Lipid droplets (LDs) are lipid storage organelles that in hepatocytes may be catabolized by autophagy for use as an energy source, but the membrane-trafficking machinery regulating such a process is poorly characterized. We hypothesized that the large GTPase Dynamin 2 (Dyn2), well known for its involvement in membrane deformation and cellular protein trafficking, could orchestrate autophagy-mediated LD breakdown. Accordingly, depletion or pharmacologic inhibition of Dyn2 led to a substantial accumulation of LDs in hepatocytes. Strikingly, the targeted disruption of Dyn2 induced a dramatic four- to fivefold increase in the size of autolysosomes. Chronic or acute Dyn2 inhibition combined with nutrient deprivation stimulated the excessive tubulation of these autolysosomal compartments. Importantly, Dyn2 associated with these tubules along their length, and the tubules vesiculated and fragmented in the presence of functional Dyn2. These findings provide new evidence for the participation of the autolysosome in LD metabolism and demonstrate a novel role for dynamin in the function and maturation of an autophagic compartment.

Introduction

Autophagy is a catabolic process by which cytoplasmic material is recycled in a lysosome-mediated manner for the purposes of energy production and survival in response to cell stress. An important energy reservoir common to most cells is the cytoplasmic lipid droplet (LD), a triglyceride- and cholesterol-rich organelle that sequesters esterified lipid into a readily accessible source of substrates for numerous physiological processes (Guo et al., 2009; Walther and Farese, 2012). Recently, hepatocytes have been shown to engage the autophagic machinery to support the breakdown of LDs under nutrient-limiting conditions (Singh et al., 2009; Walther and Farese, 2012). Lipid droplets (LDs) are lipid storage organelles that in hepatocytes may be catabolized by autophagy for use as an energy source, but the membrane-trafficking machinery regulating such a process is poorly characterized. We hypothesized that the large GTPase Dynamin 2 (Dyn2), well known for its involvement in membrane deformation and cellular protein trafficking, could orchestrate autophagy-mediated LD breakdown. Accordingly, depletion or pharmacologic inhibition of Dyn2 led to a substantial accumulation of LDs in hepatocytes. Strikingly, the targeted disruption of Dyn2 induced a dramatic four- to fivefold increase in the size of autolysosomes. Chronic or acute Dyn2 inhibition combined with nutrient deprivation stimulated the excessive tubulation of these autolysosomal compartments. Importantly, Dyn2 associated with these tubules along their length, and the tubules vesiculated and fragmented in the presence of functional Dyn2. These findings provide new evidence for the participation of the autolysosome in LD metabolism and demonstrate a novel role for dynamin in the function and maturation of an autophagic compartment.

Lipid droplet breakdown requires Dynamin 2 for vesiculation of autolysosomal tubules in hepatocytes

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perturbation, vesiculation resumes from the ALR tubules. We hypothesize that Dyn2 normally participates in the liberation of protolysosomes from autolysosomal tubules. These results suggest a novel, central, and direct role for this mechanoenzyme in the maintenance of lysosomal homeostasis and the autophagic clearance of hepatic lipid content.

