mTOR complex 1 activity is required to maintain the canonical endocytic recycling pathway against lysosomal delivery

Received for publication, December 19, 2016, and in revised form, February 10, 2017 \ Published, JBC Papers in Press, February 14, 2017, DOI 10.1074/jbc.M116.771451

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Edited by Thomas Söllner

The plasma membrane of mammalian cells undergoes constitutive endocytosis, endocytic sorting, and recycling, which delivers nutrients to the lysosomes. The receptors, along with membrane lipids, are normally returned to the plasma membrane to sustain this action. It is not known, however, whether this process is influenced by metabolic conditions. Here we report that endocytic recycling requires active mechanistic target of rapamycin (aka mammalian target of rapamycin) (mTORC1), a master metabolic sensor. Upon mTORC1 inactivation, either by starvation or by inhibitor, recycling receptors and plasma membrane lipids, such as transferrin receptors and sphingomyelin, are delivered to the lysosomes. This lysosomal targeting is independent of canonical autophagy: both WT and Atg5−/− mouse embryonic fibroblasts respond similarly. Furthermore, we identify hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), an endosomal sorting complexes required for transport (ESCRT)-0 component, as a downstream target of mTORC1. Hrs requires mTORC1 activity to maintain its protein expression level. Silencing Hrs without decreasing mTORC1 activity is sufficient to target transferrin and sphingomyelin to the lysosomes. It is thus evident that the canonical recycling pathway is under the regulation of mTORC1 and likely most predominant in proliferating cells where mTORC1 is highly active.

It is well established that endocytic recycling is important to sustain nutrient uptake and regulate cell surface protein expression. For example, transferrin (Tf) receptors bind to iron-loaded Tf in the medium and are endocytosed together with bound Tf. After endocytosis, Tf releases iron because of the acidic pH of the endosomal lumen before returning to the plasma membrane. Recycling receptors are usually sorted with high fidelity so that their half-life is far longer (~20 h) than a single cycle of endocytosis. They efficiently recycle back to the plasma membrane and, therefore, are normally undetectable in the lysosomes (2). Bulk plasma membrane lipids, which form the limiting membranes of early endosomes after initial internalization, are also efficiently recycled with similar kinetics as recycling receptors (3). This gives rise to the concept that recycling of the membrane is a default pathway for early endocytic membrane components, including receptors, in most mammalian cells. In contrast, the contents of the early endosomal lumen appear to progress to the lysosome by default. Specific signals are thought to be necessary to deliver membrane proteins or lipids to the lysosomes.

One of the best characterized signals for lysosomal targeting is ubiquitination. Ubiquitinated cargoes interact with the endosomal sorting complexes required for transport (ESCRT) machinery, resulting in their removal from the limiting membrane of the endosomes and incorporation into vesicles budded into the endosomal lumen. These proteins are thereby sorted apart from recycling components and are targeted to the lysosomes instead (4). EGF receptor is a well-studied example (5). Many G protein-coupled receptors similarly interact with ESCRTs and are consequently targeted to the lysosomes (6).

In recent years, it has become increasingly apparent that the metabolic state of the cell has a significant impact on cellular processes of mammalian cells. One example is autophagy. Upon nutrient starvation, cells turn on a normally dormant mechanism to digest non-essential components of the cell. This process provides much needed nutrients and also limits non-essential cellular processes, particularly those involved in cell proliferation (7). One of the major molecular switches is mechanistic target of rapamycin complex 1 (mTORC1). mTORC1 is known to integrate at least four metabolic inputs to regulate cell growth, such as nutrient availability, growth factor signaling, cellular energy status, and cellular stress levels (8, 9). Through phosphorylation of p70-S6 kinase, for instance, mTORC1 promotes mRNA maturation and protein translation (10). mTORC1 also phosphorylates unc-51 like kinase (ULK1) to suppress autophagy (11). In proliferating cells, particularly in...
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cultured cells, mTORC1 activity is generally high. Only when acutely exposed to specific medium that lacks either amino acids, glucose, or both do cultured cells suppress mTORC1 activity and turn on autophagy (12).

