Mitochondrial homeostasis is regulated by a balance between mitochondrial biogenesis and degradation. Emerging evidence suggests that mitophagy, a selective form of autophagy that degrades mitochondria, plays a key role in the physiology and pathophysiology of mitochondria-enriched cells, such as brown and beige adipocytes. This review discusses findings regarding the roles of autophagy and mitophagy in cellular development, maintenance, and functions of metabolic organs, including adipose tissue, liver, and pancreas. A better understanding of the molecular links between mitophagy and energy metabolism will help to identify promising targets for the treatment of obesity and obesity-associated disorders.

Introduction: Autophagy and Mitophagy

Mitochondria are double-membrane organelles that serve as the central source of ATP (adenosine 5’-triphosphate) with which cells carry out various functions. The cell must undergo both mitochondrial biogenesis and mitochondrial degradation to maintain “healthy” mitochondria in response to varying energetic demands of the cell (Fig. 1). On one end, mitochondrial biogenesis is tightly regulated by various transcriptional regulators encoded by nuclear genes, including peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α), nuclear respiratory factor 1 (Nrf1) and Nrf2, and mitochondrial transcription factor A (Tfam). Transcriptional control of mitochondrial biogenesis has been previously discussed in detail (1).

On the other end, mitochondrial degradation is carried out through autophagy, a process of intracellular degradation to break down unwanted or damaged cellular components. The main hallmark of autophagy that distinguishes it from other degradation processes is the formation of a double-membrane vesicle, the autophagosome, to deliver large cytoplasmic components to the lysosome for degradation. The detailed processes of autophagosome formation are described elsewhere (2, 3). In short, the molecular signal from the mechanistic target of rapamycin complex 1 (mTORC1) triggers the activation of unc-51–like autophagy activating kinase 1 (ULK1) complex, consisting of ULK1, autophagy-related protein 13 (ATG13), and focal adhesion kinase family interacting protein of 200 kDa (FIP200), to initiate the formation of the isolation membrane from existing membrane sources such as the endoplasmic reticulum (ER) or Golgi (Fig. 2A). The membrane further expands to produce a completely enclosed, double-membrane vesicle known as the autophagosome. Autophagosome formation is orchestrated by a number of core autophagy-related proteins. A key step for autophagosome formation is the conjugation of phosphatidylethanolamine (PE) to microtubule-associated protein 1 light chain 3 (LC3), an ATG8 homolog, to generate a lipidated form of LC3, LC3–PE. This conjugation is mediated in part by ATG7 and the ATG5–ATG12–ATG16L1 complex (3). LC3 is retained inside the autophagosome and, when expressed as a green fluorescent protein (GFP) fusion protein, serves as a common marker of autophagy (4). Once the autophagosome is developed, it fuses with the lysosome, forming an autolysosome, a single-membraned acidic vesicle where lysosomal hydrolytic enzymes, such as cathepsins, degrade the enclosed contents. Lysosome biogenesis is an important component of autophagy machinery and regulated by the microphthalmia/ transcription factor E (MiT/TFE) family of transcription factors, which includes microphthalmia-associated transcription factor (MITF), transcription factor EB (TFEB), and transcription factor binding to IGHM enhancer 3 (TFE3) (5–8). Although the later elements of the autophagy machinery are pivotal in the regulation of degradation, the initial selective degradation of cytoplasmic components through autophagy is worth exploring in depth.

