THE DYNAMIC INTERPLAY BETWEEN NUTRITION, AUTOPHAGY AND CELL METABOLISM

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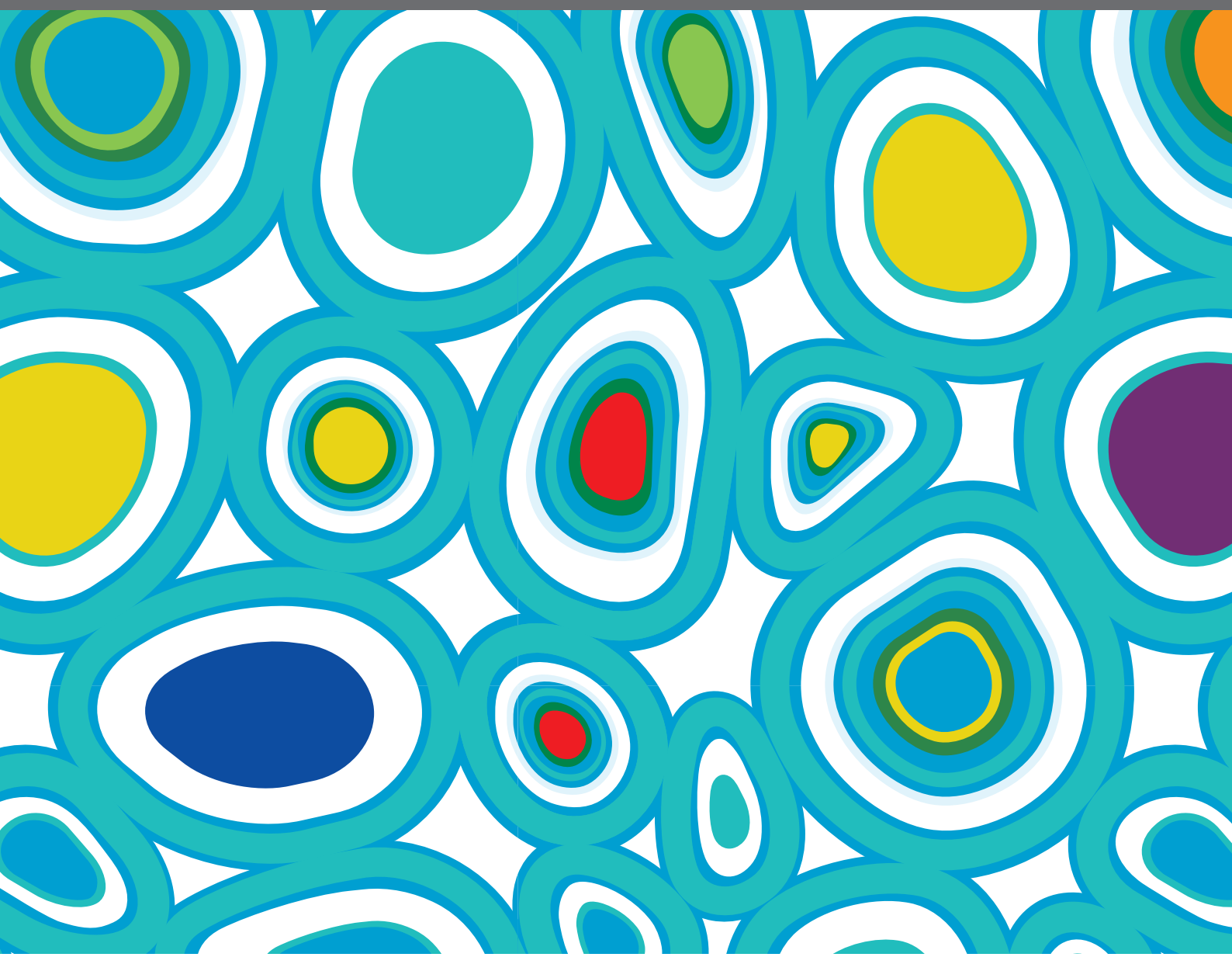

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THE DYNAMIC INTERPLAY BETWEEN NUTRITION, AUTOPHAGY AND CELL METABOLISM

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EDITORIAL
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Editorial: The Dynamic Interplay Between Nutrition, Autophagy and Cell Metabolism

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Editorial on the Research Topic

The Dynamic Interplay Between Nutrition, Autophagy and Cell Metabolism

Autophagy (derived from Greek words *"auto"* meaning self and *"phagy"* meaning eating) is a physiological cellular program that removes unnecessary or dysfunctional components in an orderly fashion (Dikic and Elazar, 2018; Platt, 2018). Autophagy allows the degradation and recycling of cellular components. It modulates metabolism of lipids, proteins, or carbohydrates, prevents accumulation of protein aggregates, removes damaged molecules and intracellular organelles and therefore autophagy plays a prominent function in maintaining cell homeostasis, inflammation, neurodegenerative disease and aging (Parenti et al., 2015; Dikic and Elazar, 2018; Platt, 2018) (**Figure 1**).

The orderly degradation of cytoplasmic content generates by-products that are directly used in many metabolic pathways, therefore autophagy is intimately linked to many metabolic pathways. To emphasize the importance of autophagy in health and disease, we are presenting a Research Topic containing a diversity of Review and Research Articles that shed new light on the molecular mechanisms regulating the interplay between autophagy and cell metabolism in physiological and pathological conditions.

Autophagy has been described in yeast, animals and plants and most of the autophagy related genes (ATG) are conserved among different living organisms (Dikic and Elazar, 2018). In the opening article of this Research Topic, Bu et al., discuss about the role of ATG8 in plant autophagy. Multiple ATG8 orthologs have been identified in plants and the diversity within the ATG8 family may suggest that they are involved in various functions in plants autophagy. More specifically, ATG8 proteins undergo conjugation to phosphatidylethanolamine and regulate membrane elongation during autophagosome biogenesis. This is a process that serves as an important catabolic mechanism in plant growth and development, as well as in plant responses to stress. ATG8s play a role in cargo recognition for selective autophagy by interacting with autophagy receptors/adaptors to target specific substrates for degradation. But the exact roles of ATG8 in plants intracellular trafficking are still poorly characterized. In addition, ATG8 proteins play non-autophagic roles, such as plant senescence control, by interacting with specific proteins. Nevertheless, the broad spectrum of ATG8 functions is yet to be completely clarified.

In another review article focusing on lipid metabolism, Xie et al. discuss the complex interplay between lipid metabolism and autophagy. This interplay is important for the maintenance of cell homeostasis through modulation of cell survival and death. Although lipid metabolism is crucial in the formation of membrane structures that constitute the autophagosome—the double-membrane

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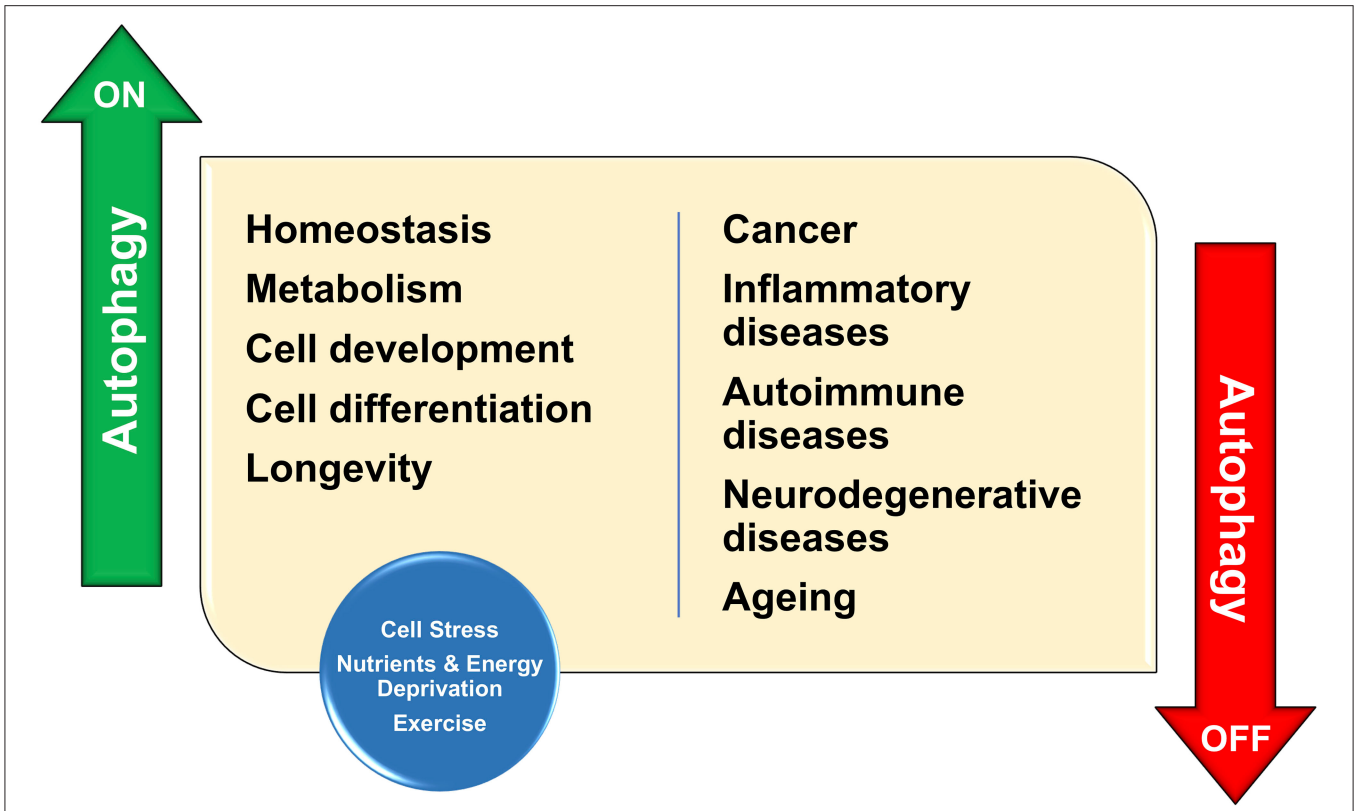

FIGURE 1 | Autophagy has important functions in organelle maintenance, protein turnover, and cellular stress; Therefore, autophagy contributes to the maintenance of cellular homeostasis. As a consequence, any defect in such protective functions would result in the development of many human conditions including cancer, inflammation, and neurodegenerative disease.

sequestering vesicles hallmark of autophagy—autophagy *per se* has been found to promote lipid catabolism and lipid peroxidation-induced cell death, such as ferroptosis. A complex connection linking fatty acids, triglyceride, cholesterol, membrane lipids biosynthesis, nutrition status, and autophagy has been also found. The dysfunction of autophagy-dependent lipid catabolism is implicated in several pathologic conditions including steatosis, non-alcoholic fatty liver disease, Parkinson's disease and metabolic syndrome. On this regard, autophagy has been taken in consideration as a target for the treatment of several diseases. Here, the authors suggest that a better understanding of the mechanisms underlining the interplay between autophagy and lipid metabolism would promote potential treatments for lipid metabolism-related disorders. However, many questions remain to be addressed including: how autophagy functions in regulating lipid metabolism in different cells? How autophagy switch from pro-survival to pro-death signal? How selective is autophagy in controlling cell-death?

A review article by Stacchiotti and Corsetti provides an in-depth view of the age-related neurodegeneration, that leads to severe diseases affecting motility, memory, cognitive function, and social life. To date, there is a lack of effective treatments for neurodegeneration and the consequent irreversible neuronal loss. Considering that aberrant autophagy is involved in aging and neurodegeneration, its targeting may represent a possible strategy to fight or prevent age-related neurodegeneration. Many natural compounds such as polyphenols, flavonoids, polyamine, and sugars, limit brain damage *in vitro* and *in vivo* and are modulators of autophagy. Their activity leads to restoration of efficient autophagy thus promoting degradation of misfolded proteins and of dysfunctional mitochondria. Many studies have reported the efficacy of natural compounds in enhancing or restoring autophagy in Alzheimer, Parkinson and Huntington disease preclinical rodent models. However, an enhanced autophagy may be deleterious in forebrain axons, affecting retrograde flux and function. These evidences indicate that in the brain an enhanced autophagy may not be always beneficial. Unfortunately, a limitation to the development of autophagy modulators for therapeutic intervention in humans is that the currently available methods to measure the autophagic flux are not efficient and consequently the generation of natural drug's efficacy in modulating autophagy is complex.

We were also enthusiastic to read a research article by Marzetti et al. discussing physical frailty and sarcopenia (PF&S), a human condition characterized by reduced physical function and low muscle mass in the elderly. In their work, the authors analyzed the relationship between three processes that are thought to be involved in PF&S: systemic inflammation, amino

acid dysmetabolism and mitochondrial dysfunction. The studies took advantage of a cohort of old adults recruited in the "BIOmarkers associated with Sarcopenia and Physical frailty in ElderRly pErsons" (BIOSPHERE) study to evaluate inflammatory biomolecules, amino acids and derivatives, and mitochondrial-derived vesicle (MDV) cargo molecules as possible biomarkers for PF&S. The idea behind this primary research was the evaluation of these biomarkers in large cohorts and their changes over time or in response to clinical interventions in order to unveil specific pathogenetic pathways of PF&S and identify new biological targets for drug development. In particular, the retrieval of MDVs in serum of older adults with PF&S has been associated with an innate immune response. According to this theory, MDVs may function as antigen-presenting vesicles carrying harmful material. Similar to damage-associated molecular patterns (DAMPs), released from injured cells, the MDV cargo can trigger caspase-1 activation and the secretion of pro-inflammatory cytokines. Impaired mitochondrial quality control in skeletal myocytes may therefore generate a vicious circle favoring further mitochondrial damage and the propagation of sterile inflammation through DAMPs release. On this regard, the search of circulating mitochondrial DAMPs in the elderly might be exploited for the development of therapeutic interventions for PF&S.

In their mini-review, Kitada et al. discuss about the close relationship existing between nutrients, autophagy and lifespan. In particular the authors focussed their discussion on methionine, an essential amino acid, because of its ability to modulate autophagy through modulation of mTORC1 activity. Recent studies have reported that protein restriction, rather than calorie or dietary restriction, is strongly involved in the lifespan extension and cardiometabolic health. Furthermore, dietary methionine restriction may have a beneficial effect on lifespan extension and metabolic health. Methionine may activate mTORC1 and suppress autophagy. However, it has been reported that mTORC1 may be activated by sensing *S*-adenosyl methionine (SAM) rather than methionine and SAM, rather than methionine, may be the main contributor to the aging process. SAM has been also associated to insulin resistance and truncal adiposity in elderly individuals. Therefore, the upregulation of SAM associated with overfeeding or metabolic dysfunction may be involved in whole-body metabolic impairment. Hence, the suppression of mTORC1, induced by decreasing SAM levels, may represent a therapeutic target for aging, age-related diseases and metabolic impairment.

Last but not least, Thomas et al. report that many clinical trials are evaluating the effectiveness of compounds known to regulate autophagy in patients receiving anti-cancer chemotherapy. In this primary study the authors aimed to assess the effect of amino acid starvation on doxorubicin-treated breast cancer cells by assessing the modulation of autophagy and apoptosis. Indeed, many cancers are known to respond to certain chemotherapeutics or to radiation therapy by increasing autophagic activity. Although the role of autophagy in these circumstances are still debatable, it appears that autophagy acts predominantly as cell survival mediator. In their cell culture-based studies the authors elegantly demonstrated a differential protection of non-cancer cells and increased apoptosis in cancer cells during chemotherapy when these cell lines with high basal autophagy activity were starved of amino acids. Altogether, these results suggest that short-term starvation during doxorubicin chemotherapy may be a possible strategy for adjuvant therapy, especially for the protection of non-cancerous cells.

In conclusion in this Research Topic we have collected a series of review and primary research article describing the close relationships between autophagy and metabolism and we have learnt that many questions are still unanswered and therefore we encourage our colleagues to dedicate quality time onto this fascinating area of research.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Interplay Between Lipid Metabolism and Autophagy

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Autophagy is a self-eating process of using lysosomes to degrade macromolecular substances (e.g., proteins and organelles) that are damaged, degenerated, or aging. Lipid metabolism is the synthesis and degradation of lipids (e.g., triglycerides, steroids, and phospholipids) to generate energy or produce the structural components of cell membranes. There is a complex interplay between lipid metabolism (e.g., digestion, absorption, catabolism, biosynthesis, and peroxidation) and autophagy machinery, leading to the modulation of cell homeostasis, including cell survival and death. In particular, lipid metabolism is involved in the formation of autophagic membrane structures (e.g., phagophores and autophagosomes) during stress. Moreover, autophagy, especially selective autophagy (e.g., lipophagy, ferritinophagy, clockophagy, and mitophagy), promotes lipid catabolism or lipid peroxidation-induced ferroptosis through the degradation of various substances within the cell. A better understanding of the mechanisms of autophagy and possible links to lipid metabolism will undoubtedly promote potential treatments for a variety of diseases.

Keywords: autophagy, lipid, metabolism, lipophagy, ferritinophagy, clockophagy, mitophagy, disease

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INTRODUCTION

The morphological changes of macroautophagy were first observed using electron micrographs of rat liver after perfusion with glucagon for 4 h by Thomas Ashford and Keith Porter in 1962 (Ashford and Porter, 1962). Later, Christian de Duve coined the term "autophagy" from the ancient Greek language to describe the process of "self-eating" (Klionsky, 2008). It is now known that macroautophagy is one of the lysosome-mediated degradation pathways that plays a critical role in maintaining homeostasis (Yang and Klionsky, 2010). In general, increased macroautophagy can promote cell survival in response to various stresses, such as starvation, radiation, hypoxia, and oxidative stress. Macroautophagy can remove injured organelles, unused proteins, or invading microorganisms for normal cell activity and metabolism during aging, differentiation, or infection (Mizushima, 2007; Kaur and Debnath, 2015). However, deficient, excessive, or dysfunctional macroautophagy is implicated in various human diseases and pathologic conditions (Levine and Kroemer, 2019).

Lipids are one of the important nutrients of the body, providing it with energy and essential fatty acids (FAs) or their derivatives. There are three types of lipids, namely triglycerides (TGs), steroids, and phospholipids (Fahy et al., 2005, 2009). TGs have a chemical name of triacylglycerols (TAGs), built from one glycerol molecule and three FAs. Steroids include hormones and cholesterol. Notably, cholesterol, the most abundant steroid lipid in the body, also plays a role in the production of hormones. Phospholipids form double-layered membranes with water-soluble molecules on

the outside of the cell membrane and water-insoluble molecules in the inside (DeBose-Boyd, 2018). The levels of lipids are controlled by lipid metabolism, which is a complex process involved in the biosynthesis and degradation of lipids. The first step of lipid metabolism is hydrolysis. As hydrophobic molecules, lipids need to be solubilized to produce free FAs (FFAs) and monoacylglycerol (MAG) (Mu and Porsgaard, 2005) through enzymatic hydrolysis in the digestive system. The second step involves the absorption, packaging, and transporting of the FAs from the digestive system into the rest of the body (Ko et al., 2020). TGs, also known as fats, are mainly obtained from daily food. Lipogenesis is the process of synthesizing TGs, mostly completed in the liver. Dysfunction in the storage or breakdown of lipids can cause cell dysfunction, even cell death (Zechner et al., 2012).

Recent years have seen a rapid growth in the study of the interplay between macroautophagy and lipid metabolism (Liu and Czaja, 2013; Caron et al., 2015; Jaishy and Abel, 2016; Thelen and Zoncu, 2017). In this review, we introduce the basic process of macroautophagy and summarize recent progress in understanding the impact of lipid metabolism on macroautophagy.

OVERVIEW OF AUTOPHAGY

Autophagy can be divided into three main types, namely chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy, according to the transporting manners of cell materials into lysosomes (Dikic and Elazar, 2018). CMA is mediated by heat shock proteins that bind the target substrates to deliver them to lysosomes for degradation (Majeski and Dice, 2004). During microautophagy, long-lived proteins can be directly engulfed by lysosomal membrane to degrade in lysosomes (Li et al., 2012). Macroautophagy (hereafter autophagy) is a well-studied dynamic process, which is involved in the formation of several specific membrane structures, such as phagophores, autophagosomes, and autolysosomes (Dikic and Elazar, 2018) (**Figure 1**). The phagophores, also known as the isolation membranes, can engulf and isolate the cytoplasmic components to produce subsequent autophagosomes, a double membrane structure. The autophagosome further fuses with the lysosome to yield autolysosomes, leading to the degradation of the sequestered cytosolic material via the lysosome hydrolases.

At the molecular level, the formation of membrane structures of autophagy is controlled by autophagy-related (ATG) genes, which are conserved genes from yeast to humans (Levine and Kroemer, 2019). The ATG-coded proteins can form different complexes that are regulated by their posttranslational modifications (Xie et al., 2015). The ATG proteins associated with other regulators play a complex role in the autophagic process of induction, nucleation, elongation, fusion, and degradation (Dikic and Elazar, 2018).

Induction

Autophagy is initiated by the formation of a phagophore that originates in the membranes of Golgi apparatus, endoplasmic reticulum (ER), endosome, mitochondria, or the plasma membrane. The induction of autophagy is controlled by the unc-51–like autophagy-activating kinase 1 (ULK1, a homolog of Atg1 in yeast) kinase complex, including the core component of ULK1, ATG13, and RB1 inducible coiled-coil 1 (RB1CC1, also known as FIP200). In addition to ULK1, ULK2 may have similar function in autophagy induction. Notably, two upstream kinases, namely mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK), can inhibit or promote, respectively, the ULK1 kinase complex in response to environmental stresses (Holczer et al., 2019).

Nucleation

The class III phosphatidylinositol 3-kinase (PtdIns3K) complex, mainly containing phosphatidylinositol 3-kinase, catalytic subunit type 3 (PIK3C3)/VPS34, BECN1 (also known as Atg6 in yeast), and ATG14 (also known as beclin-1-associated autophagy-related key regulator [Barkor] or ATG14L), plays a key role in the nucleation of phagophores (McKnight and Zhenyu, 2013). One of the key functions of the PtdIns3K complex is the generation of phosphatidylinositol-3-phosphate (PtdIns3P), a phosphoinositide that serves as a landmark on the membrane to recruit other factors involved in the process of autophagosome formation (Bernard and Klionsky, 2014). BECN1 is a multifunctional protein that not only promotes autophagy, but also controls cellular sensitivity to regulated cell death, such as apoptosis and necroptosis, through its binding partners (Kang et al., 2011). ATG14 plays an important role in the formation of autophagosomes (Zhong et al., 2009). ATG14 acts as a specific targeting factor for PI3KC3 to autophagosome membranes to maintain membrane curvature (Fan et al., 2011). In addition, ATG14 blocks connexins-mediated inhibitory effect on autophagy during autophagasome formation (Bejarano et al., 2014).

Elongation

Subsequent to nucleation, the phagophore expands by membrane addition, which is accomplished by 2 ubiquitin-like (Ubl) conjugation systems, the ATG12-ATG5 conjugation system and the microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3) conjugation system (Ohsumi, 2001). The ATG12-ATG5 conjugate can further bind ATG16L1 (also known as Atg16 in yeast) to form a ATG12-ATG5-ATG16L1 complex at phagophores. MAP1LC3 exhibits two forms, namely MAP1LC3-I and MAP1LC3-II. At baseline, most MAP1LC3 is MAP1LC3-I. In contrast, the production of MAP1LC3-II is increased in response to autophagic stimulus that is essential for the formation of the autophagosome and subsequent degradation of cargos through the binding to autophagy receptors, such as sequestosome 1 (SQSTM1, also known as p62) and calcium-binding and coiled-coil domain 2 (CALCOCO2, also known as NDP52). In addition to Ubl conjugation systems, ATG9-mediated cycling systems contribute to the elongation of the phagophore. ATG9 is thought to move from the *trans*-Golgi network or late endosomes to the phagophore and is regulated by the activity of ULK1, PtdIns3K, and mitogen-activated protein kinase 14 (MAPK14, also known as p38)

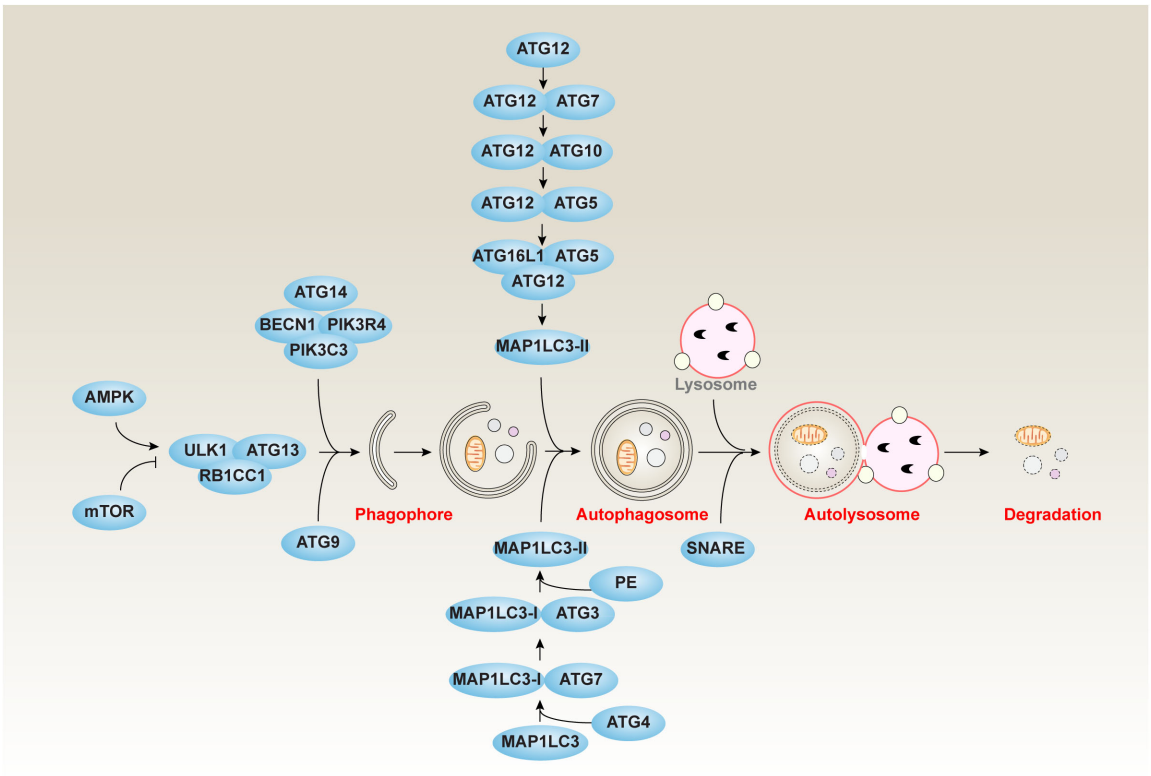

FIGURE 1 | The core molecular machinery of autophagy. Autophagy is a dynamic process involving the formation of several specific membrane structures, such as phagophores, autophagosomes, and autolysosomes. The ATG proteins associated with other regulators play a complex role in the autophagic process of induction, nucleation, elongation, fusion, and degradation.

(Young et al., 2006; Webber and Tooze, 2010). In addition to MAP1LC3, other orthologs of yeast Atg8, such as GABA type A receptor-associated protein (GABARAP) and GABA type A receptor-associated protein-like 2 (GABARAPL2, also known as GATE-16), also contribute to autophagosome formation in some cases (Schaaf et al., 2016).

Fusion and Degradation

Once autophagosome formation is complete, the outer membrane of the autophagosome fuses to lysosomes to produce autolysosome, and the cellular materials (e.g., mitochondria and ER) and invading pathogens are destroyed by enzymes in lysosomes (Pankiv et al., 2007). Although many factors affect the fusion between autophagosome and lysosome, the soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) family seems to play a key role in the formation of autolysosomes (Nakamura and Yoshimori, 2017). In addition, ATG14 binds and stabilizes the SNARE complex, thereby promoting autophagosome-lysosomal fusion (Diao et al., 2015). The autophagosome marker MAP1LC3-II protein can be finally degraded with cargos or autophagy receptors through lysosomes (Mizushima and Yoshimori, 2007; Pankiv et al., 2007). Thus, autophagic flux is an important factor in monitoring the formation and degradation of autophagosomes (Klionsky et al., 2016; Yoshii and Mizushima, 2017). The cell membrane, one of resources of the phagophore, can be eventually digested by lysosomes or self-decomposed through autolysosome formation.

LIPID DIGESTION AND AUTOPHAGY

The digestion of lipids takes place mainly in the small intestine. As pre-digested (orally- and stomach-digested) food enters the small intestine, the lipids in the food are emulsified, thereby promoting the release of FAs from TAGs, and other lipids (e.g., phospholipids and cholesterol) are also dispersed in the small colloidal particles containing water and oil that are called mixed micelles. Emulsification increases the surface area between enzymes and lipids, thereby increasing the lipolytic effect of lipase. These enzymes include pancreatic lipase, colipase, cholesterol esterase, and phospholipase A2 (PLA2). The emulsified FAs are further catalyzed by the pancreatic lipase, the phospholipids by the PLA2, and the cholesterol ester by the cholesterol ester enzyme. As a result, the lipids in the food produce glycerides, FAs, cholesterol, and phospholipids, which significantly increases the solubilization of the mixed micelles (Ko et al., 2020).

Although the autophagy-lysosomal system is not directly involved in the digestion of intestinal lipids, it plays a central role in cellular food degradation (also known as intracellular

digestion) (McVeigh et al., 2006). Digestion produces the biosynthetic precursors needed to regenerate partially disrupted structures, thereby generating the energy necessary for anabolic processes. Some core components of autophagy machinery have lipid kinase modulation activity, such as PIK3C3/VPS34 and BECN1, which are required to initiate autophagy during fasting (Pozuelo-Rubio, 2012; McKnight and Zhenyu, 2013). Consequently, this would affect the rate of energy maintenance upon acute starvation.

LIPID ABSORPTION AND AUTOPHAGY

In the small intestine, mixed micelles containing FAs, glycerol, cholesterol, and phospholipids are transported to intestinal epithelial cells for absorption. The uptake and absorption of glycerol and FAs are affected by chain length. Short-chain FAs ($\leq$12 C) can be directly absorbed into the blood by binding to albumin. Long-chain FAs (>12 C) and other lipids need to be transported across cell membranes through the action of transporters. Inside the cell, they will be resynthesized into TAGs in the ER and then transported into the Golgi apparatus, where they combine with cholesterol, phospholipids, and apolipoproteins to form a lipoprotein called chylomicrons protein. Lipoproteins are transporters that are responsible for transport from the origin to the destination through the blood and lymph. The solubility of lipoproteins in the bloodstream is due to the coating of apolipoprotein (Ko et al., 2020).

As mentioned above, intestinal epithelial cells are absorption cells of the small intestine and mediate the absorption of fats in the diet by secreting TAGs into the circulation. Generally, TAGs are stored in cytoplasmic lipid droplets (LDs) and are sequentially hydrolyzed for secretion according to changes in fat levels. The transfer and hydrolysis of TAG-containing LDs degraded by lysosomes are mediated by autophagy, a process called lipophagy (Singh et al., 2009). Therefore, LDs act as lipid reservoirs in the anabolic pathway, while lysosomes are dedicated to the degradation of intracellular components (Dugail, 2014). Diacylglycerol *O*-acyltransferase-1 (DGAT1) synthesizes TAG and is necessary for dietary fat absorption and storage. Recent studies have found a unique intestinal phenotype, abnormal TAG accumulation, and intestinal epithelial LD mobilization in DGAT1-deficient mice, resulting in delayed fat absorption and resistance to diet-induced obesity (Hung and Buhman, 2019). A high-fat diet results in increased lipid intake and intestinal fat deposition in yellow catfish, which adversely affects their lipid absorption. The underlying mechanism is that a high-fat diet upregulates lipogenesis, lipolysis, and FA transport, and it induces ER stress and activates autophagy. These effects on fat-induced changes in intestinal lipid uptake play an important regulatory role in the model of yellow catfish (Ling et al., 2019).

LIPID CATABOLISM AND AUTOPHAGY

Triglycerides and phospholipids are first broken down by lipase or phospholipase, respectively, which results in the release of FA chains from the glycerol carbon backbone. Glycerol can be phosphorylated to glycerol-3-phosphate and then converted to glyceraldehyde 3-phosphate by glycolysis. The released FAs are catabolized in a process called β-oxidation, which in turn removes two carbon acetyl groups from the end of the FA chain, thereby reducing NAD^{++} and FAD to produce NADH and FADH2, respectively. Electrons generated during β-oxidation can be used to make ATP through oxidative phosphorylation (Adeva-Andany et al., 2019). The acetyl groups produced during β-oxidation are carried into the Krebs cycle by coenzyme A, which causes them to degrade to CO_2, generate ATP through substrate-level phosphorylation, and generate additional NADH and FADH2 molecules (Adeva-Andany et al., 2019).

The catabolism of stored lipids in LDs is related to a variety of metabolic pathways that provide molecules used to generate energy, membrane building blocks, and lipid signaling (Wang, 2015). Generally, autophagy is induced for cell survival during LD degradation, which is controlled by multiple molecules (Cabodevilla et al., 2013; Parray and Yun, 2017). In particular, lipophagy-mediated LD degradation via patatin-like phospholipase domain-containing 2 (PNPLA2, also known as ATGL) can release FFAs under starvation conditions. FFA produced by LD catabolism is either transported to mitochondria for β-oxidation, or converted back to LDs. The biogenesis of LDs under starvation is mediated by autophagy degradation of membrane organelles, and DGAT1 is required as an adaptive cytoprotective mechanism against lipotoxicity (Li et al., 2017). PNPLA2-mediated signaling through sirtuin 1 (SIRT1) is necessary and sufficient to induce lipophagy for subsequent LD catabolism and FA oxidation in hepatocytes (Sathyanarayan et al., 2017). The overexpression of perilipin 2 (PLIN2, also known as adipophilin), one of the most abundantly expressed LD proteins, protects LD from autophagy-dependent degradation, while its deficiency stimulates TG catabolism through autophagy, protecting mice against fatty liver diseases (Tsai et al., 2017). Sphingosine kinase 2 (SPHK2) is also required for the autophagy-mediated catabolism of intracellular LDs to prevent the development of atherosclerosis by reducing sphingosine content in macrophages (Ishimaru et al., 2019). Thyroid hormones induce FA β-oxidation through autophagy, which is associated with an increased delivery of FAs into mitochondria. Blockage of autophagy significantly reduces thyroid hormone-mediated FA β-oxidation *in vitro* and *in vivo* (Sinha et al., 2012).

Autophagy-mediated lipid catabolism can be regulated by transcription factors. The upregulation of transcription factor forkhead homeobox protein O1 (FOXO1) or lysosomal acid lipase (LIPA) increase autophagy-dependent LD degradation and subsequent FA release through AMPK-dependent β-oxidation in adipocytes upon nutrient restriction (Lettieri Barbato et al., 2013). Another transcriptional mechanism that links autophagy to lipid catabolism is the activation of transcription factor EB (TFEB) during starvation (Li et al., 2016; Napolitano and Ballabio, 2016). TFEB-mediated transcriptional induction of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) and peroxisome proliferator-activated receptor alpha (PPARA) serves as a prosurvival response to

nutrition deprivation (Settembre et al., 2013). Moreover, PPARA-induced TFEB activation or microRNA-33–mediated TFEB inhibition may form a feedback loop to further regulate lipid catabolism and FA β-oxidation (Ouimet et al., 2016, 2017; Kim et al., 2017). This process is also implicated in the response to ethanol-induced liver injury in mice (Thomes et al., 2019).

In addition to transcription factors, phosphoinositide-3-kinase regulatory subunit 4 (PIK3R4, also known as VPS15 in yeast) is critical for regulating PPARA activation. The loss of PIK3R4 inhibits autophagy and lipid catabolism through the accumulation of PPARA repressors, such as histone deacetylase 3 (HDAC3) and nuclear receptor corepressor 1 (NCOR1) (Iershov et al., 2019). CCAAT enhancer-binding protein alpha (CEBPA) also plays an essential role in promoting cell survival and FA β-oxidation during liver injury (Lu et al., 2015), although the mechanism remains unclear. Moreover, the activation of the small guanosine triphosphatase (GTPase) family (e.g., Rab7 and Rab18), BCL2 family (e.g., BIF1), or methionine metabolism plays a context-dependent role in the regulation of FA β-oxidation during autophagy (Schroeder et al., 2015; Liu et al., 2016; Zubiete-Franco et al., 2016; Bekbulat et al., 2019).

