Mapping Autophagy on to Your Metabolic Radar

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Autophagy, which literally translates into "eating one's own self," is an evolutionarily conserved cellular recycling program that maintains "in-house" quality control by turning over cytoplasmic components within lysosomes (1). Although the discovery of lysosomes dates back to the 1950s through the electron microscopic work of Christian De Duve, recent years have seen a growing interest in autophagy research, and reports now link compromised autophagy to a wide array of common human pathologies, for instance, neurodegenerative disorders, metabolic alterations, microbial pathogenesis, and cancers, to mention just a few (2). These studies support the idea that the "housekeeping" role of autophagy, in fact, translates to key physiological functions. For instance, recycling of oxidized proteins and aged organelles through autophagic degradation protects against cellular toxicity and death (3). Recent findings now highlight roles for autophagy in mobilization of diverse cellular energy stores (4) and in adipocyte differentiation (5,6), thus presenting autophagy as an emerging player in the metabolic arena. As novel functions for autophagy continue to unfold, it becomes critical to be able to precisely monitor autophagy in diverse physiological systems. This article comments on the fundamental developments on roles for autophagy in metabolic regulation and discusses currently available methods to monitor autophagy.

AUTOPHAGY: THE MACHINERY AND REGULATORY ELEMENTS

Mammalian cells exhibit three distinct forms of autophagy to deliver cytosolic cargo to the lysosomes, namely, macroautophagy, chaperone-mediated autophagy, and microautophagy (1). Traditionally, autophagy was considered a one-lane system for protein turnover and a mechanism for replenishing the intracellular amino acid pool during starvation. However, it is now becoming increasingly clear that autophagy, in particular macroautophagy, exhibits significant versatility in its ability to degrade mitochondria (mitophagy), endoplasmic reticulum (reticulophagy), ribosomes (ribophagy), and peroxisomes (pexophagy) (1). The second form of autophagy, chaperone-mediated autophagy, displays functional selectivity for the lysosomal targeting of specific soluble cytosolic proteins with the KFERQ signature (7) and requires the lysosome-associated membrane protein-2A receptor (7). Microautophagy sequesters cargo within single-membraned vesicles that originate from lysosomes per se and then pinch off within the lysosomal lumen to degrade the contents (8). Macroautophagy will remain the prime focus of this article.

Following early studies that displayed similarities between yeast and mammalian macroautophagy (hereafter autophagy) (9), extensive studies in yeast revealed more than 30 *atg* genes that orchestrate autophagy. These Atg proteins form distinct functional complexes that regulate each step of the process, including induction of autophagy, generation of the nucleation complex, autophagosome formation, and cargo recognition (1). Although in most mammalian cells autophagy occurs at basal levels, stress or starvation strongly upregulate this pathway (4). Autophagy requires the de novo formation of the nucleation complex, which in turn requires Beclin (Atg6 in yeast) to dissociate from its binding partner Bcl-2 (1,4) (Fig. 1). The release of Beclin allows it to form a transient complex with Atg14, vacuolar protein sorting (vps) 15, and the lipid kinase vps34 that generate the functional class III phosphatidylinositol 3-kinase (PI3K) complex (1) (Fig. 1). The lipid kinase activity of this complex produces phosphatidylinositol 3-phosphate that facilitates the targeting of additional Atg molecules to the nucleation complex. Recruitment of membranes through the shuttling of Atg9, the sole transmembrane Atg, to the nucleation complex promotes formation of the limiting membrane (10). The activation of two independent ubiquitin-like conjugation cascades, the Atg5-Atg12 and the light chain-3 (LC3) systems contributes to membrane elongation and autophagosome formation (1) (Fig. 1). Briefly, activation of the ubiquitin-like Atg12 requires Atg7, a crucial E1-like activating enzyme that catalyzes the covalent binding of Atg5 with Atg12. Subsequent interactions between Atg5-Atg12 and Atg16 recruit these molecules to the limiting membrane (11). In parallel, LC3 is first processed by the cysteine protease Atg4 to expose a COOH-terminal glycine (12), following which Atg7-activated LC3 is transferred to Atg3. In ensuing reactions requiring Atg5-Atg12 (13), LC3 is lipidated to membrane-associated phosphatidylethanolamine. The limiting membrane eventually seals upon itself to sequester cargo within double-membraned autophagosomes (1). The fusion of autophagosomes with lysosomes allows the acquisition of a battery of hydrolases and proton pumps that facilitate lumen acidification and cargo hydrolysis. The individual constituents of the degraded cargo are transferred back to the cytosol via lysosomal permeases and transporters.