A particularly interesting example is mitophagy, the selective clearance of mitochondria through autophagy. Selectivity is driven by specific proteins that physically connect the intended target (such as mitochondria) with the autophagosomal protein LC3. These receptors interact with the autophagosome through the LC3-interacting region (LIR) (Fig. 2B). Mitochondrial damage is a major physiological trigger for selective mitochondrial clearance. Damage-induced mitophagy can occur through two different mechanisms: (i) adapter-mediated, ubiquitin-dependent mitophagy and (ii) direct, ubiquitin-independent mitophagy (Fig. 2B). Adapter-mediated mitophagy, which is mediated by phosphatase and tensin homolog (PTEN)–induced putative kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin, requires the ubiquitination of the target. Damage to the mitochondria leads to reduced mitochondrial

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**Fig. 1. Regulation of mitochondrial dynamics.** Mitochondrial content is regulated by a balance between mitochondrial biogenesis and degradation. Nuclear-coded transcriptional regulators, such as PGC-1α, Nrf1 and Nrf2 (Nrf1/2), and Tfam, control mitochondrial biogenesis, whereas autophagy removes damaged or unwanted mitochondria. Sirt1, sirtuin 1.
domain for cargo recognition and an LIR domain that interacts with LC3 to promote encapsulation by the autophagosome. Whether adapter proteins have tissue- or cell type–specific functions have yet to be revealed. Damage-induced mitophagy can also occur through the direct interaction of mitochondria-localized proteins with LC3 independent of ubiquitination. For example, BCL2/E1B 19 kDa–interacting protein 3 (BNIP3) and FUN14 domain-containing protein 1 (FUNDC1) directly interact with LC3 to promote mitophagy in response to hypoxia-triggered mitochondrial damage (14, 15). The mitophagy receptor BCL2-like 13 (BCL2L13), which is a mammalian homolog of Atg32, directly interacts with LC3 through the LIR domain, but the mechanism that activates BCL2L13 remains to be determined (16).

Mitophagy can take place independently of mitochondrial damage during developmental processes, although fewer models of this process have been established. For instance, the BNIP3 homology NIP3-like protein X (NIX; also known as BNIP3L) is required for mitochondrial clearance during erythrocyte maturation (17, 18). NIX mediates mitophagy in a ubiquitin-independent manner, and blocking the direct interaction between NIX and LC3 leads to accumulation of mitochondria in maturing erythrocytes (19). In addition, mitochondrial degradation occurs when sperm mitochondria are removed during fertilization. The mechanism of paternal mitochondrial degradation is not conserved between species. In Caenorhabditis elegans, this process requires autophagosome formation and is independent of ubiquitination (20, 21). In Drosophila, paternal mitophagy also requires autophagosome formation that is ubiquitin- and p62-dependent but does not require Parkin (22). Examples in C. elegans and Drosophila suggest that there are uncharacterized proteins that target mitochondria for degradation. In mammals, including the mouse, pig, and rhesus monkey, mitochondrial degradation appears to occur through the ubiquitin-proteasome system independently of LC3-mediated autophagy (23–25).

In a slightly different vein, we have identified a developmentally important process of mitochondrial clearance: selective mitochondrial degradation during beige adipocyte conversion to white adipocytes after the withdrawal of cold exposure or β3-adrenergic receptor (β3-AR) stimulation. The mechanism underlying the recognition of mitochondria for selective degradation awaits future investigation.
**The Role of Autophagy in Adipose Biology**

Mammals have two functionally distinct types of adipocytes: white adipocytes, which store excess energy as triglycerides, and brown adipocytes, which dissipate energy in the form of heat and thus can counteract obesity and obesity-associated diseases such as type 2 diabetes (26). Adult humans and rodents have a “recruitable” form of brown adipocytes, termed “beige adipocytes,” the development of which can be induced by certain environmental stimuli such as chronic cold exposure (27–29). Although brown and beige adipocytes have similar biochemical and morphological characteristics, including the brown and beige fat-specific protein uncoupling protein 1 (UCP1), high mitochondrial content, and multilocular lipid droplets, their developmental origins are distinct (29–34). Brown adipocytes arise early during development from a subset of dermomyotome precursors, and their development is stalled during postnatal stages. On the other hand, beige adipocytes arise postnatally in part through the action of the transcription factor progesterone receptor (PR) (PRD1-BF1-RIZ1 homologous) domain-containing 16 (PRDM16) in response to environmental cues from precursors that are positive for early B cell factor 2 (Ebf2), platelet-derived growth factor receptor α (Pdgfra), and stem cell antigen–1 (Scal1) (Fig. 3) (35–37). Cold exposure, which stimulates the β3-AR signaling pathway, is a dominant activator of brown and beige adipocyte development. Notably, beige adipocytes lose their morphological and thermogenic characteristics and acquire “white-like” characteristics shortly after the requisite stimuli (cold exposure or β3-AR stimulation) are removed (38, 39).

