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Sterol-dependent regulation of mTORC1 activation at the lysosomal surface

By

Brian Megia Castellano

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

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Abstract

Sterol-dependent regulation of mTORC1 activation at the lysosomal surface
by
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The mechanistic target of rapamycin complex 1 (mTORC1) is a multi-subunit protein kinase that regulates growth and metabolism in response to nutrients and environmental signals. Since its discovery in 1991, intensive studies have shown that these signals regulate mTORC1 in experimental systems ranging from yeast to fruit flies, mice and human cells. Only recently has research identified molecules responsible for the mechanisms involved in mTORC1 regulation, especially by amino acids. I led a collaboration with others in my research group, by which I identified cholesterol as a novel positive input for mTORC1 activation and characterized components that the cell uses to sense and convey the cholesterol signal. I showed that sterol levels correlate with mTORC1 activity in vivo, and that the lysosome has the machinery necessary for sterol-dependent regulation of mTORC1 in vitro. I found that a putative lysosomal cholesterol transporter, Niemann-Pick C1 (NPC1), is necessary for modulating mTORC1 activity in response to changing cholesterol levels. In cells lacking NPC1, or expressing NPC1 mutants incapable of transporting cholesterol, mTORC1 activity is elevated and no longer sensitive to changes in sterol levels, suggesting that NPC1 functions as a negative regulator. Conversely, an apparent amino acid transporter, SLC38A9, was identified as necessary for sterol-dependent activation of mTORC1. SLC38A9 contains conserved cholesterol responsive motifs that I showed are necessary to convey cholesterol responsiveness to mTORC1 because mutagenesis of these motifs decoupled the effect of amino acids and cholesterol in regulation mTORC1 activity. Overall, this work elucidated a novel mechanism by which cells integrate cholesterol- and amino acid-dependent signals to precisely regulate mTORC1 function for optimal cell growth.
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Chapter 1: Introduction

1.1 Nutrient balance for cellular growth and proliferation

Cells constantly balance their anabolic and catabolic processes in order to adapt to changing conditions (1). Critical to this adaptation is the ability of every cell to precisely monitor its nutrient availability and in response to this information, mount the appropriate metabolic programs (2). Nutrients such as amino acids and lipids can be synthesized from simpler precursors, but metazoan cells are incapable of synthesizing de novo ten proteinogenic amino acids and two membranogenic lipids. These essential nutrients, along with a significant fraction of the non-essential nutrients, are derived from extracellular nutrient uptake and by intracellular degradation of macromolecules (3).

Extracellular nutrient uptake is mediated through plasma membrane transporters and receptor-mediated endocytosis (3). Growth factor signaling increases uptake of soluble nutrients, such as glucose and amino acids, by increasing the number of transporters at the plasma membrane (4). This is achieved through post-translational regulation of transporter trafficking to the plasma membrane and also through transcriptional regulation of transporter expression (5, 6). In addition to increased uptake, growth factor signaling also increases the activity of enzymes such as hexokinase, which phosphorylates glucose in order to increase its intracellular retention and subsequent breakdown (7, 8).

The uptake of water-insoluble nutrients such as lipids is accomplished through a process known as receptor-mediated endocytosis (9). Proteins package lipids and act as carriers that are recognized by dedicated receptors at the plasma membrane, which then promote their selective endocytosis. The uptake of low-density lipoprotein (LDL) is the main route for acquisition of cholesterol, triglycerides and phospholipids (10). The LDL particles are trafficked to the lysosome, which is one of the main sites for intracellular macromolecular degradation (11).

The lysosome is a membrane-bound organelle that contains acidic hydrolases within its lumen. These hydrolases degrade a variety of macromolecules down to simpler components. For instance, cathepsin proteases break down proteins into single amino acids or di- and tripeptides. These amino acids and small peptides are then released to the cytoplasm by membrane proteins called permeases that span the lysosomal membrane. Permeases transfer and export simple nutrients out of the lysosome for cellular use (11). Lysosomal hydrolases require a low pH (in the 4.0 to 5.0 range) for their function. The lysosomal lumen is acidified by the vacuolar H+-ATPase (v-ATPase), which utilizes ATP hydrolysis to drive protons against the gradient from the cytoplasm into the lysosome (12). In addition to endocytosis, which delivers extracellular cargo to the lysosome, a membrane-based pathway known as autophagy captures intracellular components such as macromolecules and organelles, and delivers them to the lysosome for degradation. Autophagy involves sequestration of portions of the cytoplasm or entire organelles within a double membrane, the autophagosome, which then fuses with the lysosome for degradation. This process can be selective to recycle damaged or unneeded organelles and other macromolecules, but can also be a non-selective means to regenerate nutrients when a cell is under stress or starvation (13).

Derangement of lysosomal function has been implicated in many diseases, especially cancer, which is driven by aberrant nutrient metabolism, and neurodegeneration, which often stems from
defective quality control (4, 14). A multitude of neurodegenerative diseases are associated with lysosomal storage disorders (LSDs) where genetic mutations in hydrolases or permeases impair lysosomal degradation and/or lysosomal export. These mutations in hydrolases or permeases impair the processing, storage and trafficking of their specific macromolecular or nutrient substrates. In addition, high levels of these substrates within in the lysosome can affect the storage and trafficking of other nutrients due to coupling in transport and retention (15). For example, in the neurodegenerative disease Niemann-Pick disease type C1 (NPC) multiple lipids accumulate such as cholesterol, sphingosine, sphingomyelin and glycosphingolipids (16).

Many cancer types exhibit high proliferation rates under limiting nutrients and this is attributed, in part, to altered lysosomal function and nutrient metabolism (4). This is especially true in cancers that reside in poorly vascularized tissues where available extracellular nutrients are scarce. KRAS-driven cancers such as pancreatic and lung adenocarcinomas rely on macropinocytosis, which is a process where cells engulf the surrounding extracellular matrix for lysosomal degradation. In these cancers macropinocytosis and autophagy coupled with high lysosomal function provide additional sources of nutrients to support high growth rates in poor nutrient environments. Unlike non-transformed cells, which inversely regulate catabolic and anabolic processes, cancer cells have gained the ability to simultaneously activate anabolism and catabolism in order to gain maximal growth advantage (17).

1.2 mTORC1 is a master nutrient regulator of cellular growth and proliferation

Cellular growth and proliferation are energetically demanding processes that depend critically on the availability of nutrients and energy. Cells integrate multiple pro-growth signals through a few master sensors to ensure that growth and proliferation are activated only when intracellular and extracellular conditions are favorable (1). One of these master sensors is an ancient protein kinase conserved from yeast to man, known as the mechanistic target of rapamycin complex 1 (mTORC1). mTORC1 is a master nutrient regulator that integrates long-range signals carried by growth factors with short-range signals from intracellular environments that includes nutrients, energy and oxygen (18).

Growth factors, like insulin, regulate mTORC1 via the phosphoinositide 3-kinase (PI3K)-Akt pathway. The insulin receptor belongs to the receptor tyrosine kinase (RTK) family. Insulin binding induces receptor dimerization, which promotes autophosphorylation. These phosphorylation sites recruit scaffolding adaptors IRS1/2 (insulin receptor substrate 1/2). PI3K is recruited to the plasma membrane directly by the tyrosine receptors or through IRS1/IRS2. At the plasma membrane PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-triphosphate (PIP3). The pleckstrin homology (PH) domain of Akt binds to PIP3 and this induced proximity to the plasma membrane allows Akt to be activated by 3-phosphoinositide-dependent protein kinase-1 (PDPK1) (19). Akt phosphorylates and inactivates the tuberous sclerosis complex (TSC), which is a GTPase activating protein (GAP) for a small GTPase, Rheb (20, 21). Rheb in its GTP-bound form is capable of turning on mTORC1 kinase activity (22, 23).

