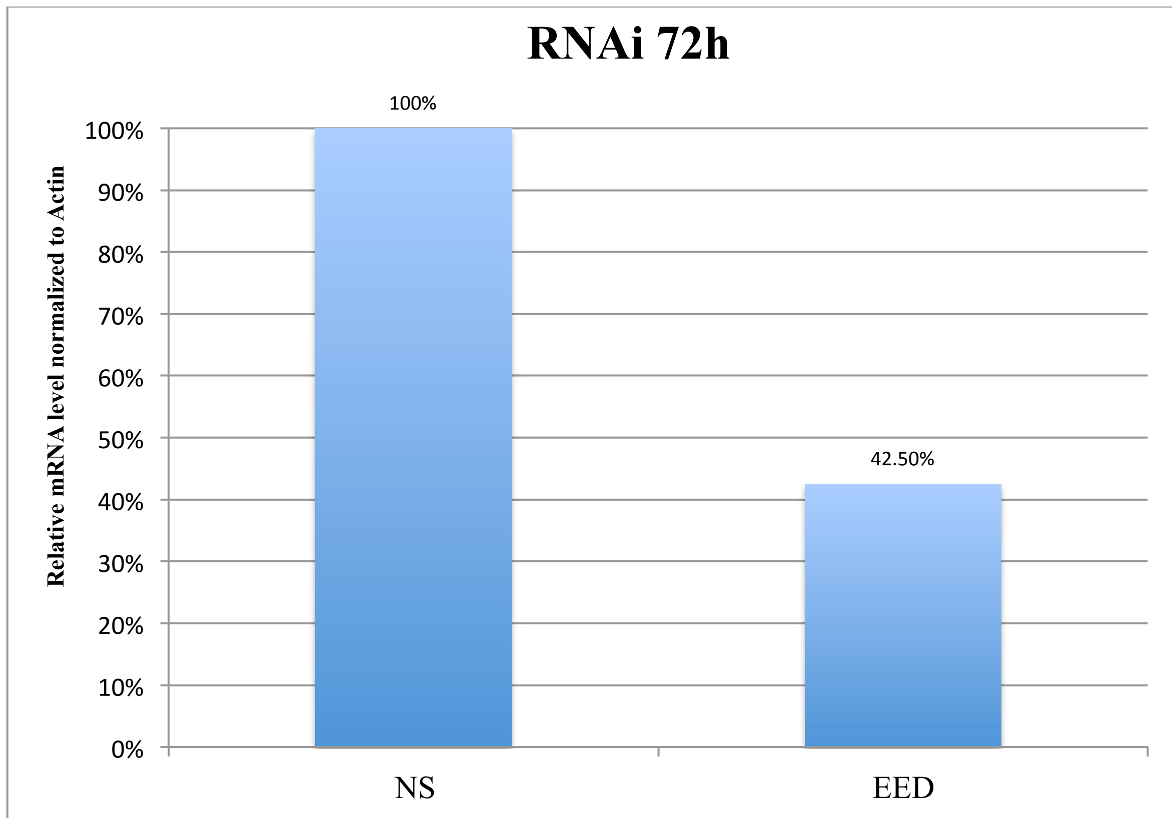

Figure 3.13: mRNA analysis of EED by RT-PCR
HeLa cells were treated with either Non Specific (NS) or EED specific oligonucleotides for 72 hours, RNA isolated and analyzed by RT-PCR. mRNA was normalized to actin. Quantification was done with Gel Doc XR+ software (n=1).

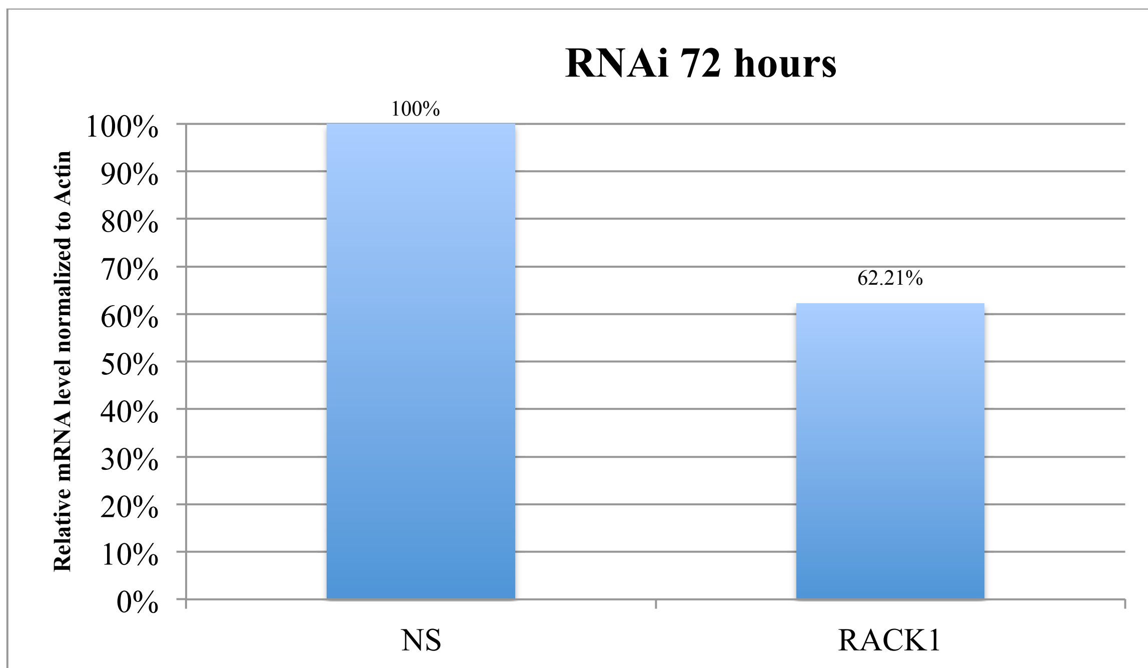

Figure 3.14: mRNA analysis of RACK1 by RT-PCR
HEK293 cells were treated with either Non Specific (NS) or RACK1 specific oligonucleotides for 72 hours, RNA isolated and analyzed by RT-PCR. mRNA was normalized to actin. Quantification was done with Gel Doc XR+ software (n=1).

Table 3.1: List of altered cellular lipids in *beige$_j$* or FAN $^{-/-}$ macrophages

LC-MS lipidomic analysis of cellular lipids from C57BL/6 vs *beige$_j$*, or C57BL/6 vs FAN$^{-/-}$. Putative alter lipids were analyzed using Mass Hunter Quant to eliminate false positives. Altered lipid metabolites are highlighted in red. Yellow highlights show higher level of lipid in either *beige$_j$* or FAN$^{-/-}$ samples and green highlights show lower level of change in lipid. Red highlights p values below 0.05. Few alterations were observed between the *beige$_j$* and C57BL/6 samples.