The quest for elements in the nutritional regulation of autophagy has revealed a complex signaling network that converges upon the mammalian target of rapamycin complex 1 (mTORC1) (14) (Fig. 2). These findings form the basis for the use of rapamycin, a known inhibitor of mTORC1, to activate autophagy. Studies in yeast have shown that nutrient-activated TOR blocks autophagy by phosphorylating Atg13 (1), thus impairing formation of

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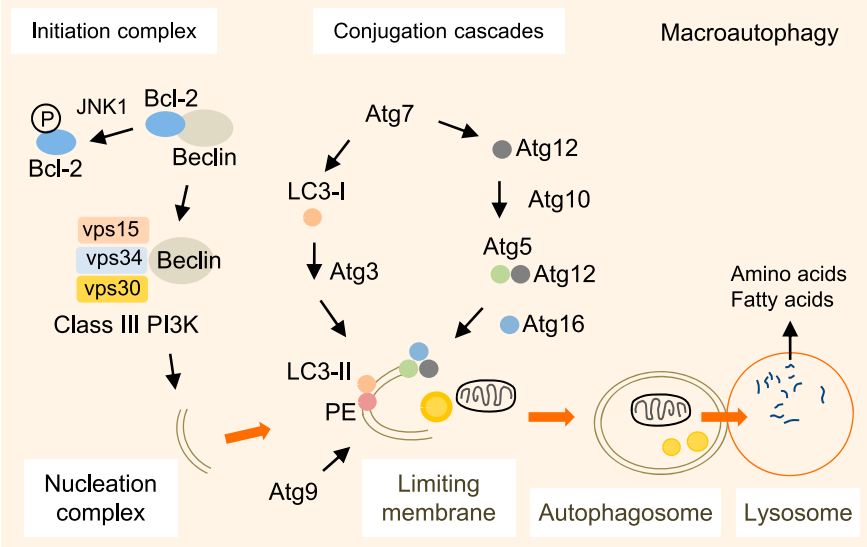

FIG. 1. Molecular constituents of autophagy. Autophagy requires more than 30 Atg proteins that orchestrate the formation of a de novo limiting membrane, which sequesters cytosolic cargo and then seals upon itself to form an autophagosome. The fusion of autophagosomes to lysosomes leads to cargo degradation and release of nutrients into the cytosol. JNK, Jun NH_2-terminal kinase 1; PE, phosphatidylethanolamine; P, phosphorylation of JNK.

the Atg1-Atg13 complex that is required for Atg9 shuttling. In mammals, mTORC1-mediated ULK1 (Atg1 in yeast) phosphorylation traps the Atg13-FIP200-ULK1 complex into an inactive form that blocks autophagy (15) (Fig. 2). In contrast, energy depletion is sensed by AMP-activated protein kinase (AMPK) that activates autophagy not only through its ability to impair mTORC1 activity but also via direct phosphorylation of ULK1 (16) (Fig. 2), which recruits it to the site of autophagosome formation. Recent studies reveal additional mechanisms that allow mTORC1 to fine-tune its regulatory effect on autophagy. For example, mTORC1 inhibits death-associated protein 1 (DAP1), a negative regulator of autophagy, which prevents the uncontrolled upregulation of autophagy during starvation (17) (Fig. 2). Furthermore, cells circumvent the inhibitory effect of mTORC1 on autophagy by raising levels of sestrins that upregulate autophagy by activating AMPK (18).

AUTOPHAGY AND THE METABOLIC CONNECTION

A rapidly evolving area of research investigates roles for autophagy in metabolic regulation, and some of these studies have highlighted metabolic functions of autophagy in liver, adipose tissue, and pancreas. Until recently, mobilization of lipids was attributed to cytosolic lipases; however, studies now demonstrate a role for autophagosomes in the "in-bulk" delivery of lipids to lysosomes for degradation (19). Studies in cultured hepatocytes lacking the autophagy gene *atg7* or following pharmacological inhibition of autophagy revealed marked accumulation of cellular lipid droplets and reduced rates of β-oxidation

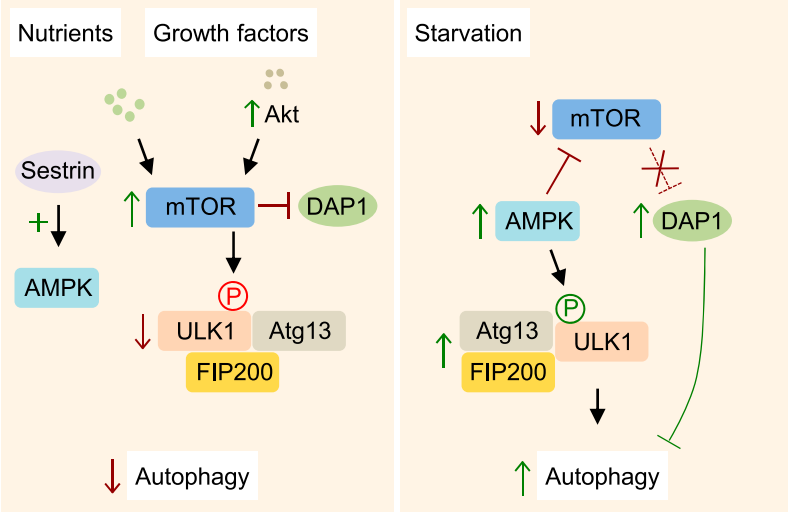

FIG. 2. Autophagy is regulated by mTOR and AMPK signaling. Nutrient availability and growth factors activate mTOR that phosphorylates ULK1 to inhibit autophagy by trapping the ULK1-FIP200-Atg13 complex in an inactive state. Starvation reduces mTOR activity, which releases its inhibition on autophagy and on DAP1, the activation of which prevents uncontrolled activation of autophagy during starvation. Energy depletion activates AMPK that phosphorylates ULK1 at distinct residues to activate autophagy. Cells may bypass chronic mTOR activation by upregulating sestrins that upregulate autophagy by increasing AMPK activity.