In contrast to growth factor activation of mTORC1, low energy levels and hypoxic conditions activate AMP-activated protein kinase (AMPK). AMPK activates TSC resulting in conversion of GTP-bound Rheb to the inactive GDP-bound form, which is incapable of activating mTORC1 (24-26). Independent of AMPK, hypoxia also induces the synthesis of REDD1 (regulated in development and DNA damage responses1) that potentially displaces inhibitory 14-3-3 proteins from TSC, again leading to conversion of Rheb to the GDP-bound state (27).
Amino acid levels regulate the subcellular localization of mTORC1 (18). In amino depleted states, mTORC1 is diffuse in the cytoplasm. Adding back amino acids triggers a rapid response that, within minutes, leads to mTORC1 translocation to the lysosomal surface (28, 29). This localization mechanism has been characterized to involve a pair of heterodimeric Rag GTPases. RagA or B, which are functionally equivalent to each other, form an obligate complex with RagC or D, which are also functionally equivalent. Mutagenesis studies have shown that, in the activated state triggered by amino acids, RagA/B and RagC/D are bound to GTP and GDP, respectively, and this heterodimer is able to recruit mTORC1 to the lysosomal surface where it is in close proximity to Rheb for kinase activation. Conversely, amino acid withdrawal is thought to convert the Rags from an active GTP:GDP bound state to an inactive GDP:GTP bound state, terminating mTORC1 recruitment to the lysosome (28-31). Interestingly, unlike other GTPases, the Rag GTPases are not post-translationally lipidated to anchor them directly to a membrane (29, 32). Instead they bind to a pentameric complex called Ragulator, which localizes to the lysosomal surface through a subunit that is myristoylated and palmitoylated (29).

Both cytoplasmic amino acids and amino acids within the lumen of the lysosome converge on the Rag GTPases through multiple complexes that ultimately modulate the nucleotide state of either Rag component (18). Three cytoplasmic amino acid sensors have been identified: Sestrin2, CASTOR and SAMTOR which sense leucine, arginine and S-adenosylmethionine (SAM, a methionine metabolite), respectively (33-39). Sestrin2 and CASTOR bind and inhibit a complex called GATOR2 when they are not bound to their respective amino acid (33-38). GATOR2 is a positive regulator of mTORC1 and inhibits another complex named GATOR1. GATOR1 is a GAP for RagA/B and promotes hydrolysis of its GTP to GDP. GATOR1-mediated GTP hydrolysis puts RagA/B in an inactive form incapable of recruiting mTORC1 to the lysosomal surface (40-42). GATOR1 also interacts with SAMTOR in a SAM-dependent manner. In the absence of SAM, SAMTOR promotes GATOR1 activity to inhibit mTORC1 localization to the lysosomal surface (39).

A pool of amino acids also within the lysosomal lumen is also thought to regulate mTORC1 activity (18, 43). A Na⁺-couple amino acid transporter at the lysosome, SLC38A9, senses arginine from within the lysosome to convey arginine sufficiency to mTORC1. SLC38A9 is in complex with other lysosomal resident proteins including the v-ATPase and a pentameric complex called Ragulator (44-46). v-ATPase function is necessary for mTORC1 activation, but the molecular mechanism of this regulation is currently unclear (43). Ragulator binds strongly to the Rag GTPases and was shown to function as a guanine exchange factor (GEF) for RagA/B (28, 29). Potentially SLC38A9 and the v-ATPase are able to regulate mTORC1 activity by modulating Ragulator’s GEF activity. RagA/B is not the only Rag GTPase that is regulated, but a Folliculin-FNIP2 complex is a GAP for RagC/D (47, 48).

### 1.3 mTORC1 increases macromolecule production and stability

Upon activation by its upstream stimuli, mTORC1 phosphorylates downstream targets to promote cellular growth and proliferation. The pro-growth activities of mTORC1 rely on regulating macromolecule production and degradation for proteins, lipids and nucleotides (18).

mTORC1 promotes protein synthesis by positively and negatively phosphorylating two key effectors, p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4E-BP), respectively (49-51). S6K1 is a step for signal amplification and has downstream targets that induce cap-dependent mRNA translation (49). 4E-BP prevents the assembly of the eIF4F pre-initiation complex and thereby, inhibition of 4E-BP by mTORC1 allows complex formation and translation initiation (50, 51).
mTORC1 promotes membrane biogenesis for cellular growth and proliferation by promoting the activity of a master regulator of de novo lipogenesis, the sterol responsive element binding protein (SREBP) transcription factors 1 and 2 (52, 53). Additionally, mTORC1 was recently shown to phosphorylate a phosphatidic acid phosphatase, Lipin1, preventing its entry into the nucleus where Lipin1 inhibits SREBP function (54). SREBP regulates sterol and fatty acid synthesis and supports lipogenesis by targeting two groups of genes: cholesterol biosynthesis and fatty acid biosynthesis genes. The cholesterol biosynthesis genes include hydroxymethylglutaryl (HMG)-CoA synthase, HMG-CoA-reductase and the low-density lipoprotein (LDL) receptor. The lipogenic enzyme genes include acetyl-CoA carboxylase and fatty acid synthase (FAS). SREBP-regulated synthesis and coordination of sterol and fatty acids help ensure membranes are generated with appropriate lipid compositions (55).

In addition to protein and lipid synthesis, mTORC1 supports RNA and DNA synthesis by stimulating the pyrimidine and purine synthesis pathways (18). mTORC1 post-translationally increases pyrimidine synthesis by increasing CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase) activity via S6K1 phosphorylation. CAD is a catalyst for the beginning steps of pyrimidine synthesis (56, 57). mTORC1 increases purine synthesis transcriptionally by increasing activating transcription factor 4 (ATF4), which in turn transcriptionally regulates MTHFD2 gene (mitochondrial tetrahydrofolate (mTHF) cycle enzyme methylenetetrahydrofolate dehydrogenase 2). MTHFD2 encodes for an enzyme involved in the tetrahydrofolate cycle, which provides one-carbon formyl units for purine synthesis (58). High levels of purine and pyrimidine nucleotides are necessary for the cell to enter into S phase of mitosis (2).

In addition to increasing protein and nucleic acid synthesis and membrane biogenesis, mTORC1 inhibits degradation of these macromolecules by phosphorylating ULK1 (unc-51 like autophagy activating kinase 1) and transcription factor EB (TFEB) (59-62). ULK1 is a kinase that promotes autophagosome formation, and its phosphorylation by mTORC1 prevents ULK1 activation by AMPK (59). TFEB is a transcription factor that induces a class of genes that promote autophagy and lysosome biogenesis. Upon mTORC1 phosphorylation TFEB is sequestered away from the nucleus in the cytoplasm preventing it from activating target genes that include the lysosome v-ATPase and hydrolases, as well as autophagy components (60-62).

1.4 Cellular mass accumulation and nutrient availability
mTORC1 is a signal integrator that drives cellular growth by increasing macromolecule production and simultaneously inhibiting their degradation. This allows cells to accumulate cellular mass (proteins, lipids and nucleic acids) and energy to enter the cell cycle (2). mTORC1 responds to the availability of amino acids to promote protein synthesis (18). Is mTORC1 also responsive to the availability of lipids to promote membrane biogenesis? The next section looks into how cells acquire lipids and how this is coordinated with biosynthesis.