In yeast, LDs can also be turned over in vacuoles/lysosomes by microlipophagy, a process morphologically similar to microautophagy (van Zutphen et al., 2014). Microlipophagy is different from lipophagy and does not involve core autophagy proteins, but requires ESCRT components and newly identified VPS proteins (Vevea et al., 2015; Oku et al., 2017). Microlipophagy-dependent LDs depletion is triggered by AMPK activation, but not glucose starvation, amino acid deprivation or rapamycin treatment (Seo et al., 2017). In contrast, mTOR (Rahman et al., 2018), amino acid (Hatakeyama et al., 2019), and glucose (Iwama and Ohsumi, 2019) are important regulators of microautophagy.

CMA deficiency can cause lipid accumulation (Qiao et al., 2020), and vice versa, a high-fat diet and excessive cholesterol intake can inhibit CMA (Rodriguez-Navarro et al., 2012). Lysosome-associated membrane protein type 2A (LAMP2A) is a key protein in the CMA pathway. The accelerated degradation of LAMP2A determines the loss of lysosomal membrane stability. Nutrient deprivation is also an activator of CMA, which selectively degrades PLIN (e.g., PLIN2 and PLIN3) and promotes the hydrolysis of LDs (Kaushik and Cuervo, 2015). These findings support the role of CMA in lipid metabolism, but the precise molecular pathway remains unclear.

The dysfunction of autophagy-dependent lipid catabolism is implicated in several pathologic conditions. Thiodigalactoside plays a role in browning and lipid catabolism by jointly inhibiting GAL1 and ATG5, so it may have potential therapeutic significance for regulating energy homeostasis through its role in white adipose tissue (Parray and Yun, 2017). Autophagy-mediated lipid catabolism is activated as a compensation for glutaminolysis inhibition, which regulates tumor cell survival (Halama et al., 2018). Enteric infection can initiate the metabolic reprogramming of enterocytes toward lipid catabolism, which is controlled by ULK1-dependent lipophagy and the subsequent activation of dual oxidase 1 (DUOX1), a member of the NADPH oxidase family (Lee et al., 2018). These findings indicate a complex interplay between lipid catabolism and autophagy.

LIPID BIOSYNTHESIS AND AUTOPHAGY

Fatty Acid Biosynthesis

Fatty acids can be saturated (like palmitic acid and stearic acid) or unsaturated (like oleic acid). FAs are synthesized by gradually adding two-carbon units in the form of acetyl-CoA (Herman and Zhang, 2016). Acetyl-CoA is an important intermediate produced by the decarboxylation of pyruvate in the glucose breakdown pathway. However, the two-carbon units are produced not only by acetyl-CoA directly, but also by a carboxylated product of acetyl-CoA or malonyl-CoA. This process is catalyzed by acetyl-CoA carboxylase (Herman and Zhang, 2016). Moreover, the synthesis of FAs from acetyl-CoA or malonyl-CoA is mediated by fatty acid synthase (FASN) (Chirala and Wakil, 2004). Acyl carrier protein (ACP), a component of the FASN complex, is the core activator for FA biosynthesis (Herman and Zhang, 2016). The acyl groups get anchored to the CoA group of ACP through a thioester linkage. In many cases, inhibition of FA synthesis promotes autophagy (**Figure 2**).

Autophagy has been shown to regulate food intake and energy balance in hypothalamic agouti-related peptide (AgRP) neurons partly through the modulation of FA biosynthesis (Kaushik et al., 2011). The levels of AgRP, a neuropeptide produced in the brain by the AgRP neuron, is regulated by starvation-induced autophagy and subsequently the production of FFAs (Kaushik et al., 2011). In contrast, an autophagy deficiency in the hypothalamus may produce a lean body phenotype due to the lack of FFA-dependent AgRP production (Kaushik et al., 2011). The inhibition of autophagy by constitutive mTOR activity makes hypoxic cells dependent on exogenous desaturated lipids because that the level of unsaturated FA synthesized is reduced under hypoxia (Young et al., 2013). An increase in *de novo* synthesis of lipids is thought to be a metabolic adaptation of cancer cells, which can promote survival and metastasis. Increased FASN expression in colorectal cancer cells is associated with the inactivation of autophagy, including increased expression of SQSTM1 (Zaytseva et al., 2015). LD-deprived cells fail to induce autophagy due to accelerated lipid synthesis (Regnacq et al., 2016). In contrast, the administration of cerulenin (a potent inhibitor of FASN) or palmitic acid can restore nitrogen starvation-induced autophagy in the absence of LDs (Regnacq et al., 2016). It is worth noting that arachidonic acid, a long-chain polyunsaturated fatty acid (PUFA), is the main synthetic product under nitrogen deprivation, whereas monounsaturated oleic acid is the main product under phosphorous deprivation (Kokabi et al., 2019). The inhibition of PI3K signaling is responsible for lipogenesis rather than lipid hydrolysis by initiating *de novo* FA biosynthesis (Ramanan et al., 2018). These findings reveal a complex connection linking FA biosynthesis, nutrition status, and autophagy.

Triglyceride Biosynthesis

There are three main ways for TGs to biosynthesize, namely the glycerol-3-phosphate (G3P) pathway [e.g., glycerol-3-phosphate acyltransferase (GPAT)], the dihydroxyacetone phosphate (DHAP) pathway, and the monoacylglycerol pathway [e.g., monoacylglycerol acyltransferase (MGAT)]. The G3P pathway,

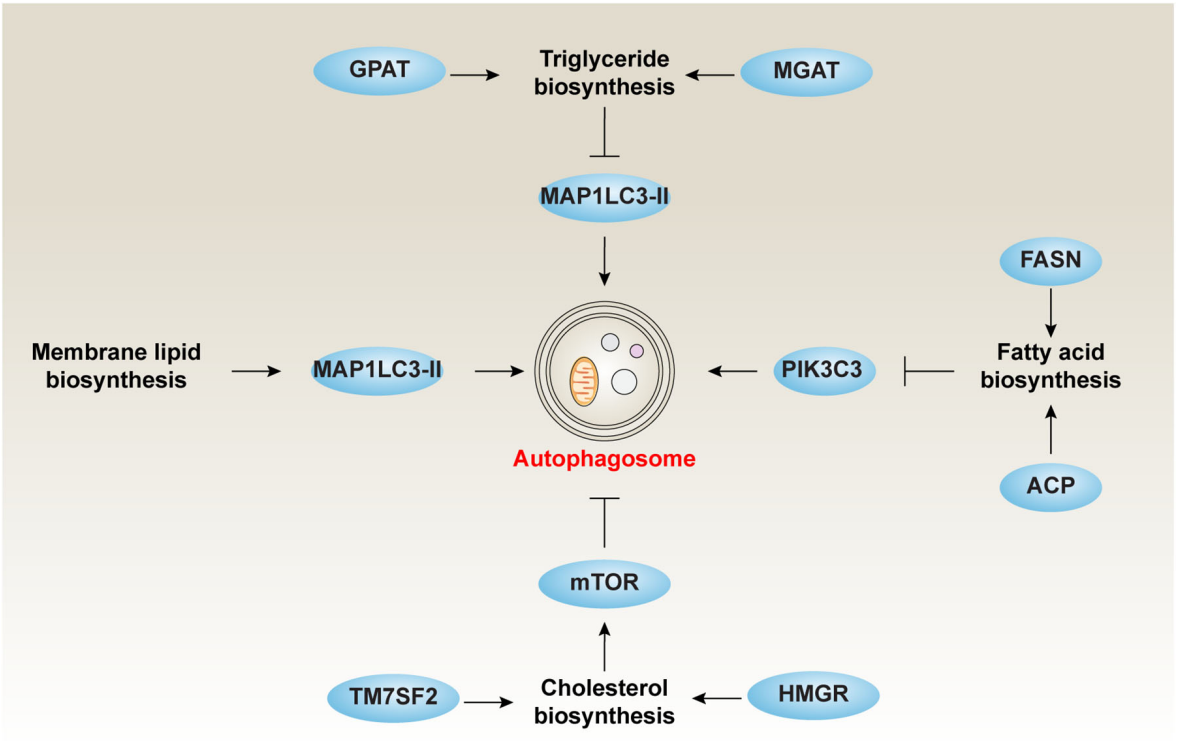

FIGURE 2 | The role of lipid biosynthesis in autophagy. Inhibiting TG, CE, and FA or increasing membrane lipid biosynthesis is responsible for the induction of autophagy.

referred to as the Kennedy pathway, was identified by Eugene Kennedy in 1960, which is responsible for 90% of TG synthesis (Chai et al., 2017). Except in the intestine and adipocytes, TG synthesis begins with G3P (Chai et al., 2017). Glycerol is first phosphorylated by glycerin kinase, and then activated FA (e.g., fatty acyl-CoA) is used as a substrate for the addition of FA to produce phosphatidic acid. The phosphate group is then removed and the last FA is added.

Autophagy is implicated in the metabolic balance of liver TG. A lack of protein in the diet reduces the expression of autophagy receptor SQSTM1, increases the expression of autophagosome marker (MAP1LC3-II) as well as ER stress marker (the spliced isoform of XBP1), which helps accumulate TG in the liver (Yokota et al., 2016). Other regulators also participate in TG metabolism via modulating autophagy activity. For example, the loss of PLIN2 inhibits lipogenesis, reduces TG synthesis, and enhances autophagy (Irungbam et al., 2020). These findings suggest that TG metabolism plays a vital role in the modulation of autophagy (**Figure 2**).

Cholesterol Biosynthesis

The biosynthesis of cholesterol generally takes place in the ER of hepatic cells and begins with acetyl-CoA, which is mainly derived from an oxidation reaction in the mitochondria (Alphonse and Jones, 2016). Acetyl-CoA is converted to 3-hydroxy- 3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. HMG-CoA is then converted to mevalonate by HMG-CoA reductase (HMGR). This reaction is completed with the aid of NADPH, a co-factor for all reduction reactions during cholesterol synthesis (Jiang et al., 2018). Mevalonate can undergo a series of phosphorylations or decarboxylations to produce isoprenoid and isopentenyl pyrophosphate (Liao et al., 2016). A squalene synthase-mediated condensing reaction leads to the production of squalene. The first of the sterols is formed following the production of squalene and lanosterol. The conversion of lanosterol to cholesterol requires additional multiple biochemistry reaction steps (Cerqueira et al., 2016). Notably, the conversion of HMG-CoA to mevalonate by HMG-CoA reductase is the rate-limiting step of cholesterol biosynthesis, which is under strict regulatory control (Cerqueira et al., 2016). Consequently, HMGR has been long-recognized as a drug target to reduce serum cholesterol levels.

It is becoming increasingly clear that the inhibition of cholesterol synthesis is responsible for the induction of prosurvival autophagy through blocking the AKT-mTOR pathway in human blood cancer cells (Vilimanovich et al., 2015) (**Figure 2**). This process can be selectively attenuated by either mevalonate or squalene, but not by isopentenyl pyrophosphate (Vilimanovich et al., 2015). The depletion of transmembrane 7 superfamily member 2 (TM7SF2), a key regulator of cholesterol biosynthesis, results in the increased expression of FA catabolic enzymes accompanied by decreased lipid accumulation, autophagy, and tissue injury in mice exposed to endotoxin (Gatticchi et al., 2015). In addition, *de novo*

sphingolipid biosynthesis is essential for autophagy induction in macrophages, which plays a protective role by clearing excess lipids from LDs through the turnover of ORMDL sphingolipid biosynthesis regulator 1 (ORMDL1) protein, a negative regulator of serine palmitoyl-CoA transferase activity (Wang et al., 2015b). Thus, the modulation of autophagy may influence cholesterol biosynthesis to reduce high-cholesterol–related diseases, such as atherosclerosis, heart disease, and stroke.

Membrane Lipid Biosynthesis

Membrane lipids are necessary to form the structure of biological membranes (such as cell membranes and intracellular membranes) and are mainly composed of phospholipids, glycolipids, and sterols (e.g., cholesterol). They can be arranged in double layers together with intact and peripheral membrane proteins. Biosynthesis of membrane lipids involves the production of major membrane lipids and their transport from the site of synthesis into the cell membrane (van Meer et al., 2008).

It is thought that isolated membranes observed during autophagy are mainly derived from pre-formed organelle membranes (e.g., ER). Instead, the phagophore membrane expands along with localized phospholipid synthesis (Schutter et al., 2020). The original separation membrane is formed on ER from locally synthesized lipids, then an increase in the biosynthesis of the bilayer-forming phospholipids [phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS)] occurs simultaneously with the induction of autophagy (**Figure 2**). PE conjugates the cytosolic MAP1LC3-I to form MAP1LC3-II, which is an important event in isolated membrane. The effect of PI3K on ER phosphatidylinositol coincides with the biogenesis of phospholipids. The two processes work together to help extend and assemble autophagosome particles (Girardi et al., 2011). The first step in *de novo* phospholipid synthesis at the ER is to make stable contact with nascent autophagosomes, which is essential for autophagy induction. Recent studies have shown that the conserved acyl-CoA synthetase FAA1 accumulates on nucleated phagophores, which is required for FA-mediated phospholipid synthesis and for promoting the assembly of phospholipids into autophagic membranes during phagophore elongation (Schutter et al., 2020). Glycosphingolipid is a key component of the eukaryotic cell membrane and is necessary for cavernous-mediated endocytosis and the function of glycosphingolipid-binding toxins (Sillence, 2007). Glycosphingolipid biosynthesis is restricted by enhanced autophagy, while its catabolism increases (Ghidoni et al., 1996). *De novo* sphingolipid biosynthesis is essential for autophagy induction (Wang et al., 2015a). Administering inhibitors to the first step of sphingolipid synthesis reduces autophagic activity by affecting autophagosome formation rather than the pre-structure formation of autophagosomes (Yamagata et al., 2011). Ceramide, a sphingolipid metabolite, serves as a strong autophagy activator (Scarlatti et al., 2004). Inhibiting synthesis of inositol phosphorylceramide reduces autophagy (Yamagata et al., 2011). Mitophagy, the degradation of mitochondria via selective autophagy, is linked to the phospholipid biosynthesis pathway for the conversion of PE to PC by the two methyltransferases, EBP cholestenol delta-isomerase (EBP, also known as CHO2) and phosphatidylethanolamine *N*-methyltransferase (PEMT) (Sakakibara et al., 2015). In addition, the autophagic digestion of LDs through lipophagy in liver is an essential process to obtain energy (Cai et al., 2016). Thus, the composition of membrane lipid seems to be a hallmark of autophagy induction.

LIPID PEROXIDATION AND AUTOPHAGY

Cell death has multiple forms, each exhibiting different molecular mechanisms and signal transductions (Tang et al., 2019). Although autophagy generally promotes cell survival through removing damaged organelles and oxidized molecules, it can also cause cell death under certain circumstances. This type of regulated cell death requires autophagy machinery and is termed as autophagy-dependent cell death by the Nomenclature Committee on Cell Death (Galluzzi et al., 2018).

Lipid peroxidation is a chain reaction of the oxidative degradation of lipids. In the reaction, an initiator radical first takes an allylic hydrogen of the unsaturated lipid and generates a corresponding radical. The free radical then reacts with an oxygen molecule to generate a corresponding peroxy radical, which captures the allyl hydrogen of another molecule and converts it into a hydroperoxide. Polyunsaturated fatty acids (PUFAs) are susceptible to peroxidation to yield various degradation products, such as malondialdehyde (MDA) and 4-hydroxy-2′-nonenal (4HNE) (Ye et al., 2016). These lipid peroxidation products influence cell fate partly through the activation of autophagy. For example, 4HNE can induce autophagy through the activation of c-Jun amino-terminal kinase (JNK) (Csala et al., 2015). The activation of JNK is accompanied by BCL2 being dissociated from BECN1 or by the induction of heme oxygenase 1 (HMOX1, also known as HO1) expression and MAP1LC3-II formation (Velez et al., 2011; Haberzettl and Hill, 2013). Other signaling associated with 4HNE-induced autophagy are the MAPK, mTOR, and protein kinase C pathways (Martinez-Useros and Garcia-Foncillas, 2016). In addition to inducing autophagy at lower concentrations, 4HNE can inhibit autophagic flux at higher concentrations (Dodson et al., 2017), indicating a negative feedback mechanism to limit excessive activation of autophagy during lipid peroxidation.

Lipid peroxidation is implicated in various kinds of regulated cell death (Kang et al., 2018; Su et al., 2019). In particular, increased lipid peroxidation is an important signal for triggering ferroptosis, an iron-dependent form of cell death that was first identified in mutated RAS cancer cells (Dixon et al., 2012). The molecular mechanism of ferroptosis is complicated, depending on the context (Xie et al., 2016; Stockwell et al., 2017; Dai et al., 2020a). There are many connections between lipid metabolism and ferroptosis. Lipid biosynthesis that depends on acyl-CoA synthetase long-chain family member 4 (ACSL4) (Yuan et al., 2016; Kagan et al., 2017) and subsequent lipoxygenase-dependent lipid (e.g., PUFAs) peroxidation (Yang et al., 2016) promotes membrane rupture during ferroptosis. NADPH oxidases (NOXs)

and other oxidases may also facilitate membrane oxidative injury during ferroptosis (Gaschler and Stockwell, 2017; Xie et al., 2017). In contrast, several antioxidant or membrane repair mechanisms can prevent ferroptosis. The main anti-ferroptosis mechanisms include system xc^--mediated glutathione peroxidase 4 (GPX4) activation (Dixon et al., 2012; Yang et al., 2014), apoptosis-inducing factor mitochondria-associated 2 (AIFM2)-mediated coenzyme Q10 production (Bersuker et al., 2019; Doll et al., 2019), endosomal sorting complexes required for transport (ESCRT)-III–mediated membrane repair (Dai et al., 2020c,d), and nuclear factor, erythroid 2-like 2 (NFE2L2, also known as NRF2)-mediated antioxidant response (Sun et al., 2016a,b; Dodson et al., 2019).

Early studies indicate that ferroptosis is different from other forms of regulated cell death, such as apoptosis, necroptosis, and autophagy (Dixon et al., 2012). However, increasing studies suggest that ferroptosis exhibits a particular relationship with autophagy during anticancer therapies, tumorigenesis, inflammatory injury, and tissue fibrosis (Kang and Tang, 2017; Zhou et al., 2019; Liu et al., 2020) (**Figure 3**). Several types of selective autophagy, such as ferritinophagy, clockophagy, lipophagy, and mitophagy, promote ferroptotic cell death through degradation of the iron-storing protein ferritin, the core circadian clock protein aryl hydrocarbon receptor nuclear translocator-like (ARNTL, also known as BMAL1), LDs, and mitochondria, respectively (Hou et al., 2016; Basit et al., 2017; Bai et al., 2019; Liu et al., 2019; Yang et al., 2019). CMA also promotes ferroptosis through HSP90-mediated GPX4 degradation (Wu et al., 2019). Moreover, BECN1 facilitates ferroptosis through directly inhibiting SLC7A11/system xc^- activity (Song et al., 2018) or inducing ferritinophagy (Zhang et al., 2018). The stimulator of interferon response cGAMP interactor 1 (STING1, also known as TMEM173), an ER-associated protein involved in immunity, infection, and coagulation, connects mitochondrial DNA stress to autophagy-dependent ferroptosis (Li et al., 2020). Nanoparticle ferritin-bound erastin and rapamycin (NFER), a nanodrug, exhibits a robust ability to induce ferroptosis and autophagy to inhibit tumor growth (Li et al., 2019). The release of damage-associated molecular patterns (DAMPs) from ferroptotic cells serves as a mediator implicated in immune cell activation (Wen et al., 2019) and tumorigenesis (Dai et al., 2020b). In addition to cancer biology, autophagy-mediated ferroptosis is also implicated in hepatic fibrosis and neurodegenerative disease (Zhang et al., 2018; Kong et al., 2019). These findings may provide a useful framework for understanding the pathological characteristics of autophagy-mediated ferroptosis in diseases.

LIPID METABOLISM DISORDERS AND AUTOPHAGY

Autophagy is tightly regulated by ATG genes. When these genes are mutated, a series of diseases, such as cancer, infectious disease, and neurodegenerative disease, can be induced. In addition, impaired autophagy is also closely related to the pathology of several lipid metabolic disorders discussed below.

Lysosomal storage diseases (LSDs) are a class of genetic disorders in which proteins responsible for digestion or absorption of endocytosed material do not function or localize properly. The resulting cellular "lipid indigestion" or "lipid digestion defects" cause a buildup of intracellular storage that contains unprocessed lipids (Kiselyov and Muallem, 2008). LSDs consist of a group of rare inherited metabolic disorder diseases, such as Niemann-Pick C1 (NPC1) disease, G(M1)-gangliosidosis, Gaucher disease, Danon disease, Pompe disease, mucolipidosis type IV disease, and neuronal ceroid lipofuscinoses (NCLs). Impaired autophagy activity is commonly responsible for these LSDs (Seranova et al., 2017). For example, NCLs can be caused by mutations in lysosomal proteases, which leads to a deficiency in the autophagy-dependent degradation of NCL proteins (Brandenstein et al., 2016). Mutated NPC1 protein can block autophagy induction through the inhibition of SNARE-dependent membrane fusion, whereas ATG5-deficient cells exhibit increased NPC1protein accumulation (Sarkar et al., 2013). Thus, the pharmacological induction of autophagy may ameliorate the phenotypes of LSDs.

Preeclampsia is a pregnancy complication characterized by high blood pressure and signs of multiple organ damage (e.g., liver and kidney). Preeclampsia is associated with increased oxidative stress, which can cause autophagy-dependent cell death in extravillous trophoblasts. Mechanistically, oxidative stress reduces lysosomal activities and enhances *de novo* sphingolipids synthesis, which finally results in ceramide overload-dependent autophagic cell death and subsequent inflammation response (Melland-Smith et al., 2015). In addition to excessive autophagy-mediated cellular damage in extravillous trophoblasts, mild levels of autophagy may promote cell survival under hypoxic and low-nutrient conditions (Nakashima et al., 2017). It remains unknown whether a systemic autophagy response affects pregnant women.

The liver is the hub of fat transport. After fat is digested and absorpted, a portion of it enters the liver, and then it is converted into body fat and stored. The liver is also one of the main organs for the synthesis of FAs, cholesterol, and phospholipids in the body. Excess cholesterol is excreted with bile. Lipid metabolic imbalance leads to lipid accumulation in the liver, resulting from steatosis due to non-alcoholic fatty liver disease (NAFLD). The level of lipids in the liver is modulated by lipophagy, and impaired lysosomal pathways are involved in the pathogenesis of NAFLD. In contrast, the activation of autophagic pathways has been shown to ameliorate steatosis and NAFLD in animal models (Ma et al., 2013; Xiao et al., 2016; Kim et al., 2019). These findings suggest that autophagy activators may have therapeutic potential in NAFLD, which includes a spectrum of hepatic disorders associated with obesity.

Altered lipid metabolism and autophagy also contribute to neurodegenerative diseases, such as Parkinson's disease (PD), a progressive disorder that affects movement. Specific gene mutations, such for as PTEN-induced kinase 1 (PINK1), increase the risk of PD. PINK1 is an important regulator of mitochondrial quality through multiple mechanisms, including mitophagy (Rub et al., 2017). Depleted or mutated PINK1 can increase mitochondrial oxidative injury, ER stress, and mitophagy

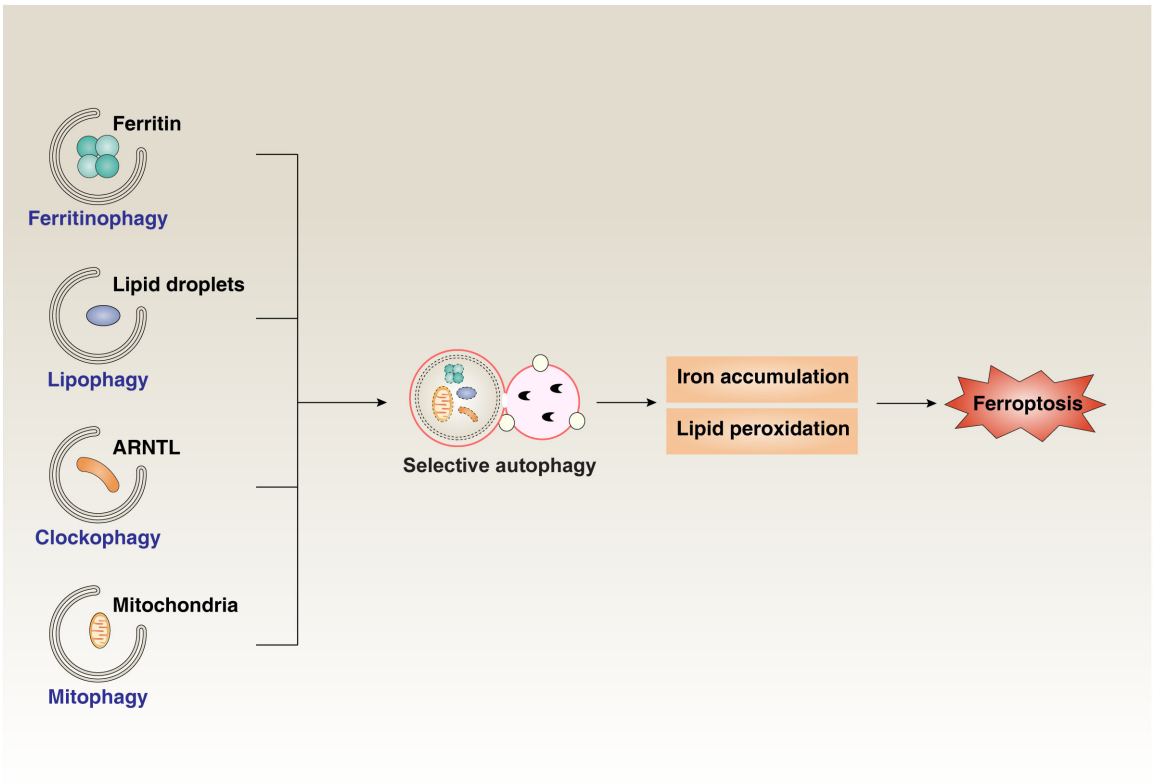

FIGURE 3 | The role of selective autophagy in ferroptosis. Ferritinophagy, clockophagy, lipophagy, and mitophagy promote the degradation of the iron-storing protein ferritin, the core circadian clock protein ARNTL, lipid droplets, and mitochondria, respectively. Activating these types of selective autophagy results in iron accumulation and lipid peroxidation, which finally induces ferroptotic cell death.

deficient, which leads to cell death, inflammation, and immune suppression in various diseases (Kang et al., 2016; Li et al., 2018). Of note, reduced hydrolase activity has shown to increase cholesterol accumulation during PD development (Garcia-Sanz et al., 2017). Thus, reducing lipid storage may restore the activity of autophagy, especially mitophagy, to alleviate mitochondrial damage in PD (Han et al., 2018).

Metabolic syndrome includes a cluster of conditions, such as hypertension, hyperglycemia, excessive waist fat, and abnormal cholesterol levels. Autophagic activity is significantly reduced in metabolic syndrome, which increases the risk of obesity, type 2 diabetes, and atherosclerosis. The inhibition of autophagy promotes lipid accumulation, mitochondria dysfunction, and ER stress (Perrotta and Aquila, 2015; Zhang et al., 2015; Martinez-Useros and Garcia-Foncillas, 2016). In contrast, the activation of autophagy may decrease metabolic syndrome-related diseases.

CONCLUSION AND PERSPECTIVE

Autophagy is a conserved adaptive response to environmental changes and plays a pivotal role in cell survival and death. It can degrade aging organelles and proteins to produce amino acids, nucleotides, and FFAs for cell survival. At the same time, it can also be used as an active mechanism to induce autophagy-dependent cell death. Generally, ceramides are involved in pro-survival autophagy, while PUFAs are involved in pro-death autophagy. The process of autophagy is regulated by a series of complex signaling molecules and metabolic pathways. Lipid metabolism plays an important role in regulating multiple cell processes. In the past 10 years, there have been major breakthroughs in understanding the crosstalk between lipid metabolism (e.g., digestion, absorption, catabolism, biosynthesis, and peroxidation) and autophagy. In particular, lipid metabolism has been found to be involved in the formation of membrane structures related to autophagy. Inhibiting TG, CE, and FA or increasing membrane lipid biosynthesis is responsible for the induction of autophagy. Moreover, autophagy promotes lipid catabolism and lipid peroxidation-induced cell death, such as ferroptosis. Targeting the autophagy pathway has received extensive attention in human diseases, including lipid metabolism-related disorders. Although these advances in knowledge have propelled the field forward, there is still much to explore. For example, how does autophagy function in lipid metabolism pathways in different cells or tissues? To what extent does the lipid context around membranes affect autophagy induction? How does autophagy switch from pro-survival mode to a pro-death one that ruptures the membranes? To what degree is selective autophagy specially linked to ferroptotic cell death? Which ATG modifications are responsible

for lipid disorder phenotypes? A better understanding of the mechanisms of autophagy and possible links to lipid metabolism will undoubtedly promote potential treatments for a variety of diseases.

AUTHOR CONTRIBUTIONS

YX and DT conceived of the topic for this review. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Multiple Functions of ATG8 Family Proteins in Plant Autophagy

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Autophagy is a major degradation process of cytoplasmic components in eukaryotes, and executes both bulk and selective degradation of targeted cargos. A set of autophagy-related (ATG) proteins participate in various stages of the autophagic process. Among ATGs, ubiquitin-like protein ATG8 plays a central role in autophagy. The ATG8 protein is conjugated to the membrane lipid phosphatidylethanolamine in a ubiquitin-like conjugation reaction that is essential for autophagosome formation. In addition, ATG8 interacts with various adaptor/receptor proteins to recruit specific cargos for degradation by selective autophagy. The ATG8-interacting proteins usually contain the ATG8-interacting motif (AIM) or the ubiquitin-interacting motif (UIM) for ATG8 binding. Unlike a single ATG8 gene in yeast, multiple ATG8 orthologs have been identified in the plant kingdom. The large diversity within the ATG8 family may explain the various functions of selective autophagy in plants. Here, we discuss and summarize the current view of the structure and function of ATG8 proteins in plants.

Keywords: autophagy, ATG8, cargo receptors, selective autophagy, AIM, UIM

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INTRODUCTION

Intracellular protein quality-control is crucial for successful cell growth and development, which requires a proper balance between protein synthesis and degradation. The two major pathways for protein quality control in eukaryotes are, autophagy and the ubiquitin-proteasome system. Autophagy is the main process for the degradation of long-lived cytosolic proteins and organelles; whereas the ubiquitin-proteasome system is responsible for the degradation of short-lived proteins (Klionsky and Emr, 2000). Three types of autophagy have been reported in plants: microautophagy, macroautophagy, and mega-autophagy (Marshall and Vierstra, 2018). During microautophagy, cytoplasmic components are engulfed by invagination of the tonoplast into the vacuole; in contrast, macroautophagy involves the trapping of target cytoplasmic constituents double-membrane vesicles called autophagosomes, in which they are delivered to the vacuole for degradation (**Figure 1**). Macroautophagy is the predominant, and most studied form of autophagy in plants. This process involves the sequestration of cytoplasmic materials by a double-membrane vesicle called autophagosome, which delivers the intracellular cargo into the vacuole for degradation (**Figure 1**). Mega-autophagy is the most extreme form of autophagy which occurs at the final stage of programmed cell death (PCD). During mega-autophagy, the tonoplast permeabilizes and ruptures, and vacuolar hydrolases are released into the cytoplasm to degrade cytoplasmic components (**Figure 1**).

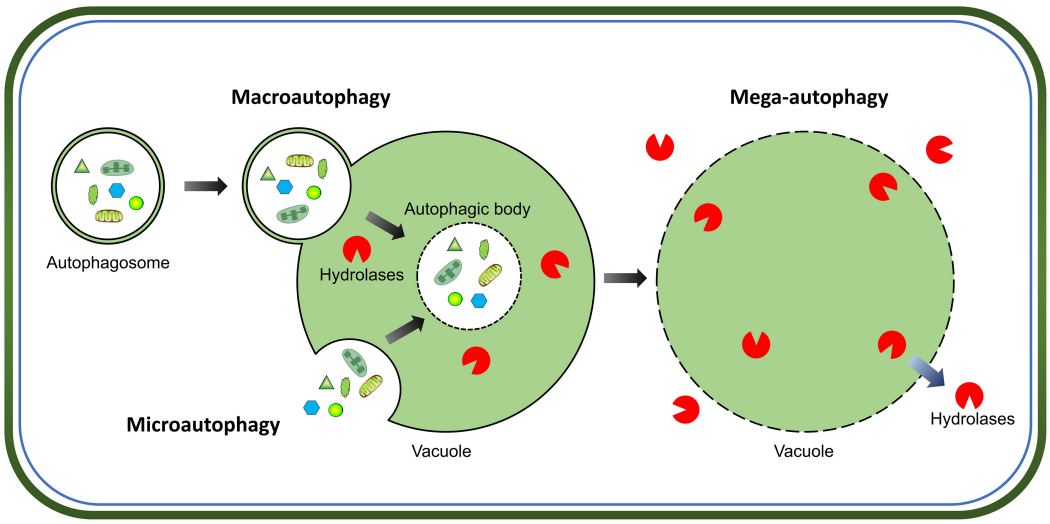

FIGURE 1 | Morphological characteristics of three types of autophagy in plants. Microautophagy is achieved via invagination of the tonoplast to engulf cytoplasmic components into the vacuole. Macroautophagy delivers target cytoplasmic constituents through autophagosomes which fuse with the tonoplast to release internal vesicle into the vacuole. Both microautophagy and macroautophagy involve the formation of autophagic body and degradation of cargos by hydrolases in the vacuole. Additionally, mega-autophagy is the most extreme form of autophagy which involves permeabilization or rupture of the tonoplast and release of vacuolar hydrolases into the cytoplasm to degrade cytoplasmic materials *in situ*.

Macroautophagy (hereafter referred to as autophagy) plays an important role in plant growth and development, as well as in various biotic and abiotic stress responses (Liu and Bassham, 2012; Signorelli et al., 2019). Under normal conditions, autophagy remains at a basal level for maintaining cellular homeostasis. Although autophagy-deficient mutants are not lethal, lack of autophagy leads to premature leaf senescence as well as reduced seed yield and quality in plants (Phillips et al., 2008; Chung et al., 2010; Barros et al., 2017). In contrast, enhanced autophagy, which is caused by the overexpression of *ATG* genes, improves plant growth, seed yield, and nitrogen remobilization efficiency (Minina et al., 2018; Chen et al., 2019). To cope with various stresses, autophagy is upregulated to promote plant survival (Signorelli et al., 2019; Su et al., 2020). During senescence or stress responses, autophagy contributes to the recycling of cellular material and remobilization of nutrients, including proteins, lipids, carbohydrates (Masclaux-Daubresse et al., 2017).

