A novel Rab10-EHBP1-EHD2 complex essential for the autophagic engulfment of lipid droplets

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The autophagic digestion of lipid droplets (LDs) through lipophagy is an essential process by which most cells catabolize lipids as an energy source. However, the cellular machinery used for the engulfment of LDs during autophagy is poorly understood. We report a novel function for a small Rab guanosine triphosphatase (GTPase) in the recruitment of adaptors required for the engulfment of LDs by the growing autophagosome. In hepatocytes stimulated to undergo autophagy, Rab10 activity is amplified significantly, concomitant with its increased recruitment to nascent autophagic membranes at the LD surface. Disruption of Rab10 function by small interfering RNA knockdown or expression of a GTPase-defective variant leads to LD accumulation. Finally, Rab10 activation during autophagy is essential for LC3 recruitment to the autophagosome and stimulates its increased association with the adaptor protein EHBP1 (EH domain binding protein 1) and the membrane-deforming adenosine triphosphatase EHD2 (EH domain containing 2) that, together, are essential in driving the activated “engulfment” of LDs during lipophagy in hepatocytes.

INTRODUCTION

Macroautophagy is a catabolic process that results in the delivery of reusable cellular material to the lysosome for the purposes of energy generation and survival in response to cellular stress. A central organelle targeted by the macroautophagic process is the lipid droplet (LD), a dynamic, triglyceride-rich structure surrounded by a phospholipid monolayer. Bound to the surface are different proteins that mediate the formation, dynamics, and catabolism of this organelle (1, 2). LDs are degraded by a selective form of macroautophagy known as “lipophagy” (3, 4). During lipophagy, a membranous isolation membrane (phagophore) envelops an individual LD, sequestering it within a double-membrane structure called the autophagosome (5), which subsequently fuses with the lysosome to form an autolysosome. Lysosomal lipases deposited into autolysosomes can hydrolyze neutral lipids contained within the captured LDs, releasing free fatty acids that can be used for the generation of adenosine 5′-triphosphate via mitochondrial β-oxidation.

Although a significant body of knowledge has been compiled regarding the molecular mechanisms of nonselective macroautophagy, surprisingly little is known about how LDs are specifically targeted and degraded by the autophagic machinery for selective lipophagy.

Several small Rab guanosine triphosphatases (GTPases) that actively regulate membrane dynamics and trafficking (6) have been observed to associate tightly with the LD phospholipid monolayer (7, 8), although the function of these regulatory enzymes in the regulation of LD function is poorly understood. We recently reported that Rab7, a regulator of endocytic vesicle maturation, also plays an instrumental role in the initial recruitment of degradative compartments to the surface of the LD in hepatocytes under nutrient-limiting conditions (9). An additional LD-associated Rab protein, Rab10, has also been shown to participate in the insulin-stimulated translocation of glucose transporter type 4 (GLUT4) in adipocytes (10), basement membrane secretion (11), and the formation and maintenance of the endoplasmic reticulum (ER) (12). Here, we report that Rab10 prominently associates with nascent LC3-positive phagophores that actively engulf LDs. Rab10 is potently activated under autophagy-stimulating conditions, such as nutrient deprivation or mechanistic target of rapamycin (mTOR) inhibition. This activity triggers the assembly of a trimeric protein complex at the autophagosome-LD interface comprising Rab10, the endocytic adaptor EH domain binding protein 1 (EHBP1), and the membrane-remodeling adenosine triphosphatase (ATPase) EH domain containing 2 (EHD2). The assembly of this complex is distinct from the lipophagic action of Rab7 and subsequent to Rab7-LD interaction. Thus, Rab10 mediates the selective targeting of autophagic machinery to the LDs through a novel trimeric complex that forms at the “lipophagic junction” between the autophagic machinery and the LD.

RESULTS

Rab10 distributes to membranous structures intimately associated with hepatocellular LDs

In an independent screen of biochemically fractionated HuH-7 human hepatocellular carcinoma cells, we found Rab10 to be a bona fide component of isolated LDs, consistent with previous proteomic reports demonstrating LD localization (13, 14) (Fig. 1A). To confirm this localization, a superfolder green fluorescent protein (sfGFP)–tagged Rab10 construct (sfGFP-Rab10) was expressed in several cell types, including HuH-7 (Fig. 1B) or Hep3B human hepatoma cells, clone 9 rat hepatocytes, and primary porcine hepatocytes (fig. S1, A to C). In all cell lines examined, we observed that sfGFP-Rab10 distributed to distinct, well-defined, crescent-shaped structures proximal to Oil Red O (ORO)–stained LDs (Fig. 1B and fig. S1). The LD-associated localization of the fluorescently tagged Rab10 was also observed by immunofluorescent detection of the endogenous protein (Fig. 1C). Although expression of a GFP-tagged constitutively active form of Rab10 (GFP-Rab10Q68L) gave rise to numerous Rab10-decorated LDs, these structures were not observed in cells expressing...
the dominant-negative form of Rab10 (GFP-Rab10T23N) (Fig. 1, D to F), indicating that the activity of this GTPase is required for its recruitment to the LD. Compared to conventional wide-field fluorescence microscopy (Fig. 1G), our observations of Rab10 association with LDs by super-resolution microscopy (Fig. 1, H and I) provided greater clarity of these interactions and revealed undulating membranous structures within the LD-proximal Rab10 structures that appeared to completely surround the LD surface (Fig. II).

**Rab10 participates in LD breakdown under nutrient-limiting conditions**

On the basis of our observation that Rab10 can accumulate on structures associated with the LD surface, we next assessed whether Rab10 might play a role in LD metabolism. Culture of HuH-7 cells in nutrient-limiting conditions (e.g., Hep3B cells) reveals the presence of prominent Rab10-positive structures (green) in close proximity to ORO-stained LDs (red). (D and E) Fluorescence microscopy images of serum-starved HuH-7 hepatoma cells expressing either the constitutively active sfGFP-Q68L (D) or dominant-negative sfGFP-T23N (E) mutant forms of Rab10. Arrowheads indicate examples of Rab10-positive LD-associated structures. (F) Quantification of the number of Rab10-positive LDs observed in n = 3 independent experiments performed on control or HBSS-starved cells (100 cells per condition). ***P < 0.001. (G to I) Comparison of images of a solitary sfGFP-Rab10-positive LD (from a HuH-7 cell HBSS-starved for 1 hour) observed using conventional wide-field epifluorescence (G) or super-resolution microscopy (H and I). The higher resolution of the latter two images (H and I) reveals a membranous structure (I) (sfGFP signal alone) that appears to extend completely around the LD surface. Scale bars, 10 μm (B and C), 5 μm (D and E), and 1 μm (G to I).