1.5 Lipid acquisition through biosynthesis and lysosomal degradation
Amino acids are water-soluble and are able to diffuse around the cytoplasm and within the lysosome to interact with sensors that activate mTORC1 (SLC38A9, CASTOR, Sestrin2 and SAMTOR) (18, 63). Lipids however are mainly hydrophobic and their diffusion around the cell relies on vesicular and non-vesicular routes (9, 64). Organelles are highly dynamic structures that undergo fission and fusion events that facilitate lipid transport throughout the cell. More directly cells are also capable of direct lipid trafficking through lipid-binding proteins that localize to
organelle contact sites. These proteins contain domains with a hydrophobic pocket that shields lipids from the aqueous environment during the transfer between organelle membranes (64).

There are two main lipid entry points within the cell: intracellular biosynthetic pathways and lysosomal degradation. The majority of lipids are synthesized within the endoplasmic reticulum (ER). The ER is able to produce cholesterol and many of the structural lipids called glycerophospholipids (65). Interestingly, lipids and lipid derivatives can be signaling molecules that regulate their own synthesis (10).

One of the most characterized signaling lipid pathways is the homeostatic regulation of cholesterol synthesis (10). When cholesterol levels in the ER are high, SREBP is sequestered there and kept inactive by binding to a complex of SREBP cleavage activated protein (SCAP) and insulin induced protein (Insig). When cholesterol levels drop, and SCAP no longer associates with cholesterol, SCAP undergoes a conformational change that dissociates it from Insig. The SREBP-SCAP complex then translocates to the Golgi apparatus where SREBP is cleaved by dedicated proteases, freeing a soluble SREBP N-terminal fragment that functions as a transcription factor with functions mentioned above (66, 67).

Apart from synthesis, cells can obtain lipids via scavenging pathways that converge on the lysosome (9). Lysosomes process exogenous lipids derived from receptor-mediated endocytosis of low-density lipoproteins (LDL) or endogenous lipids derived from autophagy-mediated degradation of lipid droplets (a process also known as lipophagy) (10, 68). Cholesterol esters within LDL and lipid droplets are degraded within the lysosome by lysosomal acid lipase type A (LipA), generating free cholesterol and triglycerides (69). Cholesterol is then transported to extra-lysosomal compartments via a two-protein system, NPC1 and NPC2, which are associated with the NPC disease mentioned above. Luminal NPC2 binds to cholesterol via a hydrophobic cavity and delivers it to a luminal cholesterol acceptor domain of NPC1, which is a multi-pass transmembrane protein (70). Following its delivery to NPC1, cholesterol may be deposited on the limiting membrane of the lysosome, which is followed by delivery to other membrane compartments including the Golgi apparatus, ER and plasma membrane (71). The mechanisms of cholesterol export from the lysosome are still unclear, but may involve interactions of NPC1 with an acceptor protein at contact sites (9).

1.6 Lipid signals to promote cellular growth and proliferation

Previous studies have shown that on a systemic level, mice kept on a diet high in fatty acids and cholesterol exhibit higher levels of mTORC1 activity than mice kept on normal chow (72, 73). Phosphoinositides are involved in regulating mTORC1 through the growth factor signaling pathway as mentioned earlier, but it is currently unclear if cells are able to sense lipids on a cell-autonomous level to regulate mTORC1 (19). Since the lysosome is a dietary lipid and nutrient gateway for the cell and the lysosomal surface is a site for mTORC1 regulation (3, 18), we hypothesize that there could be dedicated machinery that is capable of sensing and conveying lipid sufficiency to mTORC1.
Chapter 2: Materials and Methods

2.1 Materials
Reagents were obtained from the following sources: antibodies to phospho-T389 S6K1 (9234S), S6K1 (2708S), phospho-S65 4E-BP (9451S), 4E-BP (9644S and 9452S), phospho-S757 ULK (6888S), ULK (8054S), TSC2 (4308S), RagA (4357S), RagC (3360S), LAMTOR1 (8975S), LAMTOR2 (8145S), mTOR (2983S and 2972S), raptor (2280S), phospho-S473 Akt (4060S), Akt (4691), phospho-T202/204 ERK1/2 (9101S), ERK1/2 (4695S) and the FLAG (2368S) epitope from Cell Signaling Technology; antibodies to NPC1 (ab36983), ATP6V1B2 (ab73404) from Abcam; antibody to LAMP2 (sc-18822) from Santa Cruz Biotechnology; antibody to SLC38A9 (HPA043785) from Sigma Aldrich; HRP-labeled anti-rabbit (PI-1000) secondary antibodies from Vector Laboratories. RPMI, FLAG M2 affinity gel, ATP, amino acids, amino acid esters, cholesterol, filipin and methyl-beta-cyclodextrin (MCD) from Sigma Aldrich; Pierce Protease Inhibitor Tablets from Fischer Scientific; DMEM from Life Technologies; PNGaseF from New England Biolabs; Cholesterol oxidase from MilliPore; trans-sterol refers to PhotoClick Cholesterol from Avanti Polar Lipids, Inc.; Lipoprotein, low density from Alfa Aesar; oxysterols (5-CHOLESTEN-3β, 25-DIOL; 5-CHOLESTEN-3β, 7β-DIOL; 5-, 25R-CHOLESTEN-3β, 26-DIOL; 5-CHOLESTEN-3β, 20α-DIOL) and mevastatin from Steraloids. siRNA smartpools against SLC38A9 were purchased from Dharmacon. A rabbit polyclonal antibody against NPC1 was a generous gift from Linton Traub (Univ. of Pittsburgh).

2.2 Methods

Cholesterol starvation/stimulation in cells
HEK-293T, CHO or MEF cells in culture dishes were rinsed once with serum-free media and incubated in DMEM containing 0.5-1.0% methyl-beta-cyclodextrin (MCD) supplemented with 0.5% lipid-depleted serum (LDS) for 2 hours. Cells were then transferred to DMEM supplemented with 0.5% LDS and 0.1% MCD (starved condition), or to DMEM + 0.5% LDS containing 20μg/ml cholesterol pre-complexed with 0.1% MCD (resulting in MCD:cholesterol at 1:1 molar ratio, 50μM), or to DMEM + 0.5% LDS + 0.1% MCD supplemented with 25-50μg/ml low-density lipoprotein (LDL) and incubated for 1-2 hours. MCD:cholesterol complexes were prepared by diluting a 20mg/ml cholesterol stock solution (in EtOH) 1000-fold into a 15-ml falcon tube containing DMEM + 0.1% MCD + 0.5% LDS, resulting in 50μM final concentration of both cholesterol and MCD. The tube was vortexed and incubated in a 37°C water bath for 2h. Lipid-depleted serum was prepared as described (74).

Lysosome immunoprecipitation for cholesterol measurements
Confluent HEK-293T cells stably expressing LAMP1-mRFP-FLAG\textsuperscript{X2} (LRF), plated at 2 x 15cm dishes per condition in quadruplicate, were sterol-depleted for 2h in 0.5% MCD, or sterol-depleted and restimulated with 50μM cholesterol (in complex with MCD at 1:1 stoichiometry) for 2h. Following these incubations, cells were rinsed once in cold PBS, then scraped, spun down and resuspended in 750ul of fractionation buffer: 140mM KCl, 5mM MgCl\textsubscript{2}, 50mM Sucrose, 20mM HEPES, pH 7.4, supplemented with protease inhibitors. Cells were mechanically broken.
by spraying 4-5 times through a 23G needle attached to a 1ml syringe, then spun down at 2000g for 10min, yielding a post nuclear supernatant (PNS). PNS aliquots were equalized based on total protein concentration and subjected to overnight immunoprecipitation with 100μl of a 50% slurry of anti-FLAG affinity gel. The next day, beads were washed 4 times in fractionation buffer. After washing, beads were loaded on a spin column and lipids were eluted from the beads by adding hexane:ethyl acetate (1:1).