The molecular mechanisms of autophagy are sophisticated and involve a number of autophagy-related proteins that participate in autophagosome formation. ATGs can be divided into several functional groups: the ATG1 kinase complex, the ATG9 recycling complex, the phosphatidylinositol 3-kinase (PI3K) complex, and two ubiquitin-like conjugation systems, namely, the ATG8 lipidation system and the ATG12 conjugation system (Li and Vierstra, 2012). In Arabidopsis, the ATG1 kinase complex, consisting of ATG1, ATG13 and two additional subunits, ATG11 and ATG101, regulates the induction of autophagy (Suttangkakul et al., 2011; Li et al., 2014). The transmembrane protein ATG9, along with ATG2 and ATG18, recruits lipids to the expanding phagophore (Zhuang et al., 2017, 2018). The PI3K complex, including VPS34, VPS15, VPS38, and ATG6, decorates the phagophore with phosphatidylinositol-3-phosphate (PI3P), which is essential for the vesicle nucleation in *Arabidopsis* (Zhuang et al., 2018). Two ubiquitin-like conjugation systems mediate the expansion of phagophore and autophagosome maturation (Marshall and Vierstra, 2018). In particular, ATG8 plays a central role in plant autophagy. As shown in **Figure 2**, newly synthesized ATG8 is cleaved by cysteine proteinase ATG4 to expose the C-terminal glycine residue (Yoshimoto et al., 2004). Subsequently, the exposed glycine of ATG8 is conjugated to the membrane lipid phosphatidylethanolamine (PE) in a ubiquitin-like conjugation reaction catalyzed by ATG7 (E1-like enzyme), ATG3 (E2-like enzyme) and the ATG12-ATG5 complex (E3-like enzyme) (Doelling et al., 2002; Thompson et al., 2005; Phillips et al., 2008; Chung et al., 2010). Moreover, the association of ATG8 with the autophagosome is a reversible process; thus, the ATG8-PE adduct can be deconjugated from the membrane by ATG4 proteinase, whereby released ATG8 is recycled to participate in a new conjugation reaction (Yoshimoto et al., 2004; Woo et al., 2014).

Autophagy was originally considered as a non-selective process for the bulk degradation of cytoplasmic components (Thompson and Vierstra, 2005). However, increasing evidence suggests that autophagy may also be a selective process involving the degradation of specific target cargos, such as proteins, protein aggregates, malfunctioning organelles, and even invading pathogens (Li and Vierstra, 2012; Marshall and Vierstra, 2018). ATG8 is crucial for both bulk and selective autophagy. During bulk autophagy, ATG8 is involved in phagophore membrane elongation and autophagosome assembly (Nakatogawa et al., 2007). As for selective autophagy, ATG8 plays an additional role in cargo selection by providing a docking site for numerous autophagy adaptors and receptors that recruit the target cargo

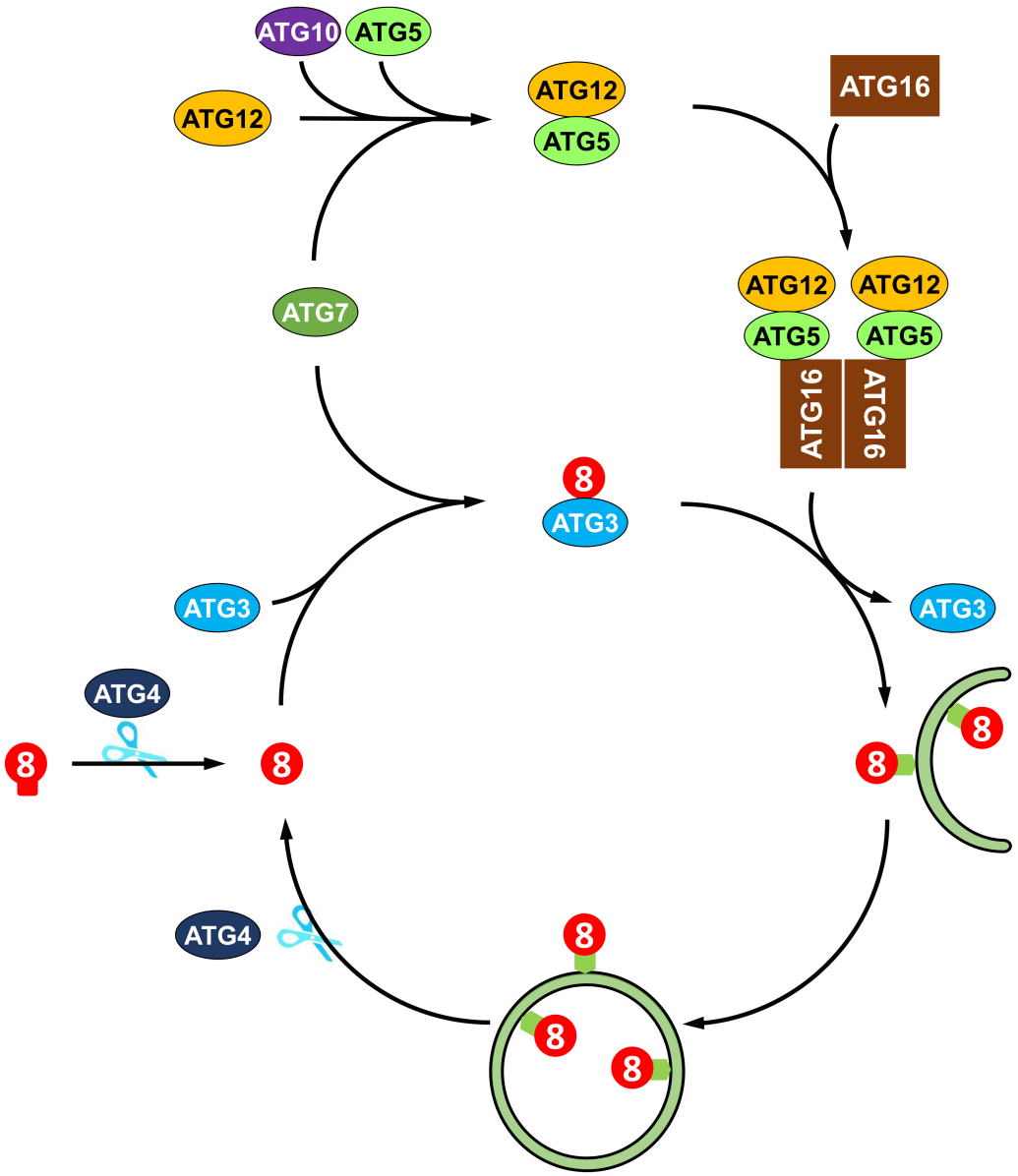

FIGURE 2 | Recycling of ATG8 protein in plant autophagy. ATG8 protein is synthesized as proprotein, which is cleaved by the cysteine protease Atg4 to expose a glycine residue at the C-terminus. Then, the processed ATG8 protein is conjugated to phosphatidylethanolamine (PE) by E2-like ATG3 and the E3-like ATG12-ATG5-ATG16 complex. The ATG8-PE adduct coats the expanding phagophore and contributes to the maturation of autophagosome. Again, ATG8-PE attached to the outer autophagosome membrane is deconjugated from PE by ATG4, thus recycling ATG8 protein.

for degradation (Johansen and Lamark, 2011). In this review, we analyze the structural features and interactions of ATG8 proteins in plants and address their fundamental roles in plant selective autophagy.

FEATURES OF ATG8 FAMILY PROTEINS IN PLANTS

ATG genes are evolutionarily conserved across eukaryotes. Core ATG genes were originally identified in yeast, and most ATG genes have been found in plants based on sequence similarity with yeast homologs. Although yeast carries only one ATG8 gene, the plant ATG8 protein family diversifies from a single copy in algae to multiple genes in higher plants (**Supplementary Table 1** and **Figure 3**). As shown in **Supplementary Table 1**, nine ATG8 isoforms have been identified in Arabidopsis, five in maize, seven in rice and potato, and eleven in soybean (Hanaoka et al., 2002; Chung et al., 2009; Perez-Perez and Crespo, 2010; Xia et al., 2011, 2012; Maqbool et al., 2016; Kellner et al., 2017; Zess et al., 2019). However, early-diverged plant lineages, such as algae, possess only a single ATG8 (**Supplementary Table 1** and

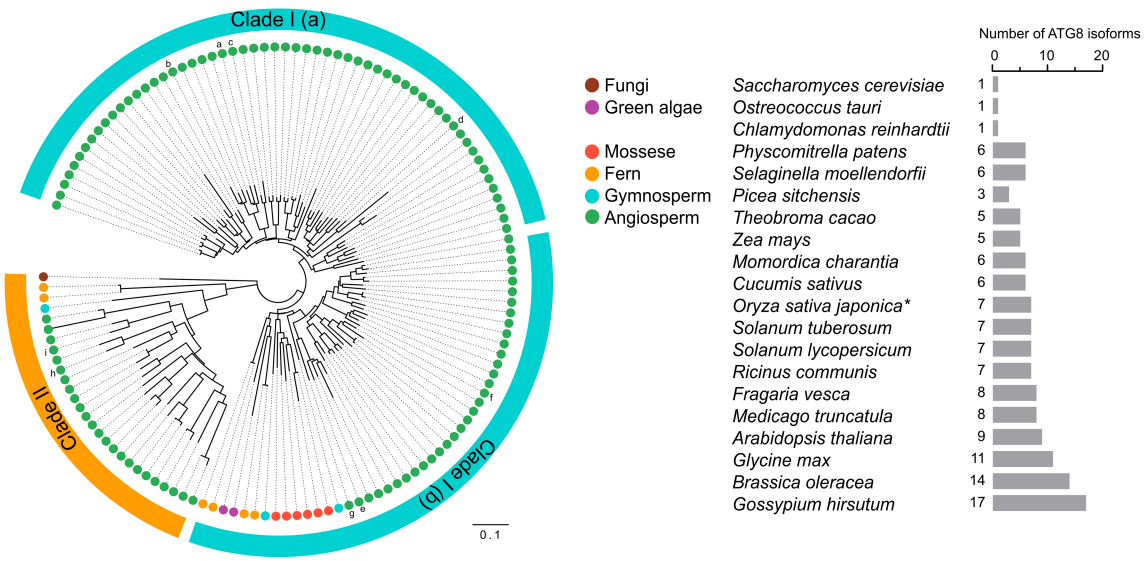

FIGURE 3 | Phylogenetic analysis of ATG8s in plants. Phylogenetic analysis of ATG8 sequences from 19 plant species and *Saccharomyces cerevisiae* (budding yeast) with circular bands highlighting clades I and II. The tree was calculated in MEGA7 from a 419 nt alignment (ClustaW) of 133 sequences applying the maximum composite likelihood matrix. Colored dots indicate plant taxonomic groups. Nine *Arabidopsis thaliana* ATG8 variants AtATG8a-AtATG8i are shown in the tree. Branch lengths reflect the average number of substitutions per site. Scale bar: 0.01 substitutions per site. The number of ATG8 isoforms across the plant lineage is indicated in the right. The asterisk indicates OsATG8f and OsATG8i are excluded from phylogenetic analysis.

Figure 3). On the other hand, a recent study aiming to estimate ATG8 diversity across green plants found that, in order to adapt to adverse and complex conditions, the ATG8 gene family has undergone large expansion in plants via multiple whole-genome duplications (Kellner et al., 2017). As a result, ATG8 expansion may have led to the current diversity of selective autophagy in plants.

Plant ATG8 genes can be grouped into two clades by phylogenetic analysis (**Figure 3**). Clade I, which includes most of the plant ATG8 family members, is closely related to fungi, whereas clade II is more similar to the ATG8 homologs in animals (Kellner et al., 2017). However, some plant ATG8 isoforms in clade II, such as ATG8h and ATG8i in Arabidopsis, lack extra amino acid residues at the C-terminus after the glycine residue, which indicates that these ATG8 proteins can interact with the autophagosome membrane without ATG4 processing (Seo et al., 2016). In addition, clade I can be further divided into two subgroups, clade I (a) and clade I (b), containing AtATG8a-d and AtATG8e-g, respectively (**Figure 3**), which is consistent with the results of previous studies (Seo et al., 2016; Kellner et al., 2017). However, there is a slight difference in gene classification compared with that reported by Slavikova et al. (2005), in which AtATG8a, AtATG8c, AtATG8d, and AtATG8f were grouped together, while AtATG8b, AtATG8e, and AtATG8g were grouped together.

Crystal structures of several ATG8 family proteins from yeast, animals and plants, have been solved (Sugawara et al., 2004; Kumeta et al., 2010; Maqbool et al., 2016). These studies indicated that ATG8 proteins conserved among eukaryotes contain an N-terminal helical domain and C-terminal ubiquitin domain (**Figure 4**). The C-terminal ubiquitin domain, similar with ubiquitin, adopts a β-grasp (ubiquitin-like) fold consisting of four β-strands (β1–β4) and two α helices (α3 and α4). However, the N-terminal helical domain is formed by two other α helices (α1 and α2), which is a unique feature of ATG8 proteins. The C-terminal ubiquitin domain is conserved among ATG8 family members and might play a crucial role in protein-protein interactions (Shpilka et al., 2011), whereas the N-terminal helical domain is not conserved and is responsible for binding specificity to ATG8-interacting proteins in animals and plants (Coyle et al., 2002; Ketelaar et al., 2004; Zess et al., 2019).

Generally, ATG8 binds to specific proteins with the ATG8-interacting motif (AIM) in yeast and plants, or the LC3-interacting region (LIR) in animals (Noda et al., 2010). The core AIM sequence is defined as W/F/Y-XX-L/I/V, an aromatic amino acid (Trp, Tyr, or Phe) followed by two random amino acids and an aliphatic amino acid (Leu, Ile, and Val). A hydrophobic patch of ATG8 known as the LIR/AIM docking site (LDS), with two distinct pockets (W and L pockets), is responsible for binding with AIM (Noda et al., 2010). The W pocket is formed at the interface between the β-grasp and the N-terminal helices and embraces the aromatic residue of AIM, while the L pocket is located in β-grasp fold and embraces the aliphatic amino acid of AIM (Maqbool et al., 2016).

Notably, there are some proteins that bind ATG8 differently from the AIM-LDS type of binding in animals and plants (Lin et al., 2013; Marshall et al., 2015). CoIP and pull-down assays demonstrated that SQST-1 (the p62 homolog in *Caenorhabditis elegans*) associated with LGG-1 (the ATG8 homolog in *C. elegans*), while mutating the LIR motif in SQST-1 had no effect on the interaction with LGG-1, which indicated that the LIR motif is not necessary for binding of SQST-1

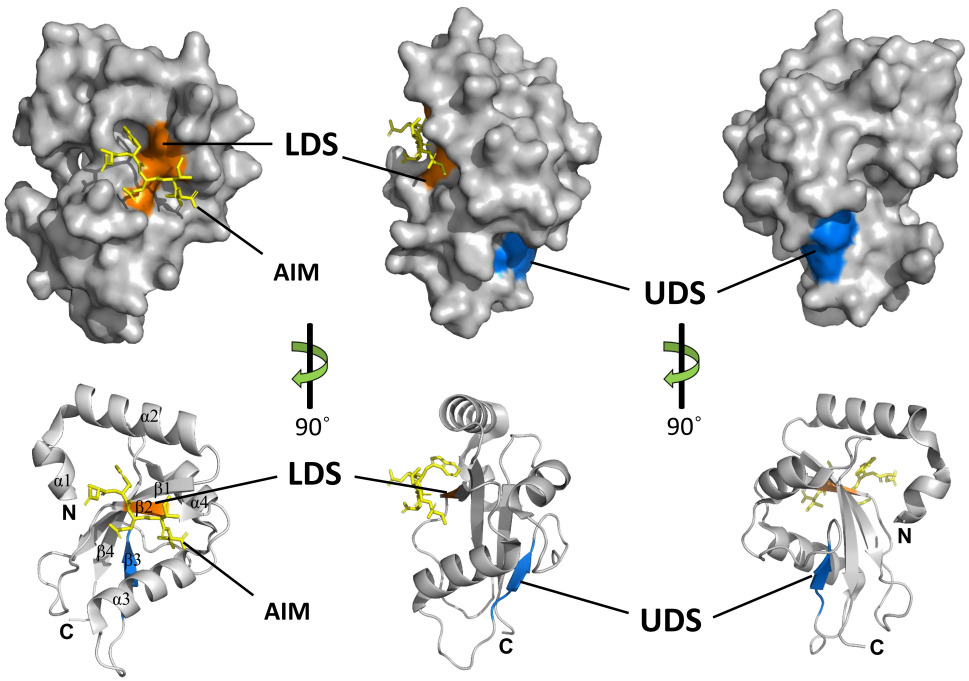

FIGURE 4 | Structures and binding motifs of ATG8 proteins. The 3-dimensional structure of potato ATG8CL (PDB: 5L83) was downloaded from the Protein Data Bank (https://www.rcsb.org/pdb). The positions of LDS (orange) and UDS (blue) are mapped with the corresponding positions of yeast ATG8. The AIM-containing peptide (residues 377–381) from *Phytophthora infestans* PexRD54 is shown as a yellow stick. α-Helices, β-strands, N-terminal and the C-terminal regions are shown in ribbon diagrams.

to LGG-1 (Lin et al., 2013). Furthermore, ubiquitin receptor RPN10 (regulatory particle non-ATPase 10) acts as an autophagy receptor for degradation of the proteasome by binding to ATG8 in Arabidopsis. However, the interaction with ATG8 depends on the ubiquitin-interacting motif (UIM), not the canonical AIM (Marshall et al., 2015). Generally, UIM is a 20-amino acid stretch of the amino acid sequence that folds into a single α helix in which a consensus sequence (φ-θ-X-A-φ-X-X-S) for ATG8 binding is defined (where φ is a hydrophobic residue, θ is a hydrophilic residue, A is alanine, S is serine and X is any amino acid, respectively) (Marshall et al., 2019). ATG8 binds to proteins with a UIM through an alternative interaction site called UIM-docking site (UDS). The UDS sequence within ATG8 is highly conserved across species. Saturating mutagenesis identified a core 4-residue motif for permitted residues of φ-F-φ-**Ω**/T within UDS, where φ represents small hydrophobic residues and **Ω** represents aromatic residues (Marshall et al., 2019). In addition, the location of UDS is opposite to that of LDS; thus, ATG8 can bind to proteins with AIM and UIM simultaneously (**Figure 4**). The UIM-UDS interface greatly widens the range of autophagy receptors and adaptors, thereby expanding the scope of selective autophagy.

FUNCTIONS OF THE ATG8 PROTEINS IN PLANTS

ATG8 proteins perform multiple functions in plants. For example, ATG8 proteins regulate membrane elongation during autophagosome biogenesis. Moreover, they play a crucial role in cargo recognition for selective autophagy by interacting with autophagy receptors/adaptors. In addition to their autophagic roles, ATG8 proteins play non-autophagic roles in plants. In this section, we summarize the current knowledge regarding the different roles of ATG8 proteins in plants.

AUTOPHAGOSOME BIOGENESIS

ATG8 is a ubiquitin-like protein required for autophagosome formation. First, mature ATG8 processed by the ATG4 protease is conjugated to the membrane lipid PE in Arabidopsis (Xie et al., 2008; Woo et al., 2014). subsequently, the ATG8-PE adduct promotes autophagosome biogenesis through membrane tethering and remodeling (Ohsumi, 2001; Nakatogawa et al., 2007). On one hand, the loss of ATG8 functionality blocks autophagosome formation in yeast and other fungi (Kirisako et al., 1999; Ren et al., 2018); however, this phenotype has not been observed in plants yet, probably due to ATG8 gene redundancy. On the other hand, overexpression of ATG8 promotes autophagosome formation in Arabidopsis and rice (Chen et al., 2019; Yu et al., 2019). In addition to ATG8 lipidation, ATG8 proteins are deconjugated by ATG4 and released from the autophagosome membrane, whereby the released ATG8 proteins appear to be recycled to facilitate autophagosome formation in plants (Yoshimoto

et al., 2004). In addition to supplying ATG8 free protein, the delipidation of ATG8-PE also plays an important role in autophagosome membrane expansion (Hirata et al., 2017). Indeed, the inability for ATG8 delipidation results in mislocalization of the autophagosome to the vacuolar membrane and in defective autophagosome biogenesis (Nair et al., 2012). Therefore, the ATG4-mediated deconjugation of ATG8-PE plays a dual role in autophagosome biogenesis, namely, during the early stage of autophagosome formation, the release of ATG8 from non-autophagosomal membranes supplies the increasing demand for ATG8, while at the later stage, the release of ATG8 from the phagophore membrane facilitates autophagosome maturation (Yu et al., 2012). In addition to inducing autophagy at the early stage in autophagosome biogenesis, ATG8 downregulates autophagy at the later stage. The ATG1/13 kinase complex plays an essential role in the initiation of autophagy across the evolutionary scale from yeast to plants (Mizushima, 2010; Suttangkakul et al., 2011). ATG8 directly binds ATG1 and ATG11 in an AIM-dependent manner. Moreover, ATG8-binding triggers self-digestion of the ATG1-ATG13 complex, thereby suppressing autophagic activity (Suttangkakul et al., 2011; Li et al., 2014).

In addition to its interaction with ATG proteins, ATG8 promotes phagophore expansion and maturation by recruiting a non-ATG protein, SH3 DOMAIN-CONTAINING PROTEIN2 (SH3P2) in Arabidopsis. During autophagy induction, SH3P2 is recruited to the phagophore assembly site (PAS) by membrane associated ATG8 protein; then, ATG8 binds to phosphatidylinositol 3-phosphate (PI3P) and coordinates with the PI3K complex to facilitate autophagosome formation. Consistently, knockdown of SH3P2 significantly suppresses autophagosome formation (Zhuang et al., 2013).

AUTOPHAGIC CARGO RECOGNITION

Autophagy was initially thought to be an non-selective, bulk degradation process of cytoplasmic contents. However, increasing evidence has shown that autophagic cargos can be selectively targeted for degradation. Subsequently, selective autophagy has been characterized according to the type of targeted substrates, such as proteins (proteaphagy), protein aggregates (aggrephagy), pathogens (xenophagy), chloroplasts (chlorophagy), endoplasmic reticulum (reticulophagy), and others. In plants, ATG8 proteins play a crucial role in selective autophagy through their interaction with various AIM/UIM-containing proteins (Marshall and Vierstra, 2018; Lei and Klionsky, 2019). Recently, an increasing number of ATG8-binding proteins, many of which are likely cargo receptors, have been identified in plants. As shown in **Figure 5**, these proteins bind both autophagic cargos and ATG8 proteins to facilitate cargo gathering. Therefore, the association of ATG8 proteins with adaptor/receptor proteins is necessary for cargo selection and degradation. Here, ATG8-interacting receptors and cargos that are essential for selective autophagy in plants are discussed.

NBR1

Protein aggregates are degraded through the ubiquitin-proteasome system (UPS) and autophagy (termed aggrephagy) to maintain cellular homeostasis (Lamark and Johansen, 2012). In animals, two selective cargo receptors, p62 and NBR1 (neighbor of BRCA1 gene 1), are responsible for autophagic degradation of ubiquitinated protein aggregates (Lamark et al., 2009); p62 was first identified as an autophagic receptor to bind both target-associated ubiquitin and LC3 (Pankiv et al., 2007). Like p62, NBR1 was shown to be an Ub- and LC3-binding protein (Kirkin et al., 2009). Both p62 and NBR1 are structurally similar, containing an N-terminal PB1 domain, a LIR motif that can interact with LC3, and a C-terminal ubiquitin-associated (UBA) domain that can bind ubiquitin (Lamark et al., 2009).

AtNBR1, the first selective autophagy receptor reported in plants, is a hybrid protein homolog to both NBR1 and p62 that harbors functions of these two proteins (Svenning et al., 2011). AtNBR1 binds ubiquitin through a C-terminal UBA domain and interacts with various ATG8 proteins via an evolutionarily conserved AIM, mediating the sequestration of ubiquitinated protein aggregates into autophagosomes (Svenning et al., 2011; Jung et al., 2019). Joka2, a homolog of AtNBR1, has been identified as a selective autophagy receptor in tobacco (*Nicotiana tabacum*) (Zientara-Rytter et al., 2011). Similar to *atg* mutants, ubiquitinated protein aggregates are highly accumulated in both Arabidopsis and tomato *nbr1* mutant under heat stress and non-stress conditions (Zhou et al., 2013, 2014; Jung et al., 2019). These results suggest that NBR1 is an autophagy receptor involved in the selective degradation of protein aggregates in plants.

In addition to its role in aggrephagy, NBR1 acts as a xenophagy receptor in plant immune responses. For example, during viral infections, AtNBR1 contributes to plant immunity by directly binding the viral capsid protein P4 and particles of the cauliflower mosaic virus (CaMV), and the viral silencing suppressor HCpro of the turnip mosaic virus (TuMV); thus, mediating their selective autophagic degradation (Hafrén et al., 2017, 2018). Further, upon bacterial infection, NBR1-dependent selective autophagy enhances host antibacterial immunity by promoting autophagic degradation of an unknown factor required for bacterial pathogenesis and infection (Üstün et al., 2018). Surprisingly, plant pathogens can manipulate the host autophagy machinery to promote infection. Thus, Phytophthora infestans effector protein PexRD54 binds potato ATG8CL to prevent interaction of ATG8CL with the autophagy cargo receptor Joka2, thereby facilitating autophagic clearance of plant or pathogen proteins that negatively affect immunity (Dagdas et al., 2016; Maqbool et al., 2016). Meanwhile, PexRD54 activates autophagy to redistribute nutrients in favor of the pathogen (Dagdas et al., 2016).

TSPO

Another autophagy receptor, TSPO (tryptophan-rich sensory protein), is a porphyrin-binding membrane protein which contains an AIM for ATG8 binding in Arabidopsis (Vanhee et al., 2011). AtTSPO is induced by abscisic acid (ABA) and

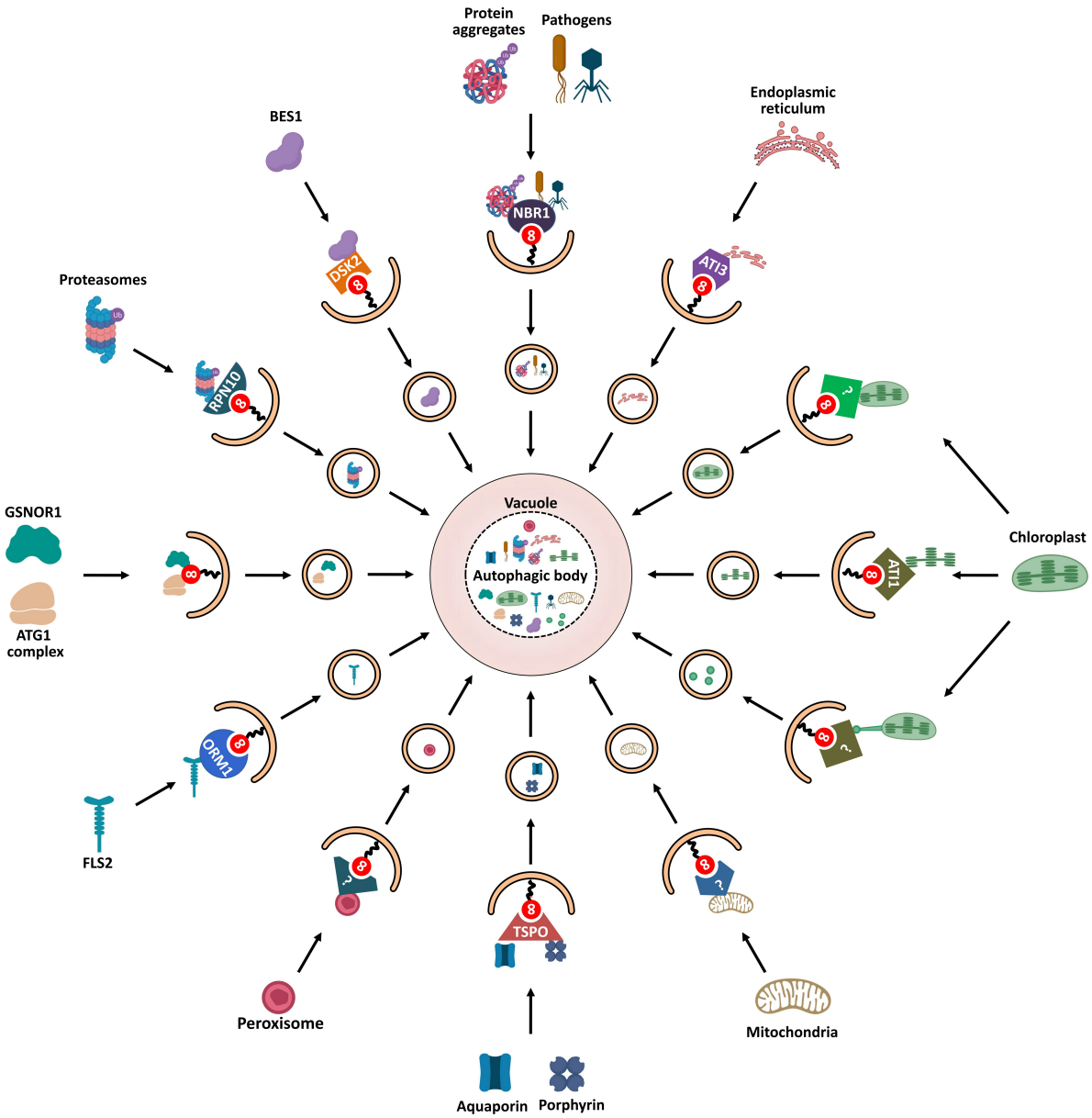

FIGURE 5 | Schematic diagram of ATG8-mediated selective autophagy pathways in plants. Various ATG8-binding proteins act as selective autophagy receptors and mediate the delivery of specific target cargos to autophagosomes for degradation. NBR1, the first autophagy receptor identified in plants, mediates selective degradation of ubiquitinated protein aggregates and virulence factors; degradation of chloroplasts is mediated by three autophagic pathways: First, ATI1 binds and transports thylakoid proteins to vacuole for autophagic degradation through ATI1-PS bodies; Then, stromal components including Rubisco are engulfed by autophagic membrane to form Rubisco-containing bodies (RCBs) that are delivered to vacuole for degradation; Finally, the whole chloroplasts are entirely engulfed by the vacuolar membrane for degradation. ATG11 is involved in the autophagic degradation of mitochondria; however, the cargo receptor of mitophagy remains unknown. PEX6 and PEX10 may function as potential pexophagy receptors. In turn, degradation of the endoplasmic reticulum is mediated by another ATI protein, ATI3. RPN10 is involved in selective degradation of inactivated proteasomes extensively ubiquitylated. The selective autophagic degradation of BR signaling pathway transcription-factor BES1 is mediated by DSK2. Aquaporin PIP2;7 and porphyrin (heme) are both recognized by receptor TSPO and subjected to selective autophagic degradation. ORM1/2 proteins mediate the degradation of plant immune receptor FLS2. In addition to cooperating with autophagy receptors, some AIM/UIM-containing proteins, such as ATG1, ATG11, and GSNOR1, also bind directly to ATG8 to mediate their degradation.

abiotic stress such as osmotic or salt stress (Guillaumot et al., 2009; Balsemão-Pires et al., 2011). Thus, for example, the level of heme increases as a result of stress response and extra unbound heme may induce ROS production. In such case, TSPO shows a high affinity for heme and acts as a heme scavenger via ATG8-mediated selective autophagy, thus regulating heme levels in Arabidopsis cells (Vanhee et al., 2011). This result suggests that TSPO might modulate redox homeostasis through heme binding

and scavenging during stress. Moreover, TSPO binds to the plasma membrane aquaporin PIP2;7 (plasma membrane intrinsic protein 2;7) for autophagic degradation (Hachez et al., 2014). This TSPO-mediated selective degradation of PIP2;7 has been proposed as a mechanism to protect plant cells from water deficit.

RPN10

The UPS and autophagy are the two major protein quality-control pathways responsible for cellular homeostasis (Dikic, 2017). Ubiquitination serves as the degradation signal in both UPS and autophagy. Thus, UPS and autophagy functionally interconnect with each other (Ji and Kwon, 2017). Moreover, the 26S proteasome can be selectively degraded by autophagy (termed proteaphagy), which was first discovered in Arabidopsis (Marshall et al., 2015). Proteaphagy is induced by nitrogen starvation and proteasome inhibition. When 26S proteasomes are inactivated by a proteasome inhibitor or by a genetic mutation, proteasomes are extensively ubiquitylated and selectively degraded by autophagy mediated by the proteasome subunit RPN10. RPN10 does not contain the canonical AIM as other autophagy receptors but harbors three UIMs, in which UIM1 is responsible for binding ubiquitin and UIM2 for binding ATG8. Upon inhibitor-induced proteaphagy, ubiquitylated proteasomes are recruited by the free form of RPN10 through UIM1; meanwhile, RPN10 also binds membrane-associated ATG8 by UIM2 to form a stable tripartite complex which is engulfed in autophagosomes and then degraded (Marshall et al., 2015). The interaction between RPN10 and ATG8 is necessary for inhibitor-induced proteaphagy and is highly conserved across plant species. However, this binding is absent in yeast and animals because the orthologs of RPN10 in yeast and humans lack the UIM-related ATG8-binding motif. Alternatively, yeast proteaphagy employs a CUE-domain protein, namely, Cue5, as a ubiquitin-ATG8 adaptor (Marshall et al., 2016); Cue5 simultaneously binds ATG8 and ubiquitin through the AIM and the CUE domain, respectively (Lu et al., 2014).

ATI Proteins

Five plant-specific ATG8-interacting (ATI) proteins were found to interact with ATG8 in Arabidopsis. These proteins can be divided into two groups, ATI1/2 and ATI3a/b/c (Honig et al., 2012; Zhou et al., 2018). ATI1 and ATI2 are transmembrane proteins which contain two putative AIMs. However, only the N-terminal AIMs are responsible for interacting with ATG8 (Sjogaard et al., 2019). Meanwhile, ATI1 also interacts with chloroplast-associated proteins, such as NPQ4 and APE1 (Honig et al., 2012; Michaeli et al., 2014). Upon carbon starvation, the ATI1-decorated plastid-associated bodies (ATI1-PS bodies) deliver chloroplast components including stromal proteins, envelope, and thylakoid proteins to the vacuole for degradation, which is referred as chlorophagy (Michaeli et al., 2014). The interaction between ATI1 and ATG8 contributes to the targeting of the ATI1-PS bodies to the autophagosomes (Michaeli et al., 2014). Interestingly, ATI1 bodies are mainly localized in endoplasmic reticulum (ER)-associated vesicles and are distinct from autophagosomes (Honig et al., 2012). To date, whether ATI1 is involved in the selective autophagic degradation of ER (reticulophagy) remains unknown.

Additionally, ATI3 was identified as a specific receptor of reticulophagy in dicot plant species (Zhou et al., 2018). Three related ATI3 proteins (ATI3a/b/c) were found in Arabidopsis, all of which contain a C-terminal LIR motif and interact with ATG8. Additionally, ATI3a interacts with ER-localized UBAC2a/b (Ubiquitin associated proteins 2a/b), which are involved in ER-associated degradation (Zhou et al., 2018). Further research has shown that NAI2, an ER body component, interacts with an unknown protein encoded by the *At4g15545* gene, which is a potential UBAC2-interacting protein (Wang et al., 2019). These results suggest that ATI3 plays an important role in selective autophagy degradation of ER components.

ORM Proteins

Orosomucoid (ORM) proteins are known as negative regulators of sphingolipid biosynthesis (Breslow et al., 2010). A recent study reported that ORM proteins act as selective autophagy receptors to mediate the degradation of plant immune receptor FLS2 (FLAGELLIN-SENSING 2) (Yang et al., 2019). Two ORM proteins, ORM1 and ORM2, were identified in Arabidopsis (Li et al., 2016). ORM1 contains an N-terminal AIM, and ORM2 contains two AIMs at both N and C terminuses. ORM proteins bind both ATG8 and FLS2 simultaneously. Furthermore, ORM downregulation increases FLS2 accumulation and FLS2-dependent immune responses, while overexpression of ORM causes FLS2 degradation and suppression of FLS2-dependent signaling (Yang et al., 2019). These results suggest that ORM-mediated selective autophagy plays a key role in plant immunity.

DSK2

DSK2 (dominant suppressor of KAR2) is a ubiquitin-binding receptor protein related to protein degradation pathways in eukaryotes (Lee and Brown, 2012). Two DSK2 proteins (DSK2A and DSK2B) were identified in Arabidopsis (Farmer et al., 2010). AtDSK2 acts as an autophagy receptor for transcription factor BES1 (BRI1-EMS suppressor 1) of the brassinosteroid (BR) pathway. Under drought and starvation conditions, BES1 is ubiquitinated by the E3 ubiquitin ligase SINAT2 (SEVEN-*IN ABSENTIA* 2), which promotes binding of BES1 to DSK2. Concomitantly, DSK2 is phosphorylated by kinase BIN2, which enhances the interaction between DSK2 and ATG8. Thus, during abiotic stress, DSK2 recruits BES1 to the ATG8-located autophagosomes for degradation. DSK2 decreases BR signals by selective degradation of BES1 through autophagy to switch plant metabolism from growth to stress mode (Nolan et al., 2017).