The endocytic recycling pathway is one of the major mechanisms by which cells take up nutrients from the extracellular space. It is plausible that this pathway is integrated with other pathways responsive to the nutritional needs or metabolic status of the cell. However, previous studies have not addressed whether endocytic recycling is subject to regulation by mTORC1 in mammalian cells. Here we report that the recycling of both recycling receptors and membrane lipids is dramatically responsive to mTORC1 activity. Active recycling requires mTORC1 activity. This action of mTORC1 is through hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), an ESCRT-0 component.

Results

Starvation suppresses recycling and targets receptors to the lysosomes independent of canonical autophagy

We first exposed mouse embryonic fibroblasts (MEFs) to full growth medium (fed) or amino acid-free medium (starved) for 4 h and characterized Tf uptake, targeting, and recycling. MEF cells were then incubated with Alexa 488-Tf and LysoTracker Red for 30 min at 37 °C. In growth medium, Tf brightly labeled puncta throughout the cell (Fig. 1A, a), presumably the early, sorting, and recycling endosomes, respectively (13). There was little Tf in the late endosomes/lysosome compartments, marked by LysoTracker Red (Fig. 1A, b and c). However, in starved cells, large numbers of Tf were detected in LysoTracker-positive compartments (Fig. 1A, d–f), demonstrating lysosomal targeting. Indeed, quantification shows that the colocalization between Tf and LysoTracker was greatly increased upon starvation (Fig. 1B). Remarkably, nearly all LysoTracker-positive compartments contained Tf. In cells maintained in normal medium, little Tf was detected in the lysosomes. This trend remained throughout all experiments below.

The delivery of Tf to the lysosomes could be due to enhanced fluid phase uptake during starvation. We indeed observed more BSA uptake upon starvation compared with fed conditions (supplemental Fig. 1). However, Alexa 488-Tf uptake could be successfully competed off by excess unlabeled Tf in both fed and starved cells (supplemental Fig. 2), indicating that receptor-mediated endocytosis is fully responsible for Tf lysosomal uptake even under starvation conditions. In addition to the rerouting of Tf in starved cells, we also detected a substantial amount of Tf receptors in the lysosomes, labeled by either LysoTracker or Lamp-1 (a lysosomal membrane protein) (supplemental Fig. 3, A and B). We further verified that the LysoTracker was reliably marking lysosomes in starved cells, as overnight-loaded AF488-BSA was completely colocalized with the LysoTracker added to the starved cells during the last 30 min of 4-h starvation (supplemental Fig. 4A). The LysoTracker also colocalized with fluorescein dextran, another commonly used lysosome marker, in starved cells (supplemental Fig. 4B).

Consistent with lysosomal targeting of recycling receptors, we found decreased protein levels of Tf and LDL receptors, another long-lived recycling receptor, in starved cells (Fig. 1C). We also observed that bafilomycin, which neutralizes lysosomal pH, prevented the degradation of recycling receptors (supplemental Fig. 5). The time course of Tf uptake is shown in Fig. 1D. Tf uptake was significantly increased in starved cells at all time points measured in comparison with cells kept in full growth medium. This increase in Tf uptake seemed to reach a plateau after 20–30 min, possibly because of the decrease in Tf receptors on the cell surface of the cells and Tf degradation.

To further confirm Tf lysosomal targeting, cells were incubated with Tf for 30 min, followed by a 45-min chase in the presence of deferoxamine (see Fig. 2A for a schematic). Deferoxamine prevents recycled Tf from re-entering the cells by chelating iron. Indeed, cells in full growth medium completely cleared out Tf after the chase (Fig. 2A, a–c), confirming efficient Tf recycling. In starved cells, however, a large amount of Tf was retained in the lysosomes after the chase (Fig. 2A, d–f; quantification in Fig. 2B). The identical pattern could also be seen when lysosomes were labeled by dextran (supplemental Fig. 4C). In addition, this starvation-induced Tf lysosomal targeting is not limited to MEF cells; we observed the same phenomenon in other cultured cell types, such as HEK cells (supplemental Fig. 6). Also, MEF cells from several different origins showed a similar response to starvation. Taken together, these results demonstrate that starvation conditions significantly altered the endocytosis pathway and delivered normally recycling receptors to lysosomes for degradation.