Name	Possible ID's	Fold Change *beige$_j$* Vs C57BL/6	p-value *beige$_j$* Vs C57BL/6	Fold Change FAN $^{-/-}$ Vs C57BL/6	p-value FAN $^{-/-}$ Vs C57BL/6
497.3823_1.0899055	unknown	2.965164018	0.035581594	2.227001955	0.05946134
272.2343_4.685147	2-hydroxy palmitic acid	0.543375214	0.196797837	0.120965879	0.040404999
440.3061_5.9858084	unknown	0.718325744	0.252121994	0.261214751	0.025732393
582.4651_7.052325	unknown	0.586911324	0.22148379	0.123411971	0.041186971
530.4318_7.0854125	unknown	0.701554305	0.377051824	0.061410521	0.042772567
254.2242_7.0966735	palmitoleic acid	0.533319935	0.084116867	0.126270375	0.01320699
555.3902_7.488754	unknown	0.701615535	0.377104854	0.067759424	0.04353791
268.24_7.9912376:1	2-hydroxy 17:0 FA	0.832074217	0.471435235	0.2803071	0.026795393
633.5702_8.147067	cer(t20:0/18:0) or cer(t18:0/20:0) or cer(d20:0/18:0(2OH)) or cer(d18:0/20:0(2OH));	1.037180412	0.782154655	0.354811296	0.001611473
422.2799_9.004133	PA(P-18:0/0:0)	0.570409909	0.077173726	0.177512544	0.013677897
282.2554_9.012176	oleic acid	0.716790716	0.199110021	0.279827659	0.021379078
1186.9279_8.99351	unknown	0.606083804	0.117015354	0.134065964	0.012379705
326.1919_9.008625	unknown	0.666354991	0.078422385	0.244995702	0.007237286
340.2094_9.03116	unknown	0.913958495	0.759523482	0.27018871	0.00855246
599.4691_15.007718	Cer(d18:2/18:1) or CerP(d18:1/16:0) or Cer(d14:2(4E,6E)/22:1(13Z)) or Cer(d16:2(4E,6E)/20:1(11Z))	4.906910634	0.101371602	2.87566406	0.034133479
366.3495_16.366756	24:1 FA	0.981168953	0.939028676	0.361624345	0.041927542

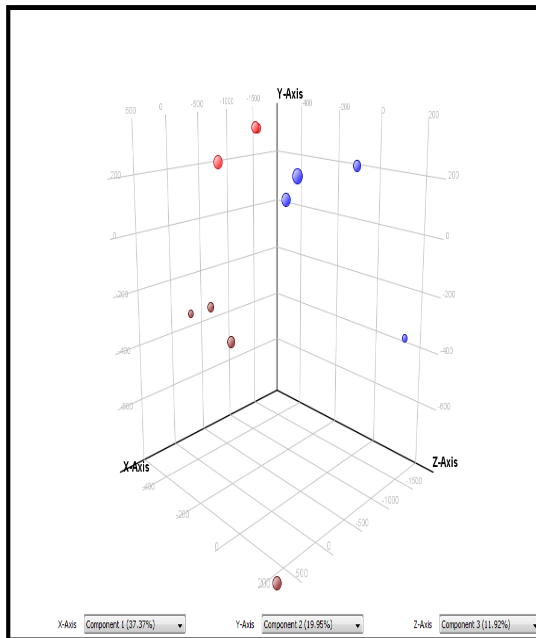

Figure A

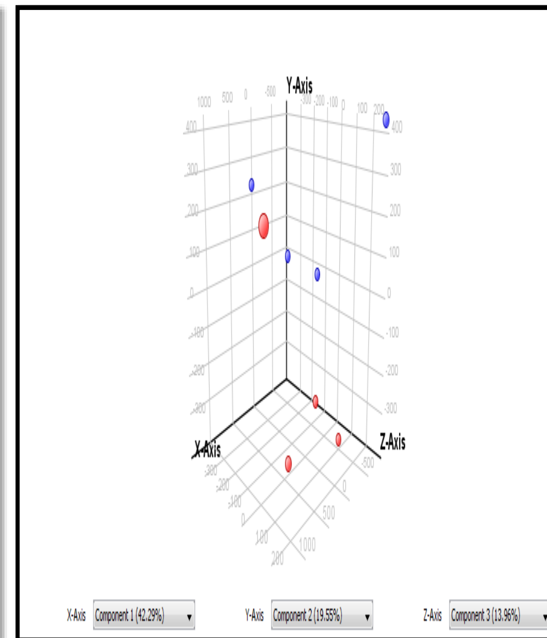

Figure B

Figure 3.15: PCA analysis of C57BL/6, *beige$_j$* and FAN$^{-/-}$

A. PCA plot of C57BL/6 (blue), *beige$_j$* (red) and FAN $^{-/-}$(brown) shows clear class separation.
B. PCA plot of FAN $^{-/-}$ versus C57BL/6. Class separation is observed primarily in the first component.