**Lysosomal cholesterol measurement using mass spectrometry**
The samples were extracted by modified Bligh-Dyer method in the presence of internal standard d7-cholesterol (20 μg per sample). The samples were further derivatized to improve the mass spectrometric detection sensitivity of cholesterol. Measurement of cholesterol was performed with a Shimadzu 10A HPLC system and a Shimadzu SIL-20AC HT auto-sampler coupled to a Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer operated in SRM mode under ESI(+). Data processing was conducted with Xcalibur (Thermo). A quality control (QC) sample was prepared by pooling the aliquots of the study samples and was used to monitor the instrument stability. The QC was injected eight times in the beginning to stabilize the instrument, and was injected between every four study samples. The data was accepted if the coefficient variance (CV) of cholesterol in QC sample was < 15%. The quantification of cholesterol was calculated as the peak area ratio of cholesterol to d7-cholesterol multiplied by the quantity of d7-cholesterol (20 μg).

**NPC1 Knockout using Cas9 nucleofection in HEK-293T cells**
Knockout of NPC1 was modified from published protocols (34). Briefly, sgRNAs targeted to the first exon of NPC1 were designed using online software ([http://crispr.mit.edu](http://crispr.mit.edu)). sgRNAs were generated by HiScribe (NEB E2050S) T7 *in vitro* transcription using PCR-generated DNA as a template. 100 pmol of Cas9-2NLS (Macrolab) was diluted to a final volume of 5 μL with Cas9 buffer (20 mM HEPES (pH 7.5), 150 mM KC1, 1 mM MgCl2, 10% glycerol and 1 mM TCEP) and mixed slowly into 5 μL of Cas9 buffer containing 120 pmol of L2 sgRNA. The resulting mixture was incubated for 10 min at RT to allow RNP formation. 2 × 10^−5 HEK293t cells were harvested, washed once in PBS, and resuspended in 20 μL of SF nucleofection buffer (Lonza, Basel, Switzerland). 10 μL of RNP mixture, 100 pmol of donor DNA, and cell suspension were combined in a Lonza 4d strip nucleocuvette. Reaction mixtures were electroporated using setting DS150, incubated in the nucleocuvette at RT for 10 min, and transferred to culture dishes containing pre-warmed media. Single clones were plated and colonies were screened for NPC1 deletion via filipin staining (Sigma-Aldrich) and high throughput microscopy. Hits were validated by immunoblotting.

**Immunofluorescence assays**
HEK-293T, CHO or MEF cells were plated on fibronectin-coated glass coverslips in 6-well plates (35mm diameter/well), at 300,000-500,000 cells/well. 12-16 hours later, cells were subjected to sterol or amino acid depletion/restimulation and fixed in 4% paraformaldehyde (in PBS) for 15 min at RT. The coverslips were rinsed twice with PBS and cells were permeabilized with 0.1% (w/v) Saponin in PBS for 10 min. After rinsing twice with PBS, the slides were incubated with primary antibody in 5% normal donkey serum for 1 hr at room temperature, rinsed four times with PBS, and incubated with fluorophore-conjugated secondary antibodies produced in goat or donkey (Life Technologies, diluted 1:1000 in 5% normal donkey serum) for 45 min at room temperature in the dark, washed four times with PBS. Coverslips were mounted
on glass slides using Vectashield (Vector Laboratories) and imaged on a spinning disk confocal system (Andor Revolution on a Nikon Eclipse Ti microscope).
3.1 Results

**Cholesterol at the lysosome regulates mTORC1 activity**

In cultured human embryonic kidney cells (HEK)-293T cells, I used methyl-β-cyclodextrin (MCD) to manipulate cellular cholesterol content. MCD is a cyclic oligosaccharide that is capable of depleting cholesterol or, if pre-complexed with cholesterol (MCD:cholesterol), can deliver cholesterol to the cell (75). MCD was used to deplete cellular cholesterol and I observed that mTORC1 signaling was suppressed upon MCD treatment that was rescued by LDL in a dose- and time-dependent manner (Fig. 1 A and B top). The timing of mTORC1 activation closely parallels LDL trafficking to the lysosome for degradation suggesting that components of LDL are capable of activating mTORC1 activity once they are released in the lysosomal lumen. LDL degradation involves de-esterification of cholesteryl-esters by LipA (69). I used Lalistat to inhibit LDL degradation and observed mitigated mTORC1 activation (Fig. 1C). Lalistat is a thiadiazole carbamate and is a specific inhibitor of LipA, the lysosomal hydrolase for cholesterol esters (69, 76). LDL-dependent activation of mTORC1 is specific to cholesterol and not other LDL components or cholesterol derivatives including oleic acid, 25-hydroxycholesterol and cholesterol enantiomer (ent-cholesterol) (Fig. 1 D and E) (77). Since LDL degradation is necessary for rescuing mTORC1 signaling upon MCD treatment, we hypothesized that lysosomal cholesterol levels might change in response to MCD. Using a c-terminal red fluorescent protein (RFP)-FLAG\(^2\) tag fused to the lysosomal associated membrane protein 1 (LAMP1-RFP-FLAG\(^2\), LRF), we carried out affinity-based purification of intact lysosomes from HEK-293T cells that were either not treated with MCD, treated with MCD or treated with MCD followed by resupplementation with MCD:cholesterol. The cholesterol content of each treatment was determined in collaboration with the Ory Lab at Washington University School of Medicine in St. Louis (Fig. 1F). Indeed, cholesterol levels at the lysosome changed upon MCD treatment and returned to control levels after cholesterol supplementation.

**Cholesterol regulates mTORC1 localization to the lysosomal surface**

To investigate how cholesterol levels at the lysosome can regulate mTORC1 signaling, we examined the two main pathways for mTORC1 regulation: mTORC1 kinase activation and its localization to the lysosomal surface, which are mediated by the growth factor and nutrient-sensing pathways, respectively (18). To test the first pathway, TSC2-null (TSC2\(^{-/-}\)) mouse embryonic fibroblasts (MEFs) were used since these exhibit constitutive over-activation of mTORC1 downstream of PI3K-Akt pathway (78). In these cells mTORC1 signaling decreases when treated with increasing amounts of MCD (Fig. 2A). This indicates to us that cholesterol is not acting through the PI3K-Akt pathway. To investigate the localization pathway, I used immunofluorescence to see if lysosomal mTORC1 localization is impaired upon MCD treatment. First off, MCD treatment does not disrupt the integrity of the protein scaffold that recruits mTORC1 to the lysosomal membrane, as indicated by the co-localization of mTORC1-recruiting proteins, p18 and RagC, to LAMP2-positive lysosomes in MCD-treated cells (Fig. 2 B and C) (77). In contrast, endogenous mTOR becomes dispersed upon MCD treatment and re-localizes to the lysosome upon the addition of LDL or exogenous cholesterol indicating that cholesterol
plays a role in the mTORC1 localization pathway (Fig. 2 B and C) (77). To further test the involvement of this pathway in cholesterol sensing, we use RagBQ99L, a Rag GTPase mutant the recruits mTORC1 to the lysosome in an amino acid-independent manner due to its inability to hydrolyze. Indeed, mTORC1 activation and localization were largely resistant to cholesterol depletion in cells stably express FLAG-tagged RagBQ99L (Fig. 2 D, E and F) (77). This indicates to us that cholesterol is acting upstream or in parallel to the Rag GTPases in the localization pathway.