Uncertain Receptor in Several Types of Selective Autophagy

Chlorophagy is important for quality control and nutrients recycling (Jarvis and López-Juez, 2013). Besides ATI1-PS body, four other types of chlorophagy are reported in plants (Zhuang and Jiang, 2019). However, the receptors for these types of chlorophagy are undetermined. First, the whole chloroplasts are captured by autophagic vesicles and delivered

to the vacuole for degradation upon ultraviolet radiation (Izumi et al., 2017). In addition, the second pathway for whole chloroplasts degradation is mediated by microautophagy (Nakamura et al., 2018). High-intensity light triggers chloroplast envelope damage and leads to chloroplast swelling, These swollen chloroplasts are directly engulfed by the vacuolar membrane and degraded by vacuolar hydrolase, which is dependent on the core autophagic machinery. The third type of chlorophagy is mediated by rubisco-containing bodies (RCB). During leaf senescence, RCBs containing chloroplast stromal proteins including Rubisco are transported to the vacuolar lumen for recycling through an autophagy-dependent manner (Ishida et al., 2008; Izumi et al., 2010).

The autophagic degradation of mitochondria (mitophagy) is well described in mammals. However, the understanding of mitophagy is much less in plants. A recent study reported that Arabidopsis ATG11 plays an important role in plant mitophagy (Li et al., 2014). Arabidopsis ATG11 interacts with ATG13, ATG101, ATG8, and is colocalized with mitochondria (Li et al., 2014). The turnover of mitochondrial proteins is blocked in Arabidopsis *atg11* mutants (Li et al., 2014). However, the mechanism of ATG11-mediated plant mitophagy is still unknown, and the cargo receptor of plant mitophagy is still waiting to be discovered.

Peroxisomes participate in various cellular processes in plants, such as lipid metabolism, photorespiration, and phytohormone synthesis. Pexophagy, the autophagic degradation of peroxisomes, is important for peroxisome quality control. The receptor of plant pexophagy remains undetermined. Atg30 and Atg36, two AIM-containing proteins, have been identified as pexophagy receptors in yeast (Farre et al., 2013; Zientara-Rytter et al., 2018). However, homologs of Atg30 and Atg36 are not found in plants. NBR1, together with p62, acts as a pexophagy receptor to recruit peroxisomes to PAS in mammals (Deosaran et al., 2013; Zhang et al., 2015; Sargent et al., 2016). Although plant NBR1 has been demonstrated as a receptor in aggrephagy and xenophagy, there is no direct evidence indicating that NBR1 is involved in pexophagy in plants. However, a recent study reported that Arabidopsis PEX6 (Peroxin 6) and PEX10 interact with ATG8, suggesting that they may function as potential pexophagy receptors in plants (Xie et al., 2016).

Receptor-Independent Cargo Recognition

In addition to cooperating with autophagy receptors, ATG8 proteins directly bind to the substrates to mediate their degradation. For instance, *Nicotiana benthamiana* ATG8f protein was demonstrated to target virulence protein βC1 of CLCuMuV (cotton leaf curl Multan virus) for autophagic degradation without the help of a cargo receptor such as NBR1/Joka2 (Haxim et al., 2017). This finding indicates that autophagy contributes to plant immunity through selective degradation of viral proteins independently of canonical autophagy receptors. Another example is NO-induced selective autophagy of GSNOR1 (*S*-nitrosoglutathione reductase 1) during hypoxia (Zhan et al., 2018). GSNOR1, a master regulator of NO signaling, is stable under normal conditions. However, hypoxia induces conformational changes in GSNOR1, whereby it exposes the AIM motif and facilitates the interaction of GSNOR1 with ATG8, ultimately leading to the selective degradation of GSNOR1 (Zhan et al., 2018).

NON-AUTOPHAGIC ROLES OF ATG8 PROTEINS IN PLANTS

In addition to their autophagic roles, ATG8 proteins fulfill functions that are not associated with autophagy. Thus, according to a recent study, MATE transporter-family protein ABS3 (ABNORMAL SHOOT 3) promoted senescence under both, normal and nutrient-deprived conditions in Arabidopsis (Jia et al., 2019). ABS3 contains two AIMs which are critical for binding to ATG8. Interestingly, this ATG8-ABS3 interaction is independent of the autophagic function of ATG8, but essential for ABS3-mediated senescence (Jia et al., 2019). In brief, ATG8 plays dual roles in controlling plant senescence. Under normal nutrient conditions, ATG8 is lipidated and activates autophagy to promote plant longevity. However, under nutrient-deprived or autophagy-deficiency conditions, ATG8 binds to ABS3 to promote ABS3 degradation and plant senescence independently of autophagy (Jia et al., 2019).

ATG8 proteins participate in diverse intracellular transport processes in animals (Shpilka et al., 2011). Animal ATG8 proteins can be divided into three subfamilies: microtubule-associated protein 1 light chain 3 (LC3), γ-aminobutyric acid receptor-associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE-16). GABARAP is involved in the intracellular trafficking of membrane proteins, such as GABA, κ-opioid, transferrin receptors and *N*-cadherin/β-catenin complex (Green et al., 2002; Leil et al., 2004; Chen et al., 2007, 2011; Nakamura et al., 2008). GATE-16 participates in intra-Golgi transport of Golgi SNARE protein 28 (GOS-28) and *N*-ethylmaleimide sensitive factor (NSF) (Sagiv et al., 2000; Muller et al., 2002). LC3 binds to FYVE and coiled-coil domain containing protein 1 (FYCO1) that interacts with Rab7 to mediate autophagosome transport to the vacuole along microtubule (Pankiv et al., 2010). In plants, the role of ATG8 in intracellular transport processes is poorly understood. Although FYCO1 homologs have been identified in Arabidopsis (Wywial and Singh, 2010), it is still unknown whether FYCO1 proteins are involved in autophagosome transport in plants. The exocyst is an evolutionary conserved protein complex mediating early tethering of secretory vesicles to the plasma membrane during exocytosis. Most of Exo70 subunits in Arabidopsis possesses widespread AIMs (Tzfadia and Galili, 2013). Despite the lack of direct evidence for ATG8 binding, Exo70B1 is colocalized with the ATG8f in Arabidopsis (Kulich et al., 2013). Further, Exo70B2 has been demonstrated to interact with ATG8, an interaction enhanced by phosphorylation of MPK3 kinase that leads to autophagic recycling of Exo70B2 (Teh et al., 2018). These results suggest that Exo70B1 and Exo70B2 may be involved in autophagic transport into the vacuole.

CONCLUSION AND PROSPECTS

Autophagy serves as an important catabolic mechanism involved in plant growth and development, and plant responses to stress. Initially, autophagy was known exclusively as a non-selective degradation process; however, increasing evidence suggests that autophagy is also a highly selective pathway to target specific substrates for degradation. ATG8 proteins play multifunctional roles in plant autophagy, promoting autophagosome biogenesis. Moreover, ATG8 proteins interact with various adaptor/receptor proteins to recruit specific targeted cargos for degradation through selective autophagy. The identification of ATG8-interacting autophagy receptor proteins helps us to understand how autophagy substrates are selected for degradation. Future identification of AIM/UIM-containing proteins should greatly expand the scope of selective autophagy. In addition, ATG8 is also involved in other intracellular processes that appear to be independent of autophagy. In conclusion, studies on ATG8 have greatly contributed to our understanding of the molecular basis for the connection of autophagy with other metabolic processes. Although extensive studies have been carried out on ATG8s in plants, there are many unanswered questions about their functions. An intriguing question relates to the presence of multiple ATG8 isoforms in plants, in contrast to a single ATG8 protein in yeasts; indeed, the reason for such great ATG8 diversity in plants remains unclear. Similarly, it is not known whether ATG8 isoforms interact with specific cargo receptors while engaging in different types of selective autophagy. In addition, the roles of ATG8 in intracellular trafficking are still poorly characterized in plants. Therefore, further study of the ATG8-interacting proteins will be important for understanding the role of ATG8s in autophagy-dependent and autophagy-independent functions.

AUTHOR CONTRIBUTIONS

LC conceptualized the review. FB, MY, and XG wrote the first draft. WH and LC critically revised the manuscript. All authors read and approved the final content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00466/full#supplementary-material

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Mechanism of Activation of Mechanistic Target of Rapamycin Complex 1 by Methionine

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Nutrients are closely involved in the regulation of lifespan and metabolic health. Cellular activities, such as the regulation of metabolism, growth, and aging, are mediated by a network of nutrients and nutrient-sensing pathways. Among the nutrient-sensing pathways, the mechanistic target of rapamycin complex 1 (mTORC1) acts as the central regulator of cellular functions, which include autophagy. Autophagy plays a significant role in the removal of protein aggregates and damaged or excess organelles, including mitochondria, to maintain intracellular homeostasis, which is involved in lifespan extension and cardiometabolic health. Moreover, dietary methionine restriction may have a beneficial effect on lifespan extension and metabolic health. In contrast, methionine may activate mTORC1 and suppress autophagy. As the mechanism of methionine sensing on mTORC1, SAMTOR was identified as a sensor of *S*-adenosyl methionine (SAM), a metabolite of methionine, in the cytoplasm. Conversely, methionine may activate the mTORC1 signaling pathway through the activation of phosphatase 2A (PP2A) because of increased methylation in response to intracellular SAM levels. In this review, we summarized the recent findings regarding the mechanism via which methionine activates mTORC1.

Keywords: methionine, *S*-adenosyl methionine, mechanistic target of rapamycin complex 1, autophagy, SAMTOR, phosphatase 2A methylation

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INTRODUCTION

All organisms adapt and respond to the nutrients available in the environment. Cellular activities, including the regulation of metabolism, cell growth, and aging, are mediated by a network that comprised nutrients and nutrient-sensing pathways (Efeyan et al., 2015). Dietary interventions, such as calorie or dietary restriction and protein restriction, have been widely explored for their impact on lifespan extension or the prevention of age-related diseases through effects on cardiometabolic health. Calorie or dietary restriction without malnutrition has been demonstrated to extend the lifespan of organisms and improve their cardiometabolic health (Colman et al., 2009; Fontana et al., 2010; Mattison et al., 2012, 2017). However, recent studies have reported that protein restriction, rather than calorie or dietary restriction, is more strongly involved in the lifespan extension and cardiometabolic health (Nakagawa et al., 2012; Levine et al., 2014; Solon-Biet et al., 2014; Simpson et al., 2017; Kitada et al., 2019). Moreover, accumulated evidence from experimental studies indicates that the restriction of specific amino acids, such as branched-chain amino acids (BCAAs) or methionine, promotes longevity and cardiometabolic health (Fontana et al., 2016;

Lee et al., 2016; Cummings et al., 2018; Kitada et al., 2019), which possibly mediates the benefits of protein restriction.

Among the nutrient-sensing pathways, the mechanistic target of rapamycin complex 1 (mTORC1) is a serine/threonine protein kinase that acts as the central regulator of cell growth and metabolism in response to the changes in nutrients or growth factors (Kim and Guan, 2019). Numerous studies on the pharmacological inhibition of mTORC1 by rapamycin have demonstrated the lifespan-extension benefit of this approach (Harrison et al., 2009; Anisimov et al., 2011; Wilkinson et al., 2012; Miller et al., 2014; Zhang et al., 2014), which suggest that mTORC1 is closely involved in lifespan regulation. The mechanism via which the suppression of mTORC1 leads to lifespan extension includes the induction of the autophagy (Kim and Guan, 2019). Autophagy is a lysosomal degradation pathway that plays an important role in the removal of protein aggregates and damaged or excess organelles, such as mitochondria, to maintain homeostasis and cell function (Mizushima et al., 2008). An appropriate autophagy may protect cells against various age-related stress conditions, which results in lifespan extension and cardiometabolic health (Wong et al., 2020). mTORC1 has been recognized as a crucial regulator of autophagy, and amino acids are one of the strong factors that affect mTORC1 activation (Kim and Guan, 2019). Thus, the beneficial effect of protein restriction on lifespan extension may be mediated through the induction of autophagy via the suppression of mTORC1 under amino-acid restriction. Recent findings have clarified that essential amino acids, including BCAAs or methionine, are possibly related to the regulation of the aging process, lifespan, and cardiometabolic health through multiple physiological and molecular mechanisms. In particular, the mechanisms underlying the role of methionine in the regulation of aging or lifespan have been widely investigated through dietary intervention via the application of a methionine restriction diet. Among these mechanisms, the involvement of methionine in the regulation of mTORC1 and autophagy has been elucidated based on the results of those studies. In the current review, we summarized the recent findings regarding the mechanism of mTORC1 activation by methionine.

ROLE OF RAGS ON THE REGULATION OF MTORC1 ACTIVITY BY AMINO-ACID SENSING

The mTORC1 activity is regulated by several molecules in response to changes in nutrients, including amino acids and growth factors. Moreover, the upstream component of the amino-acid–sensing pathway of mTORC1 is complicated (Kim and Guan, 2019). The regulation of mTORC1 activity by amino acids occurs through the translocation and localization of mTORC1 to lysosomes. The heterodimers of low-molecular-weight GTPases, RagA or B, and RagC, or D (Kim et al., 2008; Sancak et al., 2008; Anandapadamanaban et al., 2019), which are localized in lysosomes, play an important role in the activation of mTORC1 by amino acids. RagA and RagC exist as a dimer, and the GTP-bound form of RagA is its active form, whereas the GDP-bound form of RagC is its active form. In the presence of amino acids, these proteins function as activated GTP-RagA or GDP-RagC. In contrast, under amino-acid starvation, they function as a combination of inactivated GDP-RagA or GTP-RagC. The activated Rag dimer binds to Raptor, which is a major component of mTORC1, and participates in the translocation and localization of mTORC1 from the cytoplasm to lysosomes. Thereafter, in the lysosome, mTORC1 is activated by GTP-Rheb.

The GATOR1 and GATOR2 complexes are recognized as Rag regulators and are localized in the cytoplasm (Bar-Peled et al., 2013). GATOR1 is a complex composed of three proteins, DEPDC5, NPRL2, and NPRL3, and has RagA-binding ability and GTPase-activating protein (GAP) activity for RagA (Shen et al., 2018). DEPDC5 of GATOR1 contains a GAP domain, which binds directly to RagA, thus inactivating it. However, deletion of the GATOR1 component results in the amino-acid–independent localization and activation of mTORC1 in the lysosome, which demonstrates that GATOR1 is a negative regulator of mTORC1. In contrast, GATOR2 is a complex consisting of five proteins, Sec13, Seh1L, WDR24, WDR59, and Mios (Bar-Peled et al., 2013). GATOR2 binds to GATOR1; GATOR2 acts as the positive regulator of mTORC1 by suppressing the GAP activity of GATOR1. Leucine and arginine bind to sestrin1/2 and CASTOR1, respectively, and sestrin1/2 and CASTOR1 are also recognized as sensors of leucine or arginine (**Figure 1A**). Amino-acid–bound sensor proteins dissociate from GATOR2, thus losing their ability to inactivate GATOR2 (**Figure 1A**; Chantranupong et al., 2014, 2016; Parmigiani et al., 2014; Saxton et al., 2016a,b; Wolfson et al., 2016). Consequently, the activated GATOR2 triggers the activation of mTORC1 through the inactivation of GATOR1. Conversely, during leucine or arginine starvation, sestrin1/2 and CASTOR1 bind to GATOR2 and inactivate GATOR2, which results in mTORC1 inactivation via an increase in the RagA GAP activity of GATOR1 (**Figure 1B**).

In addition to the cytosolic amino-acid–sensing branch, Shen and Sabatini reported that Ragulator and SLC38A9 are two critical regulators of the activation of mTORC1 as the lysosomal amino-acid–sensing branch (Shen and Sabatini, 2018) (**Figure 1C**). Ragulator tethers the Rag heterodimer to the lysosomal surface, and the SLC38A9 transmembrane protein is a lysosomal arginine sensor that stimulates mTORC1 activity through the regulation of Rags. Ragulator and SLC38A9 are guanine exchange factors that lead the Rags toward the active form (Shen and Sabatini, 2018). Ragulator triggers GTP release from RagC, thus lifting the locked inactivated state of the Rags (Shen and Sabatini, 2018). Upon arginine binding, SLC38A9 converts RagA from the GDP- to the GTP-bound state, leading to the activation of the Rags (Shen and Sabatini, 2018). Thus, Ragulator and SLC38A9 activate mTORC1 by recruiting it to the lysosomal surface via Rag activation in response to arginine levels in the lysosome. Moreover, v-ATPase interacts with Ragulator, Rags, and SLC38A9 and is involved both in amino-acid–sensing and in efflux from the lysosome (Zoncu et al., 2011; Abu-Remaileh et al., 2017; Wyant et al., 2017). However, it remains unknown whether v-ATPase senses amino acids.

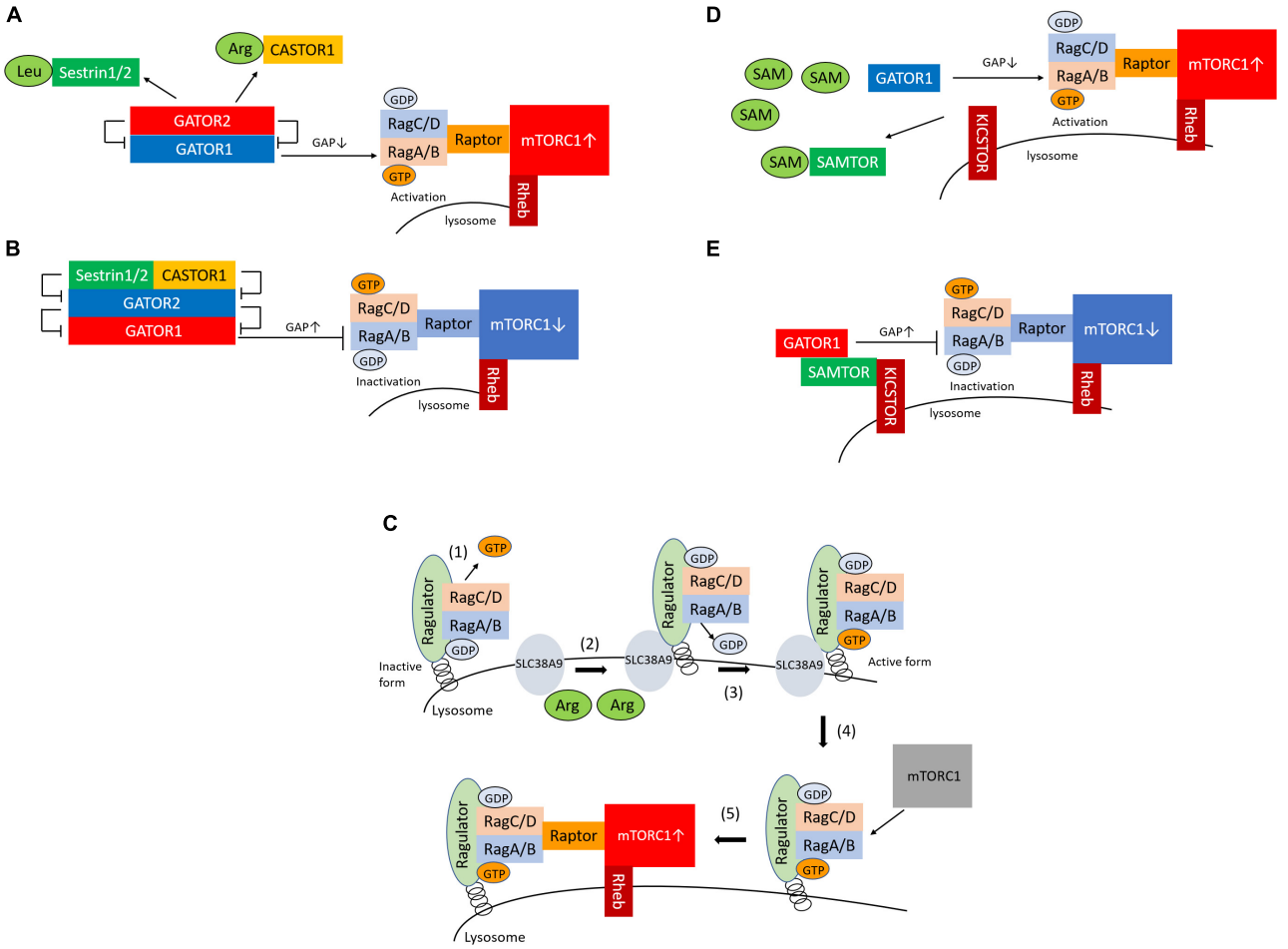

FIGURE 1 | Sestrin1/2, CASTOR1, and SAMTOR are cytosolic sensors of leucine, arginine, and SAM for the regulation of mTORC1. **(A)** Sestrin1/2 and CASTOR1 sense leucine and arginine in the cytoplasm, respectively. Upon binding of leucine or arginine to sestrin1/2 and CASTOR1, these proteins dissociate from GATOR2, which releases their suppressive effect on GATOR2. GATOR2 suppresses GATOR1, which consequently activates mTORC1 via GTP-bounded RagA formation by GAP inactivation. **(B)** In conditions of low leucine and arginine levels, sestrin1/2 and CASTOR1 suppress GATOR2 (the negative regulator of GATOR1), which leads to the activation of GATOR1 and results in the suppression of mTORC1 via GAP activation of RagA. **(C)** (1) Ragulator releases GTP from RagC; (2) SLC38A9 is activated by arginine in the lysosome; (3) SLC38A9 converts RagA from the GDP- to the GTP-bound state, leading to the activation of the Rags; (4) Ragulator and SLC38A9 recruit the mTORC1 to the lysosomal surface; and (5) mTORC1 is activated. **(D)** SAMTOR senses SAM in the cytoplasm. Upon the binding of SAM to SAMTOR, SAMTOR dissociates from GATOR1. The disruption of the SAMTOR–GATOR1 complex leads to the inactivation of GATOR1, which results in mTORC1 activation via the inhibition of GAP activation and increased GTP binding to RagA. **(E)** In conditions of low levels of SAM, the SAMTOR–GATOR1 complex suppresses mTORC1 activity. SAM, *S*-adenosyl methionine; mTORC1, mechanistic target of rapamycin complex 1; GAP, GTPase-activating protein.

METHIONINE-INDUCED MTORC1 ACTIVATION AND THE ROLE OF SAMTOR AS A SAM SENSOR THAT PROVIDES A LINK TO THE METHIONINE METABOLISM

The KICSTOR complex is one of the regulators of mTORC1 and comprises kaptin (KPTN), the integrin-α FG-GAP repeat-containing protein 2 (ITFG2), C12orf66, and seizure threshold 2 (SZT2) (Wolfson et al., 2017). C7orf60 was identified as an interacting protein of GATOR1 and was subsequently renamed SAMTOR (Gu et al., 2017). The overexpression of SAMTOR suppresses mTORC1 activity, which indicates that SAMTOR is a negative regulator of mTORC1. SAM is converted from methionine, and methionine starvation reduces the concentration of SAM in the cytoplasm. When present of SAM, SAM binds to SAMTOR, which then dissociates from GATOR1 (**Figure 1D**). The disruption of the SAMTOR–GATOR1 complex leads to the inactivation of GATOR1, which then results in mTORC1 activation through the inhibition of GAP activation and increased binding of GTP to RagA. In contrast, methionine starvation reduces SAM levels below the dissociation constant of the SAM–SAMTOR complex, thus promoting SAMTOR–GATOR1 binding and, in turn, suppressing mTORC1 activity (**Figure 1E**). However, loss of SAMTOR activates mTORC1, even in conditions of methionine starvation. In addition, SAMTOR mutants that cannot bind to SAM fail to transmit methionine

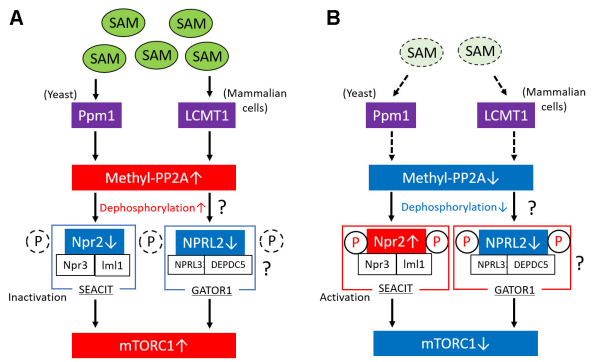

FIGURE 2 | Regulation of mTORC1 via the methylation of PP2A in response to SAM. **(A)** In conditions of high levels of intracellular SAM in yeast, Ppm1 induces the methylation of the catalytic subunit of PP2A in response to SAM concentration. The activated (methylated) form of PP2A suppresses Npr2 through its dephosphorylation. The complex consisting of Npr2, Npr3, and Iml1 (SEACIT) is a negative regulator of mTORC1; therefore, the suppression of SEACIT via the dephosphorylation of Npr2 results in the activation of mTORC1. In mammalian cells, LCMT1 induces the methylation of the catalytic subunit of PP2A in response to SAM concentration, leading to the activation of mTORC1, possibly through the activation of GATOR1. Moreover, in mammalian cells, PP2A possibly regulates the phosphorylation levels of NPRL2 in response to SAM levels. **(B)** Lower SAM levels reduce the methylation levels of PP2A in yeast and mammalian cells and promote the activation of Npr2 via its phosphorylation, which results in the suppression of mTORC1 activity. In mammalian cells, PP2A possibly regulates the phosphorylation levels of NPRL2 in response to SAM levels. mTORC1, mechanistic target of rapamycin complex 1; PP2A, phosphatase 2A; SAM, *S*-adenosyl methionine; GAP, GTPase-activating protein; LCMT1, leucine carboxyl methyltransferase 1.

sufficiently to mTORC1, therefore suppressing mTORC1. These results indicate that SAMTOR serves as a SAM sensor in the methionine-mediated mTORC1 activation.

ROLE OF THE INDUCTION OF THE METHYLATION OF PP2A BY SAM IN MTORC1 ACTIVATION

The study performed by Sutter et al. also showed that methionine regulates the mTORC1 signaling pathway and autophagy through the regulation of the methylation status of phosphatase 2A (PP2A) in yeast (Sutter et al., 2013; Laxman et al., 2014). In the presence of high levels of intracellular SAM, Ppm1 induces the methylation of the catalytic subunit of PP2A in response to SAM concentration. PP2A is activated by its methylation; thereafter, methylated PP2A can suppress Npr2 through its dephosphorylation, which results in mTORC1 activation and the suppression of autophagy (**Figure 2A**). The complex consisting of Npr2, Npr3, and Iml1 (NPRL2, NPRL3, and DEPDC5 in mammals, respectively) is termed SEACIT in yeast (GATOR1 in mammals, as described above) (Panchaud et al., 2013) and functions as a negative regulator of mTORC1 via a GAP activity toward the yeast Rag orthologs, that is, Gtr1/2 (Rags family in mammals) (Gao and Kaiser, 2006). Therefore, suppression of SEACIT by the dephosphorylation of Npr2 induced by the activation of PP2A results in the activation of mTORC1. In contrast, lower SAM levels in cells reduce the methylation levels of PP2A and promote the phosphorylation of Npr2, which results in the suppression of mTORC1 activity and the induction of autophagy (**Figure 2B**). In mammalian cells, the methylation of PP2A is catalyzed by a specific *S*-adenosyl methionine (SAM)–dependent methyltransferase, the leucine carboxyl methyltransferase 1 (LCMT1) (Stanevich et al., 2011). Activated PP2A possibly dephosphorylates NPRL2 and results in mTORC1 activation in mammalian cells; however, no report has shown whether PP2A is directly involved in the regulation of the phosphorylation state of NPRL2. Therefore, further studies are necessary to clarify this issue.

We also reported that a low-protein diet ameliorates diabetes-induced kidney injury and that dietary methionine abrogates the beneficial effects of a low-protein diet in diabetic kidneys (Kitada et al., 2020). More specifically, diabetic rats that were fed a low-protein + methionine diet exhibited increased expression of LCMT1 and methyl-PP2A compared with control (standard-diet–fed) and low-protein-diet–fed diabetic rats, which was accompanied by an increase in renal SAM levels. Although the expression of glycine *N*-methyltransferase (Gnmt), which is a SAM-converted enzyme, was decreased in diabetic rat kidneys, changes in renal SAM levels were dependent on the dietary methionine content (Kitada et al., 2020). Consistent with the alteration of LCMT1 and methyl-PP2A, mTORC1 activation and autophagy suppression were observed in standard-diet–fed and low-protein + methionine–fed diabetic rats. Furthermore, we also used cultured human kidney-2 cells to confirm that the administration of SAM-induced methylated PP2A increased the expression of methyl-PP2A and activated mTORC1 (Kitada et al., 2020). However, the involvement of SAM-induced methylated PP2A in mTORC1 activation through NPRL2 and the activation of the negative regulator of mTORC1 by its increased phosphorylation, such as that observed for Npr2 in yeast, remain unknown.

METHIONINE ACTIVATES MTORC1 THROUGH TAS1R1/TAS1R3

Nelson et al. previously identified a mammalian amino-acid taste receptor, the taste 1 receptor member 1 (TAS1R1)/taste 1 receptor member 3 (TAS1R3) heterodimer, which is a cell-surface G-protein–coupled receptor (Nelson et al., 2002). This receptor broadly functions as an amino-acid sensor that responds to most of the 20 standard amino acids. Upon sensing amino acids, this receptor activates mTORC1 through the activation of phospholipase C, the increase in intracellular calcium, and the activation of the mitogen-activated protein kinase 1/mitogen-activated protein kinase 3 (Wauson et al., 2015). TAS1R1–TAS1R3 is required for the amino-acid–induced mTORC1 localization to the lysosome, which is a necessary step in mTORC1 activation. Several reports have demonstrated that TAS1R1–TAS1R3 may serve as a sensor of extracellular methionine and that it activates mTORC1 in cultured C2C12 cells and bovine epithelial cells (Zhou et al., 2016, 2018).

DISCUSSION

In this study, we described the recent findings regarding the mechanism via which methionine induces the activation of mTORC1. mTORC1 may be activated by sensing SAM rather than methionine. A previous report by Obata et al. provided evidence that SAM, rather than methionine, may be the main contributor to the aging process (Obata and Miura, 2015). Those authors showed that increasing SAM catabolism via the action of glycine *N*-methyltransferase (Gnmt) extends the lifespan in *Drosophila*. In particular, SAM is upregulated in older flies, even if the transcription of Gnmt is induced in a forkhead box O (FOXO)–dependent manner. However, overexpression of Gnmt suppresses the age-dependent increase in SAM and extends lifespan in *Drosophila*. In addition, metabolic impairment, such as insulin resistance in obesity, is closely involved in the aging process. A previous report demonstrated that plasma SAM concentrations were related to higher fasting insulin levels, the homeostasis model assessment of insulin resistance, and the tumor necrosis factor α in a cross-sectional study that involved subjects with metabolic syndrome (Lind et al., 2018). Another report also revealed that plasma SAM, and not methionine, is independently related to fat mass and truncal adiposity in a cross-sectional study involving elderly individuals (Elshorbagy et al., 2013); in contrast, overfeeding increases serum SAM in proportion to the fat mass gained (Elshorbagy et al., 2016). Thus, the upregulation of SAM associated with overfeeding or metabolic dysfunction may be involved in whole-body metabolic impairment. These data indicate that increased levels of SAM in the process of methionine metabolism may be related to the stimulation of aging and metabolic impairment, including insulin resistance, which is particularly associated with obesity. Previous reports have shown that dietary methionine restriction extends the lifespan or improves cardiometabolic health (Orentreich et al., 1993; Miller et al., 2005; Hasek et al., 2010; Plaisance et al., 2011; Johnson and Johnson, 2014; Lee et al., 2014; Stone et al., 2014). The effect of methionine restriction on lifespan extension or cardiometabolic health may be exerted through multiple mechanisms, including antioxidative stress, the production of hydroxy sulfates, the downregulation of GH/insulin growth factor 1 signaling, the production of fibroblast growth factor 21, the suppression of mTORC1, and the induction of autophagy (Kitada et al., 2019). Among them, the suppression of mTORC1 is induced by decreasing SAM levels. Therefore, the regulation of SAM levels and sensing of SAM in the cytoplasm may be key factors in the mechanism of lifespan extension, which may be mediated by the regulation of mTORC1. Because the selective suppression of mTORC1 induced by SAM may be a therapeutic target for aging, metabolic impairment, or aging-related disease, further studies are necessary to address these issues.

AUTHOR CONTRIBUTIONS

MK designed the manuscript, the guarantor of this work, and wrote and edited the manuscript. JX, YO, IM, and DK contributed to the discussion. All authors contributed to the article and approved the submitted version.

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REVIEW
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Natural Compounds and Autophagy: Allies Against Neurodegeneration

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Prolonging the healthy life span and limiting neurological illness are imperative goals in gerontology. Age-related neurodegeneration is progressive and leads to severe diseases affecting motility, memory, cognitive function, and social life. To date, no effective treatments are available for neurodegeneration and irreversible neuronal loss. Bioactive phytochemicals could represent a natural alternative to ensure active aging and slow onset of neurodegenerative diseases in elderly patients. Autophagy or macroautophagy is an evolutionarily conserved clearing process that is needed to remove aggregate-prone proteins and organelles in neurons and glia. It also is crucial in synaptic plasticity. Aberrant autophagy has a key role in aging and neurodegeneration. Recent evidence indicates that polyphenols like resveratrol and curcumin, flavonoids, like quercetin, polyamine, like spermidine and sugars, like trehalose, limit brain damage *in vitro* and *in vivo*. Their common mechanism of action leads to restoration of efficient autophagy by dismantling misfolded proteins and dysfunctional mitochondria. This review focuses on the role of dietary phytochemicals as modulators of autophagy to fight Alzheimer's and Parkinson's diseases, fronto-temporal dementia, amyotrophic lateral sclerosis, and psychiatric disorders. Currently, most studies have involved *in vitro* or preclinical animal models, and the therapeutic use of phytochemicals in patients remains limited.

Keywords: autophagy, polyphenols, alkaloids, terpenes, spermidine, trehalose, Alzheimer's disease, Parkinson's disease

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INTRODUCTION

The mammalian central nervous system (CNS) is a crowded environment of highly specialized neurons surrounded by glial cells, fibroblasts, and pericytes, with smooth muscle cells and endothelial cells lining associated vessels (Stevens, 2003; Zeng and Sanes, 2017; Laredo et al., 2019; Matias et al., 2019). The unique post-mitotic nature of neurons, with scarce regenerative ability, means that they must have an efficient oxidative metabolism to support their specialized functions and resist cell death (Misgeld and Schwarz, 2017; Area-Gomez et al., 2018). Indeed, proper synapse function strongly depends on mitochondria, endoplasmic reticulum, lysosomes, and axonal flux of calcium ions and neurotransmitters (Wilhelm et al., 2014; Carmona-Gutierrez et al., 2016; Krols et al., 2016; Wu et al., 2017; Eisner et al., 2018). Primary inherited but also adult mitochondrial dysfunctions in neurons are not only linked to mtDNA or mtRNA changes but also to disrupted Krebs cycle, and related biochemical pathways up to reduced ATP availability. Therefore, altered morphology and signaling of crucial organelles, like mitochondria and lysosomes, dramatically

initiate neurodegeneration (Lee et al., 2018; Cowan et al., 2019; Lie and Nixon, 2019) and vulnerable neurons' death (Andreone et al., 2019).