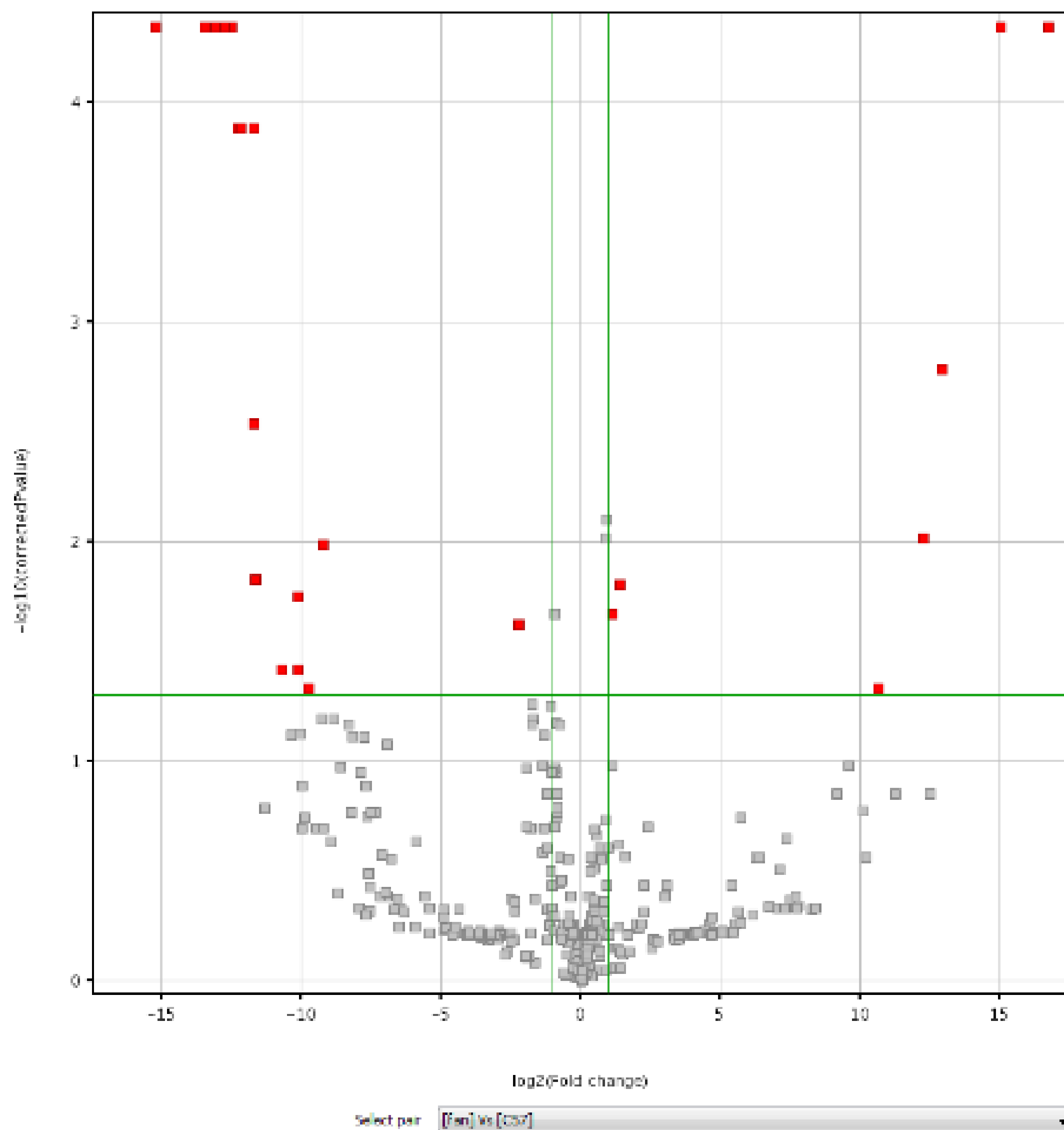

Figure 3.16: Volcano plot of FAN$^{-/-}$ vs. C57BL/6
The volcano plot derived from these data with 23 possibly altered lipids indicated by red squares above the horizontal line.

CHAPTER 4

DISCUSSION

Loss of Lyst and FAN Shows an Additive Effect on Altering Lysosome Size

Lyst and FAN proteins belong to the conserved BEACH family. The loss of either Lyst or FAN gives rise to enlarged lysosomes and a defect in cutaneous barrier repair (Möhlig et al.). The biochemical pathway for regulating lysosome size is not known for Lyst or FAN. To learn more about the biochemical pathway in regulating the size of the lysosome by Lyst and FAN, FAN$^{-/-}$ mice were crossed with *beige$_j$* mice and bred to homozygosity (FAN$^{-/-}$*;beige$_j$/beige$_j$)*. Notably, loss of both FAN and Lyst exacerbates the enlarged lysosome phenotype seen in either single deletion. Further, the pigmentation defect seen in the *beige$_j$* mouse was exacerbated by the loss of FAN. This is the first report that FAN may be involved in regulating LRO size or biogenesis and provides genetic evidence that Lyst and FAN work in different pathways to regulate LRO size. These results also suggest that there are at least two biochemical pathways involved in regulating lysosome size.

Effect of NSMase Activity on Lysosome Size

FAN binds to the TNF receptor and signals the activation of NSMase, which generates phosphocholine and ceramide. NSMases are found at the plasma membrane and other organelles including lysosomes. The cytosolic protein FAN interacts with the TNF receptor at the plasma membrane in response to ligand binding and activates NSMase2 (Philipp et al.). Overexpression of NSMase2 does not affect TNFR-mediated signaling. We determined that there were no changes in lysosome size in NSMase2 overexpressing cells, suggesting that NSMase activity is also possibly regulated at the

lysosome. Controlling the activity of NSMase on different membranes allows for localizing signaling and possibly regulating the amount of signal. Further, this suggests that molecules that regulate NSMase activity may act at different membranes and that FAN, an activator of NSMase (Adam-Klages et al.), might be rate limiting for regulating the lysosome size. In support of this hypothesis, we observed that inhibition of all NSMases with the specific NSMase inhibitor GW4869 or 3-O-Methylsphingomyelin gave rise to a time dependent enlargement of lysosomes. These results support a role for NSMase activation in regulating lysosome size.

Role of FAN in Regulation of Lysosome Size

Previous studies have shown that Lyst is limiting for regulating lysosomes size (Perou, Justice, et al.). That is, overexpression of Lyst gives rise to smaller than normal lysosomes. In the current study, we determined that increasing FAN levels also reduces lysosome size. Overexpression of FAN, however, did not reduce the size of lysosomes in the absence of Lyst. Since the loss of both Lyst and FAN is additive in affecting the enlarged size of lysosomes supporting two pathways, that overexpression of FAN in the absence of Lyst does not reduce lysosome size may reflect the absence of a factor necessary for FAN function. What that factor may be still needs to be elucidated.

The regulation of lysosome size can be mediated by fusion or fission events. Studies have suggested that Lyst is a negative regulator of fusion (Harris et al.) or a positive regulator of fission (Durchfort et al.). How FAN acts to regulate lysosome size has not been investigated. Our studies suggest that FAN acts to activate NSMases, perhaps at the lysosome but possibly at an earlier compartment. Activation of NSMase

would result in the localized generation of ceramide. Increased levels of ceramide at an endosomal/lysosomal membrane would be predicted to cause changes in the curvature of the lipid bilayer. A role for NSMase2 at the Multivesicular Body (MVB) has been suggested to drive inward membrane budding (Mittelbrunn and Sánchez-Madrid), and a defect in this pathway could lead to enlarged late endosomes fusing with lysosomes and thereby resulting in an enlarged lysosome. FAN could be acting to control NSMase activity at the MVB. *fro/fro* cells, however, do not show enlarged lysosomes. As demonstrated, FAN overexpression reduces lysosome size even in the absence of NSMase2. Other NSMases may compensate for the loss of NSMase2. Our RNAi experiments have not ruled out the possibility that FAN works through another NSMase. Future studies will be focused on efficiently reducing the levels of NSMase1 and 3 and then looking at the effects of FAN overexpression. We will also examine if FAN is working at an earlier compartment to affect the size of vesicles fusing with lysosomes.

Overexpression and RNA Interference of FAN Binding Partners

In the TNF-receptor pathway, NSMase2 is activated by TNF-R55 in response to TNF. The NSD within the TNF-R55 serves as the binding site for FAN, which recruits RACK1 and the nuclear EED protein (Philipp et al.). Our studies demonstrate that overexpression of RACK1 or EED did not affect lysosome size. We did note that a small fraction of RACK1 localized on lysosomes, so it is possible that RACK1 may play a role in regulating lysosome size. EED was seen predominantly in the nucleus as expected (Philipp et al.). Our preliminary RNAi experiments for RACK1 and EED, which showed about 50% reduction in mRNA, suggest that we may be able to reduce the levels of these

proteins sufficiently to determine if they play a role in FAN-mediated reductions in lysosome size. It should be noted, however, that the absence of RACK1 or EED is embryonic lethal (Volta et al.; Schumacher, Faust and Magnuson) and results of efficient silencing and examination of lysosome morphology changes must be analyzed with care to make sure changes in size are not the result of cells dying.

The Absence of FAN Alters the Lipid Profile

One possible explanation for changes in lysosome size when FAN is overexpressed or absent is that there is a change in the lipid composition. We performed lipidomic profiling using LC-MS and MS-MS analysis on wild type C57BL/6, *beige*$_j$ and FAN$^{-/-}$ cells to determine if the absence of Lyst or FAN alters cellular lipid content. Lipidomic analysis demonstrated a strong difference between C57BL/6 and FAN$^{-/-}$ samples but very little difference between *beige*$_j$ and C57BL/6 samples. The identification of the metabolites found was difficult. Free fatty acids like 2-hydroxy palmitic acid, palmitoleic acid, oleic acid, 24:1 fatty acid, margaric acid and nonadecanoic acid were all decreased in the FAN$^{-/-}$ samples. In addition to free fatty acid alterations, a number of possible ceramides were also decreased in the FAN$^{-/-}$ samples but their identity is not known. These results demonstrate that the absence of FAN alters lipid composition and leaves open the question of whether or not these changes would result in alterations in lysosome size. Future lipidomic analysis will be performed on lysosomes isolated from wild type C57BL/6, *beige*$_j$ and FAN$^{-/-}$ cells. These studies will allow us to determine if the whole cells changes are also reflected in the lipid content of the lysosomes.