mTORC1 scaffolding complex is dynamically regulated by cholesterol in vivo and in vitro

To gain mechanistic insight into how cholesterol regulates mTORC1 localization, I examined its ability to activate the Ragulator-Rag GTPase scaffolding complex at the lysosome surface, which is responsible for recruiting mTORC1. I used a co-immunoprecipitation assay that reads out the activation state of Rag GTPases as a function of their affinity to Ragulator. Previous studies showed that, as amino acids stimulate Ragulator GEF activity towards RagA/B, they cause the interactions between Ragulator and the Rag GTPases to weaken (28, 43). In HEK-293T cells expressing a FLAG-tagged component of Ragulator, p14, I observed that cholesterol regulates FLAG-p14 interactions with RagA and RagC in a manner similar to amino acids (Fig. 3A). This suggests that cholesterol levels are being conveyed to the mTORC1 scaffolding to regulate Ragulator GEF activity. To test if the lysosome harbors all the machinery necessary for cholesterol-mediated regulation of mTORC1, I developed in vitro assays that allow direct manipulation of lysosomal cholesterol (Fig. 3 B and D). In the first assay I used MCD to deplete cholesterol direction from a light organelle preparation containing lysosomes and FLAG-p14. Using co-immunoprecipitation, I observed that MCD-mediated cholesterol depletion enhanced the interactions between Ragulator and the Rag GTPases compared to a cholesterol-replete state (Fig. 3C). In the second assay I supplemented light organelle preparations containing FLAG-p14 with recombinant cholesterol oxidase to convert cholesterol into 4-cholesten-3-one, which is a cholesterol derivative that is unable to activate mTORC1 (Fig. 1E). In the in vitro assay, cholesterol oxidase enhanced Ragulator binding to the Rag GTPases, which was blocked by free cholesterol in a dose-dependent manner or by heat-inactivation of cholesterol oxidase (Fig. 3E). Thus, in cells and in in vitro reconstituted organelle systems, lysosomal cholesterol modulates the mTORC1 scaffolding complex to an activated state to recruit mTORC1.

The lysosomal cholesterol transporter, NPC1, interacts with the mTORC1 scaffolding complex

My results so far indicate that cholesterol derived from LDL degradation within the lysosome activates mTORC1. As mentioned above, LDL-derived cholesterol needs to be transferred from the lysosomal lumen to the limiting membrane via an NPC2 and NPC1 pathway (71). Thus, we wanted to test if this cholesterol trafficking machinery is also involved in mTORC1 regulation. I immunoprecipitated FLAG-tagged NPC1 that is stably expressed in HEK-293T cells and used immunoblotting to check for candidate interacting proteins. NPC1 pulled down multiple endogenous components of the mTORC1 scaffolding complex, including Ragulator, RagA, v-ATPase and SLC38A9 (Fig. 4 A and B). Conversely, a member of the scaffolding complex, FLAG-tagged SLC38A9, also pulls down endogenous NPC1 in a specific manner (Fig. 4C). SLC38A9 appeared to be necessary for the ability of NPC1 to interact strongly with the rest of the mTORC1 scaffolding complex since in SLC38A9-null HEK-293T cells generated via CRISPR/Cas9 technology (sgSLC38A9 cells), FLAG-tagged NPC1
immunoprecipitated Ragulator, Rag GTPases and the v-ATPase to a lower extent as compared to control cells (Fig. 4D).

**NPC1 is necessary for sterol-dependent regulation of mTORC1**

Based on the ability of NPC1 to physically interact with the mTORC1 scaffolding complex, I next analyzed the requirement for NPC1 in mTORC1 regulation by cholesterol. Surprisingly, in NPC1-null HEK-293T cells generated via CRISPR/Cas9 technology (sgNPC1 cells) mTORC1 signaling is resistant to sterol depletion as shown by persistent phosphorylation of multiple substrates in MCD-treated sgNPC1 cells (Fig. 4D). Consistent with previous studies, filipin staining also shows that sgNPC1 cells have intracellular cholesterol puncta as compared to wild type cells, which have cholesterol distributed across the plasma membrane (Fig. 5A top). Moreover, we confirmed the role of NPC1 in mTORC1 regulation by cholesterol in both MEFs from NPC1−/− mice and in Chinese hamster ovary (CHO) cells bearing a homozygous, naturally occurring deletion in the NPC1 gene (77). In sgNPC1 cells, immunofluorescence shows that mTOR remains co-localized with lysosomes during MCD-treatment suggesting that NPC1 is necessary for cholesterol-regulation of mTORC1 (Fig. 5B and D). In contrast, NPC1 is not necessary for amino acid-mediated regulation since mTOR became cytoplasmic upon amino acid starvations in both wild type and sgNPC1 cells (Fig. 5C and D). To further investigate the role of NPC1 in cholesterol-mediated regulation of mTORC1, NPC1-null (NPC1−/−) MEFs were rescued with an NPC1 construct (NPC1P691S) that has a mutation in a putative sterol-sensing domain (SSD) of unknown function that is similar to bona fide cholesterol sensing domains of HMG-CoA reductase and SCAP (80). NPC1P691S localizes to lysosomes, but is unable to rescue mTORC1 sterol sensitivity (Fig. 5E and F). This suggests that NPC1 may have a sterol-sensing function that is necessary for proper mTORC1 regulation.

In addition to studying the role of NPC1 in cholesterol-regulation of mTORC1, we also identified SLC38A9 as a positive regulator for cholesterol-activation of mTORC1 in addition to its previously identified role in arginine signaling, which are both upstream of the Rag GTPases. This work was done in close collaboration with another graduate student in the lab, Ashley Thelen and this will be discussed further in the discussion (44-46, 77). To test the epistatic relationship between SLC38A9 and NPC1, I knocked down SLC38A9 in the sgNPC1 cell lines (Fig. 5G and H). In MCD-treated sgNPC1 cells we find that the cholesterol-independent, constitutive mTORC1 signaling is mitigated when SLC38A9 is knocked down (Fig. 5G). In contrast, control cells and sgNPC1 cells were identically affected by SLC38A9 depletion during arginine starvation and restimulation (Fig. 5H). These results are consistent with a specific role of NPC1 in cholesterol-dependent, but not arginine-dependent, mTORC1 regulation.

**3.2 Discussion**

Cells must be able to dynamically integrate multiple nutrient signals into cellular pathways to ensure appropriate cellular growth, but the molecular basis of this integration remains poorly understood (1). Previous studies have shown that long- and short-range nutrients regulate mTORC1 activity including: growth factors, energy levels and amino acids regulate mTORC1 activity (18). Here we show that lysosomal cholesterol directly regulates mTORC1 by modulating mTORC1 localization to the lysosomal surface. I hypothesize that this mechanism may couple mTORC1-mediated membranogenesis to the availability of dietary cholesterol.
Cholesterol-dependent activation of mTORC1 via SLC38A9 and NPC1

Our findings identify a possible mechanism via which cells integrate cholesterol levels with other nutrient inputs to regulate mTORC1. Initially we find that cholesterol depletion inhibits mTORC1 signaling and is rescued by LDL and exogenous cholesterol. At the subcellular level, cholesterol regulates mTORC1 activity by controlling its localization to the lysosomal surface, where mTORC1 kinase activation occurs. Utilizing RagB\textsuperscript{Q99L}, a constitutively active mutant, we find that mTORC1 remains on the lysosome even when cholesterol is depleted suggesting that cholesterol is sensed upstream of the Rag GTPases.