We have reported that the conversion from beige adipocyte to white adipocyte is direct, circumventing an intermediate precursor state, and involves active mitochondrial clearance (Fig. 3 (39)). Given the crucial role of the mitochondria in the thermogenic function of beige and brown adipocytes, it is important to understand the mechanisms underlying the regulation of mitochondrial homeostasis in brown and beige adipocytes.

Autophagy has been implicated in remodeling mitochondrial contents and thus regulating adipocyte differentiation as well as the maintenance of differentiated adipocytes. Accordingly, it is important to use an appropriate Cre mouse line that can target specific adipocytes at different differentiation stages to dissect the roles of autophagy in defined cell types (for example, preadipocytes or differentiated adipocytes). Several genetic autophagy-deficient animal models have been used to study the function of autophagy in adipose tissues but exhibit inconsistent phenotypes (Table 1). For instance, a total knockout of Atg5 results in a differentiation defect of white adipose tissue (WAT), whereas deletion of Atg7 through a muscle- and brown adipocyte–specific Cre, Myf5-Cre, promotes beige adipocyte development and impairs brown adipocyte differentiation (40, 41). These models assess the role of autophagy during adipogenesis and preclude insight into autophagy function in terminally differentiated adipocytes. Similarly, aP2-Cre–mediated deletion of Atg7 or p62 affects not only mature brown and white adipocytes but also some nonadipose tissues (42–44). Nonspecific expression of aP2-Cre in skeletal muscle, liver, brain, and macrophages can cause indirect effects on adipocyte differentiation and/or function (45, 46). In addition, inhibiting autophagy in proopiomelanocortin (POMC) neurons and skeletal muscle causes browning of WAT (41, 47). We have used Ucp1-Cre to selectively target autophagy in differentiated brown and beige adipocytes to show that autophagy is required specifically for beige-to-white adipocyte conversion after cold or β3-AR withdrawal (39).

Another potential cause for various phenotypes observed in autophagy-deficient models may reside in the promiscuity of autophagy machinery components, many of which have broad cellular functions beyond autophagy (48). Deletion of either Atg5 or Atg12 using Ucp1-Cre results in a consistent phenotype: retention of mitochondrial content and high UCP1 abundance in inguinal WAT containing beige adipocytes even after withdrawal of β3-AR stimulation (39). In contrast, deletion of p62, a cargo receptor that mediates selective autophagy, results in impaired mitochondrial function including reduced UCP1 abundance in both brown adipose tissue (BAT) and inguinal WAT (44). These apparently conflicting phenotypes can be explained by the role that p62 plays in various signaling pathways, including those of nuclear factor κB (NF-κB), extracellular signal–regulated kinase 1 (ERK1), and nuclear factor erythroid 2–related factor 2 (Nrf2) (49, 50). Assessing multiple components of the autophagy process using the mature adipocyte–specific Cre lines, such as Adiponectin- or Ucp1-Cre, would clarify the specific biological processes that are specifically regulated by each autophagy regulator.

**Physiological Regulation of Autophagy in Adipose Tissues**

Autophagy is initiated in concordance with responses to nutrient availability and is thus tightly regulated by the mTORC1 complex of the mTOR signaling pathway, which acts as a nutrient sensor to coordinate cellular responses (Fig. 4). High nutrient abundance leads to the activation of mTORC1 and its downstream targets, including ribosomal S6 kinase 1 (S6K1) to promote protein synthesis for anabolic functions. In the absence of nutrients, mTORC1 is inhibited, leading to autophagy activation through the regulation of the ULK1, FIP200, and ATG13 complex (Fig. 4A) (51).