Summary

Lyst and FAN proteins belong to the conserved BEACH family. Loss of either Lyst or FAN gives rise to enlarged lysosome. Generation of a *beige*$_j$/FAN$^{-/-}$ mouse supports a role of FAN in LRO homeostasis and that the loss of both proteins is additive. The enlarged lysosome size observed in the *beige*$_j$/FAN$^{-/-}$ mouse suggests that there are at least two biochemical pathways involved in regulating lysosome size. The overexpression of FAN in absence of Lyst did not correct the size of lysosome.

FAN binds to the TNF receptor and signals the activation of NSMase. The NSMase are found at the plasma membrane and on other organelles including lysosomes. Inhibition of NSMase with the specific NSMase inhibitor GW4869 or 3-O-Methylsphingomyelin resulted in enlarged lysosome. The overexpression of FAN resulted in the reduction of lysosome size but the overexpression phenotype was blocked by the addition of the NSMase inhibitor GW4869. This suggests that the FAN is working through an NSMase to decrease lysosome size.

The role of FAN-binding partners RACK1 and EED in regulating the lysosome morphology is not resolved and future experiments are necessary to determine if they play a role in regulating lysosome size.

The activation of NSMases is predicted to change the lipid composition of cells. Lipidomic profiling using LC-MS and MS-MS analysis of C57BL/6 and FAN$^{-/-}$ cells showed that the levels of free fatty acids and ceramides were significantly decreased in FAN$^{-/-}$ cells. Future studies will examine if these changes are also reflected on the lipid composition of lysosomes. Changes in cellular lipid composition in the FAN$^{-/-}$ cells may

also alter other membrane trafficking events. Future studies may inform whether there are alterations in trafficking in other pathways.

APPENDIX

CONVENTIONAL AND SECRETORY LYSOSOMES

Note: this portion of the manuscript is going to be published in Encyclopedia of Cell Biology.

Abstract

Lysosomes are membrane bound organelles that contain hydrolases capable of degrading proteins, lipids and carbohydrates. They are involved in nutrient sensing and storage and retrieval. Lysosomes are highly dynamic and are capable of fusion and fission events with other organelles and plasma membranes. Higher eukaryotes possess a specialized group of lysosomes termed secretory lysosomes that are involved in pigmentation, coagulation, wound repair and immunologic functions. Understanding the molecular machinery that regulates lysosome and secretory lysosome homeostasis is underscored by the fact that mutations in the genes that regulate the biogenesis, movement and delivery of lysosome and secretory lysosome contents result in human diseases.

Introduction

Lysosomes were first described by Dr. Christian de Duve in 1955 as the major degradative compartment within eukaryotic cells (Appelmans, Wattiaux and De Duve). Structurally they appear as dense bodies that often are found associated with the Microtubule-Organizing Center (MTOC) near the nucleus of the cell. They can vary in size, shape and number depending upon the cell type. Recently, the quantity of lysosomes was found to be regulated by the Transcription Factor EB (TFEB) (Sardiello et al.). TFEB recognizes a DNA sequence (GTCACGTGAC) termed coordinated lysosomal expression and regulation or CLEAR sequence found in many genes that encode for lysosomal proteins (Settembre et al.). TFEB controls the biogenesis of lysosomal hydrolases and membrane proteins. It exists in the cytosol and under altered lysosomal

conditions such as nutrient starvation or hyperaccumulation of substrates, TFEB translocates to the nucleus to activate lysosome biogenesis gene transcription, thus generating increased numbers of lysosomes. Lysosomes possess an acidic lumen containing many types of hydrolases responsible for the degradation of substrates. The high H^+ content is driven by the presence of the vacuolar ATPase H^+ pump. Lysosomes also contain high levels of calcium (Ghislat and Knecht), second in amount compared to the ER. The lysosomal membrane contains highly glycosylated proteins including Lysosome-Associated Membrane Proteins (LAMPs) and Lysosomal Integral Membrane Proteins (LIMPs). This high level of glycosylation is thought to protect the proteins from the degradative enzymes stored in lysosomes. Cytosolic facing lysosomal proteins are involved in regulating the transport of material to and from the lysosome (Saftig and Klumperman; Luzio, Hackmann, et al.). Transport to the lysosome is primarily achieved through vesicular delivery of endocytosed or phagocytosed material. Along this delivery pathway to the lysosome, lipids and proteins can be recycled from endosomal compartments or they can be targeted to lysosomes for degradation. Lysosomes can also obtain cytosolic components such as proteins, lipids and organelles destined for degradation through regulated autophagy (Schneider and Cuervo; Parzych and Klionsky). The digestive nature of lysosomes allows for the reuse of amino acids and lipids as building "material" for generating new nucleic acids, proteins, lipids, and organelles.

Along with their degradative functions, specialized lysosomes termed Lysosome-Related Organelles (LROs) or secretory lysosomes can be found in most cells types. Examples of secretory lysosomes include melanosomes, lytic granules, platelet-dense granules and Major Histocompatibility Complex class II (MHCII) compartments. They

share many properties of lysosomes but also have cell-specific functions such as pigmentation, host defense, coagulation, and innate and adaptive immunity. This review will focus on the characteristics of lysosomes and cell-specific secretory lysosomes, their biogenesis and functions, including an emphasis on the importance of conventional and secretory lysosomes in human disease.

Conventional Lysosomes

All eukaryotes possess organelles that are ascribed to have lysosomal functions, the storage and degradation of molecules. In plants and fungi such as the model organism *Saccharomyces cerevisiae*, lysosomes are called vacuoles. Much of what is know about lysosome biogenesis and delivery to lysosomes can be attributed to the exquisite genetic studies done in yeast (Schekman; Rothman et al.; Klionsky, Herman and Emr; Bryant and Stevens; Wickner; Wickner and Haas; Efe, Botelho and Emr). Historically, the lysosomal compartment has been considered to be an endpoint organelle and many reviews on the endocytic system still depict this organelle as an endpoint. Over the past 20 years it has been appreciated that this organelle is not the endpoint of the endocytic pathway but rather is another membrane compartment where sorting occurs, pathogens can be degraded or persist, signaling can be initiated and energy metabolism can be assessed (Luzio, Hackmann, et al.; Borlace et al.; Barrias, de Carvalho and De Souza; Baxt, Garza-Mayers and Goldberg; Settembre et al.). Lysosomes and secretory lysosomes can be distinguished from late endosomes by the absence of Mannose-6-Phosphate Receptors (M6PR; Brown, Goodhouse and Farquhar), receptors responsible for the delivery of lysosomal hydrolases to the developing lysosome. The delivery of molecules to

lysosomes can be mediated either by transport vesicles from the Trans-Golgi Network (TGN) fusing with late endosomes followed by the recycling of components required for further rounds of delivery from the TGN or by direct delivery of LAMPs from the TGN to lysosomes through an Adaptor Protein (AP). This AP-mediated delivery provides a mechanism for coated vesicles to sort cargo specific for lysosomal delivery. The delivery of molecules from late endosomes to lysosome was coined "kiss-and-run," in which late endosomes briefly fuse with lysosomes. It forms a hybrid organelle that fissions, reforming lysosomes and recycling late endocytic vesicles (Bright et al.; Luzio, Pryor and Bright; Luzio, Pryor, et al.; Luzio, Rous, et al.; Mullock et al.). Biochemical and microscopic evidence has also shown that lysosomes are capable of fusing with other lysosomes (homotypic fusion) and they can subsequently fission (Patterson and Lippincott-Schwartz; Ward, Leslie and Kaplan; Ward et al.; Durchfort et al.; Perou, Leslie, et al.; Michaillat, Baars and Mayer; Michaillat and Mayer; Peters et al.; Zieger and Mayer; Schulze et al.). This dynamic nature allows for the redistribution of substrates among lysosomes for efficient degradation and regulates the size of the lysosomes.