A portion of my findings was generated in close collaboration with a fellow graduate student, Ashley M. Thelen (77). This collaborative work characterized another protein involved in sterol-dependent mTORC1 regulation. A bioinformatics approach was taken to search the mTORC1 scaffolding complex for putative cholesterol-binding or cholesterol-interacting motifs. Surprisingly SLC38A9, an arginine transporter characterized in arginine-regulation of mTORC1, contains a putative Cholesterol Recognition Amino acid Consensus (CRAC) motif within transmembrane 8. In addition to this CRAC motif, transmembrane domain 8 of SLC38A9 also contains an inverse CRAC motif termed a CARC motif. These putative tandem motifs span the lipid bilayer and together would be able to interact with cholesterol on both membrane leaflets (81, 82). Utilizing \textit{in vivo} and \textit{in vitro} assays, we showed that these CRAC and CARC motifs do interact with cholesterol and mutation of critical residues impairs these interactions (77). Functionally, deletion of SLC38A9 abolishes mTORC1 activity overall, but cells rescued with SLC38A9 constructs that harbor individual or concurrent mutations of the CRAC or CARC motifs negatively effects sterol-activation of mTORC1, but arginine regulation is maintained. These results show that SLC38A9 is a signal integrator and is able to convey both arginine and lysosomal cholesterol levels to mTORC1 to modulate its localization and activation at the lysosomal surface.

Cholesterol from receptor-mediated endocytosis and autophagy is trafficked to other cellular compartments through the lysosome. The putative lysosomal cholesterol transporter, NPC1, was found to interact with components of the mTORC1 scaffolding complex, which include the Rag GTPases, Ragulator, the v-ATPase and SLC38A9. These interactions likely allow NPC1 to modulate cholesterol levels around the scaffolding complex, where SLC38A9 is poised to sense cholesterol with its tandem CRAC and CARC domains. In NPC1-null cells or cells rescued with a putative SSD mutant, NPC1\textsuperscript{P691S}, mTORC1 signaling is largely unresponsive to cholesterol depletion. This suggests that NPC1 is a negative regulator of mTORC1 and that NPC1 cholesterol transport activity is necessary for mTORC1 to be sensitive to cholesterol levels. This gives rise to two potential models for NPC1-mediated regulation of mTORC1. The first model requires active transport of cholesterol and this is sensed and conveyed through conformational changes within NPC1. Another model requires cholesterol transport activity to deplete the lysosomal pool of cholesterol to sensitize SLC38A9 to cholesterol levels. Interestingly, NPC1 loss of function or haploinsufficiency is associated with diseases such as neurodegeneration, obesity and diabetes. mTORC1 over activation is also implicated in these diseases and our work suggests a possible causative link between NPC1, mTORC1 activity and the progression of these diseased states (9, 83, 84).

Regulation of mTORC1 by SLC38A9 and NPC1 occurs upstream of its localization to the lysosomal surface. A knockdown of SLC38A9 in NPC1-null cells is able to delineate where the relationship between these two proteins and mTORC1 regulation. SLC38A9 is downstream of NPC1 as knocking down SLC38A9 abolishes mTORC1 activity regardless if NPC1 is present.
or not. This result is consistent with a model in which NPC1 regulates the amount of cholesterol available for the sensor, SLC38A9 to activate mTORC1.

**mTORC1 generates regulatory feedback mechanisms for appropriate cellular growth**

In our final model, sterol-dependent regulation of mTORC1 requires SLC38A9 to sense lysosomal cholesterol and NPC1 to distribute cholesterol to other cellular compartments. Cells preferentially utilize lipoproteins for membrane precursors, but LDL has a higher ratio of cholesterol to fatty acids as compared to cellular membranes (85). This increase in lysosomal cholesterol activates mTORC1, which in turn promotes fatty acid synthesis through the master lipogenic transcription factor, SREBP1c. mTORC1 is capable of generating this regulatory feedback mechanism to couple dietary cholesterol from LDL to fatty acid synthesis to produce membranes with the correct lipid composition to ultimately support cellular growth. Interestingly, mTORC1 generates other regulatory feedback loops from positive inputs such as growth factors, energy and amino acids.

In the context of growth factor signaling, mTORC1 inhibits the PI3K-Akt pathway through inhibition of IRS1 and stabilization of Grb10 (growth factor receptor-bound protein 10) (86-89). IRS1 is negatively phosphorylated by both mTORC1 and S6K1, which results in less PI3K recruited to the plasma membrane thereby mitigating insulin signaling (86, 87). mTORC1 directly phosphorylates Grb10, which increases its stability and allows Grb10 to bind the insulin receptor and inhibits insulin signaling upstream of Akt (88, 89).

The increase in protein synthesis promoted by mTORC1 also triggers regulatory feedback mechanisms to maintain adequate levels of energy and amino acids. Translation is one of the most energetically expensive processes in the cell (90). Translation hydrolyzes GTP and utilizes aminoacyl-tRNAs (aa-tRNAs) as building blocks for proteins. GTP is generated from the tricarboxylic acid (TCA) cycle and nucleoside-diphosphate kinase, which can transfer the terminal phosphate from ATP to GDP to produce ADP and GTP (91). aa-tRNAs also utilizes ATP through synthetases that hydrolyze ATP to AMP to conjugate amino acids to their cognate tRNAs (92). So the process of translation consumes ATP, GTP and amino acids, which are positive regulators of mTORC1. This regulatory feedback modulates mTORC1 activity to replenish amino acid and energy stores after depletion. Previous studies show that mTORC1 signaling increases when translation is halted with cyclohexamide treatments even in full nutrient conditions (93).

Cells utilize mTORC1 as an elaborate coincidence detector so when long- and short-range nutrients levels are high, proliferation can begin. mTORC1 generates regulatory feedback mechanisms to ensure that these nutrient levels stay above thresholds to ensure and continue appropriate cellular growth (Fig. 6). mTORC1’s capacity for self-regulation support its role as a master nutrient regulator of cellular growth. Understanding mTORC1 on both a mechanistic molecular level and systems level can shed insight into disease progression for cancer and neurodegeneration.
3.3 Figures