One of the best documented events during nutrient starvation is autophagy. To determine whether autophagy plays a role in lysosomal targeting of recycling receptors, we performed identical experiments in Atg5−/− MEFs. These cells fail to form classical autophagosomes or fuse with lysosomes during starvation because of the lack of Atg5 (14). However, we found that Atg5−/− MEFs responded to starvation identically as WT MEFs in terms of Tf endocytotic trafficking (Fig. 3A), lysosomal targeting (Fig. 3B), and recycling receptor degradation (Fig. 3C). It is thus apparent that starvation-induced lysosomal delivery of recycling receptors is largely independent of Atg5-related autophagy.

Starvation also delivered normally recycling bulk lipids to the lysosomes

It is known that plasma membrane lipids, such as sphingomyelin (SM), constitutively undergo bulk flow recycling, which is believed to be the major mechanism for receptor recycling (3). Therefore, we next tested whether membrane flow is also altered during starvation by analyzing SM endocytosis in fed and starved MEFs using NBD-C6-SM (15). In control cells, NBD-C6-SM rapidly populated endosomal structures (5 min) but did not enter the lysosomes (Fig. 4A, a–c), and, even after 30 min, there was little colocalization between NBD-C6-SM and LysoTracker (Fig. 4B, a–c). However, when cells were exposed to starvation medium, NBD-C6-SM rapidly appeared in LysoTracker-positive compartments (5 min) (Fig. 4A, d–f) and con-
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Figure 1. Tf uptake and recycling under fed and starved conditions. A, WT MEFs were incubated in full growth (a–c) or starvation (d–f) medium for 4 h and subsequently labeled with AF488-Tf and LysoTracker Red for 30 min. B, relative colocalization between Tf and LysoTracker was quantified by Pearson’s coefficient analysis from ~50 cells. Data are presented as means ± S.E. ****, p < 0.0001. C, Western blotting analysis of Tf receptor (Tf-R) and LDL receptor (LDL-R) protein levels in MEF WT cells incubated in full growth or starvation medium for 6 h. mTORC1 activity is indicated by phospho-S6 levels. D, Tf uptake time course in MEF WT cells. Cells were incubated with AF488-Tf for 5, 10, 15, 20, 30, and 60 min. FI per cell was quantified. Each experiment was repeated at least three times, and representative images are shown. Scale bar = 10 μm.

continued to accumulate there for at least 30 min (Fig. 4B, d–f). Indeed, the amount of NBD-C6-SM in LysoTracker-positive compartments steadily increased with time in starved cells, whereas little NBD-C6-SM appeared in the lysosomes of fed cells (Fig. 4C). Thus, membrane bulk flow was also altered by starvation and diverted to the lysosomes. Given that both Tf and NBD-C6-SM appeared in lysosomes similarly, it is highly probable that endosomes, which contained bulk lipids and recycling receptors from the plasma membrane, were directly delivered to lysosomes upon nutrient deprivation.
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To understand how starvation alters the recycling pathway, we first asked whether a master metabolic regulator, transcription factor EB (TFEB), was involved. TFEB is known to be phosphorylated upon starvation and, consequently, translocates to the nucleus to turn on a large set of genes, including those involved in autophagy and lysosomal biogenesis (16). We indeed observed TFEB nuclear translocation in starved cells (supplemental Fig. 7A). However, subsequent experiments with the protein synthesis inhibitor cycloheximide showed that Tf was still targeted to the lysosomes upon starvation even though new protein synthesis was prevented (supplemental Fig. 7B). This suggested that synthesis of autophagic/lysosomal proteins was not necessary for altered lysosomal targeting during starvation.