Cell-type Specific Secretory Lysosomes

Secretory lysosomes are a subset of organelles that are indistinguishable from lysosomes, or they can be a distinct subset of organelles that are present in cells that also contain "traditional" lysosomes. The function of these LROs varies depending upon the cell type. Most cell types contain secretory lysosomes involved in plasma membrane repair (Jaiswal, Andrews and Simon; Bansal et al.; Keefe et al.; Zhang et al.; Laulagnier et al.; Xu et al.; McNeil; McNeil and Kirchhausen). Plasma membrane injury can occur

due to mechanical stress (e.g., muscle, skin, endothelial cells and adipocytes) or by pathogens trying to gain access to host cells (e.g., pore-forming toxins generated by bacteria). Scientists have taken advantage of plasma membrane repair mechanisms to introduce reagents (DNA, immunoglobulins, drugs) into cells by electroporation, glass-bead disruption or scrape-loading methods. The repair of the plasma membrane occurs within seconds/minutes and is essential for survival. This process involves the influx of calcium, which binds to the phospholipid-binding protein synaptotagmin VII (Andrews, Almeida and Corrotte), triggering interaction with soluble N-ethylmaleimide-Sensitive Factor (NSF) Attachment protein Receptors (SNAREs) such as SNAP-23, which are necessary for membrane fusion and cytoskeletal rearrangement. The fusion of lysosomes at the wound site results in lysosomal hydrolases being released extracellularly and the presence of lysosomal membrane proteins including LAMPs, LIMPs and the vacuolar H^+-ATPase localized at the cell surface. A lysosomal enzyme, acid sphingomyelinase, has been shown to be important for the restoration of plasma membrane integrity (Tam et al.). Data suggest that the transient plasma membrane localization of the vacuolar H^+-ATPase may provide the necessary pH conditions for acid sphingomyelinase to function at the outer leaflet of the plasma membrane generating ceramide (Xu et al.), which produces an inward bulging of the plasma membrane and the dynamin-independent internalization of the wound repair site (Idone et al.; Lariccia et al.; Sinha et al.; Jiang and Chen). The endocytosis of the wound patch allows for the recovery of the lysosomal membrane proteins. Defects in plasma membrane repair are associated with a broad range of human diseases including neurodegeneration, muscular dystrophy, lysosomal storage diseases, heart disease, skin disorders and obesity.

Hematopoietic-lineage Secretory Lysosomes

Distinct subsets of secretory lysosomes are found in the cells that belong to hematopoietic lineages such as lymphocytes, granulocytes (eosinophils, neutrophils, basophils, mast cells), platelets and macrophages. These vesicles contain cell type-specific molecules that are involved in disease-related processes including pathogen killing, lysing virally or bacterially infected cells and lysing tumourigenic cells. The secretory lysosomes are also involved in homeostatic processes including promoting coagulation and antigen presentation in innate and adaptive immune responses.

In Cytotoxic T Lymphocytes (CTLs), the LROs or lytic granules are the only lysosomes present in the cells. They store acid hydrolases to degrade endocytosed material but also contain secretory molecules such as perforin and granzymes. They function at neutral pH to insert into target cell membranes at the immunologic synapse forming a pore that can result in target cell killing (Holt, Gallo and Griffiths; Luzio, Pryor and Bright; Pattu, Halimani, et al.; Figure A.1). Another important molecule contained in small vesicles within these secretory lysosomes is Fas Ligand (FasL), a transmembrane protein that is released in small vesicles when the lysosomes fuse with the plasma membrane at the immunologic synapse (Zuccato et al.). The small vesicles then fuse with the target antigen-presenting cell that contains Fas and this recognition triggers apoptosis. Loss of FasL gives rise to Autoimmune Lymphoproliferative Syndrome, or ALPS1B (Wu et al.). The release of the lytic granule contents at the immunologic synapse provides a restricted “barrier” to protect against unwanted killing of neighboring cells.

Some of the mechanisms for lytic granule release have been elucidated through the use of inhibitory molecules (e.g., microtubule depolymerizing agents such as

nocodozole or nonhydrolyzable nucleotides (GTPgS or ATPgS) and through the identification of human mutations in genes encoding required components. Two such components are the ras related small GTPase (Rab) Rab27a, which is required for docking of the lytic granule at the plasma membrane, and Munc13-4, which is an effector molecule of Rab27a that regulates fusion with the plasma membrane. Mutations in the genes that encode Rab27a (Menasche, Pastural, et al.; Desnos et al.; Menasche, Feldmann, et al.) or Munc13-4 (Feldmann et al.; Yamamoto et al.; Garrett et al.) result in defective cytotoxic T cell killing and human diseases (Griscelli syndrome and hemophagocytic lymphohistiocytosis, respectively). Several SNARE molecules (proteins on cognate donor and acceptor membranes that act as addressing molecules) required for lytic granule exocytosis in CTLs include Vamp8 (Loo et al.), Vti1b (Dressel et al.), Syntaxin7 (Pattu, Qu, et al.), Syntaxin11 (Bryceson et al.; Halimani et al.), Munc18-2 and Synaptobrevin2 (Matti et al.). Mutations in many of these genes in humans give rise to type 3-5 familial hemophagocytic lymphohistiocytosis, a life-threatening disease where the immune system produces too many activated immune cells that can damage major organs of the body (Faitelson and Grunebaum; Sieni et al.). Similar regulatory mechanisms for LRO secretion appears to exist in Natural Killer (NK) cells, although NK cells also have non secretory lysosomes that function to degrade endocytosed material (van der Sluijs, Zibouche and van Kerkhof).

Neutrophils contain primary and secondary granules (LROs) that assist in the uptake and killing of bacteria. During phagosome formation the cell undergoes a change in the localization of the Microtubule-Organizing Center (MTOC) toward the forming phagosome (Tapper, Furuya and Grinstein). The primary granules can fuse with the

phagosomal membrane and release of granule content assists in bacterial killing. The contents of the granules are very specific. Primary or azurophilic granules contain proteases such as elastase, Cathepsin G, antimicrobial peptides and myeloperoxidase. Secondary granules contain lactoferrin and lysozyme, which are directed, respectively, at limiting bacterial iron acquisition and lysis of pathogen cell walls.