However, autophagy, literally from ancient Greek "*self-eating,*" is an evolutionary conserved mechanism to maintain neuronal homeostasis during the development and in mature cells (Kulkarni et al., 2018). This "cleaning" pathway based on lysosomes' activity, maintains nutrient recycling in starvation, neurotransmitter release, synaptic remodeling and pruning during development in axons and dendrites (Geronimo-Olvera and Massieu, 2019; Lieberman and Sulzer, 2019; Lieberman et al., 2019; Stavoe and Holzbaur, 2019). Recently, Tomoda et al. (2019) outlined a novel essential role of autophagy, at synaptic level, regulating information processing, memory, mood, and cognitive functions in mouse models. Moreover, Lieberman et al. (2020) discovered another essential function of autophagy in the regulation of potassium channels in neurons in the striatum of mice, necessary for excitability and motor learning. However, autophagy is not only a peculiarity of neurons but also present in astrocytes, oligodendrocytes and microglia in aging and neurodegenerative disorders (Kim et al., 2017; Plaza-Zabala et al., 2017; Belgrad et al., 2020; Wang and Xu, 2020).

Since 2006, Mizushima's and Tanaka's group reported dysfunctional autophagy in mice lacking fundamental autophagy genes (ATG5 and ATG7 knockout) associated to locomotor abnormalities, neuronal loss in brain and cerebellum, behavioral defects and death within 28 weeks of birth (Hara et al., 2006; Komatsu et al., 2006).

Currently, the idea of a strict interdependence between autophagy and diseases in the CNS is well defined and aberrant autophagy is associated to aging and the pathogenesis of neurodegenerative diseases characterized by abnormal proteostasis (Morimoto and Cuervo, 2014; Tanaka and Matsuda, 2014; Nikoletopoulou et al., 2015). However, in addition to macroautophagy, other degradative mechanisms are involved in the clearance of misfolded proteins in neurons such as the molecular chaperones and the ubiquitin-proteasome (Nixon, 2013; Taylor et al., 2014; Ungelenk et al., 2016; Cristofani et al., 2017; Mogk and Bukau, 2017). Recent evidence indicates that chaperones and ubiquitin-proteasome are mainly required to dismantle short-lived soluble proteins, while autophagy dismantles large misfolded aggregates in non-selective or selective manner (Chu, 2019). This last mechanism, called *aggrephagy*, implies a receptor and a substrate connection to best recognize abnormal inclusions in damaged neurons (Gatica et al., 2018). Indeed, toxic proteic aggregates impair neurotransmission, calcium flux, mitochondria activity, membrane permeability and are commonly detected in animals and post-mortem brain in patients affected by neurodegenerative diseases like Alzheimer's (AD), Parkinson's (PD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and Huntington's (HD) (Chung et al., 2018; Cheon et al., 2019; Malampati et al., 2020). However, in addition to protein aggregates, selective autophagy removes damaged organelles like mitochondria, ribosomes and the endoplasmic reticulum to maintain neuronal homeostasis (Morishita and Mizushima, 2019; Evans and Holzbaur, 2020).

Consequently, there is extensive evidence that dysfunctional autophagy and/or mitophagy have been implicated in the onset and progression of neurodegenerative and psychiatric disorders like bipolar disorder and schizophrenia (Fujikake et al., 2018; Mizushima, 2018; Bar-Yosef et al., 2019; Kuang et al., 2019; Malik et al., 2019; Palikaras and Tavernarakis, 2020).

In recent years, the belief that autophagy is a druggable target and, consequently, its modulation a promising therapeutic opportunity for CNS diseases have been reported in several authoritative reviews (Morel et al., 2017; Scrivo et al., 2018; Condello et al., 2019; Djajadikerta et al., 2019; Mputhia et al., 2019; Peng et al., 2019; Thellung et al., 2019). However, the complex pathogenesis of neurodegeneration and a better knowledge of autophagy steps and upstream signaling pathways are necessary to extend promising results obtained in animal models to patients. Remarkably, autophagy modulation seems particularly favorable in an early phase of neurodegeneration and long-term autophagy modulators without side effects are urgently required (Li et al., 2015; Menzies et al., 2017; Giampieri et al., 2019).

For all these reasons, we conceived this critical review focusing on recent studies on dietary natural products and herbs able to regulate autophagy and limit neurodegeneration *in vitro*, in rodent models and eventually in patients. Before analyzing the specific role of natural products in neurodegenerative diseases, a brief explanation of the autophagic mechanisms activated in the brain is shown below.

AUTOPHAGIC SIGNALING

There are three crucial types of autophagy deeply characterized in neurons (Bento-Cuesta et al., 2017) and less in glia (Strohm and Behrends, 2019), defined macroautophagy, chaperone-mediated autophagy (CMA) and endosomal microautophagy.

Macroautophagy

Macroautophagy (simply referred as "autophagy" hereafter), the most studied dynamic mechanism of recycling macromolecules and organelles, may be beneficial or detrimental for neurons, depending on its intensity, speed (called "the autophagic flux") and regulation (Feng et al., 2014; Button et al., 2015; Hansen et al., 2018). Indeed, there is a basal beneficial autophagy necessary for proper development to maintain life span and prolong longevity, and a detrimental excessive autophagy, called autophagic cell death or autosis (Galluzzi et al., 2018). This last type is predominant in the hippocampus, where the loss of adult stem neurons, consequent to insulin withdrawal, induces cognitive deficits (Yu et al., 2008; Jung et al., 2020). Moreover, also selected autophagy of mitochondria, called mitophagy, whenever excessive becomes detrimental leading to neuronal death, such as reported in ischemic and hypoxic events *in vitro* and in rat spinal cord (Feng et al., 2018; Yu et al., 2018). Recent studies in preclinical animal models and in post-mortem brain samples from patients indicated a strict connection between defective autophagy and mitophagy to the pathogenesis of Parkinson's

(Gao et al., 2017; Arotcarena et al., 2019; Lin et al., 2019) and Alzheimer's disease (Chakravorthy et al., 2019; Xie et al., 2020).

However, it is essential to precisely modulate and monitor each step of the autophagy process to avoid detrimental irreversible effects instead of benefits in neurons (Mariňo et al., 2011). Therefore, we resumed below different stages of mammalian autophagy, considering that for each step it is possible to foresee a genetic or a pharmacologic regulation.

The first step of autophagy starts in the cytoplasm with the "phagophore," a peculiar double membrane that subsequently elongates, and closes on itself to produce an "autophagosome," filled with misfolded proteins, lipidic materials or damaged organelles. The "autophagosome" progressively matures and merges with lysosomes becoming an "autolysosome" to complete clearing. In the last final step, all cargo is dismantled by lysosomal hydrolases and eventually a new phagophore is reformed.

Different upstream machineries control the induction of mammalian autophagy and the first initiation step. The most studied are the serine/threonine protein kinase ULK1 (unc-51-like kinase 1), which forms an assembly with autophagy-related proteins 13 and 101 (Zachari and Gauley, 2017), and phosphatidylinositol 3-kinase (PI3K), essential for starting all the process (Devereaux et al., 2013). The following elongation step involves a detailed genetic program and autophagy-related proteins (ATGs), directly regulated by two pathways: the mTOR complex 1 (mTORC1) and Bcl2/Beclin 1, the mammalian ortholog of ATG6. Intriguingly, mTORC1 downregulates ULK1, so inhibiting autophagy, but may be further positively regulated by Akt or negatively by the AMP-activated protein kinase (AMPK). In this last case, autophagy is stimulated. Other kinases regulate Bcl2 by inhibiting its binding to Beclin 1 and stimulating autophagy (He and Klionsky, 2009). Furthermore, selected transcription factors influence the formation of autophagosomes, like the master transcriptional regulator of autophagy/lysosomal biogenesis (TFEB), and peroxisome proliferator-activated receptor alpha (PPAR alpha) (Fullgrabe et al., 2016). Remarkably, to treat devastating neurodegenerative syndromes many efforts have been addressed to modulate TFEB and, consequently, to restore proper autophagy (Cortes and La Spada, 2019).

Different set of ATG proteins regulate the maturation and the closure of the autophagosome, and mainly the ATG8/LC3, or microtubule-associated protein 1 light-chain 3 (LC3I), is necessary for the final formation of the autophagic vacuole. Indeed, for this step, the cytosolic LC3I become lipidated and associated with phosphatidylethanolamine to generate LC3II linked to the autophagosomal membrane (Mizushima, 2018). The final degradation of the cargo involves the fusion with lysosomes, strictly dependent on the sequestosome (SQSTM1 or p62) protein, that is a reliable marker of an effective autophagic flux (Sanchez-Martin and Komatsu, 2018).

Mitophagy, the selective autophagy of mitochondria, requires specific receptors, able to select damaged mitochondria for the removal (Gatica et al., 2018). The most studied are PTEN-kinase 1 (PINK1) and Parkin. PINK1, located in the outer mitochondrial membrane, recruits parkin, an ubiquitin ligase, from the cytoplasm to the depolarized mitochondria, making them recognizable by the autophagosome for dismantling. Altered PINK1/parkin signaling in dopaminergic neurons has been strictly associated to the pathogenesis of PD (Truban et al., 2017; Lin et al., 2019; Noda et al., 2020), and defective mitophagy is an additional hallmark of AD, FTD and ALS diseases (Cai and Jeong, 2020; Xie et al., 2020). Recent evidence indicates in autosomal recessive PD the recruitment of PINK1 to mitochondria-associated membrane (MAM) and the regulation of mitochondria-ER distance and mitophagy (Gelmetti et al., 2017). Intriguingly, an abnormal tethering and consequent disrupted mitophagy have been described in long projecting axon neurons and glia in ALS (Bernard-Marissal et al., 2018). Curiously, mice lacking PINK1 or Parkin do not present severe PD evidences like neuronal loss or locomotor dysfunctions, typical of humans, but probably due to their limited life span (Evans and Holzbaur, 2020). However, in hypoxia, different mitophagy pathways driven by novel receptors like NIX (Nip3 like protein X)/BNIP3 (Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3) or FUNDC1 (FUN14 domain containing 1) are activated. Recently, in mutant dopaminergic neurons, an *in vitro* model of PD, Ryan et al. (2018) demonstrated that cardiolipin exposure in the outer mitochondrial membrane is necessary for activating mitophagy and refolding of toxic alpha synuclein. Remarkably, all above receptors joined LC3 or gamma-aminobutyric acid receptor-associated protein (GABARAP) on the autophagosome for final mitochondria dismantling (Palikaras et al., 2018). A resumptive plot indicating the progressive macroautophagy signaling and its regulation is shown in **Figure 1**.

Chaperone-Mediated Autophagy (CMA)

Compared to macroautophagy, chaperone-mediated autophagy (CMA) presents three fundamental peculiarities: it removes only misfolded cytoplasmic proteins and not organelles; it does not require mature autophagosomes but only lysosomes; the recognition of aggregated proteins and the targeting to lysosomes are performed by a cytosolic chaperone HSC70 (Kaushik and Cuervo, 2018). For proper dismantling, all cytosolic proteins must contain an amino acidic sequence related to KFERQ. The complex obtained is then up-taken by the lysosome-associated membrane glycoprotein 2 (LAMP2) and degraded. Failure in CMA has been detected in PD to dismantle abnormal alpha-synuclein aggregates (a favorite CMA substrate), but alsoin AD, TD and HD to remove excessive tau protein, TAR DNA-binding protein 43 (TDP-43) and huntingtin protein, respectively (Martinez-Vicente et al., 2010; Cuervo and Wong, 2014; Wu et al., 2015; Tripathi et al., 2019). For these reasons, targeting CMA can be considered another therapeutic opportunity if autophagy is unresponsive (Xilouri et al., 2013). Intriguingly, two pathways modulate CMA in neurons: mTORC2 that inhibits LAMP2A assembly and lysosomal p38 MAPK during ER stress response (Li et al., 2019). Indeed, CMA pathway works together with other homeostatic mechanism like the ER stress response and reduced interaction between these processes results in loss of dopaminergic neurons in the *substantia nigra* in mice (Li

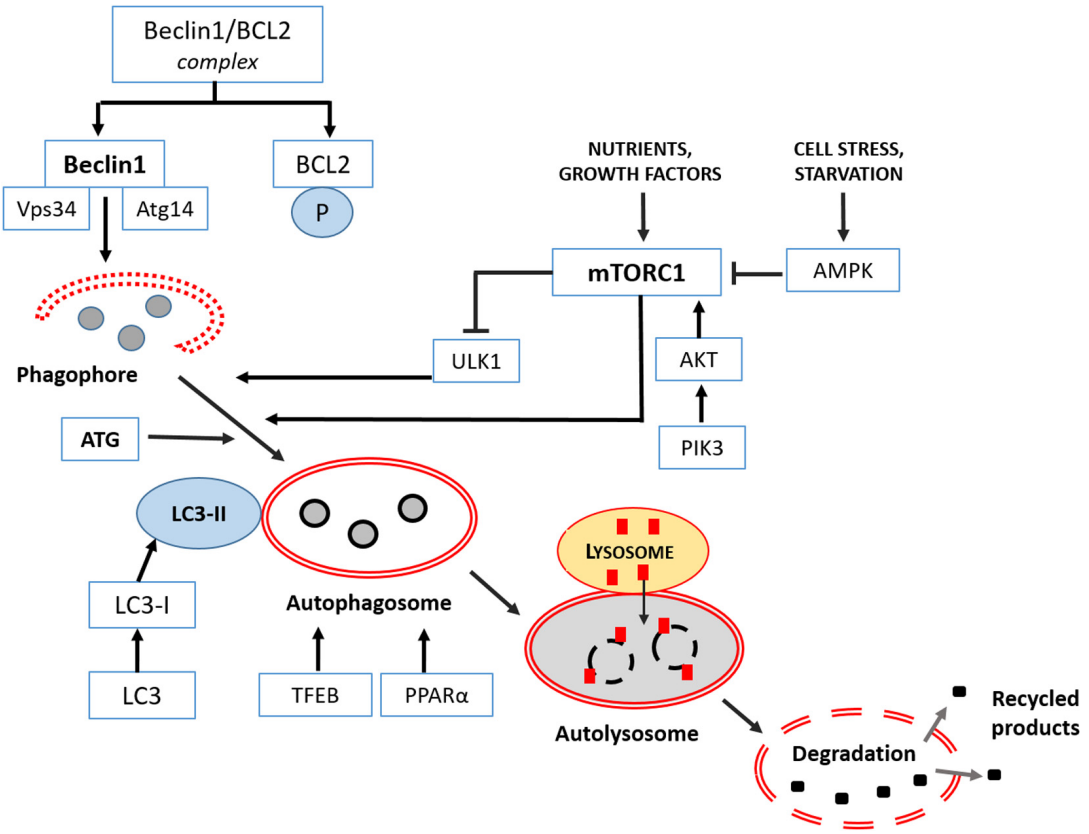

FIGURE 1 | Macroautophagy signaling in the mammalian brain. It consists in three main phases: formation-elongation of a round structure, maturation then its content degradation-recycling. The first step of autophagy starts in the cytoplasm with the "phagophore," a peculiar double membrane structure derived from ER, Golgi complex or plasmalemma that subsequently elongates, and closes on itself to produce an "autophagosome," filled with proteins or lipidic material and damaged organelles. Then the "autophagosome" progressively matures and merges with lysosomes becoming an "autolysosome" or "amphisome"to complete clearing. In the last final step, all cargo is dismantled by acidic lysosomal hydrolases and eventually a new phagophore is reformed. Major regulators of different phases are class III PI3-K complex and Beclin 1 protein, Atg proteins cascade resulting in Atg3/Atg7 mediated LC3I into LC3II conversion as indicated and discussed in the text. Extensive details on macroautophagy machinery are reported by Feng et al. (2014).

et al., 2017). Conversely, proper restoration of both CMA and macroautophagy, by the upstream regulation of the nuclear erythroid 2-related factor 2 (Nrf2) in astrocytes, promotes effective alpha-synuclein degradation and rescue in a PD mice model (Gan et al., 2012).

Microautophagy

This mechanism, still poorly defined in mammalians, involves the direct recruitment of cytosolic proteins containing the sequence KFERQ via Hsc70 into a membrane invagination of a late endosome or lysosome then their full degradation (Malik et al., 2019). This peculiar autophagosome-independent transfer occurred at synaptic level and was necessary for the renovation of synaptic proteins. Sato et al. (2019) reported that rapamycin, a known activator of macroautophagy, stimulated microautophagy in cells by TFEB stimulation and hypothesized its involvement in neurodegenerative diseases. The delivery of proteins to be degraded within vesicles resembled a "secretory autophagy" mechanism and intraluminal exosomes formation (Buratta et al., 2020).

NUTRACEUTICALS EFFECTIVE IN NEURODEGENERATION VIA AUTOPHAGY

Chronic neurodegenerative diseases are untreatable and few approved synthetic drugs reduced adverse symptoms but not cure them. In this discouraging scenario, oral supplementation with vegetal bioactive derivatives or the Mediterranean diet are promising to postpone the irreversible progression of AD, PD, ALS, FTD (Vauzour et al., 2017; Hornedo-Ortega et al., 2018; Pohl and Lin, 2018; Fernandez-Sanz et al., 2019).

However, considering that the relation "one-drug, one-target" for the multifactorial pathogenesis of neurodegenerative diseases is clinically unsuccessful, traditional medicinal herbs or plants with beneficial pleiotropic effects may represent a plausible preventive auxiliary therapeutic opportunity (Cummings et al., 2014; Park et al., 2018; Di Paolo et al., 2019). Plants derived compounds or "nutraceuticals" are secondary metabolites, produced by the plants to defend themselves from pathogens or adverse environmental conditions and have been proposed as

complementary "herbal Medicine" to treat Alzheimer's (AD) and Parkinson's disease (PD) (Ahn and Jeon, 2015; Perez-Hernandez et al., 2016; Naoi et al., 2019; Renaud and Martinoli, 2019; Chiu et al., 2020).

Nutraceuticals are largely present in fruit, vegetables, cereals, herbs used in the human nutrition, with pleiotropic anti-oxidant, anti-inflammatory, glycemia-regulating properties able to preserve brain (Kennedy and Wightman, 2011; Gonzalez et al., 2019) with fewer side-effects than synthetic drugs (Georgiou et al., 2011; Forni et al., 2019). Recently, an important source of neuroprotective compounds like phytosterols, carotenoids, fucoidans and polyphenols have been characterized also in seaweeds consumed in China and Indonesia (Schepers et al., 2020). Most of them are effective anti-inflammatory and anti-oxidants and preserve dendritic spine density in hippocampal neurons. However, the harvest and purification of seaweeds are crucial to obtain bioactive compounds to use at a dosage effective for the brain activity.

Considering the pathogenetic role of aberrant autophagy in neurodegenerative diseases, emerging evidence indicates that nutraceuticals modulators of autophagy may be promising "functional foods" (Fan et al., 2017; Prieto-Dominguez et al., 2018; Xie et al., 2019; Zeng et al., 2019). Moreover, autophagy and mitophagy are involved in the removal of aggregated proteins and dysfunctional mitochondria hallmark of AD, PD, frontotemporal dementia (FTD) and Huntington disease (HD) (Menzies et al., 2017).

In the following subheadings, we discussed and commented studies published in the last decade on nutraceuticals as regulators of autophagy and their role in neurodegenerative diseases. Natural compounds have been subdivided according to their chemical structure into four categories: polyphenols, alkaloids, terpenes and terpenoids. Finally, the last subheading, entitled "Others," is dedicated to chemically heterogeneous compounds that are emerging against neurodegeneration and aging, like the pineal indole melatonin, the disaccharide trehalose and the polyamine spermidine, a well-known caloric restriction mimetic (Kiechl et al., 2018; Lee et al., 2018; Madeo et al., 2019).

Polyphenols

Phenolics compounds are commonly present in human diet, due to their large presence in plants. They derived from phenylalanine and contained almost one phenol ring that contributed to different subclasses like: phenols acids, flavonoids, stilbenes and lignans (Tsao, 2010). Compounds belonging to this category deeply involved to attenuate neurodegeneration are: polyphenols like resveratrol in wine and in virgin olive oil; flavanols in cocoa, tea, apples, beans; hydroxycinnamates in coffee; flavonoids in tea, apples, onions, chocolate; anthocyanins in berries (Angeloni et al., 2017; Potì et al., 2019). The effects of polyphenols on autophagy are rapidly emerging as specific to a single step of the process for each neurodegenerative syndrome (Kou and Chen, 2017).

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), largely present in plants and in red wine as derivative from Vitis viniferas, regulated AMPK/mTORC1 pathway and activated the first step of autophagy to alleviate cognitive impairment in AD mice. However, dysfunctional enhanced autophagy has been reported as pathogenic hallmark of AD in dystrophic neurites with autophagic vacuoles, upregulated mTOR and reduced Beclin 1 (Pickford et al., 2008; Lipinski et al., 2010; Bordi et al., 2016). All these studies highlighted the controversial role of autophagy in AD (Castellazzi et al., 2019) and its dependence on neuronal topology and on the stage of cognitive impairment (Liu and Li, 2019).

In a recent proteomic study Lachance et al. (2019) reported in post-mortem brain reduced gene expression for autophagy kinase complex in the para-hippocampal area and hippocampus. Remarkably, the same evidence occurs in mice deprived of BECN1-PIK3C3 complex that showed memory deficits and impaired autophagy. Conversely, the activation of the nuclear receptor binding factor 2 (NRBF2), associated to PI3K complex, greatly influenced autophagy flux progression and demolition of toxic amyloid aggregates (Yang C. et al., 2017). Recently, Reddy and Oliver (2019) reported reduced autophagy and mitophagy caused by excessive amyloidosis and tau deposition in AD. Other authors reported that resveratrol is effective to reduce abnormal beta amyloid deposition in APP/PS1 mice activating AMPK (Di Meco et al., 2020) and sustaining Beclin 1 and LC3II via a Sirtuin1 signaling. Remarkably, its low bioavailability and difficulty to cross the brain blood barrier must be taken in account for its pharmacological efficacy (Salehi et al., 2018). In fact, only the trans isoform is effective in the hippocampus of AD mice by sustaining autophagy (Porquet et al., 2014). *In vitro*, in PC12 cells, resveratrol promoted autophagy via Sirtuin 1 protein and subsequent LC3I deacetylation (Deng and Mi, 2016). Resveratrol rescued ischemic damage in the rat brain during middle cerebral artery occlusion or excitotoxicity induced by glutamine (Pineda-Ramirez et al., 2020). In these models the stilbene stimulated mitophagy by activation of AMPK, Beclin 1 and LC3II conversion. Resveratrol potentiated motoneuron recovery and decreased apoptosis, after spinal cord injury in mice, through promotion of Beclin 1, LC3II and autophagy (Hu et al., 2017). Similar results were reported by Wang P. et al. (2018) in rat spinal cord injury, where resveratrol activated AMPK and autophagic flux, and consequently inhibited mTOR pathway, and *in vitro* in PC12 cells. Moreover, resveratrol induced autophagy, in dopaminergic SH-SY5Y cells challenged with rotenone via haeme oxygenase signaling (Lin et al., 2014).

As for PD mouse model triggered by methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MTP), resveratrol ameliorated locomotor activity and the number of dopaminergic neurons in the substantia nigra pars compacta, promoting Sirtuin 1/ LC3II and reducing p62/SQSTM (Guo et al., 2016). Sirtuin 1 was crucial to maintain tolerance to neurotoxic aggregates in AD and PD by inhibiting mTOR and promoting autophagy (Lee, 2019). Finally, resveratrol restored ATG4 and stimulated LC3 II and autophagy in neuroblastoma SH-SY5Y cells expressing mutant-Huntingtin treated by dopamine (Vidoni et al., 2018).

Curcumin, the main active component of curry spices, and curcuminoid from *Curcuma longa* plants have been reported to induce autophagy in AD mice limiting mTOR/Akt signaling and sustaining LC3 (Wang C. et al., 2014; Shakeri et al., 2019; Voulgaropoulou et al., 2019). *Curcumin* protected *in vitro*

an AD neuronal cell model (N2a/APP695swe), enhancing a retrograde axonal flux and restoring autophagy via Beclin 1 (Liang et al., 2019). Similarly, solid lipid nanoparticles driving *curcumin* stimulated CMA in human neuroblastoma cells and in mice neuronal cells challenged with toxic beta-amyloid inducing molecular chaperones and lysosomal activity (Maiti et al., 2017). Unfortunately, despite benefits in experimental animal models (Perrone et al., 2019), the clinical efficacy of curcuminoids in AD is still controversial (Zholos et al., 2019). However, due to the strong ability of curcumin to bind β-amyloid fibrils, it has been successfully adopted as fluorescent biomarker in the retinas at early stages of AD in patients and mice (Koronyo-Hamaoui et al., 2011). Intriguingly, Zhang et al. (2018) demonstrated that *curcumin* protected hippocampal neurons in amyloid treated mouse by limiting abnormal Beclin 1 and autophagosomes formation. *Curcumin* was also effective in PD *in vitro* model of dopaminergic neurons where modulated autophagy and cleaned α-synuclein aggregates (Jaroonwitchawan et al., 2017; Li et al., 2017). Moreover, a curcumin analog compound stimulated autophagy via TFEB-lysosome signaling, independently of mTOR, in murine neuroblastoma cells and *in vivo* (Song et al., 2016).

Green tea cathechins, mainly epigallocatechin gallate (EGCG) from *Camelia sinensis*, are diffuse phenols with recognized multiple antioxidant, anti-inflammatory and neuroprotective properties, activating beclin 1, autophagy and mitophagy as reviewed by Prasanth et al. (2019).

Phenolic oleosides, in particular oleuropein aglycone (OLE) from *Olea europea*, are safe antiaging, antioxidants and neuroprotective substances present in extra virgin olive oil (Casamenti et al., 2015). OLE supplementation for 3 months to the diet is able to sustain autophagy, at the last step of fusion of autophagosomes and lysosomes, so ameliorating cortical neuronal damage in transgenic AD and PD mice (Lauretti et al., 2017). Rigacci et al. (2015) demonstrated that OLE, *in vitro* to neuroblastoma cells and in AD TgRND8 mice, was able to intensify autophagy via calcium release from sarcoplasmic reticulum, activation of calmodulin-dependent kinase kinase β (CAMKKβ) and AMPK but mTOR inhibition. Intriguingly, OLE is a pleiotropic molecule able to regulate sirtuins and consequently Atg genes activation in AD mice (Cordero et al., 2018). Recently, Al Rihani et al. (2019) reported that extra virgin olive oil derivatives are able to cross the brain blood barrier, to reduce inflammation, amyloidosis and plaques in AD mice via AMPK signaling and autophagy restoration. Moreover, OLE, tested in PC12 cells exposed to a parkinsonian toxin, reduced mitochondrial damage and accelerated autophagic flux, so reducing neuronal death (Achour et al., 2016).

Phenolic pomegranate extracts, added *in vitro* to dopaminergic SH-SY5Y cells, upregulated autophagy and mitophagy after toxic challenge. Remarkably, the mechanism of mitophagy involved the recruitment of PINK1 and parkin to mitochondria during chemical stress (Tan et al., 2019).

Phenolic bacosides, active biocomponents from *Bacopa monnieri*, were effective to reduce ROS production and lipofuscin aggregation but preserved mitochondria in aged rats. Furthermore, they ameliorated cognitive ability and memory in humans in health and AD (Manap et al., 2019) even their influence on autophagy has not been studied yet.

Flavonoids like quercetin coupled to nanoparticles, silibinin and wogonin have been successfully used *in vitro* in SH-SY5Y neurons, and *in vivo* in rodent and human AD, where they induced autophagy by ULK1/mTOR, beclin 1 production and clearing amyloid substance (Wang D. et al., 2014; Ashrafizadeh et al., 2019b; Liu et al., 2019; Zhang et al., 2020). *Silymarin,* a lignan extracted from seeds of *Silibum marianum*, has been employed as anti-inflammatory and anti-oxidative agent in stroke by transient forebrain ischemia in rat. The neuroprotection by silymarin was due to reduced autophagic death in the CA1 region of the hippocampus (Hirayama et al., 2016). *Baicalein*, another flavonoid from *Scutellaria baicalensis*, protected rotenone treated neuroblastoma cells and mice, a well-known PD model, promoting autophagy and preventing mitochondrial damage (Kuang et al., 2017).

Alkaloids

Alkaloids are neuroprotective agents extracted by different plants and herbs, such as Solanacee, Papaveracee, Ranuncolacee, Amaryllidacee (Hussain et al., 2018).

Berberine, an alkaloid derived from *Berberis* species herbs, ameliorated autophagic flux and removed tau aggregates in AD mice, recovering memory and spatial learning (Chen et al., 2020). Intriguingly, it was also effective in removal of abnormal ubiquitinated TDP-43 deposits in frontotemporal degeneration (FTD) and amyotrophic lateral sclerosis (ALS) by autophagy (Ling et al., 2013; Chang et al., 2016). Moreover, berberine attenuated neuronal damage in a spinal cord injury model in mice, by triggering autophagy in oligodendrocytes (Wang et al., 2017). Berberine has also an important anti-cancer effect in glioblastoma by activating autophagy via AMPK signaling decreased glycolytic activity and invasive potential of cells (Chang et al., 2016; Wang et al., 2016). Oral berberine enhanced life span and stimulated autophagic markers in the brain and cerebellum of transgenic N171-82Q mice, a HD model (Jiang et al., 2015).

Caffeine, one of the most common alkaloids in the world, modulated autophagy in SH-SY5Y neuroblastoma cells exposed to prion derived protein, so protecting them against apoptosis (Moon et al., 2014). Recently, Luan et al. (2018) demonstrated that caffeine, supplemented in drinking water for 120 days, in PD mice triggered autophagy and CMA enhancing LC3 and LAMP2 and reversing toxic α-synuclein deposits.

Conophylline, an alkaloid derived from *Ervatamia microphylla*, similarly stimulated autophagy *in vitro* neurons in HD and PD mimetic models (Sasazawa et al., 2015; Umezawa et al., 2018).

Dendrobium nobile, an alkaloid derivative from Orchidacee very common in China, has been recently demonstrated to protect hippocampal neurons exposed to β-amyloid by promoting Beclin 1 and accelerating autophagic flux (Li et al., 2017).

Terpenes and Terpenoids

Terpenes extracted by essential aromatic oils have a recognized anti-inflammatory and antioxidant role (Quintans et al., 2019).

Monotherpenes have been recently considered also modulators of autophagy (Ashrafizadeh et al., 2019a).

Bergamot essential oil from *Citrus bergamot* and its derived terpene, D-limonene, have been successfully added *in vitro* to human neuroblastoma cells SH-SY5Y where they induced autophagy, increasing LC3II, and accelerated autophagic flux but independently from Beclin1 (Russo et al., 2014).

Cubeben, a sesquiterpene from *Piper cubeba*, reduced beta amyloid toxicity *in vitro* in primary neuronal cells recovering autophagy via PI3K/AMPK signaling and inhibiting mTOR (Li et al., 2019a).

Ginaton, an extract from *Ginko biloba* leaves, is a mixture of terpenoids, flavonoids and organic acids with pleiotropic roles as antioxidative and neuroprotective product, mainly after 4 h after brain ischemia in stroke (Tian et al., 2017). Li et al. (2019b) induced ischemic stroke in rats, by middle cerebral artery occlusion, and treated animals with ginaton 24 h after reperfusion once a day for 14 days. Neurological symptoms ameliorated and the infarct site decreased, together with enhanced autophagic markers via AMPK and inhibition of apoptosis.

Geraniol, an acyclic monotherpene present in several aromatic plants, was effective to protect neurons from rotenone stress, an *in vitro* PD model, by recovering mitochondria and decreasing α-synuclein and improving autophagic flux (Rekha and Sivakamasundari, 2018).

Cucurbitacin E, a terpenoid phytosterol from *Ecballium elaterium* (Cucurbitacee), partially protected PC12 neurons, treated with toxins to simulate PD, but remarkably, reduced Beclin 1 autophagy, and ameliorated autophagosomes necessary for dismantling toxic deposits (Arel-Dubeau et al., 2014).

Carotenoids, known also as tetraterpenoids, are common bioactive pigments present in fruits and vegetables in the human diet, such as peach, watermelon, tomato, spinach, carrots, broccoli, and seaweeds with antioxidant and anti-inflammatory activities, useful against neurodegeneration (Cho et al., 2018). Fucoxanthin, a carotenoid from brown seaweeds, has been an

TABLE 1 | Nutraceuticals effective against neurodegeneration via autophagy *in vitro*.

Nutraceuticals	Disease model	Dose/Signaling	References
Polyphenols			
Resveratrol	AD-Aβ25-35 treated PC12	20 μM- Increased PARP1-SIRT1	Lin et al., 2014; Deng and Mi, 2016; Guo et al., 2016; Vidoni et al., 2018; Pineda-Ramirez et al., 2020
	PD-Rotenone treated SH-SY5Y	20 μM-Increased Heme oxygenase	
	HD-Dopamine treated SH-SY5Y	100 μM-ATG4-LC3 activity	
	Glutamate-treated neurons	30 μM-AMPK-LC3	
Pomegranate extract	PD-SH-SY5Y	300 μg/ml for 6 and 24h- TFEB activation for mitochondrial quality control.	Tan et al., 2019
Curcumin	AD-N2a/APP695swe AD-hippocampal neurons	1–10 μM-TFEB binding	Wang C. et al., 2014; Song et al., 2016; Li et al., 2017; Zhang et al., 2018; Liang et al., 2019; Zholos et al., 2019
	PD-primary neurons	50 μM-AMPK activation;10–12 M-Restored autophagic flux	
Oleuropein aglycone	AD-SH-SY5Y	50 μM for 4 h-Free Ca^{2+} flux-CAMKKβ–AMPK activation	Rigacci et al., 2015
	PD-PC12 cells	10^{-12} M for 3 h- Activated autophagic flux	Achour et al., 2016
Flavonoids			
Quercetin	AD-SH-SY5Y	5 mg/ml gold-palladium nanoparticles for 24 h-Enhanced autophagosomes	Ashrafizadeh et al., 2019b
Baicalein	PD-Rotenone treated SH-SY5Y	10 μM for 24 h-activated LC3	Kuang et al., 2017
Alkaloids			
Caffeine	Prion treated- SH-SY5Y cells	2–8 mM-LC3II induction	Moon et al., 2014
Conophylline	HD and PD-PC12 neurons	3.5 ng/ml- Enhanced autophagic flux	Umezawa et al., 2018
Dendrobine	AD-Aβ hippocampus neurons	10^{-8}M/L for 24h-LC3 II, enhanced autophagic flux	Li et al., 2017
Terpenes			
D-Limonene	Starved SH-SY5Y	0.005–0.3%-LC3II and autophagic flux activation	Russo et al., 2014
Cubeben	AD-Aβ neurons	5–20 μM for 48 h-Inhibition of PI3K/Akt	Li et al., 2019a
Geraniol	PD-Rotenone treated SK-N-SH cells	100 nM for 24 h-Increased Atg5-7-12	Rekha and Sivakamasundari, 2018
Cucurbitacin E	PD- PC12 neurons	10–10 M-regulated autophagy-lysosomal pathway	Arel-Dubeau et al., 2014
Others			
β-Asarone	AD-PC12 neurons	24–72 μM-LC3II -Beclin 1 induction	Wang et al., 2020
Melatonin	Senescent SH-SY5Y	1 μM L-1- Beclin 1-autophagic flux activation	Nopparat et al., 2017
Trehalose	NSC34 cells	100 mM for 24 h-TFEB activation/Akt inhibition	Rusmini et al., 2019

activator of main autophagic markers, and a neuroprotector in mice model of traumatic brain injury via Nrf2 pathway (Zhang et al., 2017).

Others

Spermidine, a safe polyamine present in several seeds and plant-based food, like soy and wheat germ (Muñoz-Esparza et al., 2019), prolongs lifespan in lower organisms like flies, yeast and worms, and ameliorate cognitive ability in mice and old humans (Schwarz et al., 2018). Moreover, in addition to induce autophagy by removing inhibitory acetyltransferase EP300 (Pietrocola et al., 2015), spermidine was an anti-inflammatory drug that alleviated experimental autoimmune encephalomyelitis in mice (Yang Q. et al., 2016).