Another major consequence of starvation is mTORC1 inactivation. The phosphorylation of ribosomal protein S6, an mTORC1 substrate, was diminished in starved WT and Ag5−/− MEF cells (Figs. 1C and 3C) and so was phosphorylation of p70-S6 kinase (supplemental Fig. 8). To delineate whether the absence of mTORC1 activity was responsible for lysosomal targeting of Tf, cells were maintained in normal growth medium but with a specific mTORC1 inhibitor, rapamycin (17). When subjected to the inhibitor, cells kept in normal growth medium efficiently delivered Tf to the lysosomes (Fig. 5, A and quantification in B), similar to starved cells. Furthermore, rapamycin also targeted SM to the lysosomes in cells maintained in normal growth medium (Fig. 6, A and B). Thus it is mTORC1 activity that is required for maintaining the recycling pathway. Interestingly, mTORC1 activities are generally high in rapidly proliferating cells (18), including tissue culture.

Figure 2. Tf is delivered to the lysosomes during starvation conditions. A, WT MEFs were preincubated (top panel) with full growth (a–c) or starvation (d–f) medium for 4 h and subsequently labeled with Tf for 30 min, followed by a 45-min chase in the presence of deferoxamine mesylate (50 μM). Scale bar = 10 μm. B, quantitation of FI per cell of Tf retained after chase. Data are presented as means ± S.E. ****, p < 0.0001.
cells, but low in many mature animal tissues (see supplemental Fig. 9 for examples).

While searching for mTORC1 downstream targets that directly regulate recycling, we noted a previous study showing that turning off TOR, the yeast homologue of mTORC1 and mTORC2, facilitates delivery of a set of proteins from the Golgi apparatus to the vacuole (the lysosome equivalent) via down-regulation of the expression of Vsp27, an ESCORT-0 component in yeast (19). Interestingly, another study reported that silencing Hrs, the mammalian homologue of yeast Vsp27, delivered large amounts of cholesterol, a major lipid of the plasma membrane in mammalian cells, to the lysosomes (20). This is reminiscent of SM accumulation in the lysosomes described above. We therefore examined Hrs expression in response to starvation. Indeed, starvation significantly decreased Hrs protein levels in WT and Atg5−/− MEF cells (Fig. 7, A and B), likely through proteasome-mediated degradation (supplemental Fig. 10), similar to that in yeast (19). Also, consistent with a central role for mTORC1 in Tf lysosomal targeting described above, the mTORC1 inhibitor rapamycin led to decreased Hrs protein levels in fed cells (Fig. 7C). Thus, mTORC1 is likely upstream of Hrs and responsible for maintaining the Hrs protein expression level in mammalian cells.

Silencing Hrs is sufficient to deliver Tf to the lysosomes

To examine the impact of Hrs on the recycling pathway, we next silenced Hrs in MEF cells using siRNA, which decreased Hrs protein levels by approximately 80% (Fig. 8, A and B). Silencing Hrs significantly decreased the protein levels of both Tf and LDL receptors compared with scrambled siRNA (Fig. 8A). This was similar to cells exposed to starvation medium (Fig. 1C). Noticeably, mTORC1 activity, marked by phosphorylation of the ribosomal protein S6K, remained largely unchanged in Hrs knockdown cells (Fig. 8B). Nevertheless, in Hrs-silenced cells (cyan fluorescent protein (CFP) as transfection marker), Tf was targeted to the lysosomes in MEFs (Fig. 8C, e–h), similar to starved cells or fed cells treated with mTORC1 inhibitor, even though cells were kept in normal growth medium with active mTORC1. In contrast, scrambled siRNA had little effect on the recycling pathway, and Tf remained in

Figure 3. Starvation-induced lysosomal delivery of recycling receptors is largely independent of Atg5-related autophagy. A, Atg5−/− MEFs were treated with either full growth or starvation medium as in Fig. 1A. B, Tf and LysoTracker colocalization was quantified, and Tf-R and LDL-R levels were examined by Western blotting analysis (C). Data are presented as means ± S.E. ****, p < 0.0001. Scale bar = 10 μm.
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A

Lysotracker Red  NBD-Ce-SM  NBD-Ce-SM / Lysotracker Red

fed

starved

5 min

d

B

Lysotracker Red  NBD-Ce-SM  NBD-Ce-SM / Lysotracker Red

fed

starved

30 min

d

C

SM in lysosomes (FI)

starved  fed

time [min]

0  5  10  15  20

0  1000  2000  3000
the endosomes (Fig. 8, C, a–d, and quantification in D). Again, nearly all of the lysosomes contained Tf in Hrs-silenced cells. Thus, decrease of Hrs alone is sufficient for lysosomal targeting of Tf, even with normal levels of mTORC1 activities. Furthermore, Hrs silencing targeted SM to lysosomes as well (supplemental Fig. 11). Based on these results, we conclude that mTORC1 is necessary for the normal recycling process and that Hrs functions downstream of mTORC1 to maintain recycling.