(19). The liver-specific deletion of *atg7* in mice increased hepatic triglycerides, which did not occur from increased lipogenesis, since inhibiting autophagy did not modify triglyceride biosynthesis. This ability to degrade lipids by autophagy, termed lipophagy, may in fact be a generalized mechanism to turnover lipids in cells as diverse as neurons (20,21) and macrophages (22). A recent study in hypothalamic agouti-related peptide neurons revealed a role for starvation-induced lipophagy in the generation of neuronal free fatty acids that increased agouti-related peptide levels and food intake (21).

Although starvation and acute lipid loading of cells activate lipophagy (19,21), chronic high-fat feeding inhibits autophagic turnover of lipids, at least as observed in liver (19) (Fig. 3*A*). This sets up a relentless cycle that promotes steatosis, the hepatic manifestation of the metabolic syndrome (Fig. 3*B*). Subsequent studies have now shown that livers from both dietary and genetic mouse models of obesity display decreased autophagy resulting in endoplasmic reticulum stress–mediated insulin resistance (23) (Fig. 4). Although, the mechanism for reduced autophagy as a consequence of chronic lipid stress is not entirely clear, it is plausible that reactive intermediates in the background of lipid accumulation may modify membrane lipid chemistry to affect autophagosome-lysosome fusion, as recently observed in fibroblasts and liver (24). In addition, activation of nuclear factor-κB, which has been shown to reduce autophagy in macrophages (25) and initiate fatty acid–induced hepatic inflammation (26) or the hyperactivation of mTOR during obesity (27), may also block autophagy and promote hepatic insulin resistance (Fig. 4).

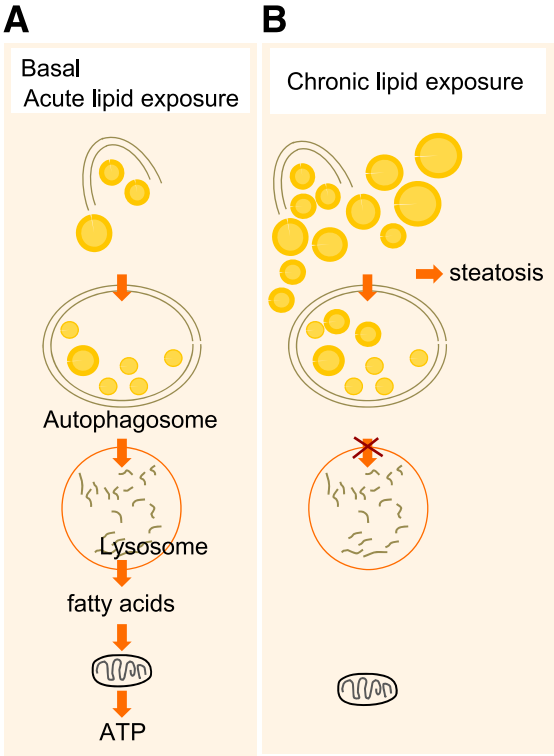

FIG. 3. Autophagic degradation of lipid droplets. Autophagy degrades hepatocellular lipid droplets under basal conditions or following an acute exposure to lipids by delivering droplets to lysosomes. *A*: Breakdown of lipid droplets releases free fatty acids that undergo β-oxidation in the mitochondria. *B*: Chronic lipid stimulus impairs delivery of lipids to lysosomes and promotes hepatic steatosis.

Jun NH2-terminal kinase 1 contributes to hepatocellular injury and insulin resistance (28), and it is conceivable that increases in reactive intermediates in autophagy-deficient steatotic livers may dysregulate Jun NH2-terminal kinase signaling and affect insulin sensitivity (Fig. 4). It remains to be seen whether decreased autophagy and its consequences on cellular energy balance (4) may be a key element determining the development of insulin resistance during aging.

The demonstration of autophagy-regulated lipid mobilization questions the existence of lipophagy in adipose tissue, an organ dedicated to fat storage. In clear contrast to findings in liver, inhibiting autophagy in adipose tissues reduced adipose lipid content and markedly improved glucose tolerance (5,6) (Fig. 4). Analyses in autophagy-deficient 3T3-L1 preadipocytes revealed decreased triglyceride content, reduced levels of key adipogenic transcription factors, CCAAT/enhancer-binding protein α and β (5), and the failure to differentiate into adipocytes. A possible explanation for reduced adiposity in the adipose-selective autophagy-deficient mice was acquisition of brown adipose-like features within their white adipose tissues (5,6). In fact, loss of autophagy in white adipose tissues resulted in increased expression of brown adipose markers, increased mitochondrial content, and raised β-oxidation rates (5) (Fig. 4). These findings demonstrate a central role for autophagy in regulation of energy balance through effects on adipose differentiation and function. Interestingly, studies examining autophagy levels in the different fat depots in humans now correlate increased autophagic activity to expansion of subcutaneous and omental fats in obese individuals (29). Remarkably, autophagy was excessively raised in the omental fat, and particularly so in insulin-resistant individuals (29) (Fig. 4). These results suggest that while autophagy is required for adipose differentiation and lipid droplet biogenesis during early development (5,6), it may also function to maintain adipose mass and lipid storage during adulthood.