Results

Knockdown of Dyn2 reduces starvation-mediated breakdown of LDs

Nutrient limitation is known to promote hepatic LD breakdown for use of free fatty acids as a cellular energy source (Singh et al., 2009). To assess a potential role for Dyn2 in LD catabolism, we first applied a knockdown/re-expression approach in cultured Hep3B hepatocellular carcinoma cells. Cells were depleted of Dyn2 by siRNA treatment, lipid-loaded overnight with 150 µM oleic acid to promote LD formation, and then starved in low-serum media (containing 0.1% FBS) for 48 h. Lipid loading and LD breakdown was assessed using Oil Red O staining. Cell boundaries are outlined and those cells re-expressing GFP, GFP-wDyn2, or GFP-K44A are denoted with asterisks. Bars, 20 µM. (B) Representative blot showing the efficiency of the Dyn2 knockdown in these cells. (C and D) Quantitation of the average LD number and area (in pixels²) per cell from three independent experiments. The data are represented as mean ± SE. *, P ≤ 0.05; **, P ≤ 0.01. NS, not significant.
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that were first oleate-loaded overnight and subsequently starved in the presence of various Dyn2 inhibitors or DMSO as indicated. Representative images of inhibitor-treated and control cells (stained with Oil Red O) are shown in A and B together with the quantitation of the average LD area per cell from three independent experiments. Pharmacological inhibitors used were: Dynasore (inhibits Dyn2 GTPase activity), MiTMAB (targets PH domain and interferes with membrane binding), Dynole 34-2 (allosteric GTPase inhibitor), and Dynole 31-2 (negative control for Dynole 34-2). Bars, 20 µM. (C) Representative images from control and Dyn2 knockout MEFs after an overnight lipid loading with 400 µM oleate for 17 h. Knockout of Dyn2 was induced by treatment with 2 µM 4-hydroxy-tamoxifen for 7 d and was confirmed by immunostaining of endogenous Dyn2 (top row) and by immunoblot (D). Bars, 20 µM. (E and F) Average LD number (E) and area (f) per cell from five independent experiments. All data are represented as mean ± SE. *, P ≤ 0.05; **, P ≤ 0.01. (G) Whole-cell lysates and LD fractions isolated from HuH-7 hepatocytes under resting or starved (2 h HBSS starvation) conditions. (H) Primary hepatocyte expressing Dyn2-GFP, showing an absence of colocalization with the LD surface (stained with Oil Red O). Bar, 20 µM. Inset shows magnification of boxed region [bar, 2 µM].

(Fig. 1 A; Fig. S1, A–C). Whereas the 48-h starvation significantly reduced the LD content of siNT-treated cells, LD breakdown was greatly impacted by Dyn2 depletion. Knockdown of Dyn2 (knockdown efficiency >90%, shown in a representative blot in Fig. 1 B) resulted in an approximately twofold increase in both the average LD number per cell as well as average LD area per cell compared with control cells (Fig. 1, C and D, respectively). Importantly, re-expression of GFP-tagged wild-type Dyn2 (GFP-Dyn2) rescued this phenotype, whereas the GTPase-defective Dyn2-K44A mutant did not (Fig. 1, A, C, and D). In general, we observed that control cells reduced their lipid content under these experimental conditions by ~60%, whereas Dyn2-depleted cells exhibited only a ~10–30% reduction in LD area and number, respectively (Fig. S1 C). Taken together, these data suggest that an active Dyn2 enzyme plays an important role in LD breakdown.

To further test these findings, pharmacological inhibitors of Dyn2 were also applied to HuH-7 and Hep3B hepatocytes that were first oleate-loaded overnight and subsequently starved in the presence of various Dyn2 inhibitors for 48 h. Interestingly, only the drugs interfering with the Dyn2 GTPase activity (Dynasore and Dynole 34-2) resulted in a reduction in LD breakdown in HuH-7 cells, whereas MiTMAB and the Dynole 31-2 negative control had no effect (Fig. 2 A). Similar results were obtained in Hep3B cells (Fig. 2 B). These data further support a role for Dyn2 activity in LD breakdown.

As an additional method to examine the role for Dyn2 in LD metabolism, we took advantage of the inducible Dyn2 knockout fibroblast cell line that was developed by Ferguson, de Camilli, and colleagues (Ferguson et al., 2009). In this cell line, Cre recombinase–mediated knockout of Dyn2 is induced by incubating cells with 4-hydroxy-tamoxifen for ~7 d, monitored by immunofluorescence (Fig. 2 C, top row) and Western blotting (Fig. 2 D). Interestingly, when cells were loaded for 17 h with 400 µM oleate, a significant increase in both the average LD
number and total LD area in the Dyn2-depleted cells was observed (Fig. 2 C, bottom row; and Fig. 2, E and F). In summary, three independent approaches (knockdown, pharmacological inhibition, and knockout) indicate a role for the large GTPase Dyn2 in hepatic LD metabolism.

As a result of our findings described above, we tested if Dyn2, based on its well-known mechanochemical ability to deform membranes, might participate directly in LD breakdown by promoting tubulation and/or scission of the LD lipid monolayer. This would result in an increased number of smaller vesicles and total surface area available to cytosolic lipases such as adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). Inconsistent with this premise, however, Dyn2 did not co-purify with LD fractions isolated by density gradient centrifugation of HuH-7 cell lysates (Fig. 2 G). Further, it did not appear to associate directly with the LD surface, as expression of GFP-tagged Dyn2 in primary hepatocytes demonstrated localization to membranous compartments surrounding or adjacent to LDs, suggesting association with a proximal organelle (Fig. 2 H).