**Fig. 1. Rab10 distributes to LD-associated structures upon serum starvation.** (A) Western blot analysis of a purified LD preparation isolated from HuH-7 hepatoma cells, probed with antibodies targeting a variety of organelle markers, including LAMP1 (lysosome), protein disulfide isomerase (PDI) (ER), PLIN2 (LD), and Rab10. Lanes indicate the whole-cell lysate (WCL), postnuclear supernatant (PNS), and LD fraction (LD). (B) Expression of sfGFP-tagged Rab10 or (C) antibody staining of endogenous Rab10 in oleate-loaded Hep3B cells reveals the presence of prominent Rab10-positive structures (green) in close proximity to ORO-stained LDs (red). (D and E) Fluorescence microscopy images of serum-starved HuH-7 hepatoma cells expressing either the constitutively active sfGFP-Q68L (D) or dominant-negative sfGFP-T23N (E) mutant forms of Rab10. Arrowheads indicate examples of Rab10-positive LD-associated structures. (F) Quantification of the number of Rab10-positive LDs observed in n = 3 independent experiments performed on control or HBSS-starved cells (100 cells per condition). ***P < 0.001. (G to I) Comparison of images of a solitary sfGFP-Rab10-positive LD (from a HuH-7 cell HBSS-starved for 1 hour) observed using conventional wide-field epifluorescence (G) or super-resolution microscopy (H and I). The higher resolution of the latter two images (H and I) reveals a membranous structure (I) (sfGFP signal alone) that appears to extend completely around the LD surface. Scale bars, 10 μm (B and C), 5 μm (D and E), and 1 μm (G to I).
Fig. 2. Rab10 function is required for LD catabolism. (A) Representative immunoblot from n = 3 independent experiments showing the efficiency (>90%) of Rab10 siRNA knockdown in HuH-7 hepatoma cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (B and C) Fluorescence imaging of HuH-7 cells subjected to 48-hour treatment with either control nontargeting (NT) siRNA or Rab10-directed siRNA and subsequently starved for an additional 48 hours. LDs are stained with ORO, and nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI). Total ORO-stained area per cell was quantified in (C) from n = 3 independent experiments (>350 cells measured per experiment). (D) HuH-7 cells were treated with control NT siRNA or Rab10 siRNA for 48 hours before re-expression of GFP-tagged forms of wild-type (WT), active (−Q68L), or inactive (−T23N) forms of Rab10. Cells were then serum-starved for a period of 48 hours to look for rescue of the LD breakdown phenotype (total ORO-stained area quantified in n = 3 independent experiments, 30 cells per repeat). (E) Quantification of a similar knockdown/re-expression experiment performed in (D), with the exception that the lysosomal protease inhibitor CQ was included in the medium during the starvation period (n = 3 independent experiments, 25 cells measured per repeat). (F to I) LDs accumulate in cells isolated from Rab10 KO mice. Cells were preloaded with 400 μM oleic acid overnight. (F) Representative immunoblot of MEFs isolated from WT or Rab10 KO embryos. (G) Measurement of total triglyceride content in WT or Rab10 KO MEFs. (H) Representative fluorescence images of oleate-loaded WT or Rab10 KO MEFs, stained with ORO and DAPI. (I) Digital quantification of average LD content per cell in WT or Rab10 KO MEFs (n = 3 independent experiments, 300 cells per repeat). (J) Quantification of the total LD area per cell in Rab10 KO MEFs transfected with either GFP vector alone or GFP-Rab10 (n = 3 independent experiments, 400 cells per repeat). Cells were preloaded with 400 μM oleic acid overnight. (K) Quantification of the release of [3H]H2O into the medium as a functional readout of mitochondrial β-oxidation in either WT or Rab10 MEFs subjected to 6 hours of growth under full-serum or serum-starved conditions. Cells were pulse-labeled with [9,10-3H]oleic acid. Data are represented as means ± SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; N.S., not significant. Scale bars, 10 μm.
autophagic isolation membranes or phagophores, known to encapsulate cargo targeted for by autophagy. This would suggest that autophagic signaling might regulate the recruitment of Rab10 to the LD. To test for a specific role of autophagy in the initial formation of these Rab10-positive structures, we expressed GFP-Rab10 in HuH-7 cells that were then subjected to HBSS starvation for 1 hour in the presence or absence of an autophagy inducer, the mTOR inhibitor Torin1 (15). Torin1-treated cells contained significantly greater numbers of Rab10-positive structures near the LD (Fig. 3, A and B). In addition, Torin1 treatment resulted in an approximately twofold increase in the number of Rab10-labeled LDs observed in resting cells, an effect that was not further increased by starvation (Fig. 3E). In contrast, treatment with the autophagic inhibitor 3-methyladenine (3-MA) completely abrogated the starvation-induced accumulation of fluorescein-tagged Rab10 near the LD (Fig. 3, D and F). To determine whether Rab10 is activated by autophagic stimulation, as suggested by the accumulation of active GFP-Rab10Q68L on LDs (Fig. 1D), we tested the effect of serum removal or Torin1 treatment on the GTPase activity of Rab10 using guanosine 5′-triphosphate (GTP)-conjugated beads to pull down active small GTPases (16, 17). We first confirmed that the active Q68L form, but not the dominant-negative T23N form of hemagglutinin (HA)–tagged Rab10, could be pulled down by these beads (Fig. 3G), validating the specificity of this assay. Lysates of HuH-7 cells subjected to HBSS starvation (Fig. 3H) or Torin1 treatment for 1 hour (Fig. 3I) were subsequently harvested for GTP pulldown. Both treatments resulted in an approximately 2.5-fold increase in the amount of Rab10 pulled down (Fig. 3J). These data indicate that Rab10 can be activated under autophagy-stimulating conditions.

LD-associated Rab10 structures are nascent autophagosomal organelles

To better define the nature of the LD-proximal Rab10-positive structures and evaluate any potential connection to autophagy, we examined the colocalization of LC3 or autophagy-related protein 16-like 1 (Atg16L1) with the LD and determined whether Rab10 colocalized with these markers of autophagic organelles (Fig. 4, A and B). Immunostaining for LC3 (Fig. 4A) or coexpression of GFP-tagged Atg16L1 (Fig. 4B) revealed that LD-localized Rab10-positive structures can be found to localize coincident with both of these markers. Consistent with this finding, we detected a very close colocalization between LD-localized Rab10 and Rab11, another small GTPase with known involvement in phagophore extension and autophagosomal maturation (Fig. 4C) (18–22). In addition, we found that LD-localized Rab10 can also colocalize with the ER marker Sec61β (Fig. 4D). This is not unexpected, because previous data show that Rab10 plays a key role in the regulation of ER morphology (12) and that the ER membrane itself may serve as a major source of membrane for the elongating phagophore (23) (Fig. 4D). Further evidence for a role of Rab10 in LD autophagy is demonstrated by the fact that a lysosomal marker, lysosomal-associated membrane protein 1 (LAMP1), also exhibits close colocalization with Rab10 near the LD surface (Fig. 4E). In support of these morphological observations, subcellular fractionation of Hep3B hepatoma cells that had been starved in HBSS revealed a significant overlap between Rab10 and LAMP1 in OptiPrep gradient fractions (Fig. 4F).