Another form of physiological regulation of autophagy is β3-AR signaling, which triggers protein kinase A (PKA) signaling. PKA signaling functions to inhibit autophagy in yeast and mammals (52), and feedback loops between autophagy and PKA have been uncovered (53, 54). Notably, the β3-AR signaling pathway is a key mediator of beige adipocyte biogenesis in the face of cold exposure. Beige adipogenesis is promoted when environmental cues, such as cold exposure, trigger the release of norepinephrine from adipose tissues.
the sympathetic nervous system, which, in turn, acts on the β3-AR, increases intracellular cyclic adenosine 3',5'-monophosphate (cAMP) concentrations, and triggers the PKA signaling pathway, leading to transcriptional activation of the thermogenic program in brown and beige fat (Fig. 4B) (55). In response to cold or β3-AR stimulation, PKA directly phosphorylates mTOR and its binding partner, regulatory-associated protein of mTOR (RAPTOR), to stimulate the activity of mTORC1, a major regulator of autophagy (Fig. 4C) (56). Our group has further shown that PKA, in turn, represses autophagy in beige adipocytes (Fig. 4D), which is partially mediated through repression of Mitf, a member of the MiT/TFE family of transcription factors that regulates lysosome biogenesis. Pharmacological inhibition of PKA relieves repression of Mitf and genes encoding autophagy and lysosome compo-

Pathological Regulation of Autophagy in Metabolic Disease

Autophagy plays a central role in the function and maintenance of metabolic tissues such as liver, pancreas, and adipose tissues. Emerging evidence suggests that dysregulation of autophagy contributes to the initiation or progression of metabolic disorders in the following organs.

Adipose tissue

Obesity (increased adiposity or body mass index) is inversely correlated with the thermogenic activity of BAT in response to cold exposure (72). Beige adipocyte biogenesis is impaired in obese mice partly because of

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the activation of negative regulators such as the transforming growth factor–β (TGF-β) and Notch signaling pathways (26). In turn, blockade of the TGF-β or Notch signaling pathways by genetic or pharmacological approaches promotes beige adipocyte biogenesis and protects mice from diet-induced obesity (73–75). Furthermore, autophagy blocks beige adipocyte development. Inhibiting autophagy through deletion of Atg7 in adipocytes leads to increased beige adipocyte differentiation, resistance to diet-induced obesity, and improved insulin sensitivity (42, 43).

Dysregulation of beige adipocyte maintenance is likely to contribute to the development of obesity. We have found that diet-induced obesity accelerates the conversion of beige adipocytes to white adipocytes after β3-AR agonist withdrawal, which correlates with increased autophagy in subcutaneous WAT. Genetic deletion of Atg12 or Atg5 specifically in UCP1-positive adipocytes substantially prolongs the retention of beige adipocytes in vivo. The maintained beige fat is thermogenically active and suppresses diet-induced obesity and obesity-induced insulin resistance (39). Notably, increased adipose tissue autophagy has been observed in human obesity and type 2 diabetes (76–79). In addition, activation of autophagy in human subjects with type 2 diabetes and obesity is partly attributed to repression of mTORC1 activity (76). These studies suggest that dysregulation of autophagy in the adipose tissues of obese subjects may contribute to the accelerated beige-to-white adipocyte conversion.

Pancreas

Defects in pancreatic islet β cell function are the fundamental cause for type 1 and type 2 diabetes. Pancreatic β cells rely heavily on mitochondria and the ER to maintain glucose-stimulated insulin production and secretion, and autophagy maintains β cell homeostasis by removing damaged mitochondria and/or ER. Mice with a β cell–specific deletion of Atg7 accumulate defective mitochondria and distended ER in their β cells, leading to impaired glucose tolerance and reduced insulin secretion (80, 81). Autophagy-deficient β cells fail to proliferate as an adaptation to increased insulin demand in obesity (81, 82). The mechanism by which autophagy controls β cell proliferation remains unclear.