Dyn2 inhibition dramatically alters the morphology and function of autophagic compartments

Hepatocytes are believed to use lysosomal components to drive an autophagy-based degradation of LDs (Singh et al., 2009; Dong and Czaja, 2011). As cells with impaired Dyn2 function exhibited a compromised breakdown of LDs under conditions of nutrient deprivation, we tested for indirect effects on lipolysis via alterations of lysosome function. Interestingly, Hep3B hepatocytes subjected to siRNA-mediated Dyn2 knockdown exhibited an exceptionally graphic redistribution of lysosomes into significantly larger membranous, juxtanuclear clusters, in contrast to control cells (Fig. 3, A–D; Fig. S2). These LAMP1-stained lysosomal aggregates also co-labeled with the autophagic marker LC3 and were similar to the persistent structures observed when autophagy is blocked at the autolysosomal stage (Rong et al., 2012). Consistent with an autophagic block in these cells is the observation that Dyn2 inhibition by siRNA knockdown or treatment with Dynasore in resting cells resulted in a four- to fivefold increase in levels of LC3-II, a classical marker of autophagosome biogenesis (Fig. 3, E and F, control lanes). To test if this LC3 increase represented an induction of the autophagic process in Dyn2-perturbed cells or, alternatively, a block in autophagic compartments of less than 1 µM in diameter (arrowheads). Dyn2 knockdown, however, induced the formation of much larger autophagosomes or autolysosomes (Fig. 4 B), in accordance with immunofluorescence images (insets), or those shown in Fig. 3. In the knockdown condition (Fig. 4, B–D), we observed a 10-fold increase (Fig. 4 E) in the number of large, dense, amorphous structures with diameters well in excess of 2 µM in size (~5 times larger than those present in control cells). Many of these organelles contained large membranous voids reminiscent of engulfed LDs (asterisks). Together, these findings suggest that Dyn2 plays an essential role in LD breakdown via a participation in lysosomal dynamics that, in turn, contribute to the autophagic process.
Dyn2 inhibition significantly impairs autophagic lysosomal reformation

To pursue the concept that Dyn2 inhibition might affect lysosomal function and hepatocyte breakdown of LDs, cells were stimulated to metabolize stored lipids by being subjected to a 24-h period of nutrient deprivation/starvation in the context of Dyn2 inhibition. Cells were then fixed and lysosomal compartments were visualized by immunostaining with antibodies to LAMP1. Under starvation conditions, untreated cells would be expected to display autolysosomal compartments with small tubular protrusions that represent the de novo formation of nascent lysosomes used for autophagy (Yu et al., 2010). Depletion of clathrin or PI(4,5)P₂ prevents the budding of these protrusions (Rong et al., 2012), resulting in the formation of aberrantly long tubules accompanied by a cessation of autophagy. We also observed the formation of remarkably long LAMP1-positive tubules in starved cells in which Dyn2 was knocked down as compared with control cells. Values represent the fold-increase in the number of autophagic structures of a given diameter (measured in microns) observed over siNT-treated control cells. Data were obtained from a single experiment with n = 6 cells examined by electron microscopy from each condition.