Together, these results suggest that Rab10 accumulates proximal to the LD following autophagic initiation and participates in the lysosome-mediated turnover of LDs. Consistent with such a model, expression of constitutively active GFP-Rab10Q68L was able to drive LD breakdown in siNT–treated hepatocytes but not in cells in which autophagic initiation had first been compromised by siRNA knockdown of Atg7 (fig. S3, A to E). The starvation-induced association of Rab10 with LDs is also prevented by Atg7 knockdown (fig. S3, F and G).

To provide greater resolution of the interaction between the putative Rab10-positive nascent autophagosomes and LDs, we conducted electron microscopy (EM) of HuH-7 hepatoma cells expressing constitutively active GFP-Rab10Q68L that had been either incubated in full-serum

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**Fig. 3. Rab10 is recruited to the LD during starvation-induced autophagy.** (A to D) Fluorescence images of HuH-7 hepatoma cells expressing sfGFP-Rab10 under resting (A), HBSS-starved (B), Torin1-treated (C), or 3-MA–treated (D) conditions. LDs are stained with ORO (red). Rab10-positive LDs are indicated by arrowheads, and average numbers of Rab10-positive LDs per cell are quantified in (E) (n = 3 independent experiments, 100 cells counted per condition). (F) Quantification of the effect of 3-MA treatment on resting or serum-starved HuH-7 cells (n = 3 independent experiments, 100 cells counted per condition). (G) Results of an anti-HA immunoblot from a GTP-agarose bead pulldown of HA-tagged Rab10Q68L or HA-tagged Rab10T23N. (H and I) Immunoblots of Rab10 showing a differential association with GTP beads in HuH-7 cells subjected under resting versus HBSS starvation conditions for 1 hour (H) or treated with DMSO (dimethyl sulfoxide) or 1 μM Torin1 for 1 hour (I). (J) Quantification of Rab10 protein levels from (H) and (I) (n = 3 independent experiments). Data are represented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. Scale bars, 1 μm.
interleukin-2 receptor functions as a Rab10 effector during the endocytic recycling of the structures observed on LDs. Recently, it was shown that EHBP1 er known Rab10 effectors modulate the biogenesis of the autophagic functions through the binding of effector proteins, we examined whether these proteins interact with Rab10 to promote autophagic turnover of LDs. Glutathione S-transferase (GST) pulldowns and Western blotting of Hep3B hepatoma cells expressing HA-EHBP1 showed that GST–Rab10 is able to pull down both HA-EHBP1 and endogenous EHD2 (Fig. 6A). Consistent with a Rab GTPase effector role for EHBP1, a GST–tagged subdomain (residues 600 to 902) of EHBP1 (Fig. 6B) is able to pull down the active Q68L form of Rab10 with greater affinity than the inactive T23N form (Fig. 6B). Autophagic induction by serum removal or Torin1 treatment, which activates Rab10 (Fig. 3, H and I), also increased the association between Rab10 and both EHBP1 (Fig. 6, C and D) and EHD2 (Fig. 6, E and F). The depletion of EHBP1 by siRNA treatment greatly reduced the associations between EHD2 and Rab10 (Fig. 6, G and H, and fig. S5), suggesting that EHBP1 is essential for the interaction between the Rab10 GTPase and the membrane-deforming ATPase. To determine whether EHBP1 and EHD2 colocalize with Rab10 at the LD surface under autophagy-stimulating conditions, we expressed HA-EHBP1 or GFP-EHD2 in serum-starved HuH-7 cells. Similar to Rab10, both proteins distribute to a structure in close proximity to the LD (Fig. 6, I and J). Cells subjected to EHD2-targeted siRNA knockdown contain similar numbers of GFP-Rab10 positive structures near LDs (Fig. 6K), indicating that Rab10 localizes to the LD independent of either EHBP1 or EHD2. In contrast, the depletion of Rab10 or EHBP1 by siRNA treatment significantly reduces the accumulation of EHD2 near LDs (Fig. 6L), indicative of a sequential recruitment of the three proteins to the surface of the LD.

**Activated Rab10 assembles together with membrane-remodeling adaptors at the LD-autophagic interface**

Because Rab10 appears to be recruited to the LD under serum starvation conditions, we tested whether this association is direct or via other membrane-adaptor proteins. Given that Rab GTPases exert their functions through the binding of effector proteins, we examined whether known Rab10 effectors modulate the biogenesis of the autophagic structures observed on LDs. Recently, it was shown that EHBP1 functions as a Rab10 effector during the endocytic recycling of the interleukin-2 receptor α chain (24). In addition, EHBP1 is known to regulate endocytosis together with the membrane-remodeling ATPase EHD2 (25). Therefore, we examined whether these proteins interact with Rab10 to promote autophagic turnover of LDs. Glutathione S-transferase (GST) pulldowns and Western blotting of Hep3B hepatoma cells expressing HA-EHBP1 showed that GST–Rab10 is able to pull down both HA-EHBP1 and endogenous EHD2 (Fig. 6A). Consistent with a Rab GTPase effector role for EHBP1, a GST–tagged subdomain (residues 600 to 902) of EHBP1 (Fig. 6B) is able to pull down the active Q68L form of Rab10 with greater affinity than the inactive T23N form (Fig. 6B). Autophagic induction by serum removal or Torin1 treatment, which activates Rab10 (Fig. 3, H and I), also increased the associations between Rab10 and both EHBP1 (Fig. 6, C and D) and EHD2 (Fig. 6, E and F). The depletion of EHBP1 by siRNA treatment greatly reduced the associations between EHD2 and Rab10 (Fig. 6, G and H, and fig. S5), suggesting that EHBP1 is essential for the interaction between the Rab10 GTPase and the membrane-deforming ATPase. To determine whether EHBP1 and EHD2 colocalize with Rab10 at the LD surface under autophagy-stimulating conditions, we expressed HA-EHBP1 or GFP-EHD2 in serum-starved HuH-7 cells. Similar to Rab10, both proteins distribute to a structure in close proximity to the LD (Fig. 6, I and J). Cells subjected to EHD2-targeted siRNA knockdown contain similar numbers of GFP-Rab10 positive structures near LDs (Fig. 6K), indicating that Rab10 localizes to the LD independent of either EHBP1 or EHD2. In contrast, the depletion of Rab10 or EHBP1 by siRNA treatment significantly reduces the accumulation of EHD2 near LDs (Fig. 6L), indicative of a sequential recruitment of the three proteins to the surface of the LD.