The regulatory function of autophagy is not limited to lipid metabolism but also extends to the maintenance of glucose homeostasis, particularly in glycogenolysis. The existence of cytosolic and lysosomal enzymes to breakdown glycogen points to important roles for autophagy in regulating cellular glycogen stores, and indeed, glucagon that regulates glycogen metabolism was the first identified hormone to activate autophagy. One physiological scenario in which autophagy is acutely required is during childbirth, wherein neonatal livers induce autophagy to mobilize glycogen stores to increase availability of glucose. In fact, the presence of lysosomal glucose-6-phosphatase facilitates the availability of glucose by modulating its efflux into the cytosol. Apart from roles for autophagy in glycogenolysis in liver, the documentation of diverse muscle diseases characterized by accumulation of glycogen granules, for instance, Danon disease (30), X-linked vacuolar myopathy with excessive autophagy (31), and Pompe disease (32), indicate the involvement of dysfunctional autophagy in these pathologies. A common feature to all of these diseases is accumulation of glycogen granules, although these diseases differ in the cellular mechanism that contributes to glycogen accumulation. For instance, Danon disease occurs from mutations in the *LAMP2* gene (33), and reduced LAMP2 function alters autophagosome-lysosome fusion (34) and impairs glycogen delivery to lysosomes. In contrast, Pompe disease patients are deficient in lysosomal acid α-glucosidase and thus are unable to break down glycogen delivered to the lysosomes. Although

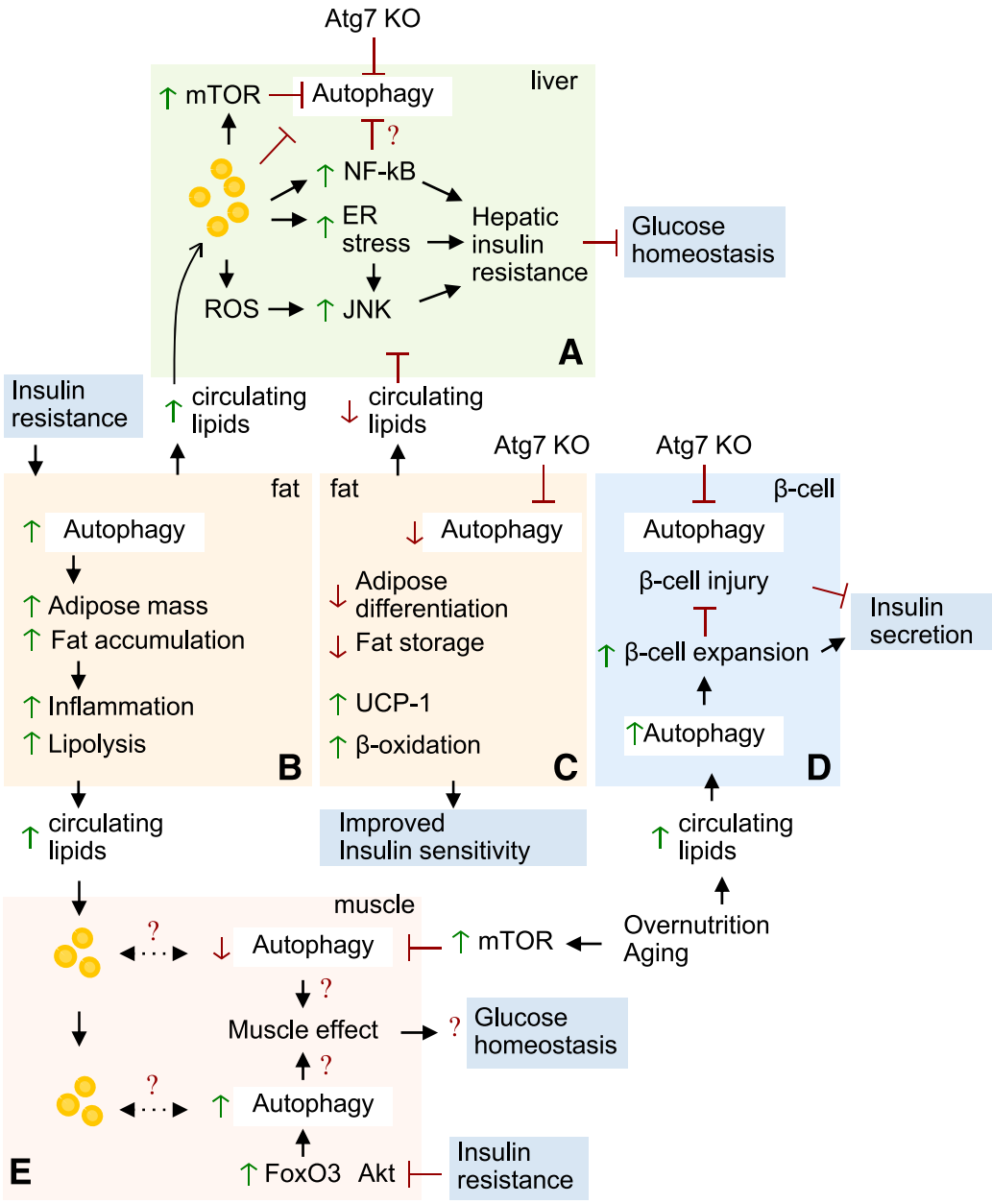

FIG. 4. Hypothetical links between autophagy and insulin resistance. *A*: Inhibition of autophagy leads to lipid accumulation that promotes hepatic insulin resistance by activating inflammatory signaling pathways and endoplasmic reticulum stress. Excessive lipids, activated nuclear factor-κB, and the hyperactivation of mTOR during obesity inhibit autophagy and further lead to hepatic steatosis and insulin resistance. *B*: Obesity activates autophagy in the adipose tissue to promote fat accumulation and inflammation, which increases circulating lipids that accumulate in ectopic sites, such as liver and muscle. *C*: Blocking adipose-selective autophagy switches adipose differentiation into brown adipose-like tissue that increases fat oxidation and improves insulin sensitivity. *D*: Induction of β-cell autophagy in response to chronic lipid stress promotes β-cell expansion and insulin secretion. Blocking β-cell–selective autophagy results in β-cell injury and reduced insulin secretion. *E*: Dysregulated skeletal muscle autophagy may occur from excessive lipid accumulation or disturbed Akt and mTOR signaling, which may affect muscle control of glucose homeostasis.