**Figure 4.** Dyn2 knockdown results in the formation of enlarged autophagic structures. (A–D) Transmission electron micrographs (TEMs) of oleate-loaded Hep3B hepatocytes treated with nontargeting control (siNT) or Dyn2 (siDyn2) siRNA for 72 h. Bars: (A and B) 2 µm; (A’ and B’) 1 µm; (C and D) 0.5 µm. Insets in A and B show fluorescent micrographs of LAMP1-stained cells (bars, 10 µM). Control cells (A) contain an abundance of small (<1 µm) electron-dense lysosomes and autolysosomes (arrowheads). Under conditions in which Dyn2 expression is suppressed (B–D), far fewer small lysosomes are observed. Instead, the cells are populated by larger autolysosomes (≥1 µm) with aberrant morphologies and containing putative LDs (*). (E) Quantitative measure of autolysosomal size from cells in which Dyn2 was knocked down as compared with control cells. Values represent the fold-increase in the number of autophagic structures of a given diameter (measured in microns) observed over siNT-treated control cells. Data were obtained from a single experiment with n = 6 cells examined by electron microscopy from each condition.
Based on the experimentation described above using siRNAs to reduce Dyn2 levels, it appears that this enzyme participates in LD breakdown indirectly by supporting lysosomal dynamics and biogenesis. A caveat of these findings is the duration (24–72 h) required for an siRNA knockdown, potentially resulting in either indirect or off-target effects. To circumvent this issue, we again used the pharmacological inhibitor Dynasore to provide a rapid, acute, and reversible block of Dyn2 function and assessed the consequences on lysosomal function. Hep3B hepatocytes expressing LAMP1-mCherry were plated onto glass-bottomed imaging dishes, starved in HBSS for 2 h, and treated with either DMSO or 40 µM Dynasore for 30 min. Still images (Fig. 6, A–D) from movies of live cells (Fig. 5 F). Indeed, some of these aberrant tubules exceeded 30 µM in length. Ultrastructural viewing by TEM revealed that these electron-dense tubules emanate from autolysosomes, and possess numerous varicosities at regularly spaced intervals along their length (Fig. 5, G and H), suggestive of membrane constrictions with defects in the final scission event. To confirm that these electron-dense structures that generated the extensive tubules were components of the late endocytic pathway, we pretreated cells with the fluid phase marker HRP, which is chased exclusively to late endosomes and lysosomes, before fixation and embedding for EM. As shown in thick EM sections (Fig. S3), tubules staining positive for HRP clearly originate from large autolysosomes in cells treated with Dyn2 siRNA.

Based on the experimentation described above using siRNAs to reduce Dyn2 levels, it appears that this enzyme participates in LD breakdown indirectly by supporting lysosomal dynamics and biogenesis. A caveat of these findings is the duration (24–72 h) required for an siRNA knockdown, potentially resulting in either indirect or off-target effects. To circumvent this issue, we again used the pharmacological inhibitor Dynasore to provide a rapid, acute, and reversible block of Dyn2 function and assessed the consequences on lysosomal function. Hep3B hepatocytes expressing LAMP1-mCherry were plated onto glass-bottomed imaging dishes, starved in HBSS for 2 h, and treated with either DMSO or 40 µM Dynasore for 30 min. Still images (Fig. 6, A–D) from movies of live cells...
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observed to undergo scission events, resulting in the release of nascent vesicles or putative protolysosomes (Fig. 6 G; Videos 3 and 4). This vesiculation process was particularly exciting as it suggested that drug-induced autolysosomal hypertubulation is a readily reversible phenomenon once Dyn2 function has been restored. Comparative analysis of movie stills taken at the beginning and end of a 45-min drug washout period showed a nearly 55% reduction in total LAMP1-positive tubule content (Fig. 6, H and I), reflecting the fragmentation process. Together, these findings demonstrate an essential role for Dyn2 activity in autolysosomal tubule disassembly and the maintenance of lysosomal homeostasis.

(Videos 1 and 2) show that, as expected, short-term starvation in HBSS with DMSO resulted in the appearance of punctate LAMP1-positive compartments (Fig. 6 A) that occasionally formed short tubular extensions (Fig. 6 B; Video 1). In contrast, the cells incubated in the presence of Dynasore for just 30 min exhibited numerous dynamic tubules emanating from enlarged LAMP1-positive compartments (Fig. 6, C and D; Video 2), similar to those observed with siRNA-mediated inhibition of Dyn2 expression (Fig. 5, C and D). These tubules were dynamic yet persistent, and importantly, upon drug washout with full-serum media, developed small varicosities or buds along their lengths (Fig. 6, E and F, arrows). Some tubules were actually observed to undergo scission events, resulting in the release of nascent vesicles or putative protolysosomes (Fig. 6 G; Videos 3 and 4). This vesiculation process was particularly exciting as it suggested that drug-induced autolysosomal hypertubulation is a readily reversible phenomenon once Dyn2 function has been restored. Comparative analysis of movie stills taken at the beginning and end of a 45-min drug washout period showed a nearly 55% reduction in total LAMP1-positive tubule content (Fig. 6, H and I), reflecting the fragmentation process. Together, these findings demonstrate an essential role for Dyn2 activity in autolysosomal tubule disassembly and the maintenance of lysosomal homeostasis.