Basophils and mast cells have secretory lysosomes that act in inflammation often associated with allergic reactions. They contain acid hydrolases along with various inflammatory mediators such as histamine, heparin, serotonin and neutral proteases (Marone et al.). Eosinophilic secretory lysosomes are associated with host defense against parasitic helminthes, allergic reactions and immunoregulation. The granules contain a variety of molecules including cationic proteins, growth factors, cytokines, receptors, and lytic molecules. The selective sorting of these molecules into eosinophil granules as well as their regulated release has identified different mechanisms that mediate release of granule contents (Muniz, Weller and Neves). These mechanisms include: 1) compound exocytosis where the entire granule fuses with the plasma releasing its contents to kill target cells or parasites; 2) selective secretion of specific contents through tubular/vesicular structures that are released from the secretory lysosomes and fuse with the plasma membrane to release their contents; and 3) whole cell eosinophil lysis, which releases intact granules that may be directed to target cells or pathogens via membrane recognition molecules.

Macrophages contain conventional lysosomes, as well as secretory lysosomes that are involved in membrane repair, pathogen killing and immunomodulation. Specialized macrophages such as osteoclasts utilize these secretory lysosomes to deliver acid

hydrolases to sites of bone resorption (Teitelbaum). These secretory lysosomes contain a subset of acid hydrolases, including cathepsin K and tartrate-resistant acid phosphatase or TRAP, two enzymes that play a role in bone resorption, which is critical for skeletal formation and remodeling (van Meel et al.). Some of the molecules that have been implicated in the release of secretory lysosomes in osteoclasts include Synaptotagmin VII (Zhao et al.), Syntaxin4, and Rab7.

The megakaryocyte, the platelet-forming cell, contains secretory and conventional lysosomes. Platelets, which are only found in mammals, are formed by the shedding or breaking off of fragments of the megakaryocyte into the bloodstream. Platelets contain three kinds of secretory lysosomes: dense core granules containing clotting factors (fibrinogen, Von Wiebrand Factor, nucleotides), alpha granules containing vasoconstricting factors (serotonin, calcium), and lysosomes containing acid hydrolases and clot remodeling factors (Plasminogen Activator Inhibitor-1 (PAI-1) and Thrombin Activatable Fibrinolysis Inhibitor (TAFI)) (Flaumenhaft et al, 2009, Italiano et al, 2008). These secretory lysosomes can also release a number of chemokines and cytokines, which promote activation of leukocytes for immune response (Ren, Ye and Whiteheart). Secretory lysosomes must be moved to the periphery of the megakaryocyte prior to shedding of platelets. A failure to appropriately move vesicles will result in coagulation defects, which is a common phenotype associated with human diseases such as Chediak-Higashi Syndrome, Hermansky-Pudlak Syndrome, Gray Platelet Syndrome and Griscelli's Syndrome. Some details of these human diseases associated with secretory lysosome defects will be described later in this chapter. The basic machinery that moves and fuses vesicles with the plasma membrane is conserved for all secretory lysosome

release, however, there are cell type specific molecules that mediate this process. Molecules required for platelet dense granule release have been characterized through human disease gene identification, mouse knockouts and platelet dense granule proteomics (Lemons et al.; Flaumenhaft et al.; Chen et al.; Lemons, Chen and Whiteheart; Reed). More recently syntaxin-binding protein 5 was shown to bind to core secretion machinery regulating when and where SNARE interactions occur, and the knockout mouse showed defective hemostasis and bleeding tendencies (Ye et al.). The general mechanisms of secretory lysosome fusion are discussed in detail below.

Nonhematologic Cell Secretory Lysosomes

While the specialization of secretory lysosomes is most pronounced in hematopoietic cell lineages, all cells have secretory lysosomes. The endothelial cell contains a specialized secretory lysosomes termed Weibel-Palade bodies (Weibel and Palade), which play a key role in wound repair and coagulation (Hannah et al.). These secretory vesicles contain the von Willebrand factor, which is important in blood clotting (Sakariassen, Bolhuis and Sixma; Ewenstein et al.; Ruggeri), as well as the membrane protein P-selectin (Bonfanti et al.; McEver et al.), which recruits leukocytes that play a role in initiating inflammation (Dore et al.; Mayadas et al.; Nolte et al.).

Astrocytes, neurons and pulmonary type II cells have also been shown to contain specialized secretory lysosomes (Zhang et al.; Tribl et al.; Weaver, Na and Stahlman) that function in ATP, pigment and surfactant storage and regulated release.

The discovery that melanosomes are secretory lysosomes in pigment cells has provided much of the basis for understanding basic LRO biogenesis and plasma

membrane release. This work was initiated by the observation that there were human diseases associated with pigmentation defects and that affected individuals often showed defects in hemostasis and immune function. Along with the more detailed characterization of these human diseases, mouse geneticists noticed that several spontaneously occurring mouse strains possessed varying levels of coat color defects and those mice often showed defects in other secretory lysosome functions (Jackson). The combination of these two observations provided the tools to identify those genes responsible for pigmentation and the biochemical pathways regulating LRO formation, movement and function. During their development, melanosomes contain enzymes and scaffolding proteins along with several ion channels necessary for the synthesis of complex pigments. These pigments provide photoprotection from ionizing radiation and regulate retinal function in the eye (Raposo and Marks). The biogenesis of these organelles follows a highly specialized endocytic trafficking pathway (Wu and Hammer), which is more specifically detailed in the chapter on LROs.

Biogenesis of Lysosomes and Secretory Lysosomes

The generation of secretory lysosomes follows the same pathway as lysosome biogenesis. The delivery of internalized membrane through endocytosis provides a pathway for selective sorting. Endosomes proceed through a series of sorting events where receptors and other plasma membrane components are recycled (e.g., transferrin receptor) as the endosome is "matured." Cargo destined for degradation remains in the endosome. The maturing endosome invaginates to form the Multivesicular Body (MVB) through a series of specific protein recruitment events that are regulated by the

Endosomal Sorting Complexes Required for Transport (ESCRT)-dependent machinery. Ubiquitin "marked" membrane proteins are sorted into intraluminal vesicles that will be delivered to the lysosome for degradation. Again, much of this work was initially done in *S. cerevisiae* (Babst, Katzmann, Estepa-Sabal, et al.; Babst, Katzmann, Snyder, et al.; Katzmann, Babst and Emr; Babst and Odorizzi; Henne, Stenmark and Emr), but a great deal of the knowledge on the mechanisms involved in MVB formation has come about through the study of virus release, specifically Human Immunodeficiency Virus (HIV) (Votteler and Sundquist) and cell abscission (Carlton and Martin-Serrano; Morita et al.). There are currently over 20 ESCRT proteins in mammals and their roles in MVB formation, sorting and trafficking and other cellular process such as abscission and exosome release are still being defined (McCullough, Colf and Sundquist; Schuh and Audhya).

Hydrolases and other soluble lysosomal components, synthesized in the ER and trafficked through the Golgi for additional glycosylation, can be delivered to the now late endosome through Mannose 6-Phosphate Receptors (M6PR), which release their cargo upon exposure to the acidic environment of the forming lysosome (Fischer et al.; Kornfeld and Mellman; Braulke and Bonifacino; Saftig and Klumperman). Other delivery mechanisms independent of M6PR are known to exist, as hydrolases can be delivered to lysosomes in the absence of M6P glycosylation, as in the human disorder I-cell disease due to a deficiency in N-acetylglucosamine-phosphotransferase (Coutinho, Prata and Alves). Membrane proteins destined for residence in lysosomes contain targeting motifs, YXXf or [DE]XXXL[LI], which are recognized by Adaptor Proteins (AP) that sort them into coated cargo vesicles for delivery with the late endocytic

machinery (Park and Guo). This can occur in the biosynthetic pathway via AP1 or AP3 or via an indirect route to the plasma membrane where the adaptor protein AP2 assists in the recognition and endocytosis of lysosomal proteins. There is also evidence that lysosomal membrane proteins such as LAMP1 and LAMP2 can be delivered to the forming lysosome in an AP-independent M6PR-independent manner (Pols et al.).