these associations reveal an important link between autophagy and glycogenolysis in the maintenance of muscle "quality control," it is unclear how autophagy per se regulates muscle function and how changes in autophagy in response to environmental stressors, such as overnutrition, affect muscle function especially during insulin resistance and diabetes. According to a previous report, control of autophagy through Akt-regulated/FoxO3-dependent transcriptional upregulation of autophagy genes modulates muscle protein turnover (35). Therefore, altered Akt signaling during obesity and insulin resistance (36) may affect autophagy function in the muscle that, in turn, may interfere with muscle functions including those related to control of blood glucose levels (Fig. 4). In contrast, hyperactivation of mTOR during overnutrition and aging may inhibit autophagy (14) to affect muscle function (Fig. 4). Further detailed investigations will be required to provide clarity into the roles for autophagy in the muscle, and how defective autophagy modifies muscle function and contributes to the development of insulin resistance and diabetes.

Autophagy may also modulate glucose homeostasis through its effects on the pancreatic β-cell (37). Although,

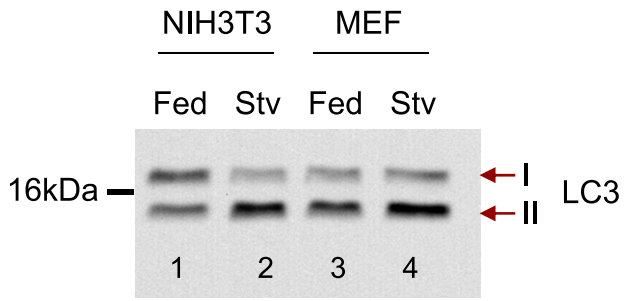

FIG. 5. Representative immunoblots for LC3. Steady state LC3 levels in NIH3T3 cells and mouse embryonic fibroblasts (MEF) cultured in serum-supplemented Dulbecco's modified Eagle's medium (Fed) or in response to serum removal for 2 h (Stv). The effect of serum starvation is increased levels of LC3-II (*lanes 2* and *4*), reflecting increased autophagosome content.

the pancreas is relatively resistant to upregulating autophagy during starvation, β-cell–selective deletion of *atg7* results in progressive β-cell injury and reduced insulin secretion (Fig. 4). A possibility exists that autophagic turnover of insulin-positive secretory granules may be a requirement for insulin secretion. In fact, studies in secretory-deficient Rab3-deficient β-cells reveal maintained intracellular insulin levels as a consequence of increased lysosomal insulin degradation (38). Studies in *db/db* mice with diet-induced obesity and insulin resistance have revealed increased autophagosome formation and β-cell expansion, and a possibility exists that autophagy protects against chronic lipid stress in the pancreas in these settings (Fig. 4), particularly since β-cell autophagy–deficient rodents failed to display similar increases in β-cell mass (37). It cannot be excluded that loss of inhibitory inputs from reduced insulin signaling may promote β-cell autophagy in this scenario.

METHODS TO MONITOR AUTOPHAGY

The inherent challenges to follow autophagy originate from the dynamic nature of this pathway. Autophagy maintains a basal flux of substrates to lysosomes that accelerates during stress or starvation, and thus, analyzing static levels of autophagosome markers may not provide information on autophagy activity. In contrast, measuring autophagic flux that reveals the net amount of substrate delivered to lysosomes per unit time will provide information on whether autophagy is active or suppressed. This section discusses essential assays for tracking autophagy and classifies these into assays that determine steady-state autophagosome content and those that reveal functional information regarding autophagic flux.

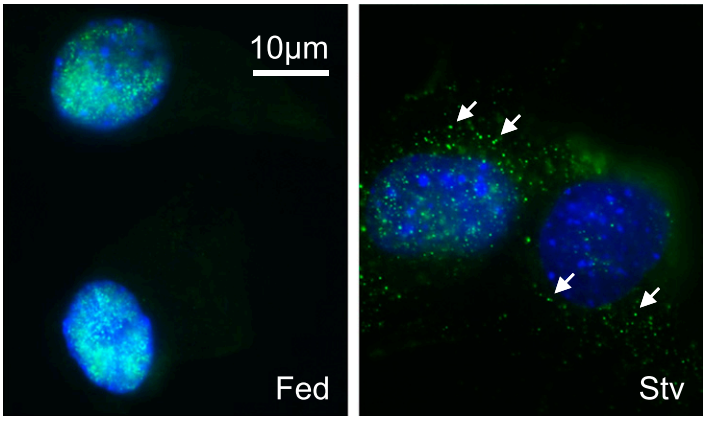

FIG. 6. Representative indirect immunofluorescence for LC3. Indirect immunofluorescence for endogenous LC3 in hypothalamic GT1–7 cells cultured in serum-supplemented medium (Fed) or following serum removal for 2 h (Stv). Distinct LC3 puncta (white arrows) are observed in response to serum removal and are in green (fluorescein isothiocyanate). Nuclei are in blue (diaminido phenylindol). (A high-quality digital representation of this figure is available in the online issue.)