**Fig. 4. Rab10-positive LD-associated structures represent nascent autophagic organelles.** (A to E) Fluorescence images of Hep3B hepatoma cells comparing the colocalization of autophagic and organelle markers with LD-localized Rab10. Boxed areas represent regions of higher magnification. Cells were transfected for 24 hours with mCherry-Rab10 or sfGFP-Rab10, preloaded with 150 μM oleic acid overnight, serum-starved in HBSS for 1 hour, and stained with the LD dye monodansylpentane (MDP) and an antibody to LC3 (A) or transfected to express the autophagy marker GFP-Atg16L1 (B), Rab11 (C), the ER marker Sec61β (D), or the lysosomal marker LAMP1-mCherry (E). Inset number represents the frequency of LD-associated marker that colocalizes with Rab10 in HBSS-starved cells averaged from three independent experiments. Scale bars, 10 μm. (F) Subcellular density gradient fractionation of oleate-loaded Hep3B cells starved in HBSS for 2 hours, lysed (WCL), and further separated into a PNS, CLF, and HSS (high-speed supernatant). The CLF was subsequently loaded onto an 8 to 27% discontinuous iodixanol (OptiPrep) gradient for separation by ultracentrifugation. Nine fractions were collected from the top of the gradient and blotted for Rab10, a mitochondrial marker (COXIV), an endoplasmic reticulum marker (PDI), and the lysosomal resident protein LAMP1.
To confirm that Rab10 associates with the LD before the recruitment of EHD2, we performed fluorescence live-cell imaging of Rab10 and EHD2 colocalization with LDs in Hep3B cells (video S1). As depicted in time-lapse stills (Fig. 6M), mCherry-Rab10 (red) is first recruited to one pole of the LD surface, followed by the appearance of EHD2 (green). This subsequent colocalization between these two proteins increases over the next 5 min (total imaging time, 35 min).

The Rab10-EHBP1-EHD2 complex promotes autophagic engulfment of LDs

Because our evidence is consistent with EHD2 and EHBP1 associating with Rab10 as a complex at an autophagic interface on the LD surface, we next tested whether these proteins functionally contributed to the process of lipophagy. Hep3B cells were treated with siRNA for 48 hours to reduce EHD2 or EHBP1 levels [knockdown efficiency, >90%; representative blots are shown in Fig. 7 (A and B)] and subsequently serum-starved in medium containing 0.2% fetal bovine serum (FBS) for 48 hours. The depletion of EHBP1 or EHD2 resulted in an approximately two-fold increase in LD area retained per cell compared to control (Fig. 7C). Re-expression of wild-type EHBP1 was able to rescue LD breakdown inhibited by siEHBP1 treatment (fig.S6A). Likewise, re-expression of wild-type EHD2, but not an ATPase-dead mutant (EHD2-T72A), was able to restore LD turnover that had been inhibited by siEHD2 treatment (Fig. 7D). In an effort to disrupt complex stability, we also overexpressed a
Fig. 6. Serum starvation potentiates the formation of a Rab10 complex together with the membrane trafficking proteins EHBP1 and EHD2 at the lipophagic junction.

(A) Immunoblot analysis of Hep3B cells transfected to express HA-EHBP1, lysed, and subjected to a GST-Rab10 pulldown. (B) Immunoblot analysis of a GST pulldown experiment in Hep3B cells coexpressing a GST-tagged form of the Rab10-interacting domain of EHBP1 (residues 600 to 902) and HA-tagged Rab10-WT, Rab10-T23N, or Rab10-Q68L. (C and D) Representative immunoblots from pulldown experiments using the same GST-EHBP1 fragment to examine the effect of EHBP1 interactions with endogenous Rab10 after serum starvation (C) or treatment with Torin1 (D) (n = 3 independent experiments for each condition). Numbers below the pulldown blot represent the mean fold enrichment in protein levels. (E and F) Representative immunoblots from pulldown experiments in Hep3B cells, probing for interactions between Rab10 and EHD2 under serum-starved (E) or Torin1-treated conditions (F) (n = 3 independent experiments for each condition). Numbers below the pulldown blot represent the mean fold enrichment in protein levels. (G) Representative immunoblot of a GST-Rab10 pulldown of EHD2 in Hep3B cells previously treated with either siNT or siEHBP1 and subjected to HBSS starvation for 1 hour (n = 3 independent experiments), quantified in (H). (I) Fluorescence images of EHBP1-positive LDs in HuH-7 cells expressing HA-EHBP1 and starved in HBSS for 1 hour before fixation and immunostaining with anti-HA antibody (green). Cells were preloaded with 150 μM oleic acid overnight. LDs are stained with ORO (red). (J) Fluorescence image of a HuH-7 cell transfected to express GFP-EHD2 (green) and mCherry-Rab10 (red) before serum starvation in HBSS for 1 hour and fixation. The boxed region shows a higher-magnification image of an example of an MDH-stained LD (blue) that is also positive for GFP-EHD2 and mCherry-Rab10. Scale bars, 10 μm. (K and L) Quantification of the appearance of GFP-Rab10-positive LDs (K) or GFP-EHD2–positive LDs (L) in HuH-7 cells following siRNA-mediated knockdown of EHBP1 and EHD2 (K) or EHBP1 and Rab10 (L) for 48 hours, followed by serum starvation in HBSS for 1 hour. Results are from n = 3 independent experiments each and are represented as mean ± SD. **P ≤ 0.01; ***P ≤ 0.001. A total of 80 cells were quantified per condition. (M) Live-cell confocal fluorescence imaging of a starved Hep3B hepatoma cell coexpressing GFP-EHD2 and mCherry-Rab10, depicting the sequential recruitment of Rab10 and EHD2 to MDH-labeled LDs (blue). Note the presence of the mCherry-Rab10–positive structure at the periphery of the LD (arrowhead) before the recruitment of GFP-EHD2, resulting in the emergence of signal overlap by 35 min. Scale bars, 5 μm.
Fig. 7. EHD2 and EHBP1 are involved in Rab10-mediated LD breakdown. (A and B) Representative immunoblots showing the efficiency of a 48-hour EHD2 (A) or EHBP1 (B) siRNA–mediated knockdown in the Hep3B hepatoma cell line. (C) Quantification of the effect of EHD2 or EHBP1 knockdown on LD breakdown in Hep3B cells following 48-hour knockdown and 48-hour serum starvation. Average ORO-stained LD area (in pixels) per cell was calculated from $n = 3$ independent experiments in 100 cells per condition. Cells were preloaded with 150 μM oleic acid overnight. (D) The dependence of LD catabolism on EHD2 activity was examined by depleting Hep3B cells of EHD2 by siRNA treatment for 24 hours and then transfecting them with GFP alone, GFP–EHD2, or GFP–EHD2–T72A (ATPase-dead) for an additional 24 hours in the presence or absence of CQ. LD breakdown is represented as the average ORO-stained area per cell calculated from $n = 3$ experiments in 25 transfected cells per condition. Cells were preloaded with 150 μM oleic acid overnight. (E) Subcellular fractionation of oleate-loaded Hep3B cells starved for 2 hours in HBSS through a 0 to 30% discontinuous iodixanol (OptiPrep) gradient. Fractions were collected from the top of the gradient and blotted for EHD2, the endosomal marker Rab5, or the lysosomal marker LAMP1. (F) Representative fluorescence images of basal or serum-starved Hep3B cells treated with siNT, Rab10 siRNA, or EHD2 siRNA and expressing a dual-fluorescent red fluorescent protein (RFP)–GFP–PLIN2 construct that had been serum-starved for 24 hours to measure the appearance of “RFP-only” PLIN2-positive puncta, indicative of interactions between the LD and the acidic lysosomal compartment. Cells were preloaded with 150 μM oleic acid overnight. (G) Quantification of the number of “RFP-only” PLIN2-positive puncta per Hep3B cell, reflective of active lipophagy, from $n = 3$ independent experiments, measuring 22 transfected cells per condition. The data are represented as mean ± SD. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. Scale bars, 10 μm.
increase to 50% of the LDs in Rab10 KO cells. In contrast, 60% of LDs in control cells were engulfed by LAMP1-positive organelles, whereas only 20% of the LDs in Rab10 KO MEFs showed a similar phenotype (Fig. 8H). Together, these data suggest that the functional role of the Rab10-EHBP1-EHD2 complex may be to regulate autophagic membrane extension and promote the engulfment of LDs during lipophagy.