Figure 1. Cholesterol at the lysosome regulates mTORC1 activity
Figure 1. Cholesterol at the lysosome regulates mTORC1 activity. (A) Dose-dependent activation of mTORC1 by LDL. HEK-293T cells were depleted of sterols with methyl-β-cyclodextrin (MCD, 0.5% w/v) for 2 hours and stimulated for 2 hours with increasing concentrations (0 to 100 µg/mL) of LDL. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and 4E-BP1 (S65) and for total protein abundance. (B) (top) Time-dependent activation of mTORC1 by LDL. HEK-293T cells were depleted of sterols for 2 hours and restimulated with LDL (50 µg/mL) for the indicated times. Cell lysates were analyzed for phosphorylation status of S6K1 (T389). (bottom) Time-course of LDL delivery to the lysosome. Cells stably expressing LAMP1-mRFP-FLAG<sup>12</sup> (LRF) were treated with boron-dipyrromethene (BODIPY)-LDL for the indicated times. (C) HEK-293T cells were depleted of sterols for 2 hours and restimulated with LDL (50 µg/mL) for 2 hours with increasing concentrations (0 to 20 uM) of Lalistat. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and for total protein abundance. (D) HEK-293T cells were depleted of sterols for 2 hours and restimulated with LDL (50 µg/mL) or with 50 uM MCD:cholesterol. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and 4E-BP1 (S65). (E) HEK-293T cells were depleted of sterols for 2 hours and restimulated for 2 hours with sterol-related ligands. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and 4E-BP1 (S65) and for total protein abundance. (F) (top) Lysosomes from HEK-293T cells stably expressing LRF were immunocaptured onto FLAG affinity beads. (bottom) Mass spectrometry measurements of unesterified cholesterol in immunocaptured lysosomes from HEK-293T cells stably expressing LRF were subjected to the indicated treatments. Shown are mean + SD; n = 4 per condition; ANOVA, *P*<0.05, followed by Tukey’s t test, *P*<0.5.
Figure 2. Cholesterol regulates mTORC1 localization to the lysosomal surface

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Figure 2. Cholesterol regulates mTORC1 localization to the lysosomal surface. (A) Cholesterol does regulate mTORC1 through TSC2. MEFs in TSC2\(^{WT}\) or TSC2\(^{-/-}\) backgrounds were depleted of sterols with increasing concentrations of MCD for 3 hours. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and 4E-BP1 (S65) and for TSC2 levels. (B) Cholesterol status does not affect Ragulator localization to LAMP2-positive lysosomes, but cholesterol status does affect mTOR localization to LAMP2-positive lysosomes. HEK-293T cells were subjected to the indicated treatments, followed by immunofluorescence for endogenous p18 or mTOR and LAMP2. Scale bar, 10\(\mu\)m. (C) Quantification of RagC-LAMP2, p18-LAMP2, and mTOR-LAMP2 co-localization under cholesterol-depleted and cholesterol-restimulated conditions. Shown are mean + SD; \(n = 15\) cells per condition; ANOVA, \(P < 0.0001\), followed by Tukey’s \(t\) test, ***\(P < 0.001\). (D) Constitutively active RagB mutant (RagB\(^{Q99L}\)) makes mTORC1 insensitive to sterol depletion. HEK-293T cells stably expressing RagB\(^{Q99L}\), along with control HEK-293T cells, were sterol-depleted for 2 hours, and restimulated for 2 hours with LDL (50 \(\mu\)g/mL) or with 50 \(\mu\)M MCD:cholesterol where indicated. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and 4E-BP1 (S65) and for total protein abundance. (E) RagB\(^{Q99L}\) makes mTOR localization to LAMP2-positive lysosomes insensitive to cholesterol levels. HEK-293T cells stably expressing RagB\(^{Q99L}\), along with control HEK-293T cells were subjected to the indicated treatments, followed by immunofluorescence for endogenous mTOR and LAMP2. Scale bar, 10\(\mu\)m. (F) Quantification of mTOR-LAMP2 co-localization under cholesterol-depleted and cholesterol-restimulated conditions in HEK-293T cells expressing RagB\(^{Q99L}\) and control HEK-293T cells.
Figure 3. mTORC1 scaffolding complex is dynamically regulated by cholesterol in vivo and in vitro
Figure 3. mTORC1 scaffolding complex is dynamically regulated by cholesterol \textit{in vivo} and \textit{in vitro}. (A) Cholesterol regulates the interaction between Ragulator and Rag GTPases in cells. HEK-293T cells stably expressing FLAG-p14 or LRF were sterol-depleted for 2 hours and restimulated with MCD: cholesterol for 2 hours where indicated. After lysis, samples were subjected to FLAG immunoprecipitations and immunoblotting for the indicated proteins. (B) Organelle-based \textit{in vitro} assay. A light organelle preparation stably expressing FLAG-tagged proteins is starved for amino acids for 45 minutes followed by restimulation with amino acid esters for 15 minutes (left) or sterol-depleted with 0.1% MCD for 1 hour followed by restimulation with MCD:cholesterol for 15 minutes (right). Interaction with endogenous proteins is determined by immunoblotting following detergent solubilization and FLAG immunoprecipitations. (C) Regulation of the interaction between Ragulator and endogenous Rag GTPases and v-ATPase by amino acids and cholesterol \textit{in vitro}. Organelle preps stably expressing FLAG-p14 or LRF were subjected to starvation/restimulation with amino acids or cholesterol. Interaction with RagA and V₁B₂ was determined by immunoblotting. (D) Organelle-based \textit{in vitro} assay. A light organelle preparation from cells stably expressing FLAG-tagged proteins is treated with cholesterol oxidase in the absence or presence of MCD:cholesterol. Interaction with endogenous binding partners is determined by immunoblotting following detergent solubilization and FLAG immunoprecipitations. (E) Cholesterol regulates the interaction between Ragulator and Rag GTPases \textit{in vitro}. Light organelle fractions from HEK-293T cells stably expressing FLAG-p14 were treated with cholesterol oxidase (2 U/mL) along with increasing concentrations of MCD:cholesterol as indicated. Samples were subjected to lysis and FLAG immunoprecipitations, followed by immunoblotting for the indicated proteins.
Figure 4. The lysosomal cholesterol transporter, NPC1, interacts with the mTORC1 scaffolding complex.
Figure 4. The lysosomal cholesterol transporter, NPC1, interacts with the mTORC1 scaffolding complex. (A) Cartoon summarizing mass spectrometry analyses of immunoprecipitates from HEK-293T cells stably expressing NPC1-FLAG. v-ATPase subunits are color-coded according to their peptide representation. Peptide counts for v-ATPase subunits from 5 independent experiments are shown in the table below. (B) Binding of NPC1 to the mTORC1 scaffolding complex. HEK-293T cells stably expressing NPC1-FLAG or LRF were lysed and subjected to FLAG immunoprecipitations followed by immunoblotting for the indicated proteins. (C) HEK-293T cells stably expressing FLAG-SLC38A9 or LRF were lysed and subjected to FLAG immunoprecipitations and followed by immunoblotting for the indicated proteins. (D) SLC38A9 is required for the interaction of NPC1 with the mTORC1 scaffolding complex. Control or SLC38A9-deleted HEK-293T cells stably expressing NPC1-FLAG were lysed and subjected to FLAG immunoprecipitations followed by immunoblotting for the indicated proteins.
Figure 5. NPC1 is necessary for sterol-dependent regulation of mTORC1