Beta-asarone (cis-2,4,5-trimethoxy-1-allyl-phenyl), a volatile oil from *Acorus tatarinowi* herb, has been successfully tested in an AD model in PC12 neurons challenged with amyloid (Aβ42), the toxic protein in plaques. Both autophagy, the autophagic flux and mitophagy pathways were triggered and ameliorated by this compound in a dose dependent manner (Wang et al., 2020). However, if *in vitro* this product sustained Beclin 1 and LC3II signaling, the same markers were inhibited *in vivo* in double transgenic APP/PS1 mice and autophagosomes decreased in hippocampal neurons (Deng et al., 2016). Probably, *in vivo* autophagy was not the main mechanism targeted by beta-asarone able to ameliorate memory and learning in mice.

Melatonin, the pineal indole, also present in vegetal food, seeds and fruit, has been considered a powerful anti-inflammatory and antioxidant dietary supplement in neurodegeneration (Shukla et al., 2019). Melatonin intake reduced experimental subarachnoid hemorrhage (SAH) in rats by blocking neuronal apoptosis and abnormal autophagy just 2 h post SAH (Shi et al., 2018). Moreover, melatonin sustained Parkin/PINK1 pathway and mitophagy but inhibited inflammasome in the same animal model (Cao et al., 2017). Similarly, *in vitro* in senescent SH-SY5Y cells, melatonin was a potent autophagy inducer and

TABLE 2 | Nutraceuticals effective against neurodegeneration via autophagy *in vivo*.

Nutraceuticals	Disease model	Dose/Signaling	References
Polyphenols			
Resveratrol	AD-AβPP/PS1 mice	1% for 10 months-AMPK/Sirtuin	Porquet et al., 2014; Guo et al., 2016; Hu et al., 2017; Zhao et al., 2017; Wang P. et al., 2018; Vidoni et al., 2018; Pineda-Ramirez et al., 2020
	Brain ischemia rat	1.8 mg/kg- AMPK/Beclin1	
	Spinal cord injury rat and mice	200 mg/kg/day i.p. for 3 days-LKB1/AMPK	
	PD-MPTP mice	100 mg/kg/day for 33 days-Sirtuin 1-LC3	
Curcumin	AD-APP/PS1 mice	160–1000 ppm for 6 months-Downregulated PIK3Akt/mTOR	Wang C. et al., 2014; Song et al., 2016; Gao et al., 2017; Li et al., 2017
	Traumatic brain injury-rat	25–100 mg/kg-Enhanced Beclin 1, LC3	Achour et al., 2016; Lauretti et al., 2017; Cordero et al., 2018;
Oleuropein aglycone	AD-TgCRND8 mice PD mice	5 mg/kg diet for 8 weeks-AMPK activation, mTOR inhibition EVOO-rich diet for 6 months-Atg5-Atg7-AMPK	Rigacci et al., 2015; Al Rihani et al., 2019
Flavonoids			
Quercetin	AD-PD mice	5 mg/kg/day for 4 weeks	Ashrafizadeh et al., 2019b; Liu et al., 2019; Zhang et al., 2020
	Traumatic brain injury-rat	50 mg/kg i.p. for 12–24h	
Silymarin	Forebrain ischemia-rat	7 mg/kg-reduced autophagic flux	Hirayama et al., 2016
Baicalein	PD-Rotenone injected mice	100 mg/kg i.p. for 5 weeks-LC3 activation	Kuang et al., 2017
Alkaloids			
Berberine	AD and HD mice	40 mg/kg-Akt inhibition	Jiang et al., 2015; Wang et al., 2017.
	Spinal cord injury-rat	20 mg/kg-AMPK activation	Chen et al., 2020
Caffeine	PD mice	1 g/L for 3 months-LC3II-LAMP2A activation	Luan et al., 2018
Terpenes			
Ginaton	Brain ischemia mice	50 mg/kg for 14 days-AMPK activation	Tian et al., 2017; Li et al., 2019b
Carotenoids	Traumatic brain injury mice	50–200 mg/kg i.g.-Beclin 1-LC3 activation	Zhang et al., 2017
Others			
β-Asarone	AD-APP/PS1 mice	10 mg/kg-Beclin 1-Akt reduction	Deng et al., 2016
Melatonin	SAH rats	5–10 mg/kg i.v.-abnormal autophagy decrease	Cao et al., 2017; Shi et al., 2018
Trehalose	Batten disease mice	2% oral-TFEB activation	He et al., 2016; Holler et al., 2016; Tien et al., 2016; Palmieri et al., 2017
	AD, PD, FTD mice		

an antagonist of NF-kB signaling by sirtuin 1 deacetylase (Nopparat et al., 2017).

Trehalose, a disaccharide present in bacteria, yeast, fungi and plants but not in vertebrates, has caught the attention as an autophagic regulator in neurodegenerative diseases (Khalifeh et al., 2019). Palmieri et al. (2017) demonstrated that trehalose, administered to a mice model of Batten disease (a neurodegenerative lysosomal disease), stimulated the clearance of toxic aggregates via activation of TFEB, the fundamental regulator of lysosomal pathway. In AD, PD and frontotemporal dementia models, the disaccharide destroyed misfolded proteins and triggered an efficient autophagic flux and lysosomal activity (He et al., 2016; Holler et al., 2016; Tien et al., 2016). Conversely, other studies on transgenic AD mice and on alpha-synuclein challenged neuroblastoma cells and primary rat cortical neurons, reported a protective activity but independent from autophagy or a block of the final step of autophagy and autolysosomes formation (Portbury et al., 2017; Yoon et al., 2017; Lee et al., 2018). A recent study on immortalized motoneurons demonstrated that *trehalose* induced TFEB nuclear translocation and autophagy and that TFEB silencing counteracted its effect (Rusmini et al., 2019). Unfortunately, *trehalose* cannot be assumed orally in humans because degraded by trehalase, an enzyme present in the gastrointestinal tract. However, recently nanolipid-trehalose conjugated have been developed as effective autophagy inducers to overcome the poor pharmacokinetics of this sugar and its efficacy at higher doses (Colombo et al., 2019).

To recapitulate, the signaling of nutraceuticals driving autophagy *in vitro* or in rodent models of CNS diseases are shown in **Tables 1**, **2**, respectively.

NON-SPECIFIC EFFECTS OF NUTRACEUTICALS IN NEURODEGENERATION

The majority of nutraceuticals reported here has been selected for their effects on the macroautophagy machinery in experimental neurodegenerative models. However, it is important to emphasize that their activity is non specific, because the same compound has multiple roles and may act as an anti-aging, anti-apoptotic, free radicals-scavenger or anti-inflammatory drug (Howes et al., 2020). For example, *ferulic acid,* a phenolic compound commonly present in fruits and vegetables, orally administered at 80–100 mg/kg in rats 30 min before middle cerebral artery occlusion, limits ischemia reducing apoptosis and activating autophagy (Cheng et al., 2019). Conversely, *crocin*, a flavonoid from *Crocus* and *Gardenia* species, ameliorated memory and behavior in AD rat model, reducing apoptosis and citochrome c release but was ineffective on autophagic markers beclin 1/LC3 (Asadi et al., 2015). Moreover, not only a single natural principle but often mixed formulation of components may be adopted in experimental and clinical trials. For example, *sailuotong*, a mixture of saffron from *Crocus sativus*, *Ginkgo biloba,* and *Panax ginseng,* has been tested successfully in old adults, with mild cognitive impairment, to ameliorate cognitive abilities (Steiner et al., 2018). The synergistic effect of *berberine* and *curcumin* was more potent than the single compound to improve cognitive function after 3 months of treatment in AD mice (Lin et al., 2020). Extra virgin olive oil, a mixture of polyphenols, α and γ-tocopherols, added for 6 months to the diet in a taupathy mice model, alleviated synaptic activity in the hippocampus and memory impairment (Lauretti et al., 2020). Currently, more intense pharmacological and pharmacokinetic studies are required to ameliorate safety, purity, bioavailability of nutraceuticals, together with the urgent requirement of a unique worldwide regulation (Helal et al., 2019).

CONCLUSION

This review focused on the regulatory role of natural dietary compounds on autophagy to postpone or alleviate neurodegeneration *in vitro* and in animal models. Unfortunately, major knowledge on the defective autophagy in humans is urgent to successfully treat patients. The importance of preventive or therapeutic autophagy regulation in neurodegenerative diseases is still debated (Bar-Yosef et al., 2019; Maiuri and Kroemer, 2019; Park et al., 2020; Suomi and Mc Williams, 2020). Negrete-Hurtado et al. (2020) reported in ATG5 KO mice brain, a non canonical role of ATG proteins and that lipidation machinery is not required for neuronal survival. Indeed, the main function of ATG-induced LC3 lipidation is to regulate microtubular dynamic in en passant boutons. According to this study, to enhance autophagy may be deleterious in forebrain axons, affecting retrograde flux and function.

Nevertheless, there is wide consensus on the efficacy of natural pleiotropic compounds able to enhance or restored insufficient autophagy in aggregated-prone proteins pathologies like AD, PD, HD in preclinical rodent models (Djajadikerta et al., 2019; Rahman et al., 2020). Remarkably, it is clear that the neuronal damage, the intensity and stage of disease greatly condition the beneficial or detrimental use of nutraceuticals and autophagy tuning in brain injury and aging (Galluzzi et al., 2017; Yessenkyzy et al., 2020). Therefore, it must be remembered that autophagy can be a double-sided process. Ferrucci et al. (2018) critically indicated that there was not a unique beneficial role of autophagy in the hypoxic brain and that may be better to inhibit autophagy to alleviate chronic ischemia. For this reason, nutraceuticals like *Leonurine*, an active extract from *Leonorus cardiaca,* inhibited ATG pathways and autophagy, so reducing neuronal damage (Liu et al., 2016).

Moreover, neurodegenerative diseases are multifactorial and many pathways must be considered (Kuang et al., 2019). Therefore, not only autophagy but also necrosis and apoptosis contributed to neuronal cell death (Leong et al., 2020). Remarkably, natural compounds able to limit neurodegeneration *in vitro* might not be effective *in vivo* and multiple products are suggested both natural and synthetic (Mazzanti and Di Giacomo, 2016). Moreover, different types of autophagy, reciprocally regulated, might concur

to alleviate abnormal proteins deposition and to clear organelles in neurons (Wang H. et al., 2018; Oshima et al., 2019; Cai and Jeong, 2020). Recently, Mizushima (2018) claimed that to measure autophagic flux in humans is still impossible and consequently the direct analysis of the efficacy of natural drugs on autophagy is lacking, due to the absence of adequate quantitative methods. However, we are confident that new discoveries on autophagy tuning in the brain may confirm the utility of safe bioactive compounds and their importance to prevent or limit unavoidable neurodegeneration. Major advances in our understanding of their mechanisms of action and pharmacokinetic and nonspecific effects are necessary for success in the struggle against neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

AS wrote the manuscript and, **Tables 1**, **2**. GC provided revised **Figure 1** and reviewed the text. Both authors conceptualized the topic, discussed the literature data, approved the final revised version of the manuscript, and ensured the accuracy of the work and intellectual content. A professional service revised the whole text in English.

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Circulating Mitochondrial-Derived Vesicles, Inflammatory Biomarkers and Amino Acids in Older Adults With Physical Frailty and Sarcopenia: A Preliminary BIOSPHERE Multi-Marker Study Using Sequential and Orthogonalized Covariance Selection – Linear Discriminant Analysis

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Physical frailty and sarcopenia (PF&S) is a prototypical geriatric condition characterized by reduced physical function and low muscle mass. The multifaceted pathophysiology of this condition recapitulates all hallmarks of aging making the identification of specific biomarkers challenging. In the present study, we explored the relationship among three processes that are thought to be involved in PF&S (i.e., systemic inflammation, amino acid dysmetabolism, and mitochondrial dysfunction). We took advantage of the well-characterized cohort of older adults recruited in the "BIOmarkers associated with Sarcopenia and Physical frailty in ElderRly pErsons" (BIOSPHERE) study to preliminarily combine in a multi-platform analytical approach inflammatory biomolecules, amino acids and derivatives, and mitochondrial-derived vesicle (MDV) cargo molecules to evaluate their performance as possible biomarkers for PF&S. Eleven older adults aged 70 years and older with PF&S and 10 non-sarcopenic non-frail controls were included in the analysis based on the availability of the three categories of biomolecules. A sequential and orthogonalized covariance selection—linear discriminant analysis (SO-CovSel–LDA) approach was used for biomarkers selection. Of the 75 analytes assayed, 16 had concentrations below the detection limit. Within the remaining 59 biomolecules, So-CovSel–LDA selected a set comprising two amino acids (phosphoethanolamine and tryptophan), two cytokines (interleukin 1 receptor

antagonist and macrophage inflammatory protein 1β), and MDV-derived nicotinamide adenine dinucleotide reduced form:ubiquinone oxidoreductase subunit S3 as the best predictors for discriminating older people with and without PF&S. The evaluation of these biomarkers in larger cohorts and their changes over time or in response to interventions may unveil specific pathogenetic pathways of PF&S and identify new biological targets for drug development.

Keywords: aging, biomarkers, cytokines, extracellular vesicles, geroscience, metabolomics, mitochondrial dysfunction, mitochondrial quality control

INTRODUCTION

Sarcopenia is the progressive decline in muscle mass and strength that accompanies aging (Marzetti et al., 2017). This condition exposes older adults to a high risk of negative health-related events, including disability, loss of independence, institutionalization, and death (Marzetti et al., 2017). The public health relevance of sarcopenia is widely recognized and so is the need for effective preventive and therapeutic interventions (Beaudart et al., 2014). Yet, the heterogeneous phenotypic presentation of sarcopenia, the insufficient understanding of its pathophysiology, and the frequent superimposition of other age-related conditions have hampered the study of sarcopenia as a standalone phenomenon (Calvani et al., 2018a). This impasse is also reflected by the lack of a unique operational definition of sarcopenia (Landi et al., 2018) and clinically meaningful biomarkers (Calvani et al., 2017). In this scenario, the recently defined "physical frailty and sarcopenia" (PF&S) syndrome has marked a major step forward for the clinical and regulatory recognition of the condition (Cesari et al., 2017).

When digging into the pathways and processes involved in PF&S pathophysiology, several factors spanning from muscle-specific mitochondrial dysfunction to systemic changes (e.g., inflammation, amino acid dysmetabolism) have been pinpointed (Marzetti et al., 2016, 2019; Picca et al., 2017a; Calvani et al., 2018b). Whether these processes share common roots and how cell-based alterations spread and are sensed at the systemic level are presently unknown. Small extracellular vesicle (sEVs) of mitochondrial origin, termed mitochondrial-derived vesicles (MDVs), have recently been proposed as possible shuttles across biological systems (Picca et al., 2020a). However, little is known about their complex regulatory network.

The pathophysiology of PF&S recapitulates all hallmarks of aging (López-Otín et al., 2013; Sierra, 2016; Justice et al., 2018). Hence, PF&S is considered to be a prototypical geroscience conditions for which a strong interdependence and non-linear relationships between biomarkers may be envisioned (Cohen et al., 2018). In such a scenario, the analysis of single pathways enlightening discrete aspects of the condition and, thus, setting aside its multifaceted nature, might neglect relevant information (Cohen et al., 2018; Justice et al., 2018). This limitation could be overcome through the adoption of multivariate analytical strategies that enable the exploitation of more comprehensive, multi-platform datasets (Calvani et al., 2015). In the present preliminary study, we took advantage of the well-characterized cohort of older adults recruited in the "BIOmarkers associated with Sarcopenia and Physical frailty in ElderRly pErsons" (BIOSPHERE) study (Calvani et al., 2018b,c; Marzetti et al., 2019; Picca et al., 2019a, 2020a) to simultaneously analyze biomediators pertaining to three different domains: inflammation, amino acid metabolism, and mitochondrial quality control (MQC). The availability of systemic inflammatory and metabolic data from this cohort (Calvani et al., 2018b; Marzetti et al., 2019) and their complementation with the analysis of circulating MDVs (Picca et al., 2020a) provided a composite dataset to explore the relationship among systemic inflammation, metabolic characteristics, and MDV trafficking in PF&S. Data analysis was performed through sequential and orthogonalized covariance selection coupled with linear discriminant analysis (SO-CovSel–LDA), an innovative analytical strategy that is particularly suited for dealing with multi-block datasets (i.e., experimental settings in which variables are assayed using different platforms and/or at different time points) (Biancolillo et al., 2020). SO-CovSel–LDA enabled selecting the variables of interest for PF&S from a large number of highly correlated candidate biomarkers. The evaluation of these biomarkers in larger cohorts and their changes over time or in response to interventions may unveil specific pathophysiological pathways of PF&S and identify biological targets for drug development.

MATERIALS AND METHODS

Participants

Participants of the present study were community-dwellers aged 70+ with PF&S and non-sarcopenic non-frail (nonPF&S) controls from the BIOSPHERE cohort (Calvani et al., 2018c). BIOSPHERE is a cross-sectional study conceived for selecting and validating a panel of candidate biomarkers for PF&S through multivariate statistical modeling (Calvani et al., 2018b,c; Picca et al., 2019a).

To diagnose PF&S, the operational definition developed in the "Sarcopenia and Physical fRailty IN older people: multi-componenT Treatment strategies" (SPRINTT) project (Marzetti et al., 2015, 2018) was applied: (a) physical frailty, based on a summary score on the Short Physical Performance Battery (SPPB) (Guralnik et al., 1994) between 3 and 9; (b) low appendicular muscle mass (aLM), according to the cut-points of the Foundation for the National Institutes of Health Sarcopenia Project (Studenski et al., 2014); and (c) absence of major mobility disability (i.e., inability to complete a 400-m walk test) (Newman

et al., 2006). Data analysis for the present investigation was conducted in a convenience sample of 21 participants (11 older adults with PF&S and 10 nonPF&S controls) for whom complete data were available for inflammatory and metabolic mediators and MDV characterization.

The Ethics Committee of the Università Cattolica del Sacro Cuore (Rome, Italy; protocol number BIOSPHERE: 8498/15) approved the study protocol. All procedures were conducted in compliance to the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Study procedures and criteria for participant selection were described thoroughly elsewhere (Calvani et al., 2018c). A written inform consent form was signed by all participants prior to enrolment.

Assessment of Appendicular Lean Mass by Dual X-Ray Absorptiometry

Appendicular lean mass was quantified through whole-body dual X-ray absorptiometry scans on a Hologic Discovery A densitometer (Hologic, Inc., Bedford, MA, USA) according to the manufacturer's directions. Criteria for low aLM were as follows: (a) aLM to body mass index (BMI) ratio (aLM_{BMI}) <0.789 and <0.512 in men and women, respectively; or (b) crude aLM <19.75 kg in men and <15.02 kg in women when the aLM_{BMI} criterion was not met (Studenski et al., 2014).

Collection and Processing of Blood Samples

Blood samples were obtained in the morning after overnight fasting by venipuncture of the median cubital vein, using BD Vacutainer® commercial tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). One tube was delivered to the centralized diagnostic laboratory of the Fondazione Policlinico Universitario "Agostino Gemelli" IRCCS (Rome, Italy) for standard blood biochemistry. The remaining tubes were processed for serum separation in the Biogerontology lab of the Università Cattolica del Sacro Cuore. After 30 min of blood clotting at room temperature, samples were centrifuged at 1,000 × *g* for 15 min at 4°C. The upper clear fraction (serum) was recovered in 0.5-mL aliquots and stored at −80°C until analysis.

Purification of Small Extracellular Vesicles From Serum

Small EVs were purified from serum through differential centrifugation and were quantified as previously reported (Picca et al., 2018a, 2019b, 2020a). In brief, serum samples were diluted with equal volumes of phosphate-buffered saline (PBS) and centrifuged at 2,000 × *g* at 4°C for 30 min. Supernatants were collected and centrifuged at 12,000× *g* at 4°C for 45 min to remove apoptotic bodies, mitochondrial fragments, cell debris, and vesicles larger than 200 nm. Supernatants were subsequently ultracentrifuged at 110,000× *g* at 4°C for 2 h. Pellets were recovered and resuspended in PBS, filtered through a 0.22-μm filter and ultracentrifuged at 110,000 × *g* at 4°C for 70 min to eliminate contaminant proteins. Pellets enriched in purified sEVs were finally resuspended in 100 μL of PBS. To quantify sEVs, total protein concentration was measured using the Bradford assay (Théry et al., 2006).

TABLE 1 | Composition of the biomarker panel.

Biological pathways	Biomolecules
Inflammation	BDNF, CRP, IL1-β, IL1-ra, IL2, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12, IL13, IL15, IL17, FGF basic, FGF21, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, CCL5, CCL11, IP-10, MPO, PDGF-BB, TNF-α, VEGF
Amino acid metabolism	1-methylhistidine, 3-methylhistidine, 4-hydroxyproline, α-aminobutyric acid, β-alanine, β-aminobutyric acid, γ-aminobutyric acid, alanine, aminoadipic acid, anserine, arginine, asparagine, aspartic acid, carnosine, citrulline, cystathionine, cystine, ethanolamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, phosphoethanolamine, phosphoserine, proline, sarcosine, serine, taurine, threonine, tryptophan, tyrosine, valine
MDVs	ATP5A, CD63, MTCOI, NDUFB8, NDUFS3, SDHB, UQCRC2

ATP5A, adenosine triphosphate 5A; BDNF, brain-derived neurotrophic factor; CCL, C-C motif chemokine ligand; CD, cluster of differentiation; CRP, C-reactive protein; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IL1-ra, IL1 receptor agonist; IP, IFN-α-induced protein; MCP-1, monocyte chemoattractant protein 1; MDVs, mitochondrial-derived vesicles; MIP-1α, macrophage inflammatory protein 1α; MIP-1β, macrophage inflammatory protein 1β; MPO, myeloperoxidase; MTCOI, mitochondrial cytochrome C oxidase subunit I; NDUFB8, nicotinamide adenine dinucleotide reduced form (NADH):ubiquinone oxidoreductase subunit B8; NDUFS3, NADH:ubiquinone oxidoreductase subunit S3; PDGF-BB, platelet-derived growth factor BB; SDHB, succinate dehydrogenase complex iron sulfur subunit B; TNF-α, tumor necrosis factor alpha; UQCRC2, ubiquinol-cytochrome C reductase core protein 2; VEGF, vascular endothelial growth factor.

Measurement of Inflammatory, Metabolic, and Mitochondrial Markers

Serum samples were assayed for a panel of 75 candidate biomarkers (**Table 1**). The panel was designed based on previous studies in older adults and their involvement in pathways and processes relevant to PF&S pathophysiology (i.e., inflammation, amino acid metabolism, and mitochondrial dysfunction) (Calvani et al., 2018b, 2020a; Marzetti et al., 2019; Picca et al., 2019a, 2020b).

Twenty-seven inflammatory mediators including cytokines, chemokines, and growth factors were assayed using the Bio-Plex Pro™ Human Cytokine 27-plex Assay kit (#M500KCAF0Y, Bio-Rad Laboratories Inc., Hercules, CA, USA) on a Bio-Plex® System with Luminex xMAP® Technology (Bio-Rad Laboratories), as described elsewhere (Ponziani et al., 2018, 2019; Marzetti et al., 2019; Picca et al., 2019a; Addolorato et al., 2020). Serum levels of C-reactive protein (CRP), myeloperoxidase (MPO), fibroblast growth factor (FGF) 21, and brain-derived neurotrophic factor (BDNF) were measured by using commercially available kits on an ELLA™ automated immunoassay system (Bio-Techne, San Jose, CA, USA). The concentration of 37 amino acids and derivatives was determined by ultraperformance liquid chromatography/mass spectrometry (UPLC/MS) as described previously (Calvani et al., 2018b).

Protein levels of tetraspanin CD63 and selected mitochondrial markers were measured by Western immunoblot analysis in purified sEVs, as detailed elsewhere (Picca et al., 2020a). According to the guidelines of the International Society of Extracellular Vesicles (Théry et al., 2018), sEV purity was ascertained by verifying the presence of the cytosolic protein flotilin (positive control) and the absence of the non-sEV component heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1, negative control). Scanning electron microscopy analyses were performed to confirm enrichment in sEVs (Théry et al., 2018).

Statistical Analysis

Descriptive Statistics

Descriptive statistics were run on all data. The normal distribution of data was ascertained via the Kolmogorov–Smirnov test. Differences in demographic, anthropometric, clinical, and biological parameters between participants with and without PF&S were assessed via *t*-test statistics or the Mann–Whitney *U*-test, as appropriate, for continuous variables. Chi-squared χ^2 or Fisher exact tests were applied for categorical variables. All tests were two-sided, with statistical significance set at $p < 0.05$. Analyses were performed using the GraphPrism 5.03 software (GraphPad Software, Inc., San Diego, CA, USA).

Sequential and Orthogonalized Covariance Selection – Linear Discriminant Analysis

In order to identify relationships among different sets of data and to identify putative markers of PF&S, a recently proposed multi-block classification approach, called SO-CovSel–LDA (Biancolillo et al., 2020), was adopted. SO-CovSel–LDA is a highly efficient multi-block classification strategy, which combines a very parsimonious variable selection algorithm to be applied on each individual block (CovSel) (Roger et al., 2011) with the sequential inclusion of data matrices, after orthogonalization with respect to the previously selected variables. This procedure reduces redundancies among the blocks and allows a clearer interpretation of results.

Here, we begin with describing the CovSel variable selection algorithm for a single block of predictors **X** to make its generalization to the case of multiple blocks easier to follow. CovSel allows selecting the minimum number of variables that provide an accurate regression model between the **X** matrix and a response **Y**. Variables are progressively selected from the **X**-block as those having maximum covariance with **Y**. Parsimony is achieved as any successive predictor is selected after both **X** and **Y** are orthogonalized with respect to the previously chosen variables, so to bring as much new information as possible. SO-CovSel is a generalization of this procedure to a multi-block case, enriched by the concept of sequential inclusion of the blocks after orthogonalization which is borrowed from methods such as SO partial least squares (SO-PLS) regression (Biancolillo and Næs, 2019; Biancolillo et al., 2019). In the case of three blocks of predictors, like in the present study (i.e., inflammatory markers, amino acids, and MDV cargo molecules), the SO-CovSel algorithm proceeds as follows. Variables are selected from the first block (amino acids and derivatives) using CovSel, then both the second block (inflammatory markers) $\mathbf{X}_2$ and the **Y** are orthogonalized with respect to the variables selected from the first block ($\mathbf{X}_{1,\text{sel}}$):

$$\mathbf{X}_{2,\,orth} = \left[\mathbf{I} - \mathbf{X}_{1,sel}\left(\mathbf{X}_{1,\,sel}^{T}\mathbf{X}_{1,sel}\right)^{-1}\mathbf{X}_{1,\,sel}^{T}\right]\mathbf{X}_2 \quad (1)$$

$$\mathbf{Y}_{orth} = \left[\mathbf{I} - \mathbf{X}_{1,sel}\left(\mathbf{X}_{1,\,sel}^{T}\mathbf{X}_{1,sel}\right)^{-1}\mathbf{X}_{1,\,sel}^{T}\right]\mathbf{Y} \quad (2)$$

where **I** is the identity matrix and the superscript *T* indicates matrix transposition. CovSel is then applied to the matrix $\mathbf{X}_{2,\,orth}$ to select variables having maximum covariance with $\mathbf{Y}_{orth}$; the selected variables are subsequently collected in the matrix $\mathbf{X}_{2,sel}$. The third block (MDV markers) and the **Y** are orthogonalized with respect to the variables selected from the first two blocks, similar to what described in equations (1) and (2). CovSel is applied to the orthogonalized third block $\mathbf{X}_{3,\,orth}$ to select variables ($\mathbf{X}_{3,sel}$) with maximum covariance with the orthogonalized response. Finally, an overall regression model is built between the selected variables from the three blocks and the **Y**:

$$\hat{\mathbf{Y}} = \mathbf{X}_{1,sel}\mathbf{B}_1 + \mathbf{X}_{2,sel}\mathbf{B}_2 + \mathbf{X}_{3,sel}\mathbf{B}_3\,\mathbf{Y} \quad (3)$$

where $\hat{\mathbf{Y}}$ is the predicted response and $\mathbf{B}_1$, $\mathbf{B}_2$, and $\mathbf{B}_3$ are the regression coefficients.

SO-CovSel–LDA is the classification analog of the SO-CovSel regression algorithm. As such, it requires the class information to be encoded in a binary-coded response vector **y**. The response vector will have the value 1 for PF&S participants and 0 for nonPF&S controls. A SO-CovSel model is built between the different blocks of predictors and the **y** as described above. Eventually, LDA is applied to the predicted response to accomplish sample classification.

To unbiasedly validate the results, a repeated double cross-validation (rDCV) procedure was used (Smit et al., 2007; Biancolillo et al., 2019). DCV consists of two loops of cross-validation nested into one another: the inner loop is used for model selection and the outer loop for external validation (i.e., contains samples that were not use at any stage of model building or optimization). Since DCV implies splitting the samples into the different cross-validation groups, to avoid the outcomes depending on a particular splitting scheme, the whole procedure is repeated a certain number of times (50 in the present study). In the context of biomarker discovery, the use of rDCV has the advantage that many different models are calculated (as many as the product of the number of DCV runs times the number of splits in the inner loop), which enables evaluating how consistently variables are selected by SO-CovSel–LDA and how robust candidate markers are. All calculations were carried out using in-house written functions running under Matlab environment (R2015b, The Mathworks, Natick, MA) and freely downloadable at: https://www.chem.uniroma1.it/romechemometrics/research/algorithms/so-covsel/.

TABLE 2 | Participant characteristics according to the presence of physical frailty & sarcopenia.

Characteristic	nonPF&S ($n = 10$)	PF&S ($n = 11$)	p-value
Age (years), mean ± SD	73.9 ± 2.7	77.7 ± 5.4	0.0557
Gender (female), *n* (%)	5 (50)	8 (73)	0.5344
BMI (kg/m^2), mean ± SD	28.1 ± 2.8	30.3 ± 4.3	0.1891
SPPB summary score, mean ± SD	12.0 ± 1.0	7.0 ± 0.3	<0.0001
aLM (kg), mean ± SD	20.21 ± 4.10	15.84 ± 3.63	0.0390
aLM_{BMI}, mean ± SD	0.81 ± 0.32	0.51 ± 0.11	0.0118
Albumin (g/L), mean ± SD	45.4 ± 12.7	39.8 ± 1.2	0.1536
Total serum protein concentration (g/L), mean ± SD	71.8 ± 4.6	75.5 ± 3.1	0.0914
Neutrophil count (10^9/L)	3.46 ± 1.40	4.07 ± 1.10	0.2757
Lymphocyte count (10^9/L)	1.76 ± 0.41	1.37 ± 0.30	0.0222
Neutrophil/lymphocyte	1.94 ± 0.57	3.13 ± 1.14	0.0076
Number of diseases*, mean ± SD	3.2 ± 1.6	3.1 ± 1.2	0.8647
Number of medications§, mean ± SD	2.9 ± 1.6	3.2 ± 1.8	0.7061

aLM, appendicular lean mass; aLM_{BMI}, aLM adjusted by body mass index (BMI); nonPF&S, non-physically frail, non-sarcopenic; PF&S, physical frailty & sarcopenia; SD, standard deviation.

**Includes hypertension, coronary artery disease, prior stroke, peripheral vascular disease, diabetes, chronic obstructive pulmonary disease, and osteoarthritis.*

§Includes prescription and over-the-counter medications.

RESULTS

Characteristics of the Study Participants

Data from 21 BIOSPHERE participants, 11 older adults with PF&S (mean age 77.7 ± 5.4 years; 73.0% women) and 10 nonPF&S controls (mean age 73.9 ± 2.7 years; 50.0% women) were analyzed in the present study. Demographic, functional, anthropometric, and clinical characteristics of the included participants were comparable to those of the whole BIOSPHERE cohort (Marzetti et al., 2019).

As shown in **Table 2**, no differences were observed in sex distribution, BMI, or number of comorbid conditions and medications between PF&S and controls participants. The latter group was slightly younger, but the age difference did not reach statistical significance. Serum albumin, total serum protein concentration, and the neutrophil count were comparable between groups. On the other hand, the lymphocyte count was lower and the neutrophil-to-lymphocyte ratio was higher in the PF&S group. As per the inclusion criteria adopted, participants with PF&S had lower SPPB scores and smaller aLM either crude or adjusted by BMI compared with nonPF&S controls.

Biomarker Selection Through SO-CovSel–LDA Analysis

Serum levels of 75 inflammatory cytokines, growth factors, neurogenesis and neural plasticity mediators, amino acids and derivatives, and MDV cargo molecules were measured through multiple analytical platforms. Of the assayed biomolecules, concentrations of anserine, carnosine, cystathionine, γ-aminobutyric acid, phosphoserine, interleukin (IL) 2, IL5, IL7, IL10, IL13, IL15, granulocyte colony-stimulating factor, vascular endothelial growth factor, mitochondrial cytochrome C oxidase subunit I, nicotinamide adenine dinucleotide reduced form (NADH):ubiquinone oxidoreductase subunit B8, and ubiquinol-cytochrome C reductase core protein 2 were below the detection limit. Serum concentrations of the assayed biomolecules are shown in **Supplementary Table 1**. SO-CovSel–LDA models were built using a multi-matrix dataset containing 59 analytes. Serum concentrations of candidate biomarkers were organized into three matrices according to the analytical approach adopted for their determination and the biological domain of pertinence (**Table 1**). Prior to data analysis, blocks were individually pretreated by autoscaling through subtracting from each variable its mean and dividing the result by its standard deviation. Afterwards, SO-CovSel–LDA models were built and validated via 50 runs of rDCV with 21 cancelation groups in the outer loop and five in the inner loop. The optimal order of blocks and the optimal number of variables from each block were selected as those returning the smallest error in the inner cross-validation loop. Since a total of 50 × 21 (i.e., 1,050) different models were built, the procedure allowed calculating the confidence intervals for the predictive ability and the consistency of selection of the optimal number of predictors as well as revealing their identity.

The predictive ability was evaluated on the samples of the outer rDCV loop, since they were completely external of the models used for their prediction, thereby providing more unbiased estimates of the discriminant capacity. Our analytical approach was able to correctly classify 87.5 ± 7.3% of PF&S participants and 83.6 ± 9.2% of nonPF&S controls, corresponding to an overall classification accuracy of 85.6 ± 5.0%. Permutation tests with 1,000 randomizations, which describe the distribution of figures of merit under the null hypothesis, showed that the discriminant ability of the model was statistically significant ($p < 0.001$).

Results appeared to be highly consistent across rDCV runs. Indeed, in all of the 1,050 models calculated, the best order of the blocks was found to be (1) amino acids and derivatives, (2) inflammatory biomolecules, and (3) MDV markers. In the large majority of iterations, the optimal model complexity was found to be 2, 2, and 1 variables, respectively. **Figure 1** illustrates the frequency of selection of discriminant markers across the 1,050 models calculated during the rDCV procedure. The figure shows that the optimal model complexity was consistently found to be 2-2-1, and that the same biomolecules—i.e., phosphoethanolamine, tryptophan, IL1 receptor antagonist (IL1-ra), macrophage inflammatory protein 1β (MIP-1β), and NDUF subunit S3 (NDUFS3)—were selected in almost all iterations, thus confirming the robustness of the proposed biomarkers in our sample.