MEASURING STEADY-STATE AUTOPHAGOSOME CONTENT

Electron microscopy. Transmission electron microscopy (TEM) remains the gold standard for the qualitative detection of autophagic components. TEM studies led to the discovery of lysosomes and have been instrumental in tracking cellular sources of autophagosomes. In fact, the elucidation that distinct endoplasmic reticulum regions, termed omegasomes, contribute to autophagosomes came through TEM studies (39). The ultrastructural definition of an autophagosome is a double-membraned vesicle that contains engulfed cytoplasmic contents. During the early stages of autophagy, the maintained morphology of sequestered material within double-membraned structures and the electron dense appearance of cargo contrasted to the cytosol help in identifying autophagosomes. The identification of autophagolysosomes or amphisomes, which are single-membraned structures originating from fusion of autophagosomes with lysosomes or endosomes, respectively, may also be relatively easy during early stages of degradation. However, once advanced degradation sets in, it may be difficult to distinguish sequestered intracellular contents from those phagocytosed from the outside of the cell or distinguishing autolysosomes from vacuoles of unclear origin. The power of TEM lies in the direct visualization of autophagosome morphology; however, TEM studies may not accurately reveal information regarding autophagic flux. Additional drawbacks include lack of objectivity and the requirement of a certain level of expertise to reproducibly distinguish between distinct autophagic structures, the lack of which often results in misinterpretation of data, as detailed in a recent review (40). Although immunolabeling of LC3 using gold-conjugated secondary antibodies may facilitate identification of autophagosomes (41), TEM still needs to be supplemented with autophagic flux studies to reveal the true dynamics of the pathway.

Biochemical detection of LC3-II. A number of approaches, for instance dyes such as monodansylcadaverine, have been used to label autophagosomes and erroneously considered as readouts for autophagic activity. Monodansylcadaverine was initially considered a specific autophagosome marker but was subsequently found to label additional organelles (42). Additionally, the acidophilic dye LysoTracker, which stains cellular acidic compartments, or biochemical detection of lysosomal membrane proteins LAMP1 or LAMP-2 isoforms or luminal cathepsins, may not reflect autophagic activity, as it is mistakenly considered to. The use of expression analyses for autophagy genes, including LC3 or Atg proteins, may not represent induction of autophagy, since it is generally considered that autophagy proteins are present in excess and that autophagy activation is primarily a posttranslational event. LC3 is the most reliable autophagosome marker (43)

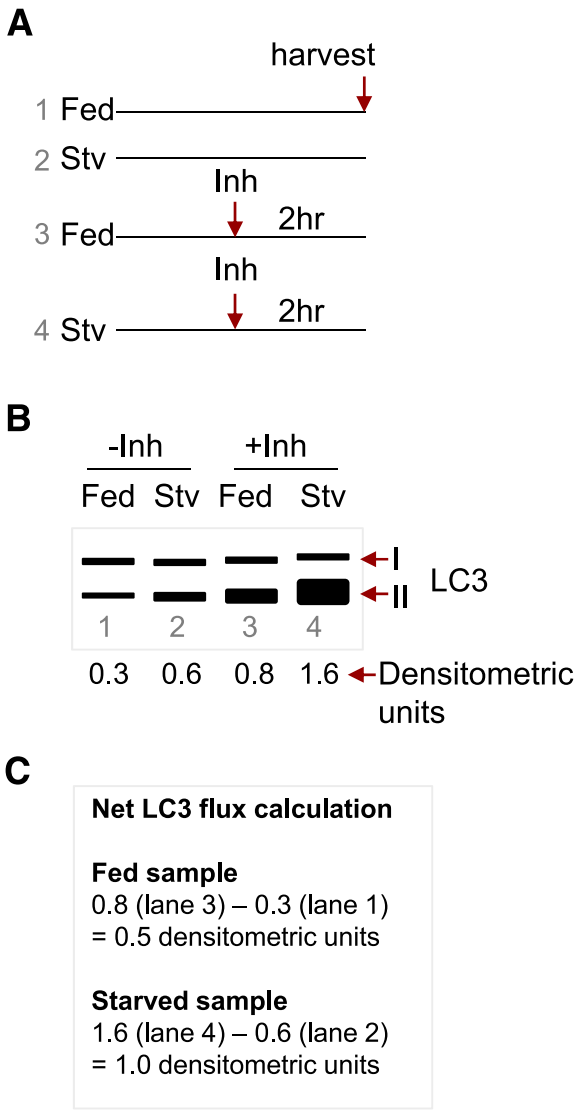

FIG. 7. Schematic representation of the LC3 flux assay. *A*: Experimental plan for the LC3 flux assay: Cells cultured in serum-supplemented (Fed) or serum-starved medium (Stv) treated in presence or absence of inhibitors of lysosomal degradation (Inh) for 2 h, following which cell lysates are subjected to immunoblotting for LC3. *B*: Cartoon depicting immunoblots and densitometry for LC3-II from cells harvested according to plan in *A*. *C*: Calculations for determination of net LC3 flux. Densitometric values of samples are subtracted from corresponding inhibitor-treated value, and these represent residual amounts of LC3-II within lysosomes. Higher values correspond to increased autophagic flux.