The mechanisms for sorting to conventional lysosomes also provide for the sorting of molecules to secretory lysosomes. There are some specialized sorting mechanisms in CTL secretory lysosome biogenesis that involve ubiquitination and phosphorylation of FasL and trafficking through the MVB for delivery to LROs (Park and Guo). Consistent with cell type-specific secretory lysosome sorting is the trafficking of elastase into neutrophils through the tetraspanin molecule CD63 (Harrison-Lavoie et al.). The sorting of molecules into the forming melanosomes is highly specific for melanocytes and deviates from typical secretory lysosome formation by sorting from the early rather than late endosomal compartment; in addition, there are no internal vesicles (Marks, Heijnen and Raposo).

Exocytosis of Secretory Lysosomes

The regulated secretion of LROs has some general principles that apply to all cell types (Figure A.2). The first step in secretion is signaling to the cell to release secretory lysosomes. This signal can be the influx of calcium, as in wound repair or it may be a receptor ligand signal, as in CTLs or NK cells where the formation of the immunologic synapse (TCR-antigen-MHC) signals the polarization of the cell and the movement or localization of “mature” secretory lysosomes near the plasma membrane. However, not

all cells polarize to release secretory lysosome contents. Secretion requires binding of the secretory lysosome to microtubules through the kinesin family of motor proteins providing long distance movements toward the cell periphery. The secretory lysosome is then released and may bind to actin through myosin motors for more subtle movements close to the plasma membrane. Once the secretory lysosome is near the plasma membrane, docking molecules on the secretory lysosome and plasma membrane tether the vesicle to the plasma membrane. Finally, SNARE pairing brings the secretory lysosome membrane in close proximity with the plasma membrane, where the SNARE pairing and Rab molecules can affect a fusion event. Thus releasing soluble secretory lysosome content, as well as delivering membrane content to the plasma membrane. The mechanisms that regulate granule release have not been fully elucidated for each cell type, however, some calcium sensing molecules such as synaptotagmin VII and tethering molecules including synaptobrevin2, Munc13-4 and Munc18-2 along with SNARE molecules Vamp7, Vamp8, Vamp2, Syntaxin4, Syntaxin7, SNAP-23, have been suggested to have a role in cell-specific secretory lysosome release (Logan et al.; Tiwari et al.; Lacy et al.; Suzuki and Verma). Further, novel SNARE regulatory molecules have recently been identified for platelet granule release (Ye et al.).

Genetic Diseases Associate with Defects in Conventional or Secretory Lysosomes

There are many human diseases associated with defects in conventional lysosome or LRO function or biogenesis and patients have been identified and described for many decades. Some of the diseases associated with conventional lysosome defects include

Lysosomal Storage Diseases (LSDs), which are classified according to the substrates they accumulate. These include mucolipidosis, sphingolipidoses, oligosaccharidoses, lipoprotein storage disorders, mucopolysaccharidosis and neuronal ceroid lipofuscinoses along with others; up to 50 have been described (Coutinho, Matos and Alves; Samie and Xu). These disorders are most often due to mutations in lysosomally targeted enzymes that degrade their specific substrates or mutations in the proteins that regulate the delivery of these enzymes to the lysosome. The resulting phenotype commonly associated with LSDs is the abnormal accumulation of undegraded substrates. Examples of LSDs that are not due to mutations in the enzymes include mucolipidosis type 4, where there is a defect in the cation channel mucolipin-1 and Niemann-Pick type C disease, which is due to mutation in either NPC1 or NPC2 (Ikonen and Holtta-Vuori; Vanier). It is unclear what ion is important in mucolipid degradation or what ion(s) mucolipin-1 transports (Dong, Cheng, et al.; Dong, Shen, et al.; Eichelsdoerfer et al.). The exact roles of NPC1 and NPC2 in cholesterol metabolism have not been fully elucidated, but they may play a role in sterol transport. Treatment for many of these LSDs is enzyme replacement therapy.

Other genetic diseases associated with the biogenesis, trafficking or homeostasis of lysosomes and secretory lysosomes that are not due to substrate accumulation include: Hermansky-Pudlak Syndrome (HPS), Griscelli's Syndrome (GS), Chediak-Higashi Syndrome (CHS), Gray Platelet Syndrome (GPS), and Familial Hemophagocytic Lymphochistiocytosis (FHL). There are nine subtypes of HPS. Phenotypes associated with HPS include pigmentation defects of the hair, skin and eyes, bleeding tendencies, neurologic disorders and in some HPS cases pulmonary fibrosis. Mutations in HPS-

associated genes encode for proteins that are involved in the biogenesis of secretory lysosomes (e.g., AP3, BLOCs, geranylgeranyl transferases). As of yet there is no cure for HPS and there is a great deal of heterogeneity of HPS-associated defects (Seward and Gahl).

There are three types of GS, which can vary in phenotype with partial albinism being the common phenotype, but can include immunodeficiency or neurologic deficits. The genes associated with GS encode for proteins involved in the movement of LROs (e.g., Myosin-Va, Rab27a and melanophilin). There is no cure for GS but the hematologic problems can be treated with bone marrow transplantion (Arico et al.; Cesaro et al.; Trottestam et al.).

CHS phenotypes include severe immune deficiency, hypopigmentation, bleeding tendency, frequent bacterial infection, oculocutaneous albinism, and progressive neurological dysfunction (Spritz). The hallmark characteristic of CHS is the presence of giant conventional and secretory lysosomes in all cells of the body (Introne, Boissy and Gahl; Shiflett, Kaplan and Ward; Stinchcombe, Bossi and Griffiths). The identification of the gene responsible for CHS was done through classic positional cloning of CHS patients and yeast artificial chromosome complementation using the *beige* mouse model (Perou, Justice, et al.; Perou, Moore, et al.; Barbosa et al.; Nagle et al.). The *CHS/LYST* gene encodes a protein of 3800 amino acids containing three to four defined domains or motif that have not informed on function (Kaplan, Domenico and Ward); although, the WIDL and WD-40 repeat domains defined a family of protein called Beige And Chediak (BEACH) and all family members that have been characterized appear to be involved in membrane trafficking (Cullinane, Schaffer and Huizing). There are two competing

hypotheses for the function of Lyst, one suggesting that Lyst functions as a negative regulator of fusion (Barrat et al.; Kypri et al.; Charette and Cosson; Rahman et al.; Kypri, Falkenstein and De Lozanne) and the other suggesting that Lyst regulates lysosome fission (Perou, Leslie, et al.; Durchfort et al.). It is of interest to note that only CHS, as opposed to HPS, GS or GPS, is associated with defects in conventional lysosomes, as well as LROs suggesting that the Lyst protein regulates the maintenance or biogenesis of both types of lysosomes. Treatment for CHS is prophylactic antibiotics early in life and bone marrow transplant, which treats the life-threatening immunologic deficits but does not treat the neurologic defects.