Figure 6. Acute inhibition of Dyn2 reversibly disrupts autophagic lysosomal reformation (ALR) and lysosomal tubule scission. (A–D) Still frames from time-lapse movies of Hep3B cells expressing LAMP1-mCherry. Cells were starved for 2 h in HBSS and subsequently treated for 30 min with either DMSO (A and B) or 40 µM Dynasore (C and D), which induced extensive tubulation of LAMP1-positive compartments. Bars (A–D): 20 µM; (A’–B’) 2 µM; (C’–D’) 10 µM. (E–G) To demonstrate the reversibility of this tubulation, Dynasore-treated cells were washed extensively with drug-free media containing 10% FBS and monitored by time-lapse microscopy for 45 min. Frequently, after drug washout, LAMP1-positive tubules exhibited noticeable varicosities (E and F, arrows; bars, 10 µM) along their length. These sites are suggestive of areas of scission and resumed budding of nascent protolysosomes from the reforming tubules (G; bars, 10 µM). (H) Tubules from cells undergoing drug washout were quantified by tracing their lengths at the beginning and end of these movies. Still frames from a representative movie show tubule content at t = 10 and 45 min after drug washout. Bars, 20 µM. (I) Analysis of five independent movies showed an average decrease in total tubulation of ~50% after drug washout. Data represent the average relative change in total tubule length between the first and last frames of the time-lapse movies. Error bars represent SE; *, P < 0.05.
fractions also contained significant levels of Dyn2, suggestive of an intimate association with the lysosomal compartment. Additional Western blotting of these fractions for a variety of subcellular components (Fig. 7 B) suggests that Dyn2 is enriched in the lysosome fractions compared with those containing markers of other organelles such as mitochondria (COXIV) or the endoplasmic reticulum (PDI). Although these fractionation experiments can only be supportive of a Dyn2 association with lysosomes, as cross-contamination of cell components by these methods is an issue, they are consistent with both the functional findings described above and the fluorescence imaging observations described below.

Using fluorescence live-cell imaging (Fig. 7, C and C’, Videos 5 and 6) and immunostaining (Fig. 7 C’’), we examined the distribution of cytoplasmic Dyn2 in relationship to Dyn2 localizes to the autolysosomal compartment and participates in ALR
With the observation that Dyn2 has a direct role in the generation and disassembly of tubulated autolysosomes, it became important to determine whether Dyn2 is physically localized to the site of action. To this end, Hep3B hepatocytes were subjected to a subcellular fractionation approach to enrich for intact lysosomes. After a 2-h HBSS starvation and treatment with 40 µM Dynasore for 30 min, the same conditions used to induce autolysosomal tubulation in Fig. 6, C and D, autolysosomes were enriched by ultracentrifugation of a crude lysosomal fraction through an iodixanol gradient. Subsequent assays for acid phosphatase activity and immunoblotting analysis of gathered fractions with an antibody against LAMP1 revealed specific fractions (1–3) that were enriched in lysosomal content (Fig. 7 A). These same fractions also contained significant levels of Dyn2, suggestive of an intimate association with the lysosomal compartment. Additional Western blotting of these fractions for a variety of subcellular components (Fig. 7 B) suggests that Dyn2 is enriched in the lysosome fractions compared with those containing markers of other organelles such as mitochondria (COXIV) or the endoplasmic reticulum (PDI). Although these fractionation experiments can only be supportive of a Dyn2 association with lysosomes, as cross-contamination of cell components by these methods is an issue, they are consistent with both the functional findings described above and the fluorescence imaging observations described below.
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**Discussion**

In this study, we have identified an important new role for Dyn2 in the autophagic degradation of hepatic LDs. Using a combination of genetic and pharmacological approaches that inhibit the function of Dyn2, we demonstrate that these perturbations alter the dynamics of the autolysosomal compartment (Figs. 3 and 4), leading to excessively long lysosomal tubules (Figs. 5–7). In hepatocytes, continued regeneration of the lysosomal pool is vital for the regulated mobilization of lipid stores in times of energetic deficit. We therefore propose a model (Fig. 8) that reflects the central importance of Dyn2 in hepatic lysosome function as it relates to lipophagy, based on the observable defects arising from Dyn2 depletion or inhibition during nutrient deprivation. Under these conditions, the ongoing liberation and formation of nascent lysosomes from the autophagosomal/autolysosomal compartment is compromised, resulting in enlarged LAMP1-positive organelles, rendering the cell unable to further catabolize LDs by lipophagy.