Discussion

Hrs is known as a part of the ESCRT pathway, where it initiates the sorting of ubiquitinated membrane proteins from the endosomes to the lysosomes (21). However, under starvation conditions or upon treatment with mTORC1 inhibitors, Hrs expression levels were diminished, and, at the same time, both Tf and bulk membrane lipid SM were targeted to the lysosomes. Ubiquitination is thus not likely involved in the targeting. Also, because TFEB and protein translation were not required, altered targeting could not be explained by increased lysosome biogenesis, i.e. recruiting of newly synthesized lysosomal proteins to the sorting endosomes. Rather, it is more likely that endosomes merged with existing lysosomes in the absence of Hrs through yet to be identified mechanisms. Another consistent observation throughout this study is that all lysosomal compartments seemed to be equally receptive to Tf or SM upon starvation, mTORC1 inhibition, or Hrs knockdown. Of interest, only silencing Hrs, but not Tsg101 (ESCRT-I), ESP20 (ESCRT-II), or CHMP6 (ESCRT-III), resulted in cholesterol accumulation in the lysosomes in the abovementioned report (20).

In yeast, starvation is known to trigger delivery and degradation of the plasma membrane proteins to supply amino acid (22, 23). The same mechanism may also be operating in mammalian cells. We showed here that, in mammalian cells, overall endocytosis may be up-regulated (increased BSA uptake), and protein receptors that normally recycle are targeted to the lysosomes by starvation. It remains to be seen whether this lysosomal targeting also applies to other plasma membrane proteins that normally do not recycle. In addition, although recycling receptors and SM are targeted to the lysosomes through diminished Hrs, we do not at present understand the precise mechanism, nor do we know the exact location where such altered trafficking occurs. At least for Tf, we did observe rather rapid delivery from the early endosomes to the lysosome (data not shown), suggesting that the change may happen rather early in the pathway. However, detailed studies are required to understand the whole process.

In summary, we provide evidence that the endocytic recycling pathway is under metabolic regulation through mTORC1 and Hrs. It is surprising and novel that diminished mTORC1 activity targets recycling receptors and bulk membrane lipids,
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which are normally constitutively recycled, to the lysosomes. Our findings are nevertheless consistent with the role of mTORC1 as a master metabolic sensor capable of directing cellular processes according to nutrient conditions. However, this regulation of the recycling pathway is distinct from classical autophagy. As mTORC1 activities are also controlled by growth factor signaling, cellular energy, and stress levels, the alteration of the recycling pathway reported here likely has much wider significance not limited to nutrient conditions. Indeed, the level of mTORC1 activity varies greatly among cell types in vivo, particularly activities involved in tissue homeostasis such as self-renewal and differentiation of stem cell (24). The results reported here, therefore, represent a major development in our understanding of the recycling pathway. It suggests that this pathway, regarded previously as default and constitutive, is intimately integrated into the metabolic regulation of cells. Thus, a paradigm shift is needed to inform future work on alterations to the endocytic recycling pathway under various tissue growth and differentiation conditions.

Figure 6. mTORC1 activity is necessary for SM recycling. A, MEFs were treated with DMSO or 250 nM rapamycin in full growth medium for 4 h prior to labeling with NBD-C6-SM for 30 min on ice. Cells were washed and subsequently incubated at 37 °C for 30 min before stripping the surface label by back exchange. B, NBD fluorescence intensity in lysosomes was quantified and is presented as mean FI ± S.E. **, p < 0.005. Scale bars = 10 μm.