GPS is a rare autosomal recessive bleeding disorder that is due to a reduction or the absence of alpha granules in platelets. The gene identified to be mutated in GPS is *NBEAL2*, which is a member of the BEACH family of proteins involved in vesicular trafficking (Kahr et al.). A mouse model of the disease demonstrated the importance of Nbeal2 in hemostasis and thrombosis and tissue repair (Deppermann et al.).

Familial Hemophagocytic Lymphochistiocytosis (FHL) patients are immunodeficient but do not exhibit pigmentation defects. FHL is manifested in the first six months of life and is historically treated with bone marrow transplantation (Jabado et al.), or more recently with stem cell transplantation (Ohga et al.). It can be caused by mutations in the *PRF1* gene, which encodes for perforin, the protein that plays an important role in CTL-mediated killing or in the gene encoding Munc13-4 (Feldmann et al.). Munc13-4 protein is essential for the secretion of secretory vesicles from CTL but not from melanocytes or neuronal cells. FHL patients' secretory lysosomes are able to transport and dock to the plasma membrane, but are not able to release their content in

immunological synapse due to the mutation of Munc13-4. The loss of perforin mediated cytotoxicity results in imbalanced immune homeostasis and deregulated activation and proliferation of CTL during antiviral immune response (Lettau et al.).

Conclusions

The biosynthesis of lysosomes and secretory lysosomes involves multiple trafficking pathways including delivery of cargo to the forming lysosome from the endocytic and biosynthetic pathways, along with recycling of membrane constituents needed for further rounds of targeting to the lysosome. There is clear evidence that these organelles are capable of fusion and fission events and the machinery that regulates this process is being elucidated. One of the major limitations in understanding the biogenesis of lysosomes and secretory lysosomes is the ability to distinguish between late endosomes and lysosomes. Currently there are no clear markers of lysosomes, as late endosomes receive "lysosomal" cargo to be delivered to the forming lysosome. Further, we do not understand many of the molecules that are involved in lysosome or secretory lysosome fission.

Many of the molecular components required for secretory lysosome biogenesis and secretion have been determined, however, it is clear that the detailed biochemical requirements for this process need to be better understood. That particular cell types have evolved mechanisms to generate and release secretory lysosomes further demonstrates the complexity of this process. The recent advances in live cell microscopy, protein tagging and proteomics should provide some of the tools necessary to identify the roles of

these novel proteins and lipids involved in the biogenesis, maintenance and secretion of lysosomes.

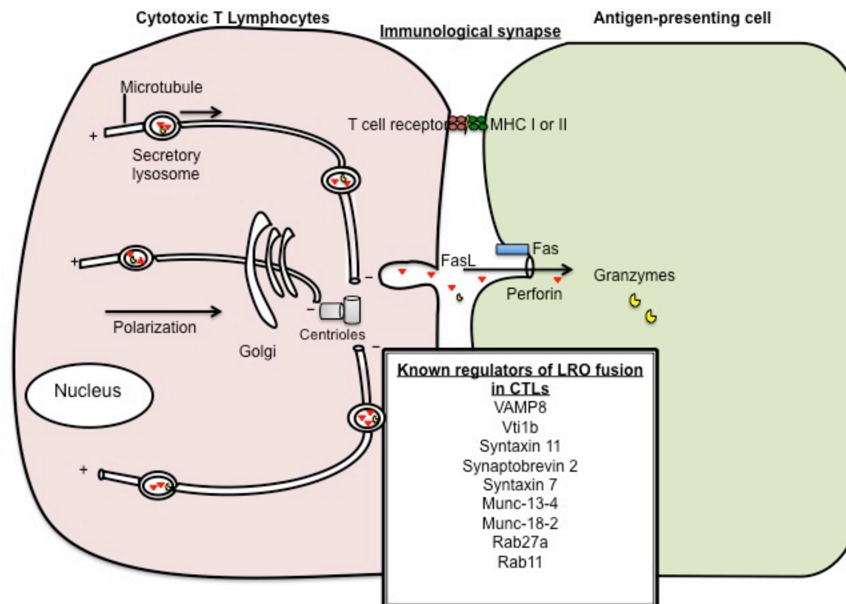

Figure A.1: A model of the immunologic synapse in Cytotoxic T Lymphocytes (CTLs) secretory lysosome release

T-cell receptors on CTL recognizes antigen on antigen presenting cell in the context of MHC forming the immunologic synapse. This recognition sends a signal that causes the CTL centromere to polarize toward the immunologic synapse. Secretory lysosomes engage the microtubule machinery to move them toward the plasma membrane at the immunologic synapse. These lytic granules contain Fas Ligand (FasL) that recognizes Fas being expressed on the antigen presenting cell. They also contain the pore forming protein perforin and granzymes, which upon release at the synapse form a pore in the plasma membrane that induces apoptosis of the antigen presenting cell. Included is a list of known regulator of CTL secretory lysosome fusion.

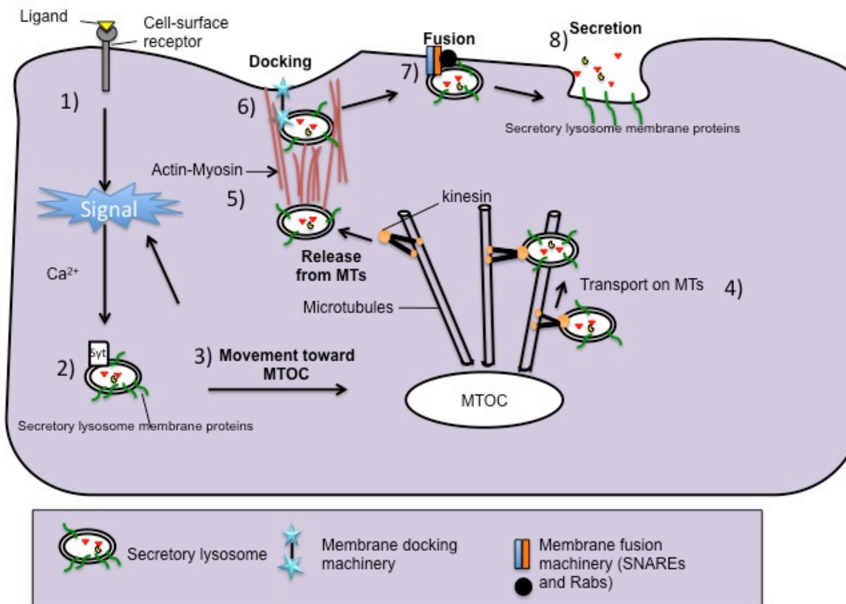

Figure A.2: A model of secretory lysosome release
1) A signal is transduced by receptor-ligand interaction, an intracellular signal or by wound formation at the plasma membrane triggering a calcium release from intracellular stores. 2) SynaptotagminVII (Syn) binds to the secretory lysosome in response to calcium and 3) the vesicle moves toward the MTOC. 4) The secretory lysosome then engages the MT through the motor protein kinesin to move to the periphery of the cell where it is released. 5) For the short movement to the plasma membrane the secretory lysosome engages actin cytoskeletal elements through myosin motors. 6) When the vesicle is in close proximity to the plasma membrane, recognition membrane docking elements (stars) bind the docking elements on the vesicle. 7) The specific vesicular and plasma membrane v and t-SNAREs bind and bring the vesicle near the plasma membrane and fusion occurs 8) releasing soluble and membrane bound secretory lysosome content.

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