**Dyn2 as a mediator of late endosomal traffic**

Dyn2 has been implicated in vesicle budding from the late endosome via a direct interaction with the endocytic adaptor...
resent points of membrane constriction and scission not unlike tent with the regularly spaced varicosities we observed by electron lysosomal tubules (Fig. 6 E) during the drug washout are consis
tently that Dyn2 is also recruited to and functions on this late endomembrane platform.

Similar to the requirement of Dyn2-mediated GTP hydrolysis at the plasma membrane to promote scission during endo
cytosis (Warnock et al., 1997), we demonstrate that the same holds true in the context of the autolysosomal reformation tubule. Indeed, pharmacological inhibition of Dyn2 enzymatic activity significantly hindered LD breakdown in cultured hepato
cytes (Fig. 2). The use of various pharmacological inhibitors of dynamin has the advantage of providing insights into the molecu
lar mechanism of Dyn2 interactions with the autolysosomal membrane. It is surprising to us that MiTMAB, which targets the pleckstrin homology domain of Dyn2 and inhibits membrane binding, does not seem to affect LD catabolism. Our finding that Dynole 34-2 (another pharmacological inhibitor of Dyn2 GTPase function), like Dynasore, does have an effect seems to suggest that Dyn2 nucleotide hydrolysis is paramount in lysosome reformation, and that membrane binding might be dispensable. An alternative explanation for why no effect was seen with MiTMAB may be due to the low concentration (2 µM) used during the 48-h period of drug treatment for this particular experiment. This low concentration was used to avoid toxicity is
sues in the hepatocyte cell lines tested (concentrations >4 µM were not tolerated for these experiments). Future analysis with other members of the MiTMAB series of inhibitors, such as OcTMB or PH domain mutants of Dyn2, may provide valu
able insights into the molecular mechanism of Dyn2 inter
actions with the autolysosomal membrane. Knockdown of Dyn2 in a non-hepatocyte cell line (Fig. 2 C) also demonstrated aberrant lipid accumulation in the absence of starvation, likely reflecting defects in basal LD turnover.

Importantly, the use of acute pharmacological inhibition complements the siRNA-based observations and reinforces the hypothesis that the observed autolysosomal tubulation is a direct consequence of inhibiting Dyn2 activity and not a response to in
hibiting the scission of clathrin-coated pits from the plasma mem
brane over a 1–3-d period during siRNA knockdown or expression of mutant Dyn2 constructs. Indeed, LAMP1-positive tubule for
mation can be observed after just 30 min of treatment with rela
tively low concentrations of the drug. Of equal importance is the near-complete reversibility of the tubulation phenotype that can be observed as soon as 30 min after media replacement. Interest
ingly, the evenly spaced puncta that form along the length of the lysosomal tubules (Fig. 6 E) during the drug washout are consist
tent with the regularly spaced varicosities we observed by electron microscopy (Fig. 5, G and H), and suggest that these sites rep
resent points of membrane constriction and scission not unlike those observed at the cell surface by a variety of different groups. Further, the localization of GFP-Dyn2 to these structures both biochemically and morphologically (Fig. 7) provides an impor
tant correlation between this novel distribution and function. Finally, thick sections of siDyn2-treated cells that were first al
lowed to endocytose HRP for several hours to label late endocytic compartments verified that these specific tubules are emanating from an endomembrane and not the PM (Fig. S3).

How Dyn2 is conditionally recruited to the autolysosomal membrane during nutrient starvation to participate in ALR re
mains unclear and will require further investigation. Under nor
mal growth conditions, the formation of and budding from ALR tubules occurs rapidly. Very few instances of LAMP1-positive compartments with persistent tubule outgrowths are evident at steady-state; in these circumstances, Dyn2 may only be tran
siently associated with the tubules. Such short-lived interactions might provide an explanation for previous difficulties in the iden
tification and localization of scission machinery at the autoly
sosomal compartment (Sridhar et al., 2013). The finding that clathrin and the AP2 adaptor complex are physically located on the ALR tubules, however, suggests that their Dyn2-mediated scission is very similar to, if not an extension of, events occurring at the PM.