Figure 7. mTORC1 activity is necessary for maintaining Hrs protein levels. A–C, Hrs protein levels were diminished by starvation treatment of WT (A) and Atg5−/− (B) MEFs and by the mTORC1 inhibitor rapamycin (C).
**Experimental procedures**

**Materials**

Cell culture growth medium, antibiotics (penicillin/streptomycin), and FBS were from Invitrogen (Burlington, ON, Canada), Thermo Fisher Scientific (Mississauga, ON, Canada), and Wisent Bioproducts (St-Bruno, QC, Canada), respectively. Other reagents used were as follows: protease inhibitor MG132 (80053-194) and deferoxamine mesylate salt (CA80056-274, VWR, Mississauga, ON, Canada); siRNA transfection reagents (transfection medium sc-36868 and transfection reagent sc-29528) and siRNAs (sc-41233 and sc-37007) (Santa Cruz Biotechnology, Dallas, TX); LysoTracker® Red DND-99 (L7528) and DAPI (D1306) (Thermo Fisher Scientific); cycloheximide (C7698), (2-hydroxypropyl)-α-cyclodextrin (390690), and rapamycin (R0395) (Sigma); poly-lysine-coated glass-bottom dishes (P35GC-1.5–10-C, (MatTek Corp.); NBD-C6-SM (810218) (Avanti Polar Lipids); ChemiBLOCKER (2170, Merck Millipore, Etobicoke, ON, Canada); pEGFP-N1-TFEB (38119, Addgene); and Effectene transfection reagent (301425, Qiagen, Toronto, ON, Canada).

**Figure 8. Silencing Hrs is sufficient to target Tf to the lysosomes.** A, siRNA knockdown was performed on MEFs using either scrambled or Hrs siRNA. Tf-R and LDL-R protein levels were assessed by Western blotting analysis. B, mTORC1 activity is indicated by phospho-S6K levels. C, siRNA knockdown of Hrs was performed on MEFs, and cells were stained with AF488-Tf and LysoTracker Red (a–h). D, relative colocalization between Tf and LysoTracker was quantified by Pearson’s coefficient analysis from 15–20 cells. Data are presented as mean ± S.E. ***p < 0.0005. Scale bars = 10 μm.
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Cell culture

WT and Atg5−/− MEFs were generously provided by Dr. Mizushima (University of Tokyo, Tokyo, Japan). Atg5−/− MEFs were verified by the absence of Atg5 protein expression. For WT MEFs, we used cells from several sources interchangeably; for example, from Drs. Mizushima, Kun-Liang Guan (University of California San Diego (UCSD)), and Michael McBurney (University of Ottawa). We did not notice any difference. All cell lines were maintained in high-glucose (25 mM) DMEM (31600-034) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO2. Starvation conditions were induced by incubating cells in amino acid-free RPMI medium (R9010-01) supplemented with 25 mM glucose and 1% penicillin/streptomycin.

Fluorophore labeling of Tf

Holo-transferrin (Sigma, T0665) was labeled with AF488 or AF568 protein labeling kits according to the protocol of the manufacturer (Life Technologies, A-10235 or A-10238).

Tf endocytosis and recycling

Cells were grown in glass-bottom dishes with normal growth medium for 48 h to 60–80% confluence. Cells were then incubated in either normal growth medium with FBS (fed) or amino acid-free medium (starved) for 4 h, followed by incubation with 20 μg/ml Alexa 488 Tf and 50 nM LysoTracker® Red for 30 min at 37 °C. For the time course, cells were incubated with Tf and LysoTracker for 5, 10, 15, 20, 30, or 60 min. Cells were then rinsed with PBS, fixed with 4% PFA for 10 min, and counterstained with DAPI before being viewed using a Zeiss LSM 510 Meta confocal fluorescence microscope with a ×63 objective and a ×60 objective on an inverted Nikon fluorescence microscope (TE2000-E) equipped with a Cascade 512B CCD camera (Roper Scientific). For treatment with mTOR inhibitor, cells were incubated with 250 nM rapamycin for 4 h, incubated with the SM complex (10 μM, complexed with 5 mM 2-hydroxypropyl-α-cyclodextrin) and 50 nM LysoTracker Red for 30 min on ice, washed in medium 1, and incubated in growth medium containing the inhibitors at 37 °C for 30 min. After a back-exchange with BSA, cells were fixed and viewed under a microscope (see above). For the quantification of uptake of SM into LysoTracker-positive compartments, LysoTracker images were used to create masks and NBD-SM fluorescence intensities in the LysoTracker-positive compartments were quantified using MetaMorph software.