**Rab10 acts downstream of Rab7 to facilitate autophagic degradation of LDs**

We have recently reported a new function for Rab7 as a regulator of lysosomal and multivesicular body (MVB) recruitment to the LD surface in hepatocytes (9). To test whether Rab10 and Rab7 might perform similar or distinct functions to mediate lipophagy, we first examined Rab7 and Rab10 interactions with their known effector proteins. Rab7 was found to interact closely with its well-characterized effector Rab7-interacting lysosomal protein (RILP), but not with the Rab10 effector protein EHBP1 (Fig. 9A and fig. S8, A and B). In addition to an anticipated binding of EHBP1, Rab10 was also observed to interact with RILP (Fig. 9B and fig. S8, A and B). However, the Rab7-RILP interaction was unaffected by the presence of Rab10 because the binding efficiency between these two proteins appeared identical in both wild-type and Rab10 KO cells (Fig. 9C). Furthermore, these Rabs preferentially distribute to different compartments in the cell because subcellular fractionation experiments show peak distributions of Rab7 with LAMP1-positive fractions, whereas Rab10 distribution is similar to that of LC3, a classical marker of the phagophore and autophagosome (Fig. 9D).

In agreement with these biochemical findings, we were able to use fluorescently tagged Rab10 and Rab7 fusion proteins to define differences in LD association within cells undergoing starvation-induced lipophagy. We examined HuH-7 cells expressing Rab7-GFP before serum starvation and 2 hours following HBSS serum starvation, both in the context of treatment with nontargeting or Rab10-targeted siRNA. No difference in Rab7 localization at the LD surface was observed, regardless of whether Rab10 had been knocked down with siRNA (fig. S8, C and D). Using live-cell microscopy, we observed instances of Rab7-positive membranes extending from LDs that appeared to actively recruit incoming Rab10-positive membranes to the LD surface (Fig. 9E and video S4). Of those droplets containing either Rab10 or Rab7, nearly half were positive for both Rab10 and Rab7, indicating significant interplay between the two Rabs during lipophagy (Fig. 9F).

**DISCUSSION**

Here, we have identified a novel protein complex in hepatocytes that is activated by nutrient deprivation and responsible for the autophagic engulfment of LDs during lipophagy. The degradation of these energy-rich structures under nutritionally restricted conditions is dependent on the accumulation of catalytically active Rab10 at the “lipophagic junction” between the LD and nascent autophagic membrane (Figs. 1 and 2). Moreover, the pharmacological or starvation-induced initiation of cellular autophagic pathways enhances Rab10 activity (Figs. 3 and 4). This activation leads to the assembly of a bridging complex consisting of Rab10 and two binding partners at the LD interface with the autophagic machinery:

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the adaptor protein EHBP1 and the membrane-deforming ATPase EHD2 (Figs. 5 to 7). Functional assembly of this complex promotes the subsequent engulfment of LDs by degradative organelles (Figs. 8 and 9) for the purposes of energy production and cell survival.

Rab10 is a key participant in LD autophagy

Previously considered a nonspecific housekeeping process, macro-autophagy is now appreciated to have the ability to selectively target specific organelles and protein complexes under appropriate conditions. The molecular mechanisms underlying these individual types of selective autophagy, including mitophagy (26), reticulophagy (27), and pexophagy (28), are currently of intense interest. Despite an increased focus on understanding the numerous different types of selective autophagy, surprisingly little is known about the molecular targets coordinating the process of lipophagy (3).

Recent studies have found that Rab GTPases play a role in conventional lipolysis; for example, the knockdown of Rab7 reduces β-adrenergic receptor–stimulated lipolysis in adipocytes (29). In addition, overexpression of Rab18 down-regulates PLIN2 and facilitates lipolysis in HepG2 hepatoma cells (30). Moreover, depletion of Rab32

**Fig. 8. The Rab10-EHBP1-EHD2 complex mediates the engulfment of LDs by autophagic organelles.** (A) Live-cell confocal fluorescence microscopy of two distinct LDs (stained with MDH, blue) from Hep3B cells expressing either mCherry-Rab10 (top series) or GFP-Rab10 (lower series). Imaging reveals the association of LD-bound Rab10 at early time points with phagophore/autophagosome-associated Rab10 (0 s) extending to nearly surround the perimeter of LDs at later time points. Dashed outlines provide fiducial points of reference as the envelopment of the LD by the phagophore progresses. These events are representative of data from more than 30 individual cells examined by live-cell imaging. (B and C) Quantification of the percentage of LDs associated with LC3- or Atg16L1-positive structures in Hep3B hepatoma cells after 48-hour siRNA treatment with the indicated siRNAs (Tri-siRNA, triple knockdown). Cells were preloaded with 150 μM oleic acid overnight. (D and E) Quantification of the percentage of LDs associated with LC3- or Atg16L1-positive structures after culture in low-serum conditions in WT or Rab10 KO MEFs. Cells were preloaded with 400 μM oleic acid overnight. (F) Quantification of the percentage of LDs associated with LAMP-1-positive structures after 48-hour siRNA treatment followed by 48-hour starvation from n = 3 independent experiments, measuring 20 cells per condition. Cells were preloaded with 150 μM oleic acid overnight. (G and H) LDs visualized from WT or Rab10 KO MEFs were divided into three groups on the basis of their association with LAMP-1: “none,” “attached,” or “engulfed” (G). Manual counting of LDs (H) in each group from WT and Rab10 KO MEFs. The graphs represent observations from n = 3 independent experiments, measuring 20 cells per condition. Cells were preloaded with 400 μM oleic acid overnight. Data are represented as means ± SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Scale bars, 1 μm.
Fig. 9. Rab10 acts downstream of Rab7 to facilitate the autophagic degradation of LDs. (A and B) Immunoblot of GST-EHBP1– or GST-RILP–mediated pulldowns for Rab7 (A) or Rab10 (B) in Hep3B hepatoma cells. Rab7 exhibits a specific affinity for RILP, whereas Rab10 interacts with both EHBP1 and RILP. (C) Representative immunoblotting of the results for GST or GST-RILP pulldowns of Rab7 in WT or Rab10 KO MEFs, showing that Rab10 KO does not affect the binding of Rab7 to RILP. (D) Immunoblotting of subcellular density gradient fractions of Hep3B cells following serum starvation in HBSS for 2 hours, followed by flotation of a CLF through an 8 to 27% discontinuous OptiPrep gradient. Boxes indicate distinct peak density fractions for either Rab10 (blue box, fraction 2) or Rab7 (red box, fraction 4). (E) Live-cell confocal fluorescence microscopy of a HuH-7 hepatoma cell cotransfected with both GFP-Rab7 and mCherry-Rab10 and subjected to serum starvation. LDs are stained with MDH (blue). Arrows indicate the extension of a Rab7-positive membrane away from the LD and the subsequent recruitment of a Rab10-positive structure (arrowheads) to the LD. (F) Quantification of the average percentage of LDs positive for the presence of Rab10 alone, Rab7 alone, or both Rab10 and Rab7 from n = 10 cells. (G) Quantification of the average number of Rab10-positive LDs in resting or serum-starved HuH-7 hepatoma cells treated with NT siRNA or Rab7 siRNA. (H) Quantification of the average number of Rab7-positive LDs in resting or serum-starved HuH-7 cells treated with NT siRNA or Rab10 siRNA. Data represent the means from n = 3 independent experiments, measuring >50 cells per condition per repeat. ***P ≤ 0.001. Scale bars, 1 μm.
was shown to enhance ATGL expression and lipolysis in hepatocytes (31). Roles for Rabs in macroautophagy have been demonstrated in several studies. A functional Rab1, for example, is required for the initiation of autophagosomal biogenesis (32). Rab5 (33) and Rab7 (34) appear to regulate autophagosomal formation and promote the fusion of autophagosomes with lysosomes, whereas Rab11 promotes the fusion of multivesicular bodies with autophagic vacuoles (18). More recently, we demonstrated a novel role for Rab7 as a key mediator of lysosomal and autophagic dysfunction (40). Before the degradation of essential organelles, such as mitochondria or the ER, therefore, the activation of specific Rabs could have important LD-localization at the LD following starvation or treatment with autophagic inducers such as Torin1 (Fig. 3) suggest that the pool of Rab10 residing on the LD surface under resting conditions might be in an inactive state. However, in response to autophagic signaling, it is unclear whether inactive Rab10 is activated on the LD in situ (similar to Rab7) or whether newly activated Rab10 is instead recruited to the LD. One would predict that, under nutritional stress, it would be beneficial for the cell to initially harvest a triglyceride-containing structure like the LD as an energy source before the degradation of essential organelles, such as mitochondria or the ER. Therefore, the activation of specific Rabs could have important LD-targeting functions for the autophagic machinery.