that exists in two forms: the soluble cytosolic LC3-I form that becomes lipidated to become the autophagosome-bound LC3-II during activation of autophagy (Fig. 1). The hydrophobicity of lipidated LC3-II allows its rapid migration on a SDS-PAGE (Fig. 5), and thus, LC3-II should not be considered as the processed form of LC3-I. LC3-II reproducibly follows the entire autophagic process from the limiting membrane to the lysosome; consequently, levels of LC3-II faithfully reflect autophagosome number, or more appropriately autophagic membranes positive for LC3-II (44). A common misinterpretation regarding LC3-II readouts is the consideration that increases in LC3-II reflect increased autophagic activity. Because the net cellular LC3-II content is a function of the amount of autophagosomes generated and the amount degraded, steady state LC3-II levels may only represent the absolute autophagosome content. Additionally, a number of studies have now reported the presence of a population of LC3-II that is generated independently of autophagosome formation. For instance, knockdowns of critical components of the functional PI3K complex, such as Atg14, vps34, or Beclin in a number of mammalian systems have significantly reduced autophagic activity without reducing LC3-II levels (15,45). Therefore, it remains imperative that analyses of steady state levels of LC3-II should be supplemented with functional autophagic flux assays.

Immunofluorescence. Light microscopic approaches to detect autophagosomes are now being used widely in cultured cells and in vivo systems, including *Drosophila melanogaster* (46), *Caenorhabditis elegans* (47), and mice (48). In principle, the analyses for autophagosome number rely on quantifying either endogenous LC3 signal by indirect immunofluorescence or by examining the signal of green fluorescent protein (GFP) (49) or mCherry (50) tagged to LC3. Detecting endogenous LC3 offers advantages in terms of reducing cellular manipulation and decreasing potential artifacts from an overexpression system. Regardless of the approach, LC3 either appears as a diffuse cytosolic signal representing soluble cytosolic LC3-I or as autophagosome-associated LC3-II puncta (Fig. 6). In certain systems, reduced endogenous signal may require an exogenous LC3 construct, in which case some critical considerations should be taken into account. For instance, the use of stable GFP-LC3 transformants may be beneficial over transient transfections because this will allow the selection of clones offering highest signal-to-noise ratios. This may facilitate selecting clones expressing physiological levels of GFP-LC3 that will preclude LC3 aggregation often observed in GFP-LC3–expressed systems. In experiments requiring GFP-LC3 overexpression, it may be important to include controls such as the use of GFP-LC3 C-terminus glycine mutants (GFP-LC3^{G120A}) that are defective for LC3 lipidation (51). This will allow one to distinguish between true increases in GFP-LC3 puncta as opposed to the lack of it in these mutants (51). A second consideration is determining what amounts to a LC3 puncta. As with all visual readouts, quantifying LC3 puncta is prone to errors from lack of objectivity; consequently, quantification may be performed by experiment-blind personnel via algorithms that define puncta size by thresholding (52). Because most cells display minimal amounts of LC3 puncta even under basal conditions, it may be inappropriate to express results as percent cells positive for LC3 puncta. Under most circumstances results may be expressed as average LC3 puncta count per cell or per cell area, although in the latter case caution should be exercised to exclude artifacts arising from GFP-LC3 aggregates. LC3 quantification may be used for in vivo analyses, although endogenous LC3 is particularly difficult to detect in muscle. Alternatively, transfecting GFP-LC3 construct by in vivo electroporation or else the use of conventional GFP-LC3 and tissue-specific transgenic mice may be useful (53).

FUNCTIONAL AUTOPHAGIC FLUX ASSAYS

Monitoring autophagic flux in cells and in tissues. Autophagic flux assays are typically based on the principle that LC3-II is turned over within lysosomes. The exposure of cells to lysosomal inhibitors that dissipate lysosomal pH or to lysosomal protease inhibitors will result in accumulation of LC3-II in lysosomes. The difference in levels of