Immunocytochemical staining

Cells were grown for 48 h to 60–80% confluence on glass-bottom dishes, incubated with either normal growth medium or starvation medium for 4 h, and subsequently stained with 100 nM LysoTracker Red DND-99 for 30 min. Cells were rinsed with PBS, fixed with 4% PFA, and incubated in buffer CT (5% (v/v) ChemiBLOCKER and 0.5% (v/v) Triton X-100 in PBS) for 20 min. The primary antibody (anti-Tf receptor/CD71 antibody (H68.4), Thermo Fisher Scientific, 13-6800); anti-Lamp1, Abcam, ab24170) was diluted in buffer CT and incubated for 1 h, followed by incubation of the secondary antibody (Thermo Fisher Scientific, goat anti-mouse IgG (H+L) Alexa 488 conjugate, A-11029 and donkey anti-rabbit IgG (H+L) secondary antibody, Alexa 594 conjugate, A-21207) in buffer C (5% (v/v) ChemiBLOCKER in PBS) for 30 min in darkness. The cells were then rinsed with PBS, stained with DAPI, and viewed on a Zeiss LSM 510 Meta confocal fluorescence microscope with a ×63 objective.

Image processing, analysis, and statistics

For the quantification, all fluorescence images were background-subtracted. Fluorescence intensities were then calculated, divided by the cell numbers in the image, and presented as fluorescence intensity (FI) per cell using MetaMorph software. For Pearson coefficients, confocal fluorescent images were analyzed with JAcop, a plugin in ImageJ. Each data point represents the analysis of 20–50 cells. Statistical differences were analyzed by unpaired t test using GraphPad Prism 5.0. p < 0.05 was deemed significant.

siRNA knockdown

Cells were grown for 24 h to 40–60% confluence before transfection. For each transfection, 60 pmol of siRNA and 6 μl of transfection reagent were used. The transfection was performed according to the instructions of the manufacturer.

Western blotting analysis

For Western blotting analysis, cells were washed in ice-cold PBS, lysed in radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 25 mM Tris (pH 7.6)) and centrifuged at 12,000 × g for 15 min. Mouse tissue was obtained from mature C57BL/6 mice, homogenized in radioimmune precipitation assay buffer, and centrifuged at 12,000 × g for 15 min. Protein concentration was determined via protein assay dye reagent (Bio-Rad, 5000006). SDS buffer
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(313 mM Tris (pH 6.8), 10% SDS, 0.05% bromphenol blue, 50% glycerol, and 0.1 M DTT) was added, and the samples were boiled at 100 °C for 10 min. Proteins were separated by SDS-PAGE on 10% gels. After separation, proteins were electrophoretically blotted on PVDF membranes. Membranes were blocked in 5% milk powder (in PBS, 1% Triton X-100) for 1 h and incubated with primary antibodies overnight. Membranes were probed with anti-transferrin receptor/CD71 antibody (H68.4, Thermo Fisher Scientific, 13-6800); anti- LDL receptor antibody (3143); anti-phospho-S6 ribosomal protein (Ser-235/236) antibody (Cell Signaling Technology, 2211S); anti-S6 ribosomal protein (54D2) antibody (Cell Signaling Technology, 2317S); anti-p70 S6 kinase (49D7) antibody (Cell Signaling Technology, 2708); anti-phospho-p70 S6 kinase (Thr-389) antibody, clone 10G7.1 (Millipore, MABS82); anti-Hrs antibody (M-79, Santa Cruz Biotechnology, sc-30221); and anti- β-Actin antibody (Sigma, A1978). Secondary antibodies (Cedarlane Laboratories, Peroxidase-AffiniPure donkey anti-rabbit IgG, 515-035-062) were incubated for 1.5 h. The chemiluminescence was detected via Immobilon Western chemiluminescent HRP substrate (Millipore, WBKLS0500).