DISCUSSION

According to the geroscience paradigm, the roots of most chronic diseases reside in perturbations of a discrete set

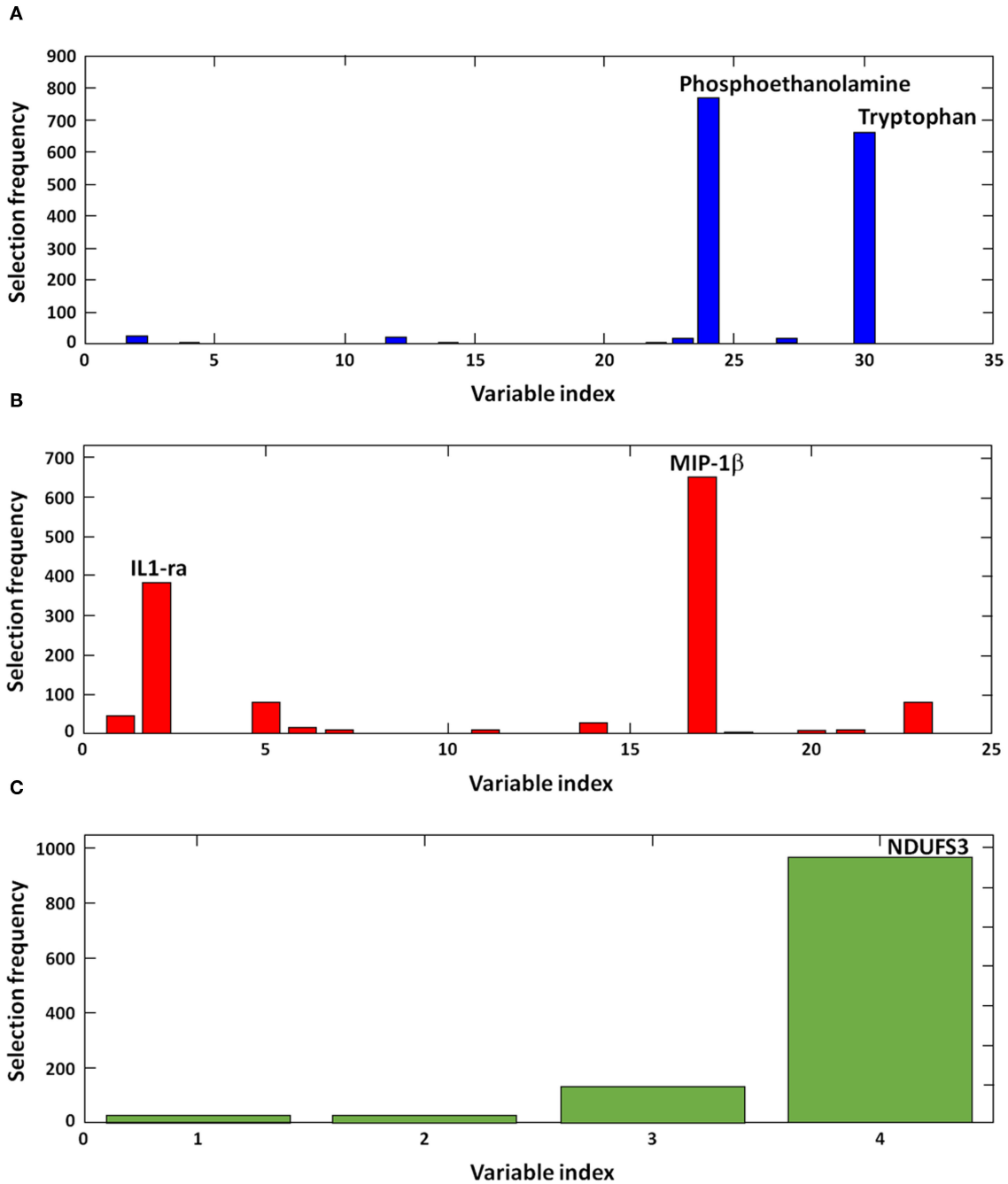

FIGURE 1 | Discriminant biomolecules selected by sequential and orthogonalized covariance selection coupled with linear discriminant analysis (SO-CovSel–LDA). Candidate biomarkers are presented according to the order in which they respective data matrices were entered into the model: **(A)** amino acids and derivatives, **(B)** inflammatory biomolecules, and **(C)** mitochondrial-derived vesicle cargo molecules. IL1-ra, interleukin 1 receptor agonist; MIP-1β, macrophage inflammatory protein 1β; NDFUS3, nicotinamide adenine dinucleotide reduced form (NADH), ubiquinone oxidoreductase subunit S3.

of biological mechanisms, collectively termed hallmarks of aging (López-Otín et al., 2013). The pathophysiology of sarcopenia involves all major biological pillars of aging and is, therefore, envisioned as a prototypical geroscience condition (Calvani et al., 2020a). In particular, derangements of skeletal myocyte quality control mechanisms are thought to play a relevant role in the development and progression of age-related muscle wasting (Iqbal et al., 2013). Indeed, altered recycling of damaged cell components and organelles via autophagy, defective mitochondrial proteostasis and dynamics, and impaired mitochondriogenesis have been described in muscles of older adults with PF&S (Picca et al.,

2018a). Systemic signatures of PF&S, including specific inflammatory and amino acid profiles, have also been identified (Calvani et al., 2018b, 2020a; Marzetti et al., 2019).

In the present study, we applied an innovative SO-CovSel–LDA analytical approach to explore the relationship among systemic inflammation, metabolic derangements, and circulating MDVs in PF&S in order to provide hints on the underlying pathogenic mechanisms. Among all assayed molecules, SO-CovSel–LDA selected a panel comprising two amino acids (i.e., phosphoethanolamine and tryptophan), two cytokines (i.e., IL1-ra and MIP-1β), and NDUFS3, a subunit of complex I of the mitochondrial electron transport chain (ETC), as the best predictors to discriminate older adults with and without PF&S.

The identification of phosphoethanolamine within the discriminant metabolites for the classification of PF&S is particularly relevant. Phosphoethanolamine is an ethanolamine derivate produced as an intermediate of the CDP-ethanolamine pathway involved in the metabolism of glycerophospholipid and biological membrane turnover (Patel and Witt, 2017; van der Veen et al., 2017). Disruption of CDP-ethanolamine pathway has been associated with mitochondrial dyshomeostasis in mouse models of muscle atrophy (Selathurai et al., 2019) and insulin resistance (Funai et al., 2016). Phosphoethanolamine has also been found to mediate mitochondrial membrane fusion and curvatures and, in combination with ethanolamine, to promote autophagy and longevity (Rockenfeller et al., 2015). The difference in phosphoethanolamine serum levels between older adults with and without PF&S might reflect impairment of autophagy in the setting of muscle atrophy (Iqbal et al., 2013; Marzetti et al., 2016). This hypothesis is in keeping with the higher secretion of sEVs previously described in older adults with PF&S (Picca et al., 2020a), which could be interpreted as an attempt to cope with deficient MQC processes (Soubannier et al., 2012; Picca et al., 2020b). Noticeably, derangements in the MQC machinery were documented in intraoperative muscle biopsies obtained from old hip-fractured patients with sarcopenia (Marzetti et al., 2016). Finally, phosphoethanolamine was also found among the mediators possibly involved in the disabling cascade in frail older persons with type 2 diabetes mellitus (T2DM) (Calvani et al., 2020b).

The amino acidic profile of participants with PF&S also included the aromatic essential amino acid tryptophan. The latter regulates several activities within the body, including growth, mood, behavior, and immune responses (Le Floc'h et al., 2011). Its metabolism is mediated by the tryptophan-kynurenine and the tryptophan-methoxyndole pathways, leading to the production of physiologically relevant bioactive compounds, such as NAD, serotonin, and melatonin (Le Floc'h et al., 2011). Changes in tryptophan circulating levels have been associated with low muscle quality (Moaddel et al., 2016), insulin resistance, and frailty in older adults with T2DM (Chen et al., 2016; Marcos-Pérez et al., 2017; Calvani et al., 2020b).

As per the inflammatory fingerprint of PF&S, IL1-ra and MIP-1β were selected by SO-CovSel–LDA as the most relevant mediators. A frailty "cytokinome" in older adults with PF&S composed of a "core" inflammatory profile with gender-specific signatures was previously described by our group (Marzetti et al., 2019). In a later study, SO-CovSel allowed restricting to only MPO and platelet-derived growth factor BB the mediators describing the contribution of inflammation to PF&S (Calvani et al., 2020a). In the same study, gender-specific models selected MIP-1β as one of the most relevant biomarkers for the discrimination between PF&S and nonPF&S participants (Calvani et al., 2020a). MIP-1β is a chemokine that regulates myoblast response to muscle injury and promotes leucocyte recruitment at the site of muscle damage (Yahiaoui et al., 2008). The presence of MIP-1β among discriminant analytes for PF&S may be interpreted as a compensatory action to impaired muscle regenerative capacity (Calvani et al., 2020a). Inflamm-aging, the chronic low-grade inflammation that develops during aging, has been involved in the pathogenesis of sarcopenia and physical disability (Wilson et al., 2017; Franceschi et al., 2018; Furman et al., 2019). In this context can be framed the selection of IL1-ra among the discriminant biomolecules associated with PF&S. IL1-ra is a natural negative modulator of IL1α- and IL1β-mediated inflammatory response and was found to be overexpressed in muscles of older adults (Przybyla et al., 2006). The inflammatory milieu of PF&S is also reflected by the higher neutrophil-to-lymphocyte ratio, an easily accessible indicator of inflammation.

Finally, the MDV cargo molecule NDUFS3 was selected among the discriminant mediators for PF&S. NDUFS3 is a nuclear-encoded subunit of complex I of the mitochondrial ETC. Mutations of NDUFS3 are associated with deficiency in mitochondrial respiration and myopathies (Bénit et al., 2004; Pereira et al., 2020). Conversely, NDUFS3 gene replacement rescued muscle structure and mitochondrial in a mouse model of mitochondrial myopathy (Pereira et al., 2020). Mitochondrial dysfunction and insufficient MDV-mediated quality control have also been hypothesized to contribute to PF&S (Picca et al., 2020a). This idea is in keeping with the altered expression of key MQC proteins described in muscles of old hip-fractured patients with sarcopenia (Marzetti et al., 2016).

Taken as a whole, our findings provide preliminary, yet novel insights into the relationship among metabolic changes, inflamm-aging, and mitochondrial dyshomeostasis in PF&S. In particular, the retrieval of MDVs in serum of older adults with PF&S allows placing this process in the context of an innate immune response as part of the "danger theory" of inflammation (Zhang et al., 2010). According to this view, MDVs may function as antigen-presenting vesicles carrying misplaced noxious material. Similar to damage-associated molecular patterns (DAMPs) released from injured cells, the MDV cargo can trigger caspase-1 activation and the secretion of pro-inflammatory cytokines (Krysko et al., 2011). This inflammatory response would be mounted through the interactions of mitochondrial DAMPs with receptors/systems including Toll-like receptors, Nod-like receptor family pyrin domain containing 3 inflammasome, and cytosolic cyclic GMP–AMP synthase-stimulator of interferon genes DNA sensing system (Picca et al., 2017b). Impaired MQC in skeletal myocytes may therefore install a vicious circle favoring further mitochondrial damage and the propagation of sterile inflammation through DAMPs release (Picca et al., 2018b). Should this hypothesis be confirmed, the scavenging of circulating mitochondrial DAMPs might be

exploited for the development of therapeutic interventions for PF&S.

Albeit presenting promising findings, our work has limitations that deserve discussion. First of all, the study was conducted in a small participant sample and results need to be validated in larger cohorts. Although participants were carefully selected and thoroughly characterized, the chance of unknown comorbidities affecting the results cannot be excluded. Also, the cross-sectional design of the study hampers establishing cause-effect or temporal relationships between the analyzed pathways and PF&S development. Finally, while the biomarker panel analyzed in the present study included a large number of biomolecules, the possibility exists that a more comprehensive appraisal of PF&S pathophysiology might be obtained through the analysis of larger sets of mediators.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Università Cattolica del Sacro Cuore, Rome, Italy. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

APi, CB, EM, FG, and RC: conceptualization. APi and FG: data curation. AB and FM: data analysis. APi, APr, FG, HC-J, and JG: methodology. APi, EM, and RC: writing—original draft preparation. CB, FG, and FL: writing—review and editing. FL and RB: supervision. CB and RB: funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.564417/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Amino Acid Starvation Sensitizes Resistant Breast Cancer to Doxorubicin-Induced Cell Death

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Many clinical trials are beginning to assess the effectiveness of compounds known to regulate autophagy in patients receiving anti-cancer chemotherapy. However, autophagy inhibition, through exogenous inhibitors, or activation, through starvation, has revealed conflicting roles in cancer management and chemotherapeutic outcome. This study aimed to assess the effect of amino acid starvation on doxorubicin-treated breast cancer cells by assessing the roles of autophagy and apoptosis. An *in vitro* breast cancer model consisting of the normal breast epithelial MCF12A and the metastatic breast cancer MDAMB231 cells was used. Apoptotic and autophagic parameters were assessed following doxorubicin treatments, alone or in combination with bafilomycin, ATG5 siRNA or amino acid starvation. Inhibition of autophagy, through ATG5 siRNA or bafilomycin treatment, increased caspase activity and intracellular doxorubicin concentrations in MCF12A and MDAMB231 cells during doxorubicin treatment. While amino acid starvation increased autophagic activity and decreased caspase activity and intracellular doxorubicin concentrations in MCF12A cells, no changes in autophagic parameters or caspase activity were observed in MDAMB231 cells. Our *in vivo* data showed that 24 h protein starvation during high dose doxorubicin treatment resulted in increased survival of tumor-bearing GFP-LC3 mice. Results from this study suggest that short term starvation during doxorubicin chemotherapy may be a realistic avenue for adjuvant therapy, especially with regards to the protection of non-cancerous cells. More research is however, needed to fully understand the regulation of autophagic flux during starvation.

Keywords: nutrient starvation, amino acids, doxorubicin, breast cancer, sensitization

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INTRODUCTION

Solid tumors make up the majority of all human cancers. Once solid neoplasms become established they can partially adapt to local micro-environmental shortages in nutrient supply by increasing autophagy (Mathew et al., 2007). Cellular nutritional status is closely associated with autophagy and several dietary factors are known to promote autophagy induction, one of the most effective being the restriction of dietary calorie intake. While dietary habits are also linked to cancer risk and progression (Popkin, 2007), caloric restriction exhibits a promising ability to extend the lifespan

of patients (Heilbronn and Ravussin, 2005) and facilitate tumor suppression (Kritchevsky, 2003). The application of short term starvation protocols in patients receiving high doses of chemotherapy has proven successful in reducing side effects in these patients (Safdie et al., 2009). In a cell culture and neuroblastoma mouse xenograft model, normal cells placed on a similar starvation protocol were shown to benefit from differential protection compared to cancer cells during high dose chemotherapy regimens (Raffaghello et al., 2008). Mice starved for 48 h had reduced chemotoxicity following high dose treatment, whereas mice fed *ad libitum* were 50% more likely to die. Tumor cell death was not compromised by the starvation protocol. The underlying mechanisms responsible for this differential protection of non-cancer cells are not yet fully understood.

Autophagy has been reported to confer resistance onto apoptosis-deficient cancer cells under metabolic stress by delaying the onset of necrotic cell death (Degenhardt et al., 2006; Sutton et al., 2019). Similarly, autophagy has also been reported to protect Caco-2 cells following exposure to toxins released by *Vibrio cholera* by engulfing and sequestering the toxins in lysosomal compartments (Gutierrez et al., 2007). More recently, high mobility group box 1 (HMGB1) release following chemotherapy-induced damage to leukemia cells caused a protective autophagy response (Liu et al., 2011a), strengthening the possibility that damage-associated molecular pattern molecule (DAMP) release during chemotherapy can increase autophagy to grant a defensive reaction (Liu et al., 2011b). In this way, damage caused by cytotoxic agents could directly result in an increased autophagic response.

Based on the premise that autophagy can promote tumor survival, it is believed that targeted and specific inhibition of autophagy could be a promising therapeutic avenue. Several *in vitro* studies have illustrated the potential of class-III phosphatidylinositol-3-kinase inhibitors such as 3-methyladenine, which prevent the formation of autophagosomes, in cancer therapy (Kanzawa et al., 2004). However, while starvation of a cervical cancer cell line resulted in apoptosis in the presence of this inhibitor (Boya et al., 2005), 3-methyladinine prevented tamoxifen-induced apoptosis in breast cancer cells (Bursch et al., 1996). Agents such as bafilomycin A1 (Baf), hydroxychloroquine and monensin (all of which prevent lysosomal fusion with autophagosomes) triggered apoptosis in HeLa cells during nutrient depletion (Boya et al., 2005), whilst Baf was also able to impede the protective effect of autophagy in several cancer lines undergoing radiation therapy (Paglin et al., 2001).

Even though Doxorubicin (Dox) is possibly the most effective anti-cancer agent available to date, it is also cytotoxic and can lead to cardiotoxicity as a result of its cumulative and dose-dependent effects (Swain et al., 2003). More effective strategies are needed to increase efficacy and protect non-cancer cells from off-target cytotoxicity. It is now also known that many anti-cancer agents and therapies increase autophagy levels in treated cancer cells at certain doses (Wu et al., 2006; Park et al., 2008). Transient, rapid and unpredictable alterations in autophagic flux could modify the way tumors respond to chemotherapy and supposedly interfere with or even augment therapy outcomes in unexpected ways. This study therefore aimed to establish the relative sensitivity of MDAMB231 and MCF12A cells to Doxorubicin, followed by the assessment of autophagy, apoptosis and the cell cycle. Furthermore, the efficiency of amino acid starvation in combination with Dox treatment was also assessed. Finally, a tumor-bearing mouse model was employed to assess whether protein starvation can sensitize tumors to Dox therapy.

MATERIALS AND METHODS

Cell Culture

Experiments were performed using two cell lines: the human metastatic mammary carcinoma cell line, MDAMB231, obtained from American Type Culture Collection (Rockville, MD, United States) and the human non-tumourigenic breast epithelial cell line, MCF12A, obtained from the University of Cape Town. The MCF7 cell line, used in selected experiments, was a donation from the University of the Western Cape.

During routine maintenance, cells were grown as monolayers in Glutamax-Dulbecco Modified Eagles Medium (DMEM; Celtic Molecular Diagnostics, Cape Town, South Africa) supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co., St Louis, MO, United States) at 37°C in a humidified atmosphere with 5% CO_2. MCF12A cell's growth medium was supplemented with Hams F12 medium (1:1), 0.5 μg/ml hydrocortisone, 10 μg/ml insulin and 20 ng/ml epithelial growth factor (EGF). All supplements were obtained from Sigma Chemical Co., St Louis, MO, United States.

Cells were first allowed to proliferate in T75 flasks (75 cm^2 flasks, Greiner Bio One, Germany) until confluency of approximately 80%, where after cells were subcultured into appropriate treatment plates or dishes. All experiments were performed using exponentially growing cells.

Drug Treatments

The following groups were used for the purpose of this study: (i) a control group, (ii) a Dox group, (iii) a siRNA control group, (iv) a siRNA control Dox group, (v) an ATG5 siRNA Dox group (ATG5 siRNA transfection 24 h prior to Dox treatment for 24 h), (vi) a Baf group and (vii) a Baf Dox group (Baf treatment 6 h prior to analysis, Dox treatment for 24 h).

Dox hydrochloride (D1515, Sigma Chemical Co., St Louis, MO, United States) stock solutions, as well as further dilutions to a concentration of 1 μM, was prepared in amino acid free medium. Bafilomycin (B1793, Sigma Chemical Co., St Louis, MO, United States) was dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions, while further dilutions to a concentration of 10 nM was performed in amino acid free medium. Based on previous experience (unpublished results), DMSO at this concentration (0.00625%) does not have any cytotoxic effects on MCF12A and MDAMB231 cells. For the siRNA group, please see the next section. For experiments conducted in the absence of amino acids, amino acid free culture medium (Highveld Biological Pty.) was used.

ATG5 siRNA Transfection

Cells were transfected using a reverse transcription protocol into 60 mm petri dishes. ATG5 siRNA duplex (20 pmol, Cell Signaling, Danvers, MA, United States, lSilence® Atg5 siRNA I #6345) was diluted into 250 μl transfection medium (containing no antibiotics or serum), where after 2 μl of Lipofectamine™ RNAiMAX (13778075; Invitrogen™, United States) was added. A volume of 250 μl from this suspension was added to each petri dish and allowed to incubate for 20 min. MCF12A cells (100 000) or MDAMB231 cells (80 000) were then plated into the culture dishes containing the RNAi duplex-Lipofectamine RNAiMAX complexes to have a final volume of 2 ml and gently mixed. Cells were incubated at 37°C until ready to treat, 48 h later. Stealth RNAi (STEALTH RNAI NEG CTL MED GC, 12935300; Invitrogen™, United States) was used as a negative control.

Caspase 3/7 Activity

Caspase-3/7 activity was measured using the Caspase-Glo® 3/7 assay (Promega, Madison, WI, United States). MCF12A cells (15 000) or MDAMB231 cells (10 000) were plated 48 h prior to treatment in white-walled 96-well plates in 100 μl culture medium. In experiments where transfection was necessary, cells were reverse transfected during plating. Following treatment, 100 μl (1:1) of Caspase-Glo® 3/7 working reagent was added to each well, and the plates mixed at 500 rpm for 30 s. The plates were then incubated at 22°C for 1 h in the dark and the luminescence measured in a luminometer (GloMax™ 96 Microplate Luminometer, Promega).

Trypan Blue Assay

Trypan blue cell viability was assessed using the Countess™ Automated Cell Counter (Invitrogen, United States). MCF12A cells (100 000) or MDAMB231 cells (80 000) were plated into 60 mm culture dishes 48 h prior to treatment. Following treatment, cells were trypsinised and pelleted, where after a 10 μl cell suspension was mixed with 10 μl 0.4% trypan blue and 10 μl loaded into a Countess™ chamber slide. Trypan blue positivity was calculated automatically and expressed as the total number of trypan blue positive cells, total number of trypan blue negative cells and absolute total cell number.

Western Blotting

Following treatment, plates containing cell monolayers were rinsed three times in 5 ml pre-lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1 mM sodium orthovanadate). Total cell protein was extracted by incubating cells on ice for 10 min in 1 ml radioimmunoprecipitation (RIPA, pH 7.4) buffer containing: 2.5 mM Tris–HCl, 1 mM EDTA, 50 mM NaF, 50 mM NaPPi, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 4 mg/ml SBTI, 10 mg/ml leupeptin, 1% NP40, 0.1% SDS and 0.5% Na-deoxycholate. Adherent cells were harvested from culture dishes by scraping, where after whole cell lysates were sonicated and centrifuged (8,000 rpm for 10 min; 4°C). Protein content was quantified using the Bradford protein determination method (Bradford, 1976) directly before preparation of cell lysates.

Fourty microgram protein extracts were prepared in Laemmli sample buffer and boiled for 5 min (95°C) prior to their separation on 12% polyacrylamide gels for LC3 II and caspase 3 and 10% polyacrylamide gels for beclin 1 by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run for 60 min at 130 V (constant) and 400 mA (Mini Protean System, Bio-Rad, United States). Following SDS-PAGE, proteins were transferred to polyvinylidine fluoride (PVDF) membranes (Immobilon, Millipore, United States) using a semi-dry electrotransfer system (Bio-Rad, United States) for 60 min at 15 V and 0.5 A. To prevent non-specific binding, membranes were blocked in 5% (w/v) fat-free milk in 0.1% Tris Buffered Saline-Tween20 (TBS-T) for 2 h at room temperature with gentle agitation. Membranes were incubated with LC3 primary antibody, caspase 3 primary antibody or beclin 1 primary antibody (all 1:1000; Cell Signaling, Danvers, MA, United States) overnight at 4°C. Goat anti-rabbit (Amersham Biosciences, United Kingdom, and Dako Cytomation, Denmark) horseradish peroxidase (HRP)-conjugated secondary antibody was added and incubated for 1 h at room temperature with gentle agitation. Antibodies were detected with the LumiGLO Reserve™ chemiluminescent substrate kit (KPL, Inc., United States) and exposed to autoradiography film (Hyperfilm, Amersham Biosciences, United Kingdom). Exposed bands were visualized and quantified by densitometry using the UNSCAN-IT© densitometry software (Silk Scientific Corporation, Utah, United States). All bands were expressed as optical density readings relative to a control present on the same blot.

Cell Cycle Analysis

Flow cytometric analysis of the cell cycle was performed using the CycleTEST™ PLUS DNA Reagent kit (Becton Dickinson, San Jose, CA, United States). MCF12A cells (200 000) or MDAMB231 cells (150 000) were plated in T25 culture flasks 48 h prior to treatment. Prior to analysis, cells were trypsinised and the cell suspensions centrifuged at 400 × *g* for 5 min at room temperature. Cells were washed in phosphate buffered saline (PBS) and 250 μl trypsin buffer was added to each tube and allowed to react for 10 min at room temperature. Thereafter, 200 μl of trypsin inhibitor and RNase buffer was added for 10 min, followed by 200 μl ice cold propidium iodide stain solution on ice in the dark for a further 10 min. Samples were filtered through a 50 μm nylon mesh. Sample fluorescence was acquired using flow cytometry within 30 min and results obtained with ModFit LT software (Verity software house, Inc., ME, United States.) on a BD FACSAria I. At least 30 000 list-mode data events were acquired for each sample. A 585/42 bandpass filter was used to analyze light emitted between 564 and 606 nm by stained cells. ModFit LT software was used to determine the percentage of cells in the g0/g1, s and g2/m phases. Mean percentages from three independent experiments were used to perform statistical comparisons.

Hoechst Nuclear Staining (Analysis of Pyknosis and Karyorrhexis)

To differentiate between normal nuclear morphology and apoptosis, characterized as nuclear condensation and fragmentation, staining with the DNA dye Hoechst 33342 (Sigma Chemical Co., St Louis, MO, United States) was employed. MDAMB2321, MCF12A and MCF7 cells were cultured and maintained as described previously, where after 60 000 MCF12A cells, 45 000 MDAMB231 cells and 45 000 MCF7 cells were plated into 35 mm culture dishes containing coverslips 48 h prior to treatment. Thereafter, the coverslips were removed, placed over glass slides, and washed with 100 μl ice cold PBS. The coverslips were treated with 500 μl ice cold acetone:methanol (1:1) and incubated at 4°C for 10 min. The fixative was removed and coverslips washed with PBS. A volume of 100 μl Hoechst in a 1:200 dilution (50 μg/ml) in sterile PBS was added directly onto the coverslips and incubated in the dark for 10 min. Coverslips were washed with PBS at room temperature before being mounted. Cells were viewed immediately using a Nikon E-400 fluorescence microscope (Nikon Microscopes, Kobe, Japan) and images acquired using a Nikon DMX1200 color digital camera (Nikon Microscopes, Kobe, Japan) with ACT-I software. Three independent experiments were conducted and four representative regions of each condition were acquired per experiment. At least 200 cells were analyzed per region. For each image, the number of condensed/fragmented nuclei was counted and expressed as a percentage of the total number of nuclei counted. In this manner, the percentage apoptosis was determined for each experimental condition.

Dox and Lysosomal-Associated Membrane 2A (LAMP-2A) Fluorescence Imaging

MCF12A cells (12 000) and MDAMB231 cells (10 000) were seeded into 8-well Nunc™ chambered plates (Nalge Nunc, Rochester, NY, United States). Cell monolayers were washed three times with sterile PBS before being fixed and permeabilised with an ice cold 1:1 methanol:acetone mixture for 10 min at 4°C. After being left to air dry for 20 min in the dark, cells were washed three times in sterile PBS and non-specific binding blocked by incubation with 5% donkey serum for 20 min. Cells were then incubated with anti-LAMP-2A primary antibody (Cell Signaling, MA, United States) for 90 min. Thereafter, a FITC-bound secondary antibody was added for 30 min and nuclei counter-stained using Hoechst 33342 (10 mg/ml in a 1:200 dilution) for 10 min at 4°C. No additional steps were required for visualization of Dox since the compound exhibits strong autofluorescence. Images were acquired with an Olympus Cell^R fluorescence 1X81 inverted microscope (Olympus Biosystems, Germany) using an F-view II camera for image acquisition and Cell^R software for image processing.

Lysotracker™ (Flow Cytometry)

MCF12A cells (200 000) or MDAMB231 or MCF7 cells (150 000) were plated into T25 culture flasks 48 h prior to treatment. Prior to analysis, cells were trypsinised and the cell suspensions centrifuged at 400 × *g* for 5 min at room temperature. Cells were then washed once in PBS. Lysotracker™ (Invitrogen™, United States) was prepared in PBS (1:10 000) immediately before use. All treated samples were split 1:1, where after cell suspensions were centrifuged at 400 × *g* for 5 min at room temperature. For each sample, one cell suspension was stained while the other was left unstained. For staining, the pellets were resuspended in 250 μl fresh Lysotracker™/PBS and incubated for 10 min at room temperature before analysis using flow cytometry (BD FACSAria I). At least 10,000 cells were collected using a 488 nm laser and 610LP, 616/23BP emission filters. Values obtained for the unstained samples were then subtracted from those obtained for the stained samples.

Tumor Establishment and Animal Protocols

The protocols in this study were carried out according to the guidelines for the care and use of laboratory animals implemented at Stellenbosch University (2009B02004). Eight week-old female C57BL6 mice (Stellenbosch University animal facility) or GFP-LC3 mice (kindly donated by Noboru Mizushima, Department of Cell Biology, National Institute for Basic Biology, Okazaki, Japan) were used in this study. The mice were maintained on standard chow diet and tap water before beginning the experiment. Mice were inoculated subcutaneously on the left pad of the fourth mammary gland with 200 μl of 2.5×10^5 E0771 cells suspended in Hanks Balanced Salt Solution (Sigma Chemical Co., St Louis, MO, United States), using a 23-gauge needle. This protocol was adapted from Ewens et al. (2006). Small tumors were evident by days 12–14 and grew to reach approximately 230 mm^2 in volume by day 33.

Drug Preparation and Administration

Doxorubicin hydrochloride (D1515, Sigma Chemical Co., St Louis, MO, United States) was dissolved in Hanks Balanced Salt Solution (Sigma Chemical Co., St Louis, MO, United States). Volumes were prepared to reflect the exact concentration required per kilogram (kg) of body weight for each mouse on the day of injection. Doxorubicin treatment was initiated on day 33 and was administered twice over a period 3 days (i.e., on day 33 and day 35) by means of i.p. injections. Mice were restrained by the scruff method and 100 μl drug suspensions were injected into the right caudal thigh (avoiding the femur and sciatic nerve) of each mouse using a 23-gauge needle. Control mice were injected with the vehicle only. Immediately following doxorubicin treatment, tumor-bearing mice were placed on either a control diet containing protein (Research Diets, Inc., New Brunswick, NJ, United States) or a diet free of protein (Research Diets, Inc., New Brunswick, NJ, United States) for a total of 24 h. These two diets were isocaloric. For the 24 h period in between interventions, mice received the control diet containing protein. Tumor size was monitored every 2 to 3 days by making measurements in two perpendicular dimensions parallel with the surface of the

mice using digital calipers. The body weight of the mice was monitored twice weekly. To assess intratumour caspase cleavage, FLIVO^{TM} *in vivo* apoptosis tracers (Immunochemistry Technologies LLC, MN, United States) were used. The SR FLIVO^{TM} red dye was prepared according to the manufacturer's protocol and 100 µl was injected into the tail vein of mice after appropriate treatments were completed. After 1 h, whole tumors were excised, digested and analyzed using flow cytometry on the BD FACSAria I.

Statistical Analysis

Data from at least three independent experiments were analyzed. Significant differences between time points and treatment groups were analyzed using either a one or two-way analysis of variance (ANOVA), together with a Bonferonni *post hoc* test. All statistical analyses were performed using Graphpad Prism version 5.01 (Graphpad Software, Inc, San Diego, CA, United States). All values are presented as means ± standard error of the mean (SEM), and the minimum level of significance was accepted as $p < 0.05$.

RESULTS

MDAMB231 Cells Are More Resistant to Dox Treatment Compared to MCF12A Cells

Dox treatment significantly increased apoptosis in MCF12A cells (**Figure 1A**). Approximately 15% of MCF12A cells presented with morphological changes to their nuclei (**Figures 1A,B**). MDAMB231 cells on the other hand displayed a relative resistance to apoptosis following treatment with Dox (**Figure 1C**). A very small, but statistically significant percentage of MDAMB231 cell's nuclei presented with morphological changes characteristic of apoptosis (**Figures 1C,D**).

Dox Treatment Increased LC3 II, but Not Beclin 1 Levels in MCF12A and MDAMB231 Cells

Both MCF12A and MDAMB231 cells responded to the presence of Dox by increasing LC3 II protein levels (**Figure 2**). Although

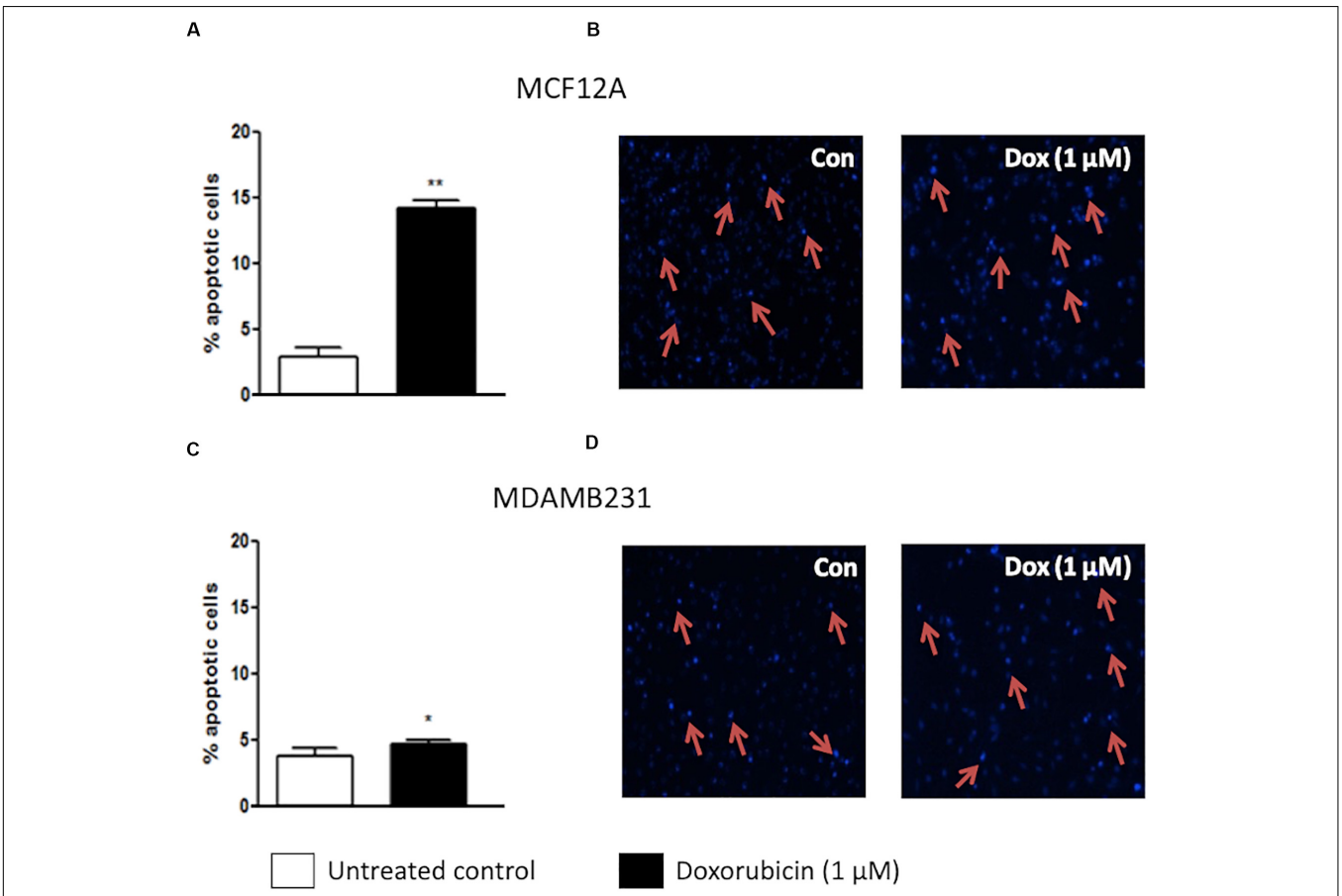

FIGURE 1 | Percentage apoptotic cells in MCF12A and MDAMB231 cultures. **(A)** Bar graph indicating the percentage of nuclei that represents morphological changes characteristic of apoptosis in MCF12A cells, **(B)** representative image of MCF12A cells stained with Hoechst, **(C)** bar graphs indicating the percentage of nuclei that represents morphological changes characteristic of apoptosis in MDAMB231 cells, and **(D)** representative images of MDAMB231 cells stained with Hoechst. Red arrows demonstrate apoptotic nuclear features. Each value represents the mean ± SEM of at least three independent determinations. Con, control; $^{*}p < 0.05$; $^{**}p < 0.01$.