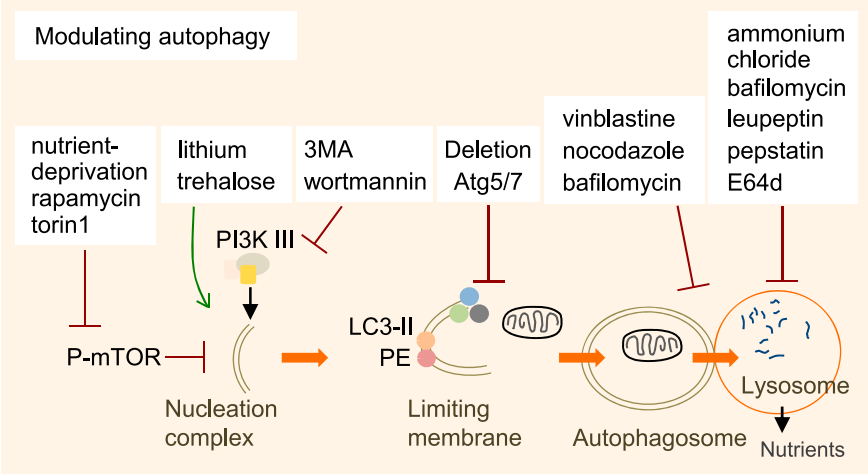

FIG. 8. Methods to modulate autophagy. Autophagosome formation can be blocked by pharmacological agents that inhibit class III PI3K (3MA, wortmannin) or through deletion of autophagy genes (*atg5* or *atg7*). Autophagosome-lysosome fusion can be inhibited by agents that affect microtubule function (vinblastine, nocodazole) or that interfere with lysosomal pH (bafilomycin). Lysosomal degradation is blocked by dissipating lysosomal pH (ammonium chloride, bafilomycin) or by inhibiting lysosomal proteases (leupeptin, pepstatin, E64d). Autophagy may be activated by inhibiting mTOR (rapamycin, torin 1) or through mechanisms independent of mTOR (lithium, trehalose). PE, phosphatidylethanolamine; P-mTOR, phosphorylated mTOR.

LC3-II in presence or absence of these inhibitors will reflect the net amount of LC3-II delivered to lysosomes, which in turn will be the measure of autophagy activity (Fig. 7). For instance, the use of lysosomal inhibitors during starvation will increase lysosomal accumulation of LC3-II over and above those observed under basal fed conditions, indicating increased autophagy during starvation (Fig. 7). The LC3-II flux assay, although a reliable indicator of autophagic activity, may still be less sensitive in cells exhibiting higher basal rates of autophagic flux. Modifying autophagosome formation by pharmacological or genetic means (Fig. 8) may serve as control experiments to confirm results obtained from the LC3-II flux assay. In addition to LC3-II, turnover of a second autophagy substrate, p62 (54), which gains access to autophagosomes through its ability to bind to LC3-II, may also be used to determine autophagic flux in a manner similar to LC3-II. However, in contrast to LC3-II, p62 molecules that are bound to polyubiquitinated substrates are degraded through the proteasome (55), and consequently, p62 flux may be used as support for results obtained from the LC3-II flux assay. The LC3-II flux assay may also be used for in vivo analyses, for instance, intraperitoneal administration of leupeptin or colchicine may be appropriate for examining LC3-II flux in liver (56) and muscle (57), respectively. An alternate approach is the ex vivo flux assay, wherein fresh tissue explants may be incubated with or without lysosomal inhibitors, and this approach may be used reproducibly in liver and oxygenated muscle tissue (E.Y., unpublished data). Since increases in LC3 and p62 gene expression during starvation might complicate data analyses, the use of additional assays for autophagy activity (detailed below) may verify results from flux assays.

Analyses for lysosomal delivery of fluorescent probes. A second approach to analyzing autophagic flux relies on the fact that GFP fluorescence is typically quenched upon delivery to the lysosome despite its relative resistance to degradation, whereas red fluorescent protein (RFP) and mCherry maintain their fluorescence in lysosomes (58). The opposing qualities of these proteins have allowed the construction of a novel chimeric mRFP-GFP-LC3 probe for monitoring autophagic flux (59). With this construct, under basal conditions, autophagosomes are observed as a yellow signal (merged mRFP and GFP signal), whereas autophagolysosomes appear red (from the quenching of GFP). The induction of autophagy can be easily tracked by observing for increases in both the yellow and red signal; however, conditions that reduce fusion of autophagosomes with lysosomes will typically increase the yellow signal. Because the method relies on lysosomal pH, it should be kept in mind that this approach may not be used in conjunction with agents that neutralize lysosomal pH, and additionally, conditions that alter lysosomal acidification may adversely affect results obtained from this assay. The relative resistance of GFP against degradation in lysosomes has led to another approach for determination of autophagic flux. Increased free GFP in lysosomes generated from cleavage of GFP-LC3 and the degradation of LC3 (the appearance of a free GFP band of reduced molecular weight by immunoblotting) will also indicate increased autophagic flux (60).

Analyses of long half-life protein degradation. The degradation rates of proteins of long half-life have often been used as a measure of autophagic flux in cultured cells (20,21). In principle, cells are treated with ^{3}H-leucine for prolonged periods of time to allow for the incorporation of radioactive label into proteins, following which cells are maintained in radioactivity-free medium to ensure complete removal of short-lived radiolabeled proteins by the proteasome. Autophagy is then induced, and the release of radiolabeled amino acids from proteolysis of radiolabeled proteins is quantified. The incorporation of pharmacological modulators of autophagy during the "chase," in particular the use of 3-methyladenine (3MA) that blocks autophagosome formation, reveals 3MA-sensitive proteolysis, which is an index of the amount of protein degradation occurring through this pathway. Although, this approach is quantitative, one caveat is that the success of the assay depends on the efficiency of 3MA to block autophagy that may vary depending on experimental conditions or the cells in which experiments are performed.

CONCLUDING REMARKS

Autophagy is a crucial mechanism that governs many aspects of cell function, including regulation of cellular

metabolism and energy balance, and alterations in autophagy have been linked to the development of insulin resistance. Although there are no specific autophagy assays tailored for diabetes research, combining multiple approaches may provide precise information regarding autophagic activity in these conditions. Although existing methods to follow autophagy in vivo are rudimentary, it is expected that more accurate means and specific reagents will emerge for the better understanding of this key degradation mechanism with the long-term goal of developing new therapies for cure or disease prevention through the modulation of autophagy.

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