It is important to add that other closely related Rab proteins may perform similar functions on the LD. For example, Rab8A and Rab10 have highly similar protein sequences (76% identity), underscoring their shared cellular functions. In adipocytes, both Rab10 (35) and Rab8A (36) mediate insulin-stimulated GLUT4 trafficking under the regulation of the same GTPase-activating protein (GAP) AS160. Most relevant is a recent study suggesting that Rab8A can mediate LD fusion and growth through an interaction with fat-specific protein 27 (Fsp27) (37). The depletion of Rab8A in Fsp27-overexpressing adipocytes reduces total LD volume. In contrast, our findings suggest that the depletion of Rab10 in serum-starved hepatocytes prevents the breakdown of LDs, resulting in an overall accumulation of LDs (Fig. 2B). It will be very interesting to test the function and contribution of Rab10 to lipophagy and lipid homeostasis in hepatocytes versus adipocytes because it appears that there may be very different roles for autophagy in liver and white adipose tissue lipid metabolism (38–42). In addition, it will be important to test whether Rab10 plays a specific role in lipophagy or whether it is also involved in other forms of selective autophagy, such as mitophagy.

The ubiquity of Rabs playing roles at multiple cellular compartments is intriguing. Multipurposing of GTPases is quite common: The conventional dynamin acts at clathrin pits, lamellapodia, focal adhesions, the trans-Golgi, autophagosomes, and the centrosome/midbody. The guanine nucleotide exchange factors (GEFs) and GAPs for Rabs are exceptionally diverse and provide distinct functional targeting for each Rab protein (43), whereas the Ras family of small GTPases, for example, were originally thought to act exclusively at the plasma membrane but are now appreciated to have important functions on a number of intracellular membranes (44). The same can now likely be said for the numerous Rab proteins that localize to the LD surface.

**A Rab10-EHBP1-EHD2 complex is essential for assembly and function of the lipophagic machinery**

Using biochemical (Fig. 6, C to F), morphological (Fig. 6, I and J), and live-cell imaging (Fig. 6M) experiments, we have observed that activated Rab10 exhibits an enhanced binding to both an effector protein (EHBP1) and an ATPase (EHD2) under autophagy-stimulating conditions, supporting the extension of an isolation membrane along the surface of the LD under conditions of nutrient deprivation. The component depletion studies (Fig. 6, K and L) suggest that Rab10 is essential for the formation of this complex because it resides on the surfaces of both LDs and autophagic organelles. Activation of Rab10 during cell starvation promotes the recruitment of the EHBP1 adaptor, required for the association of the EHD2 ATPase, as graphically depicted by live-cell
imaging (Fig. 6M). The participation of EHD2 in this complex is particularly intriguing because it shares many features with the dynamin superfamily, including its capacity to oligomerize along lipid tubules in ring-like structures (45) and actively remodel membranes in response to nucleotide hydrolysis. The use of this membrane-deforming enzyme seems particularly relevant to a process that would require substantial mechanochemical work to deform, stretch, and expand an autophagic membrane along the LD surface, as observed by fluorescence imaging (Figs. 1, G to I, and 8A) and EM (Fig. 5).

It is possible that this Rab-adaptor-ATPase complex could participate in numerous events related to lipophagocytosis, including the targeting, docking, or expansion/engulfment of the autophagic membrane along the surface of the spherical LD; alternatively, it could play important roles in all three events. In an attempt to identify the specific function of this complex, we have measured the frequency of associations between the LD and autophagic membrane markers LC3 and Atg16L1 (Fig. 8H) and the LD in Rab10 KO MEFs, and found fewer numbers of extended autophagic membranes to be associated with the LD. This suggests that the Rab-adaptor-ATPase complex is primarily involved in the regulation of LD engulfment by the autophagic membrane and that Rab10 depletion in the cell affects the “commitment” step in the autophagic engulfment of LDs during lipophagocytosis. Rab10-mediated direct interactions with LAMP1 may also reflect additional degradative mechanisms that are simultaneously occurring, such as microautophagy. These possibilities warrant further exploration.

In summary, these findings describe a novel complex that appears to mediate the interaction and engulfment of LDs by the autophagic machinery in cells exposed to nutrient stress. These observations further support an important role for autophagy in the utilization of stored cellular lipids but do not rule out a functional synergism between the action of soluble lipases and the lipophagic machinery. Future in vitro biophysical studies focusing on the mechanisms by which these components interact to deform the LD monolayer will prove informative.