A. cholesterol, p-T389 S6K1, NPC1, actin

B. sgNPC1, mTOR, LAMP2, MCD for 3h, LDL for 1h

C. sgNPC1, mTOR, LAMP2

D. Colocalization index

E. Filipin, FLAG + LAMP1, NPC1WT, NPC1P691S

F. cholesterol, LDL, p-T389 S6K1, S6K1, NPC1

G. sgNPC1, LDL, p-T389 S6K1, S6K1, actin, NPC1

H. arginine, p-T389 S6K1, S6K1, actin, NPC1
Figure 5. NPC1 is necessary for sterol-dependent regulation of mTORC1. (A) (top) Control HEK-293T cells or HEK-293T cells deleted for the NPC1 gene via CRIPSR/Cas9 (sgNPC1 clones 2 and 5) were sterol-depleted for 2 hours and restimulated with MCD: cholesterol for 2 hours where indicated. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and 4E-BP1 (S65) and for NPC1 and total protein abundance. (bottom) Filipin, a cholesterol-staining agent, staining reveals lysosomal cholesterol accumulation in sgNPC1, but not in control HEK-293T cells. (B) Lysosomal localization of mTORC1 is insensitive to sterol depletion in the absence of NPC1. sgNPC1 or NPC1-FLAG rescued sgNPC1 were subjected to the indicated treatments, followed by double immunofluorescence for mTOR and LAMP1. Scale bar, 10μm. (C) sgNPC1 or NPC1-FLAG rescued sgNPC1 cells were subjected to the indicated treatments, followed by double immunofluorescence for endogenous mTOR and LAMP2. Scale bar, 10μm. (D) Quantification of mTOR-LAMP2 co-localization under cholesterol-depleted/stimulated or amino acid-depleted/stimulated conditions in sgNPC1 or NPC1-FLAG rescued sgNPC1 cells. Shown are mean ± SD. N = 15 cells/condition. ANOVA: P<0.0001 followed by Tukey’s t test: ***p<0.001 and ****p<0.0001. (E) NPC1 WT MEFs stably expressing NPC1WT-FLAG or NPC1P691S-FLAG were subjected to filipin treatment or to double immunofluorescence for FLAG and LAMP1, as indicated. Scale bar, 10μm. (F) Requirement of the sterol-sensing domain (SSD) of NPC1 for cholesterol regulation of mTORC1. MEFs with the indicated genotypes were depleted of sterols for 2 hours and restimulated with MCD: cholesterol for 1 hour where indicated. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and for NPC1. (G) sgNPC1 cells were treated with either scrambled or SLC38A9-targeting siRNA, depleted of sterols for 2 hours and restimulated with LDL for 2 hours where indicated. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and for NPC1 and for total protein abundance. (H) sgNPC1 cells were treated with either scrambled or SLC38A9-targeting siRNA, starved for arginine for 1 hour and restimulated with arginine for 30 minutes where indicated. Cell lysates were analyzed for phosphorylation status of S6K1 (T389) and for NPC1 and total protein abundance.
Figure 6. mTORC1 generates regulatory feedback mechanisms for appropriate cellular growth

Figure 6. mTORC1 generates regulatory feedback mechanisms for appropriate cellular growth. mTORC1 is a master nutrient regulator that phosphorylates downstream targets to increase membranogenesis and protein synthesis for coordinated cellular growth. The processes of membranogenesis and protein synthesis each consume their respective nutrient inputs for mTORC1 activation and thereby generate regulatory feedback mechanisms to ensure appropriate cellular growth. Green lines indicate activation; red lines indicate inactivation or consumption.
Chapter 4: Conclusions and Future Directions

4.1 Conclusions

In this work we have identified cholesterol as a positive input for mTORC1 regulation and characterized proteins involved in conveying cholesterol levels to mTORC1 (Fig. 7). The mTORC1 protein kinase is a master nutrient regulator of cellular growth. Upon activation mTORC1 phosphorylates its downstream targets, which in turn increases anabolic processes like protein, lipid and nucleotide synthesis while inhibiting catabolic processes like autophagy and fatty acid oxidation. This results in an increase in cellular mass ensuring that appropriate growth and proliferation is achieved (18).

Understandably, mTORC1 is under tight regulation as the processes it promotes are energetically expensive and uncontrolled cellular growth can lead to diseased states (90, 94). This tight regulation is achieved by a logical AND gate where multiple nutrients like amino acids, energy and growth factors are necessary for mTORC1 activation (18). We report that cholesterol is an additional nutrient input needed to properly localize mTORC1 to the lysosomal surface for activation.

Cholesterol levels are detected at the lysosomal surface by SLC38A9, an arginine transporter, through tandem CRAC and CARC motifs in transmembrane 8 and these motifs are able to interact with cholesterol on both leaflets of the lysosomal membrane. SLC38A9 has been characterized previously in regulating mTORC1 in regards to arginine levels (44-46). The two functions of SLC38A9 help produce the logical AND gate, which integrates and conveys arginine and cholesterol levels to the Rag GTPases, which regulate mTORC1 localization to the lysosomal surface.

The putative lysosomal cholesterol transporter, NPC1, interacts with the mTORC1 scaffolding complex via SLC38A9. This close proximity allows NPC1 to traffic cholesterol near the mTORC1 scaffolding complex and thereby couples mTORC1 activation to lysosomal cholesterol efflux. In cells that are lacking NPC1 or have an NPC1 mutant that is incapable of transporting cholesterol, mTORC1 signaling is largely insensitive to cholesterol depletion while maintaining normal amino acid regulation. This suggests that an NPC1 that is actively trafficking cholesterol is necessary to regulate mTORC1.

Together, SLC38A9 and NPC1 at the lysosomal membrane generate a logical AND gate that is sensitive to both arginine and cholesterol levels to regulate mTORC1 activation dynamically and ultimately maintain appropriate cellular growth and proliferation (Fig. 7).

4.2 Future Directions

Genes associated with lipids are implicated in diseases and disease progression (64). Understanding how aberrant trafficking, localization and storage of lipids affect metabolic processes can shed light on potential therapeutics. Our findings set the stage to further investigate how lipids are sensed and how these signals are integrated into regulating cellular processes like mTORC1 signaling.

Our work demonstrates that lysosomal cholesterol is important for mTORC1 activation and impaired cholesterol efflux removes a level of mTORC1 regulation. Within the lysosome there are two pools of cholesterol: cholesterol within the lumen of the lysosome and cholesterol at the lysosomal limiting membrane. Identifying which pool or if both pools are responsible for
mTORC1 activation can lead to a better understanding of how aberrant cholesterol storage and mTORC1 over activation play a role in various disease progressions.

Cholesterol transport through the lysosome is important for appropriate mTORC1 activity and investigating processes upstream of NPC1 like LDL uptake and lipophagy can uncover other nodes of mTORC1 regulation. Further molecular and biochemical dissection of NPC1 itself can potentially decouple cholesterol transport activity and mTORC1 regulation, which would elucidate how cholesterol signals can be conveyed through different domains of a large transmembrane protein.

SLC38A9 is also able to directly integrate arginine and cholesterol levels to regulate mTORC1. Our work has shown that mutating transmembrane 8 in the CRAC and CARC motifs can decouple cholesterol and arginine regulation of mTORC1 suggesting that there are at least two domains with separate functions. Further biochemical and structural characterization of SLC38A9 can elucidate how these two domains interact with each other to dynamically transmit an integrated signal for mTORC1 activation. Also investigating SLC38A9’s protein-protein interactions with mTORC1 scaffolding complex can shed light on how the scaffold, and potentially individual components, are able to dynamically and modularly regulate mTORC1 in regards to different nutrient levels.

In addition to these biochemical and structural directions, it is apparent that on a systems level mTORC1 generates negative feedback loops with processes that it promotes like protein translation and membranogenesis. mTORC1 also promotes nucleotide synthesis and there have been links that folate, a building block of nucleotides, regulate mTORC1 activity. Mice with a folate deficiency show a decrease in mTORC1 signaling (95). Identifying how folate is sensed and integrated into the logical AND gate of mTORC1 regulation is still unclear.
4.3 Figure

Figure 7. Model of sterol-regulation of mTORC1 at the lysosome.

Figure 7. Model of sterol-regulation of mTORC1 at the lysosome. SLC38A9 stimulates Rag GTPase activation in response to cholesterol. NPC1 binds to SLC38A9 and inhibits cholesterol-mediated mTORC1 activation via its sterol transport activity.
Chapter 5: References


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