Cellular metabolism and Dyn2 function
Although numerous Dyn2 mutations have been linked to several diseases including centronuclear myopathy (CNM) or Charcot
Marie-Tooth (CMT) disease, no human Dyn2 mutations have been shown to directly result in hepatic lipid accumulation. A re
cent publication has demonstrated a role for a particular Dyn2-
CNM mutation in autophagy in newborn mice, which resulted in a significant accumulation of hepatic glycogen content (Durieux et al., 2012). It is now known that autophagic glycogenolysis is especially critical to glucose homeostasis in newborn animals (Kotoulas et al., 2006). Chemical inhibition of Dyn2 by Dynasore was recently shown to impair cholesterol trafficking in HeLa cells and macrophages (Girard et al., 2011). In that study, treat
ment of cells with Dynasore resulted in the formation of enlarged LAMP1-positive lysosomal structures (as we have also observed here) and was shown to prevent the normal efflux of free cholesterol from endolysosomal compartments to the ER. It is also worth noting that hepatocytes in which Dyn2 function is compromised also appear to possess a greater number of lipid-containing autolysosomes (Fig. 4, B–D; and Fig. S3 C), as if the LDs have progressively accumulated in these defective structures over time.

Although this study provides additional support for a role of the autophagosome and autolysosome in LD breakdown, these findings do not discount the likely and additive contribu
tions of cytosolic lipases to hepatocellular LD breakdown. ATGL and HSL, enzymes of central importance to adipose tissue biol
ogy, are also expressed in the liver (Reid et al., 2008). It will be important to define how these cytosolic lipases work synergisti
cally with the membranous autophagic pathways to mediate lipid metabolism in the hepatocyte. Defects in lysosomal homeosta
sis may also be manifest in perturbations of alternative types of
Cell culture and reagents

The HuH-7 cell line was a gift from the laboratory of G. Gores (Mayo Clinic, Rochester, MN). The Hep3B.1.7 (Hep3B) cell line was obtained from ATCC (HB-8064). The conditional dynamin knockout cell line (Dnm2*LoxP/LoxP, Cre-E230) was a gift from the laboratory of P. De Camilli (Yale University, New Haven, CT). In brief, this dynamin-depletion fibroblast cell line was isolated from conditional Dnm1/C2 knockout mice that were generated through the use of a cre/loxP recombination strategy. Conditional knockout was achieved through culturing the cells in media containing 2 μM 4-hydroxy-tamoxifen for at least 5 d before experimentation. This treatment activated a Cre recombinase-estrogen transgene, knocking out Dnm1 and Dnm2 (Ferguson et al., 2009). All cells were maintained in DMEM or MEM containing Earle's salts and glucose supplemented with 1 mM sodium pyruvate, nonessential amino acids, and 0.075% (wt/vol) sodium bicarbonate (Corning). Media for all cell lines contained 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (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.

The rabbit polyclonal antibody raised against a C-terminal peptide representing residues 761–785 of mammalian Dyn2 has been described previously (Henley et al., 1998). The mouse monoclonal LAMP-1 (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), the antibody against ADRP was from LifeSpan Biosciences, Inc. (LS-B3121), the antibody against EEA1 was from BD (610457), the antibodies against PDI and COXIV were from Cell Signaling Technology, respectively, and the antibodies recognizing actin and Rab7 were from Sigma-Aldrich (A2066 and RB779, respectively). 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). Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201306140.dv.

Online supplemental material

Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201306140.dv.

Materials and methods

The authors would like to thank members of the McNiven laboratory, especially Gina Razzillo and Zhicheng Li, for helpful discussion and critical reading of the manuscript.

Video 4 shows an autolysosomal tubule scission event after drug washout. Video 3 shows the effect of Dynasore washout. Video 2 shows the same lysosomes from under conditions of Dyn2 inhibition by Dynasore. Video 1 shows fluoroscence-labeled lysosomes in a control Hep3B hepatocyte. Video 2 shows the same lysosomes from under conditions of Dyn2 inhibition by Dynasore. Video 3 shows the effect of Dynasore washout. Video 4 shows an autolysosomal tubule scission event after drug washout. Video 5 shows colocalization of Dyn2 with LAMP-positive structures. Video 6 shows a scission event mediated by Dyn2.

Statistical analysis

All statistical tests were performed with the two-tailed Student’s t test.

The authors declare that they have no conflict of interest.

Submitted: 24 June 2013
Accepted: 19 September 2013

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