Liver

Obesity is closely associated with hepatic steatosis. Mice with diet-induced obesity or a genetically induced form of obesity (ob/ob mice) display reduced hepatic autophagy, as assessed by decreased numbers of GFP-LC3 puncta, reduced LC3-II abundance, and accumulation of p62 (85). Liver-specific deletion of Atg7, Vps34 (vacuolar protein sorting 34), or Tfeb results in lipid accumulation and enlarged livers (64, 86, 87). Conversely, overexpression of Atg7 or Tfeb by adenovirus reverses hepatic lipid accumulation, reduces liver size, prevents body weight gain in response to genetic or high-fat diet–induced obesity, and improves glucose tolerance and insulin sensitivity (85, 87). However, contrary to these studies, liver–specific deletion of Atg7 leads to reduced hepatic lipid accumulation (47, 88). Because the diverging phenotypes come from the same genetic model, the discrepancies are presumably due to differences in experimental conditions or analyses.

Starvation is a powerful stimulus that induces autophagy in the liver to control hepatic gluconeogenesis. During the early neonatal period, when the placental nutrient supply is cut off at birth, autophagy-deficient (Atg5−/−) pups die soon after birth from severe hypoglycemia and hypolipidemia (89). Dysregulation of autophagy in a liver-specific fashion in adults leads to a defect in intracellular lipid degradation, enlarged liver, and increased hepatic lipid content under starvation conditions (64, 87). In addition, liver–specific deletion of Atg7 using inducible Mx1-Cre results in hypoglycemia due to reduced gluconeogenesis (90).
Positive regulators of the starvation-induced autophagy include TFEB and PPARα; for instance, liver-specific deletion of Tfeb prevents transcription of various autophagy-related genes and leads to increased hepatic lipid content, increased circulating free fatty acids (FFAs), and impaired FFA oxidation in vitro (87). Similarly, PPARα activates transcription of autophagy components. Pharmacological activation of PPARα induces autophagy in nutrient-replete cells, which mimics a starvation response, whereas the liver of fasted Ppara−/− mice cannot induce autophagy and exhibits increased lipid content (91). On the other hand, farnesoid X receptor (FXR) functions to repress autophagy in the fed state. Pharmacological activation of FXR blunts starvation-induced hepatic autophagy, whereas Fxr−/− mice maintain high amounts of autophagy in the liver even in the fed state (65, 91). It would be important to determine whether the same regulation applies to non–starvation-induced autophagy in other metabolic organs.

Methodologies for Detecting Mitophagy in Adipocytes
Mitophagy structures were initially identified by electron microscopy. Studies from the 1950s described double-membrane vesicles, later termed autophagic vacuoles, which contained recognizable mitochondrial cristae (92). Identification and quantification of mitophagy have been challenging due largely to the substantial overlap with autophagy machinery and an absence of a universally defined marker for mitophagy per se. Despite the current limitations, there are several tools available for monitoring mitophagy as described below.

Colocalization of mitochondria-localized proteins with GFP-LC3
Mitophagy can be assessed on the basis of colocalization of the autophagosome with mitochondria. To this end, GFP-LC3 transgenic mice have been successfully used to visualize autophagosomes in vivo and cultured cells (93). Vital dyes, such as MitoTracker Red are reliable options for labeling mitochondria in vitro. However, these dyes label less than 50% of the existing mitochondria and lose signal after fixation, limitations that preclude their use in vivo (94). An alternative approach to vital dyes is to label mitochondria with mitochondria-localized proteins and assess their sequestration into the autophagosome using GFP-LC3. During the beige-to-white adipocyte conversion, we have observed the colocalization of the mitochondria protein translocon of outer mitochondrial membrane 20 (TOM20) with GFP-LC3; analyzed in conjunction with mitochondria-autophagosome structures observed by electron microscopy, as well as with changes in mitochondrial content, these data suggest that selective mitophagy takes place during the beige-to-white adipocyte conversion (39).