MATERIALS AND METHODS

Cell culture and reagents

The HuH-7 cell line was a gift from the laboratory of G. Gores (Mayo Clinic, Rochester, MN). The Hep3B2.1-7 (Hep3B) and clone 9 cell lines were obtained from the American Type Culture Collection (HB-8064 and CRL-1439, respectively). Rab10 KO MEFs were isolated as detailed below. Primary pig hepatocytes were a gift from R. Hickey (Mayo Clinic). HuH-7 and Hep3B cells were maintained in minimum essential medium containing Earle’s salts and t-glutamine supplemented with 1 mM sodium pyruvate, nonessential amino acids, and 0.075% (w/v) sodium bicarbonate (Corning). Rab10 KO MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM). Clone 9 cells were maintained in F-12K medium. Primary pig hepatocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM). Clone 9 cells were maintained in F-12K medium. Primary pig hepatocytes were maintained in DMEM. The medium for all cell lines contained 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) (Life Technologies), and cells were grown at 37°C in 5% CO2. Cells were grown on acid-washed coverslips for fluorescence microscopy and in plastic tissue culture dishes for biochemical analyses. Cells were treated with 150 µM (Hep3B and clone 9) or 400 µM (Rab10 KO MEFs) oleic acid (Sigma-Aldrich) overnight before imaging and biochemical analyses.

The rabbit polyclonal Rab10 antibody was from Sigma-Aldrich (R8906). The mouse monoclonal Rab10 (4E2) antibody was from GeneTex (GTX82800). The antibody against EHD2 (EPR9821) was from Abcam (ab154784). The antibodies against EHHB1 were from Abcam (ab97752) and Proteintech Group (17637-1-AP). The mouse monoclonal LAMP1 (H4A3) antibody was from Santa Cruz Biotechnology Inc. (sc-20011), the LC3 and LC3B antibodies were from Novus Biologicals (NB100-2220 and NB600-1384, respectively), and the antibody against PLIN2 was from LifeSpan BioSciences Inc. (LS-B3121). The antibodies against PDI and COXIV (cytochrome C oxidase subunit IV) were from Cell Signaling Technology (2446 and 4844, respectively), and the antibody recognizing actin was from Sigma-Aldrich (A2066). Secondary antibodies used for immunofluorescence were conjugated to Alexa Fluor 350, 488, or 594 (Invitrogen), and cells were mounted for microscopy in ProLong antifade reagent (Invitrogen). Secondary antibodies for Western blot analysis were conjugated to horseradish peroxidase (HRP; Invitrogen). HRP-conjugated reagents for Western blot analysis were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

The autophagy inducer Torin1 was from Tocris (4247). Macroautophagy was inhibited by the addition of 3-MA (10 mM, Sigma-Aldrich), and lysosomal proteolysis was inhibited by the addition of CQ (25 µM) (46).

Constructs, siRNA, and transfection

sfGFP was a gift from the laboratory of E. L. Snapp (Albert Einstein College of Medicine). The gene encoding full-length wild-type rat Rab10 was inserted into pEGFP-C1, sfGFP-C1, and pmCherry-C3 (a gift from D. J. Katzmann, Mayo Clinic). Cloning vector pCMV-HA was obtained from Clontech, and pEGX-4T-1 was obtained from GE Healthcare. The active human Q68L or dominant-negative human T23N forms of Rab10 were cloned into pEGFP-C1 and pCMV-HA. EHD2 and EHHB1 were cloned from a HuH-7 complementary DNA (cDNA) library and inserted into pEGFP-C1 and pEGX-4T-1. The constructs encoding LAMP1, LC3, and Atg16L1 were obtained through amplification from a rat brain cDNA library or HuH-7 cDNA library and cloned into the pmCherry-N1 (Takara Bio Inc.) or pEGFP-C1 vectors. Lipofectamine Plus reagent (Invitrogen) was used for transfection, according to the manufacturer’s instructions. The siRNA oligos targeting Rab10, EHHB1, EHD2, Rab7, and Atg7 were purchased from Thermo Fisher Scientific, and cells were transfected with the RNAiMAX reagent (Invitrogen). For re-expression studies, cells were first treated with siRNA oligos for 24 hours and then subsequently transfected with the proper expression constructs.

Generation of Rab10−/− cell lines

Rab10−/+ mice were generated using a Rab10 Genetrap embryonic stem (ES) cell line from BayGenomics (#XC820). The gene trap vector insertion resulted in a truncated Rab10 mRNA comprising amino acids 1 of 40 of Rab10 fused to β-galactosidase and neomycin transferase (β-geo). Heterozygous mice were generated by the Australian Phenomics Network ES to Mouse service at Monash University. Mice were genotyped by polymerase chain reaction (PCR) and real-time PCR using the following primers within the gene trap vector: ggcgcggcgcgctttgcgg and ggagaagttgtctctctc. Rab10−/+ mice were used for timed matings, and embryos were isolated on embryonic day 14.5. Mouse embryonic fibroblasts were generated from Rab10−/+ and Rab10−/− embryos and immortalized by serial passaging, as described previously (47).

Fluorescence microscopy

Cells were fixed and processed for immunofluorescence using standard procedures, as described previously (48). Briefly, cells were rinsed in phosphate-buffered saline (PBS), fixed in 3% formaldehyde, and permeabilized in 0.1% Triton X-100 for 2 min. Coverslips were rinsed thoroughly
with PBS between treatments. Fixed samples were blocked in buffer containing 5% goat serum and serially incubated in the appropriate primary and secondary antibodies at 37°C in blocking buffer. Images were acquired using epifluorescence microscopes (Axio Observer and Axiosvert 200; Carl Zeiss MicroImaging) fitted with a 63× Carl Zeiss Plan APOCHROMAT oil objective [numerical aperture (NA), 1.4] at room temperature. Digital images were captured with cameras (Orca II-ERG and Orca II; Hamamatsu Photonics) using iVISION software (BioVision Technologies). Total LD area was measured using the autosegmentation tool within the iVision software. The numbers of Rab10/EHD2-positive crescents or red-only PLIN2-positive puncta were both counted manually. For confocal microscopy, images were acquired with a Zeiss LSM 780 confocal microscope (Carl Zeiss Microimaging). The objective used was a 63×/1.2-NA water immersion lens. For super-resolution microscopy, images were acquired by means of a Zeiss ELYTRA Superresolution microscope using structured illumination. All images and figures were assembled using Adobe Photoshop and Illustrator CC software (Adobe Systems).

**GST pulldown binding assay**

Cells were lysed in TEN100 lysis buffer [20 mM tris (pH 7.4), 0.1 mM EDTA, 100 mM NaCl, and cOmplete protease inhibitors (Roche)]. The purified GST-fused proteins and purified GST-free proteins from *Escherichia coli* were then incubated in cell lysate for 2 hours at 4°C. After washing the beads four times with NTEN300 buffer [20 mM tris (pH 7.4), 0.1 mM EDTA, 300 mM NaCl, 0.05% NP-40, and cOmplete protease inhibitors (Roche)], samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Desired proteins were detected by immunoblotting.

**GTP binding assay**

Cells subjected to starvation or Torin1 treatment were lysed in binding buffer [1 mM dithiothreitol, 20 mM Hepes (pH 8.0), 150 mM NaCl, 10 mM MgCl₂, and cOmplete protease inhibitors (Roche)]. GTP-agarose beads (Sigma-Aldrich) were prewashed three times with binding buffer and then incubated in cell lysate for 2 hours at 4°C, followed by a further two quick washes with binding buffer. After SDS-PAGE and immunoblotting, Rab10 was detected with an antibody against Rab10 (Sigma-Aldrich).