Acknowledgments—We thank Dr. Thomas Legace for anti-Tf receptor and anti-LDL receptor antibodies, Dr. David Pickett and Keqin Yan for supplying the mouse tissues, and Dr. Rashmi Kothary for the pECPF-NI plasmid. We also thank Kevin Courtney for constructive discussions.

References

1. Hao, M., and Maxfield, F. R. (2000) Characterization of rapid membrane internalization and recycling. J. Biol. Chem. 275, 15279–15286
2. Rutledge, E. A., Mikoryak, C. A., and Draper, R. K. (1991) Turnover of the transferrin receptor is not influenced by removing most of the extracellular domain. J. Biol. Chem. 266, 21125–21130
3. Mayor, S., Presley, J. F., and Maxfield, F. R. (1993) Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. J. Cell Biol. 121, 1257–1269
4. Henne, W. M., Buchkovich, N. J., and Emr, S. D. (2011) The ESCRT pathway. Dev. Cell 21, 77–91
5. Williams, R. L., and Urbé, S. (2007) The emerging shape of the ESCRT machinery. Nat. Rev. Mol. Cell Biol. 8, 355–368
6. Giordano, F., Simoes, S., and Raposo, G. (2011) The ocular albinism type 1 (OA1) GPCR is ubiquitinated and its traffic requires endosomal sorting complex responsible for transport (ESCRT) function. Proc. Natl. Acad. Sci. U.S.A. 108, 11906–11911
7. Kaur, J., and Debnath, J. (2015) Autophagy at the crossroads of catabolism and anabolism. Nat. Rev. Mol. Cell Biol. 16, 461–472
8. Laplante, M., and Sabatini, D. M. (2013) Regulation of mTORC1 and its impact on gene expression at a glance. J. Cell Sci. 126, 1713–1719
9. Shimobayashi, M., and Hall, M. N. (2014) Making new contacts: the mTOR network in metabolism and signalling crosstalk. Nat. Rev. Mol. Cell Biol. 15, 155–162
10. Zoncu, R., Efeyan, A., and Sabatini, D. M. (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. Nat. Rev. Mol. Cell Biol. 12, 21–35
11. Kim, J., and Guan, K. L. (2011) Regulation of the autophagy initiating kinase ULK1 by nutrients: roles of mTORC1 and AMPK. Cell Cycle 10, 1337–1338
12. Korolchuk, V. I., Saiki, S., Lichtenberg, M., Siddiqi, F. H., Roberts, E. A., Imairisio, S., Jähreiss, L., Sarkar, S., Futter, M., Menzies, F. M., O’Kane, C. L., Deretic, V., and Rubinsztein, D. C. (2011) Lyosomal positioning coordinates cellular nutrient responses. Nat. Cell Biol. 13, 453–460
13. Gruenberg, J., and Maxfield, F. R. (1995) Membrane transport in the endocytic pathway. Curr. Opin. Cell Biol. 7, 552–563
14. Bampton, E. T., Goemans, C. G., Niranjan, D., Mizushima, N., and Tolkovsky, A. M. (2005) The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. Autophagy 1, 23–36
15. Koval, M., and Pagano, R. E. (1989) Lipid recycling between the plasma membrane and intracellular compartments: transport and metabolism of fluorescent sphingomyelin analogues in cultured fibroblasts. J. Cell Biol. 108, 2169–2181
16. Settembre, C., Fraldi, A., Medina, D. L., and Ballabio, A. (2015) Starvation-dependent regulation of Golgi quality control links the TOR signaling and vacuolar protein sorting pathways. J. Biol. Chem. 283, 693–698
17. Thoreen, C. C., Chantranupong, L., Keys, H. R., Wang, T., Gray, N. S., and Wang, J. E. (2015) Starvation-dependent regulation of Golgi quality control links the TOR signaling and vacuolar protein sorting pathways. J. Biol. Chem. 283, 693–698