Monitoring mitochondrial turnover: Indirect measurement of mitophagy
Mitophagy is a transient event that cannot be fully examined through snapshots of the mitochondria using mitochondria-localized proteins. MitoTimer is a fluorescent reporter that can measure the kinetics of mitochondrial biogenesis and degradation. A mutant of the red fluorescent protein is attached to the mitochondrial localization sequence of cytochrome c oxidase subunit VIII (COX VIII), which fluoresces to green when the protein is newly synthesized and then gradually transitions to red as the protein matures (95, 96). This system has been used to assess mitochondrial turnover in skeletal muscle and heart (97, 98). Quantifying the loss of red fluorescence determines the kinetics of mitophagy process. This method is an indirect assessment of mitophagy, and as such, a major concern would be that protein degradation is also measured as well as mitophagy flux.

Monitoring delivery of mitochondria to lysosomes
A more direct way of measuring mitophagy is to assess the delivery of mitochondria to lysosomes. A tandem mCherry-GFP fusion protein is attached to the mitochondrial localization sequence of the mitochondrial fission 1 protein (FIS1). Different chemical properties of mCherry and GFP allow the identification of mitochondria that are undergoing lysosomal degradation; GFP is immediately degraded in the acidic lysosomal environment, whereas mCherry persists (99). A transgenic mouse model called mito-QC (quality control), which uses this system, has been generated (100).

A similar strategy for assessing mitophagy directly has been developed using the mt-Keima transgenic mouse system. A tandem repeat of COX VIII tagged with the fluorescent protein Keima is targeted to the mitochondria. Keima fluorescence is pH-dependent; it emits at different wavelengths at neutral or acidic pH, making it possible to determine whether mitochondria are in the cytosol (neutral pH) or lysosomes (acidic pH). In addition, Keima fluorescence is resistant to lysosomal degradation because it is derived from corals (101, 102). The dual fluorescence of this reporter allows direct and quantitative assessment of mitophagic flux using fluorescence-activated cell sorting (FACS), which often is a more sensitive and quantitative tool than imaging analysis (103). At least two potential shortcomings of using mt-Keima system exist: (i) unfixed tissues are required for visualizing changes in Keima fluorescence, and (ii) some spectral overlap of the red and green fluorescence exists (101, 102). The mito-QC system mentioned above could be a better system for imaging analysis because it overcomes these limitations, but it has not yet been optimized for FACS analysis (100). These direct tools of assessing mitophagy could be applied to metabolic organs including adipose tissue.

Future Directions
An intriguing observation from our study is that autophagy-mediated degradation of mitochondria in beige adipocytes maintains the fate of these cells. Whereas the role of the nucleus in regulating mitochondrial mass and function has been examined extensively in the past, the above result indicates, in turn, that mitochondria immensely influence the cellular maintenance of beige adipocytes. What is the molecular signaling that mediates the mitochondria-to-nucleus communication? Mitochondrial clearance could regulate cellular maintenance and function by altering nuclear gene expression by limiting metabolite availability. For instance, several mitochondrial metabolites (such as α-ketoglutarate, succinate, and fumarate) regulate chromatin-modifying enzymes, such as Jumonji demethylases, which control the adipocyte differentiation program (104). In addition, mitochondrial stress in C. elegans activates another type of mitochondrial quality control, UPRmt (unfolded mitochondrial protein response), which alters the chromatin structure, causing persistent changes in gene expression (105). Further studies are needed to uncover the molecular mechanisms and biological roles of the mitochondria-nuclear communication in adipocytes.

REFERENCES AND NOTES


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