**Triglyceride measurement assay**

Lipid-loaded (400 μM oleic acid) wild-type and Rab10 KO MEFs were starved in DMEM + 0.2% FBS for 3 hours and homogenized in 100 μl 1× PBS containing 0.5% Tween 20. Cell lysates were then incubated at 70°C for 5 min, followed by spinning at 5000 rpm for 1 min at 4°C. The supernatant was transferred to a new tube and spun at 14,000 rpm for 5 min at 4°C. Triglycerides were determined with Infinity Triglyceride Reagent (Thermo Fisher Scientific), and proteins were measured with Pierce Protein BCA assay (Thermo Fisher Scientific). The normalization of triglyceride measurement was carried out by dividing triglyceride levels by protein levels.

**β-Oxidation assay**

Fatty acid oxidation was assessed on the basis of 3H₂O production from [9,10-3H]oleate. Cells were seeded into six-well plates. One day later, cells were incubated with 2 ml of DMEM/10% FBS medium containing BSA-complexed oleate [0.3 mM unlabeled plus [9,10-3H]oleate (1 μCi/ml)] overnight. Cells were then washed twice with PBS and incubated with 2.5 ml of DMEM/10% FBS or DMEM/0.2% FBS medium. After 6 hours, 0.5 ml of the medium was collected for measuring 3H₂O production.

**Statistical analysis and replication of experiments**

No statistical method was used to predetermine sample size; however, sample sizes used were based on similar experiments from previously published references. The experiments were not randomized, and the investigators were not blinded to the experimental allocations or assessments of experimental outcomes. Statistical comparisons between two conditions were assessed by using the two-tailed unpaired Student’s *t*-test, where the numerical *P* values are reported as mean ± SD and represent data from a minimum of three independent experiments. The representative images of cells and Western blots in Figs. 1 (D and E), 2 (A, B, F, and H), 3 (A to D and G to I), 6 (C to G), 7 (A, B, and F), and 9 (A to C) are examples of multiple similar fields and blots obtained in experiments that were replicated at least three times (the number of replicates are provided in the figure legends).

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/12/e1601470/DC1

fig. S1. Rab10 localizes to the LD surface during serum starvation in various hepatocyte cell models.

fig. S2. Rab10 knockdown results in LD accumulation in multiple cell types.

fig. S3. Rab10-driven autophagy of LDs is dependent on upstream autophagic initiation.

fig. S4. Additional EM of Rab10-associated autophagic-LD interactions.

fig. S5. Serum starvation potentiates the formation of a Rab10 complex together with the membrane trafficking proteins EPHB1 and EHD2 at the lipophagic junction.

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REFERENCES AND NOTES

1. Y. Guo, K. R. Cordes, R. V. Farese Jr., T. C. Walther, Lipid droplets at a glance. J. Cell Sci. 122, 749–752 (2009).
2. T. C. Walther, R. V. Farese Jr., Lipid droplets and cellular lipid metabolism. Annu. Rev. Biochem. 81, 678–714 (2012).
3. R. Singh, S. Kaushik, Y. Wang, Y. Xiang, I. Novak, M. Komatsu, K. Tanaka, A. M. Cuervo, R. Singh, S. Kaushik, Y. Wang, Y. Xiang, I. Novak, M. Komatsu, K. Tanaka, A. M. Cuervo, K. Liu, M. J. Czaja, Regulation of lipid stores and metabolism by lipophagy. J. Proteome Res. 6, 3256–3265 (2007).
4. P. Liu, R. Bartz, J. K. Zehmer, Y.-s. Ying, M. Zhu, G. Serrero, R. G. W. Anderson, Rab regulated interaction of early endosomes with lipid droplets. Biochim. Biophys. Acta 1773, 784–793 (2007).
5. A. R. English, G. K. Voeltz, Rab10 GTPase regulates ER dynamics and morphology. Nat. Cell Biol. 15, 169–178 (2013).
6. P. Liu, Y. Ying, Y. Zhao, D. I. Mundy, M. Zhu, R. G. W. Anderson, Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane trafficking. J. Biol. Chem. 279, 3787–3792 (2003).
7. S. Sato, M. Fukasawa, Y. Yamakawa, T. Natsume, T. Suzuki, I. Shiho, H. Aizaki, T. Miyamura, M. Nishijima, Proteomic profiling of lipid droplet proteins in hepatoma cell lines expressing hepatitis C virus core protein. J. Biochem. 139, 921–930 (2006).
8. C. T. Chereau, S. A. Kang, J. Wang, Q. Liu, Zhang, Y. Gao, L. J. Reichling, T. Sim, D. M. Sabatini, N. S. Gray, An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J. Cell Biol. 284, 8023–8032 (2009).
9. M. E. Maerker, J. A. Smith, B. A. Findlay, J. H. Grubmuller, M. K. W. Berman, P. E. Rieke, P. P. Pech, M. R. Schindler, A. McClelland, S. S. Pasha, G. A. Kirby, M. Foster, Z. Ahmed, J. E. Morton, D. Williams, J. G. M. R. Dobbsy, D. Musgrove, J. R. Ainsworth, P. Gissen, F. Muller, E. R. Maher, F. A. Barr, I. A. Alistair, Loss of function mutations in Rab10 cause Warburg microbead deficiency syndrome. J. Biol. Chem. 280, 2039–2045 (2005).
10. C. R. Smith, J. A. Smith, B. A. Findlay, J. H. Grubmuller, M. K. W. Berman, P. E. Rieke, P. P. Pech, M. R. Schindler, A. McClelland, S. S. Pasha, G. A. Kirby, M. Foster, Z. Ahmed, J. E. Morton, D. Williams, J. G. M. R. Dobbsy, D. Musgrove, J. R. Ainsworth, P. Gissen, F. Muller, E. R. Maher, F. A. Barr, I. A. Alistair, Loss of function mutations in Rab10 cause Warburg microbead deficiency syndrome. J. Biol. Chem. 280, 2039–2045 (2005).
11. C. T. Chereau, S. A. Kang, J. Wang, Q. Liu, Zhang, Y. Gao, L. J. Reichling, T. Sim, D. M. Sabatini, N. S. Gray, An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J. Cell Biol. 284, 8023–8032 (2009).
12. Li et al. 2016, Science Advances, 2. e1601470, 16 December 2016
48. H. Cao, F. Garcia, M. A. McNiven, Differential distribution of dynamin isoforms in mammalian cells. Mol. Biol. Cell 9, 2595–2609 (1998).

49. R. A. Iglesias, P. Wang, R. A. Coleman, Triacsin C blocks de novo synthesis of glycerolipids and cholesterol esters but not recycling of fatty acid into phospholipid: Evidence for functionally separate pools of acyl-CoA. Biochem. J. 324, 529–534 (1997).

50. J. L. Ramírez-Zacarias, F. Castro-Muñozledo, W. Kuri-Harcuch, Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. Histochemistry 97, 493–497 (2002).

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