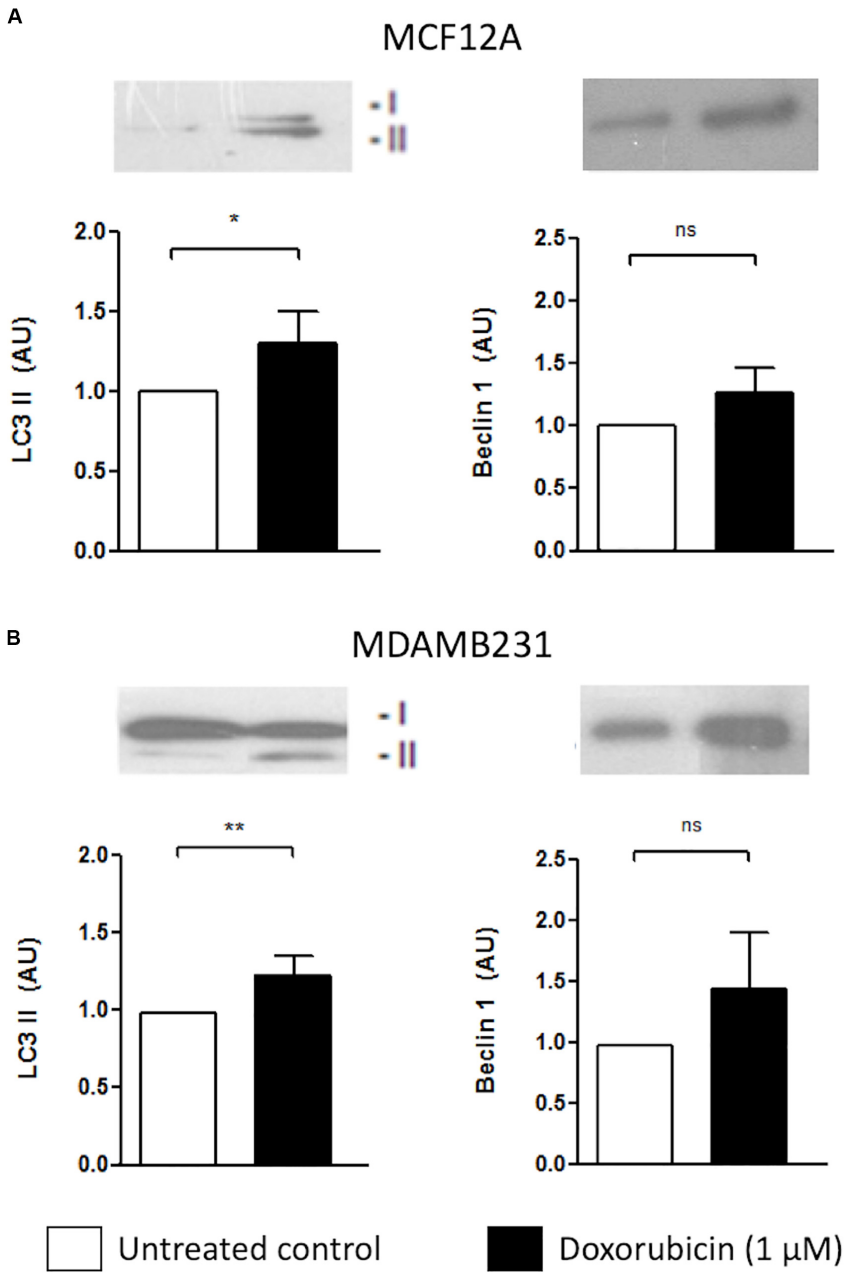

FIGURE 2 | LC3 II and Beclin 1 protein levels in MCF12A and MDAMB231 cells treated with Dox. Representative western blots and bar graphs showing densitometric representation of LC3-II and Beclin-1 protein levels in MCF12A **(A)** and MDAMB231 **(B)** cells. Each value represents the mean ± SEM of at least three independent determinations. AU, arbitrary units; Con, control; *$p < 0.05$; **$p < 0.01$; ns, no significance.

there was a slight increase in beclin-1 expression in both cell lines, these changes did not reach statistical significance.

Caspase Activity Increased Following Treatment With Dox and Dox Combinations

MCF12A cells displayed significantly increased caspase 3/7 activity following treatment with Dox for 24 h (**Figure 3A**). MDAMB231 cells also displayed a more pronounced (and significant) increase in caspase 3/7 activity following Dox treatment (**Figure 3B**). ATG5 siRNA transfection was used to inhibit autophagy. In MCF12A cells treated with Dox, ATG5 siRNA did not alter caspase 3/7 activity (**Figure 3B**), whereas in MDAMB231 cells, ATG5 siRNA significantly increased caspase 3/7 activity when administered in combination with Dox (**Figure 3E**). No changes in caspase activity was observed in either cell line when ATG5 siRNA was administered alone (**Supplementary Figure 1**). Treating either MCF12A or

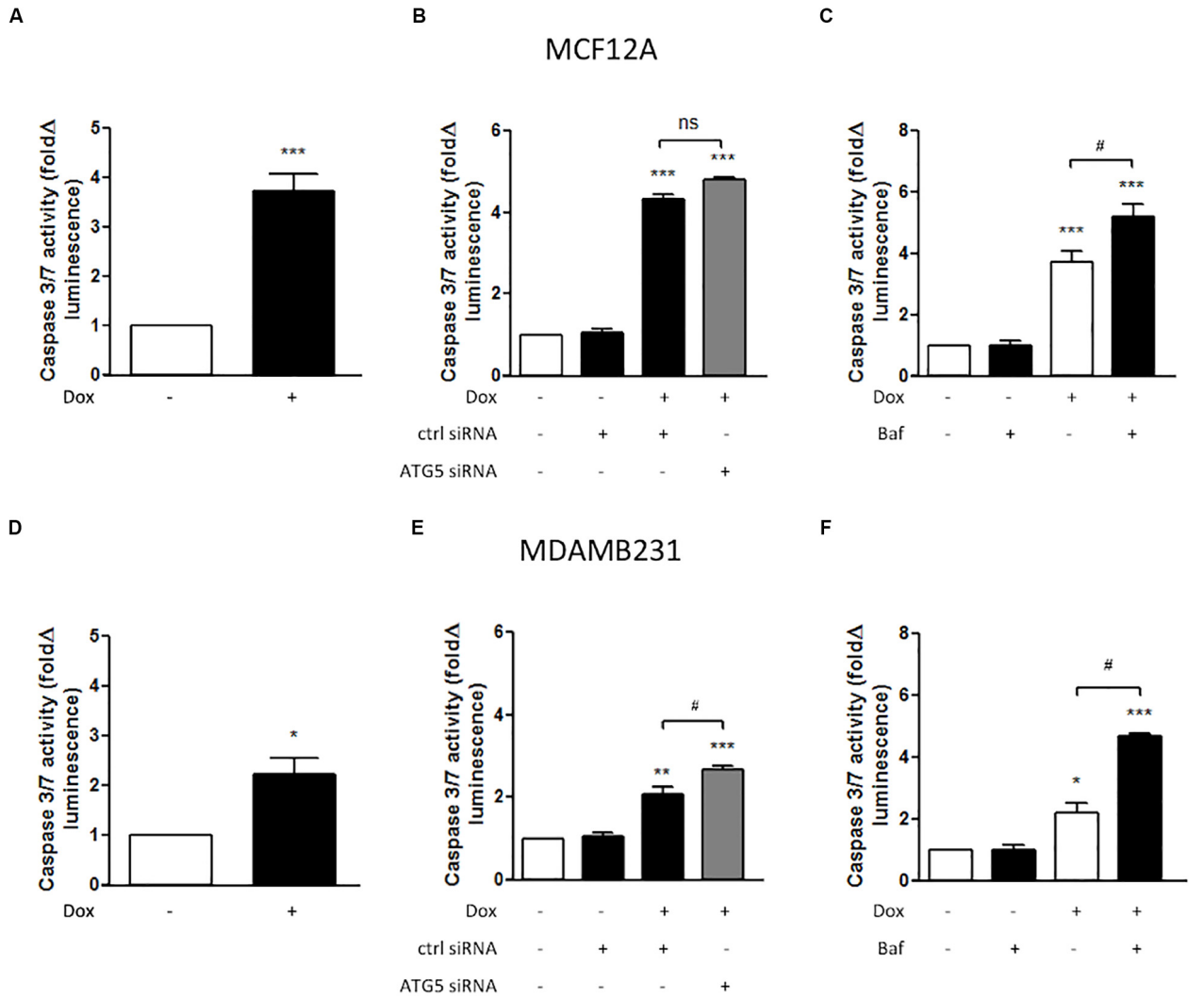

FIGURE 3 | Caspase 3/7 activity in MCF12A and MDAMB231 cells following Dox treatment and autophagy inhibition. Caspase 3/7 activity in MCF12A cells following treatment with Dox alone **(A)**, in combination with ATG5 siRNA-mediated autophagy inhibition **(B)**, and in combination with Baf-mediated autophagy inhibition **(C)**. Caspase 3/7 activity in MDAMB231 cells following treatment with Dox alone **(D)**, in combination with ATG5 siRNA-mediated autophagy inhibition **(E)**, and in combination with Baf-mediated autophagy inhibition **(F)**. Results represent fold change in luminescence in cultures incubated with Dox vs. untreated cells. Images were obtained using a 10× objective with each value representing the mean ± SEM of at least three independent determinations. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.05$, ns, no significance.

MDAMB231 cells with Baf (10 nM) 6 h prior to analysis greatly increased caspase 3/7 activity when these cells were also treated with Dox (**Figures 3C,F**).

Baf Increased Detectable Levels of Intracellular Dox in MCF12A and MDAMB231 Cells Treated With Dox

Cells were stained with LAMP-2A during Dox treatment. Intracellular localization of Dox was tracked by exploiting the compound's autofluorescence, a technique that has been utilized successfully in MDAMB231 and other cell lines (Li et al., 2010). MCF12A cells treated with Dox displayed diffuse intracellular red fluorescence associated with Dox (**Figure 4A**). Notably, small localized regions of intense red fluorescence were observed in most Dox-treated MCF12A cells (arrows), but were completely absent in cells that had also been treated with Baf. MDAMB231 cells treated with Dox displayed intense localized regions of intracellular red fluorescence associated with Dox (**Figure 4B**). This red fluorescence was associated with pooled punctate LAMP-2A signal (arrows) in Dox-treated MDAMB231 cells. Notably, LAMP-2A fluorescence appeared more diffuse if Baf was added. Importantly, MDAMB231 cells treated with Dox and Baf had significantly more observable red fluorescence within the cytoplasmic regions of these cells. However, unlike MCF12A cells, there was no Dox associated with the nuclear regions of these cells.

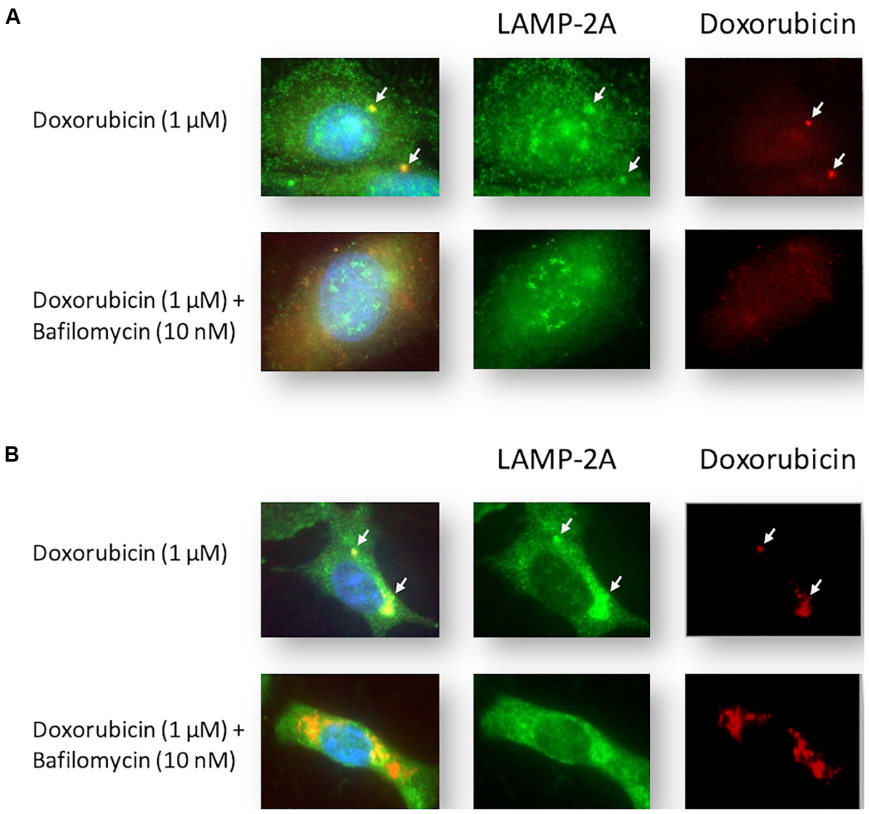

FIGURE 4 | Intracellular localization of LAMP-2A and Dox in Dox-treated MCF12A and MDAMB231 cells following autophagy inhibition. Representative images of LAMP-2A staining (green) and Dox (red) within MCF12A **(A)** and MDAMB231 **(B)** cells in the presence of Dox alone or in combination with Baf. Arrows highlight regions of localized Dox accumulation overlapping with regions of punctate LAMP-2A signal. Comparative control groups (cells treated with Dox in the presence of amino acids) are shared between **Figure 4** and **Figure 7** since treatment groups from both figures were performed together in one experiment. Images were obtained using a 40× objective.

Depleting Culture Medium of Amino Acids During Dox Treatment Protects MCF12A, but Not MDAMB231 Cells From Apoptosis

Amino acid deprivation significantly decreased caspase 3/7 activity in MCF12A cells when treated with Dox for 24 h (**Figure 5A**). This protection from increased caspase activation corresponded with increased and sustained lysosomal acidity, as measured with flow cytometry using cells stained with Lysotracker (**Figure 5C**). This is in direct contrast to the results obtained with MDAMB231 cells where incubation in culture medium deprived of amino acids, during treatment with Dox, resulted in significantly increased caspase 3/7 activity at 24 h (**Figure 5B2**), but not 12 h (**Figure 5B1**) after intervention. Amino acid deprivation during a 24 h treatment with Dox also resulted in significantly diminished lysosomal acidity levels, close to baseline, in MDABM231 cells (**Figure 5D**).

The trypan blue vital stain can traverse only cell membranes with compromised integrity, and therefore functions as a marker of necrotic and late stage apoptotic cell death and can be used as an indicator of actual cellular impairment following an intervention. MCF12A cells are extremely susceptible to Dox cytotoxicity with approximately 80% of these cells becoming trypan blue positive after a 24 h incubation with Dox (**Figure 5E**). Treatment with Dox in culture medium completely depleted of amino acids resulted in significantly less MCF12A cells becoming trypan blue positive.

MDAMB231 cells appeared to be comparatively resistant to Dox-induced membrane impairment, and amino acid starvation did not change the percentage of trypan blue positive MDAMB231 cells following treatment with Dox (**Figure 5F**).

Amino Acid Deprivation Increased Autophagy Induction and Autophagy Flux in MCF12A Cells

LC3-II decorates inner and outer membranes of autophagosomes and is the only reliable marker to analyze autophagy flux (Martinez-Lopez et al., 2016). Dox treatment during amino acid deprivation resulted in increased induction of LC3 II in MCF12A cells (**Figure 6A**). Furthermore, LC3 II protein levels were shown to accumulate if Baf was administered 6 h prior to analysis. Although a slight change was noticed in MDAMB231 cells when Baf was administered, this change was found to be non-significant (**Figure 6B**).

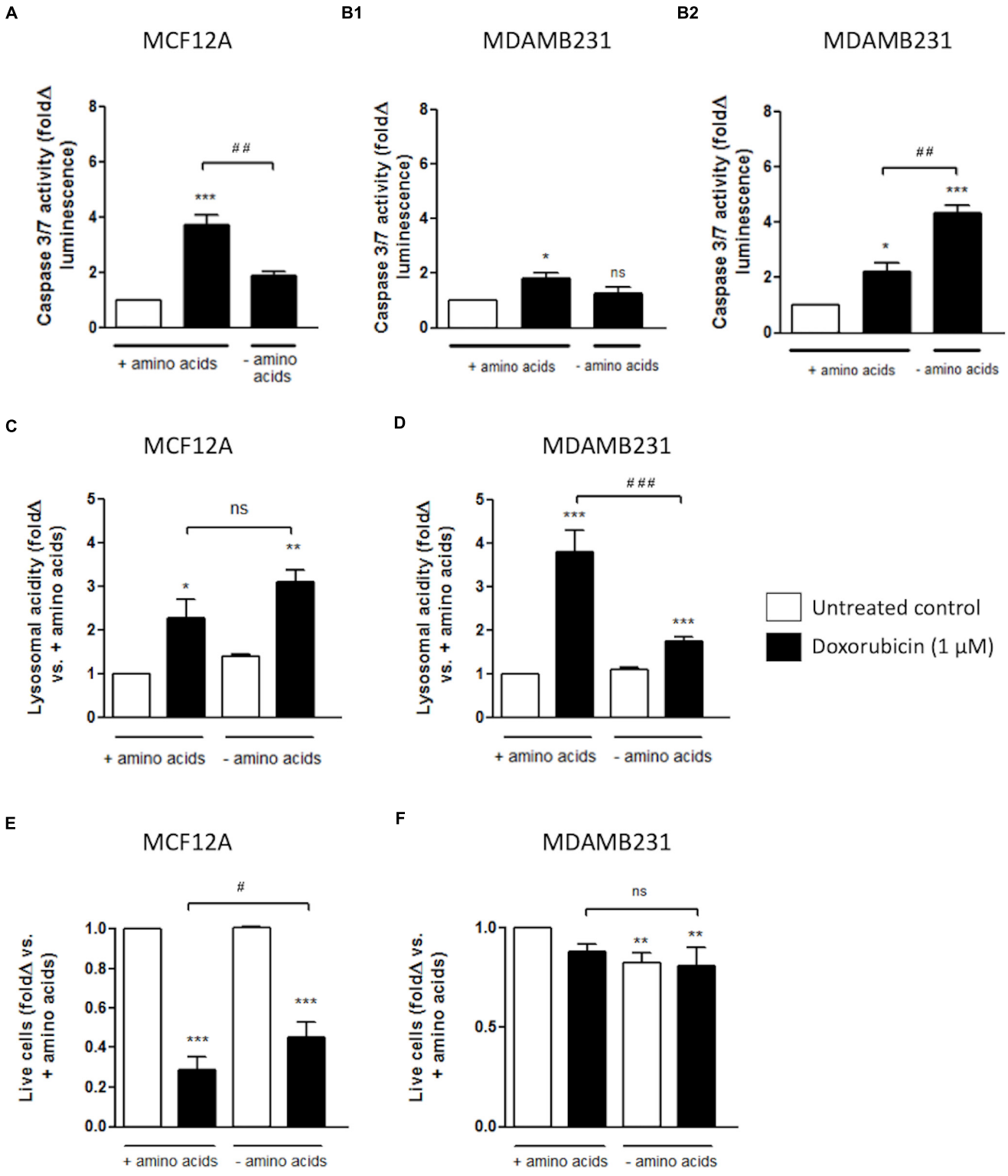

FIGURE 5 | The effect of amino acid starvation in MCF12A and MDAMB231 cells following Dox treatment. Caspase 3/7 activity in amino acid-starved MCF12A after 24 h **(A)** and MDAMB231 cells after 12 **(B1)** and 24 h **(B2)** following Dox treatment. Lysosomal acidity in amino acid-starved MCF12A **(C)** and MDAMB231 **(D)** cells following Dox treatment. Trypan blue positive cell staining in amino acid-starved MCF12A **(E)** and MDAMB231 **(F)** cells following Dox treatment. Results represent the fold change in cultures incubated with Dox vs. untreated cells. Cells were incubated in Dox and/or in amino acid deprived medium for 24 h where applicable. Each value represents the mean ± SEM of at least three independent determinations. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; ns, no significance.

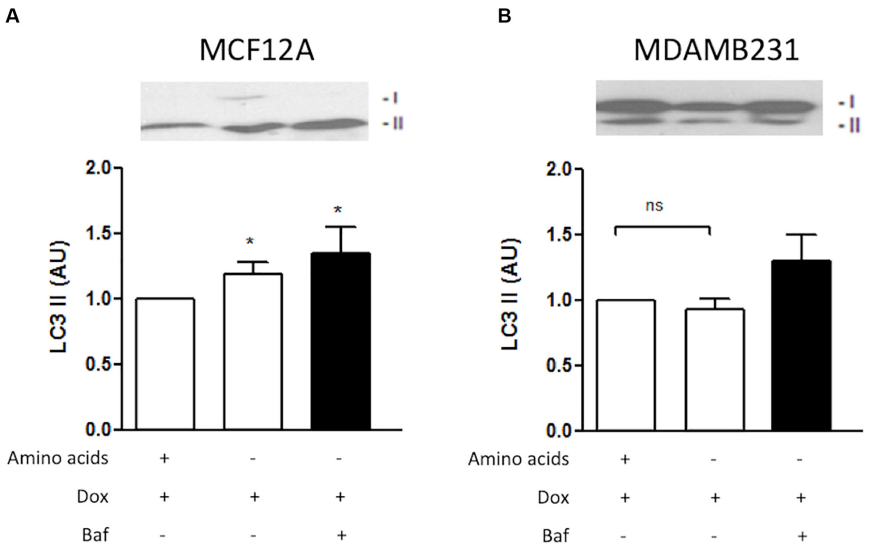

FIGURE 6 | LC3 II protein levels during amino acid starvation in MCF12A and MDAMB231 cells. Representative western blots and Bar graphs showing densitometric representations of LC3 II protein levels in MCF12A **(A)** and MDAMB231 **(B)** cells. Each value represents the mean ± SEM of at least three independent determinations. AU, arbitrary units; *$p < 0.05$ vs. amino acid control; ns, no significance.

Amino Acid Deprivation or Autophagy Inhibition With ATG5 siRNA During Dox Treatment Resulted in Increased Levels of Dox in the Nuclear Regions of MDAMB231 Cells

Amino acid deprivation from culture medium during treatment of MCF12A cells with Dox caused an apparent increase in pooled punctate LAMP-2A in the perinuclear area of these cells (illustrated by the arrow) (**Figure 7A**). Furthermore, amino acid deprivation during treatment resulted in a prominent decrease in observable Dox (red fluorescence). Inhibition of autophagy with ATG5 siRNA resulted in a dispersed pattern of LAMP-2A staining, but Dox was still readily visible in these cells.

MDAMB231 cells treated with Dox displayed intense localized regions of LAMP-2A and intracellular red fluorescence associated with each other (illustrated by the arrow) (**Figure 7B**). Amino acid deprivation during treatment resulted in a more dispersed staining pattern for the LAMP-2A marker. Furthermore, these cells displayed increased levels of nuclear Dox (red fluorescence). Inhibition of autophagy with ATG5 siRNA resulted in a prominent increase in intracellular Dox, especially at the perinuclear zone (illustrated by the arrow).

Amino Acid Deprivation During Dox Treatment Exacerbated g2/m Cell Cycle Arrest Associated With Dox Toxicity in MDAMB231 Cells

Dox treatment of MCF12A cells resulted in significant changes to the cell cycle profile. Deprivation of amino acids from culture medium during treatment with Dox resulted in the percentage of cells in the g0/g1 phase being similar to those in untreated controls (**Figure 8A**). However, the percentage of cells in g2/m phase decreased further if amino acids were absent.

Dox treatment is typically associated with an increased g2/m arrest in MDAMB231 cells (Lambert et al., 2008), although only a non-significant increase was observed in this specific model (**Figure 8B**). Treatment of MDAMB231 cells with Dox in culture medium deprived of amino acids resulted in a further significant increase in the percentage of cells in the g2/m phase of the cell cycle.

Amino Acid Deprivation Does Not Alter Lysosomal Acidity or Apoptosis Levels During Dox Treatment in Beclin 1 Haploinsufficient MCF7 Cells

MCF7 cells are autophagy incompetent due to a haploinsufficiency in the gene coding for the autophagy protein beclin 1 (Liang et al., 1999). Dox treatment, either with or without amino acids, did not significantly alter lysosomal acidity in these cells (**Figure 9A**). MCF7 cells do not express caspase 3 (Liang et al., 1999), which was confirmed by western blotting analysis (**Figure 9B**). Dox treatment of MCF7 cells resulted in increased apoptosis (**Figures 9C,D**). However, Dox treatment of these cells in culture medium deprived of amino acids, either with or without Baf, does not significantly alter apoptosis levels.

24 h Protein Starvation During High Dose Doxorubicin (10 mg/kg) Treatment Resulted in Increased Survival of E0771 Tumor-Bearing GFP-LC3 Mice

Mice with similar tumors and administered doxorubicin at a high dose (10 mg/kg) on 2 days, either side of a day without treatment had all died by day 12 after the initial treatment (**Figure 10A**). The

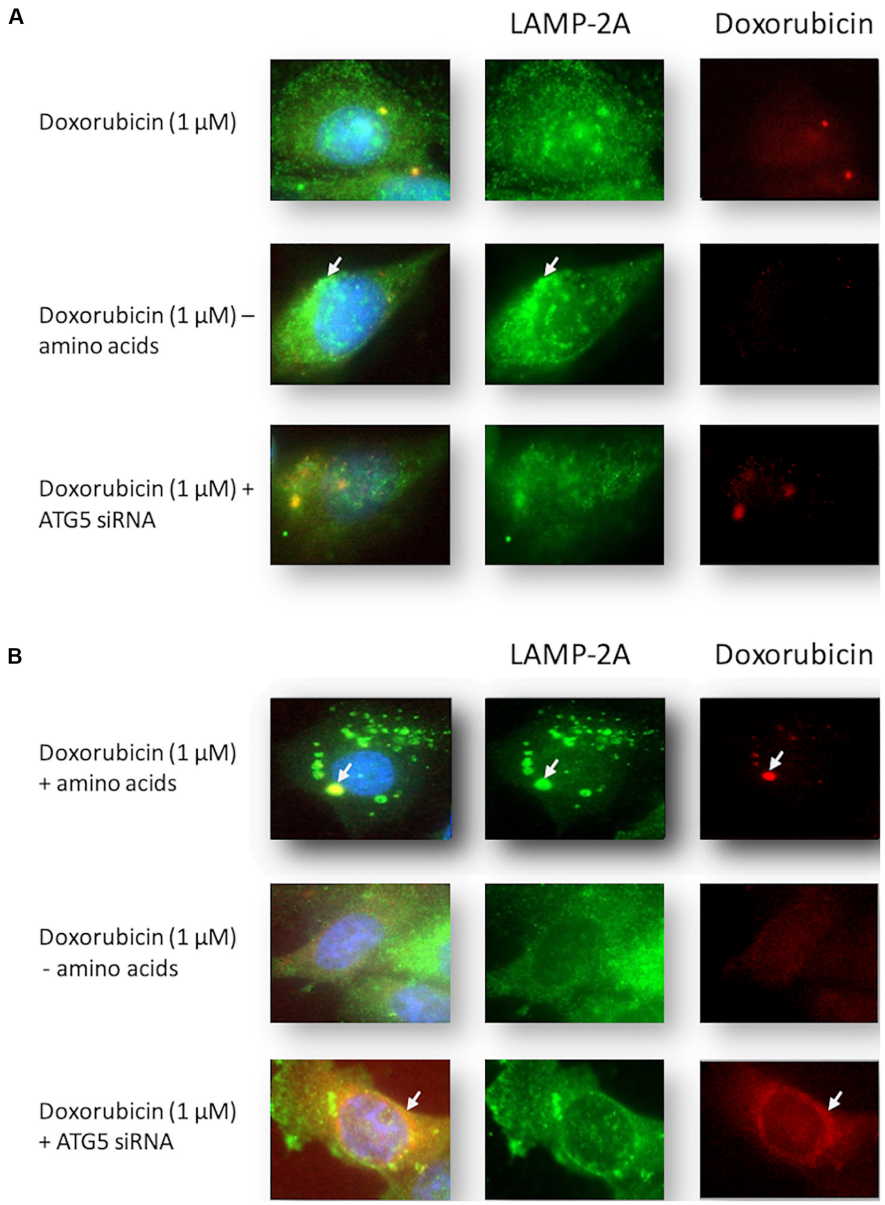

FIGURE 7 | Intracellular localization of LAMP-2A and Dox in Dox-treated MCF12A and MDAMB231 cells following amino acid starvation and autophagy inhibition. Representative images of LAMP-2A (green) straining and Dox (red) in MCF12A **(A)** and MDAMB231 **(B)** cells treated with Dox in culture medium without amino acids or with ATG5 siRNA. Arrows highlight differences in localized signals of LAMP-2A and Dox. Comparative control groups (cells treated with Dox in the presence of amino acids) are shared between **Figure 4** and **Figure 7** since treatment groups from both figures were performed together in one experiment. Images were obtained using a 40× objective.

dose of doxorubicin was selected for acute exposure on the basis of previous work that verified an inevitably toxic but not rapidly fatal dose inducing myocardial damage, which is a common side-effect of doxorubicin therapy (Sishi et al., 2013).

However, if tumor-bearing mice were placed on a diet free of protein, immediately after i.p. injection with a high dose of doxorubicin (10 mg/kg), then the survival of these mice was prolonged compared to those fed a standard diet. Furthermore, protein starved doxorubicin treated mice showed less signs of reduced mobility and ruffled hair compared to mice treated with doxorubicin but fed isocaloric protein complete diets (data not shown).

24 h Protein Starvation During High Dose Doxorubicin (10 mg/kg) Treatment Does Not Influence Changes in Tumor Volumes Attributed to Doxorubicin

Growth rates of E0771 tumors in GFP-LC3 mice were very similar in size between groups, prior to the initial interventions

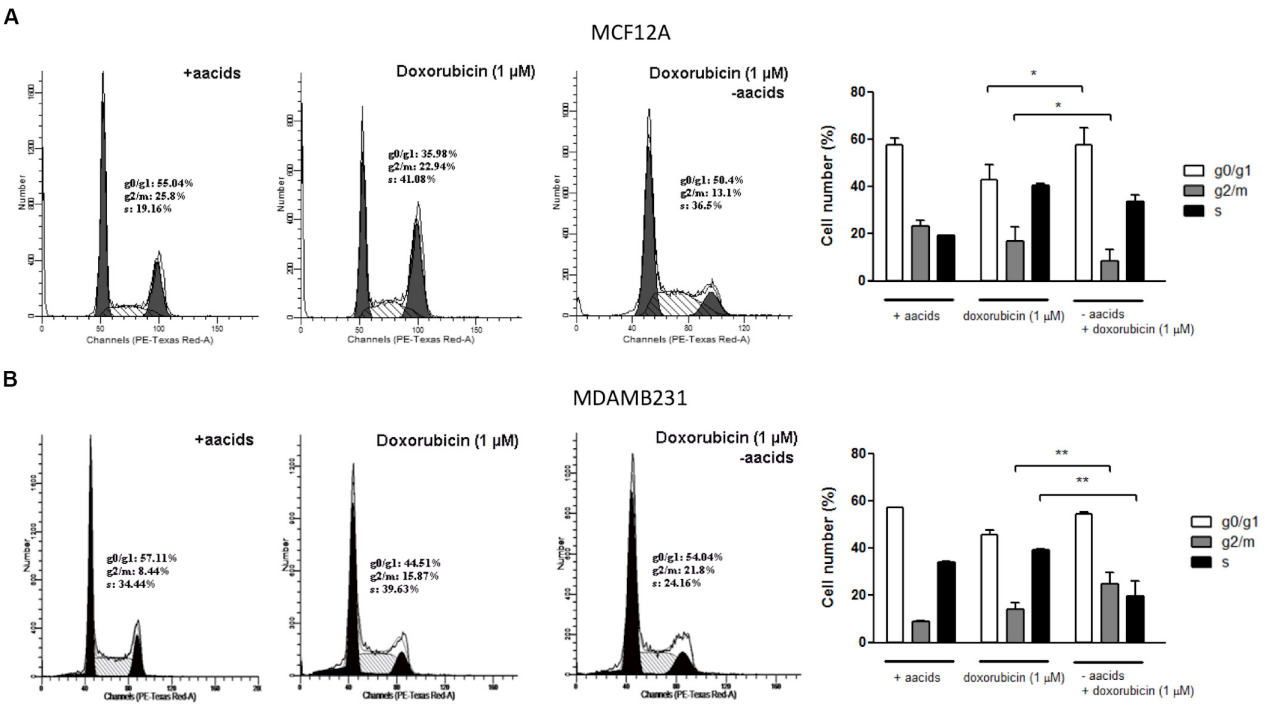

FIGURE 8 | The effect of amino acid starvation and Dox treatment on the cells cycle of MCF12A and MDAMB231 cells. Representative DNA histograms and quantified bar graphs of cell cycle progression in MCF12A **(A)** and MDAMB231 **(B)** cells. Cell cycle progression was assessed using flow cytometry. Each value represents the mean ± SEM of at least three independent determinations. aacids, amino acids; $^{*}p < 0.05$; $^{**}p < 0.01$.

(**Figure 10B**). Administration of two high dose doxorubicin (10 mg/kg) treatments over 3 days in GFP-LC3 mice bearing large E0771 tumors (>230 mm^2) resulted in significant reductions in tumor size by day eight after the first intervention (**Figure 10B**). If tumor-bearing mice were placed on diets free of protein during doxorubicin treatment (10 mg/kg), reductions in tumor size were shown to be similar to those treated with doxorubicin but fed protein complete diets (**Figure 10B**).

24 h Protein Starvation During High Dose Doxorubicin (10 mg/kg) Treatment Results in Significantly Increased Intratumour Autophagy Flux in E0771 Induced Tumors in GFP-LC3 Mice

Flow cytometry and FACS can be used to monitor autophagy in cells containing GFP-LC3 by exploiting the fact that GFP is sensitive to acidic environments, such as that of lysosomes (Martinet et al., 2006; Yang et al., 2007), and GFP fluorescence disappears immediately on it entering the reduced pH environment of a lysosome. A reduction in GFP signal therefore reflects delivery of the GFP-LC3 complex into lysosomes and can be related to the degree of autophagy flux (Shvets et al., 2008). Administration of two high dose doxorubicin (10 mg/kg) treatments over 3 days in GFP-LC3 mice bearing large E0771 induced tumors (>230 mm^2) resulted in a significant increase in GFP signal. This is inferred to indicate an increased autophagy induction and translation of the GFP-LC3 complex (**Figure 10C**). However, if mice were placed on a protein free diet for 24 h at the start of their night cycle, beginning immediately after each doxorubicin (10 mg/kg) injection, then a significant decrease in GFP signal indicates a significant increase in autophagy flux compared to mice treated but fed on protein complete diets. As only non-cancer cells contain the GFP-LC3, significantly increased intratumour autophagy flux implies that there is an increased autophagy flux in non-cancer stromal cells within the E0771 induced tumors of treated GFP-LC3 mice fed protein free diets compared to those fed protein complete diets.

24 h Protein Starvation During High Dose Doxorubicin (10 mg/kg) Treatment Results in Significantly Lower Caspase Activity Within Tumors

Administration of two high dose doxorubicin (10 mg/kg) treatments over 3 days in GFP-LC3 mice bearing large E0771 induced tumors (>230 mm^2) resulted in a significant increase in caspase activity within whole excised tumors, using a FLIVO^{TM} (FLuorescence in vIVO) *in vivo* apoptosis tracer (Immunochemistry Technologies LLC, MN, United States) and FACS flow cytometry (**Figure 10C**). However, if mice were placed on a protein free diet for 24 h at the start of their night cycle, beginning immediately after each doxorubicin injection, then caspase activity was observed to be significantly lower in excised tumors than in those mice treated but fed on protein complete diets.

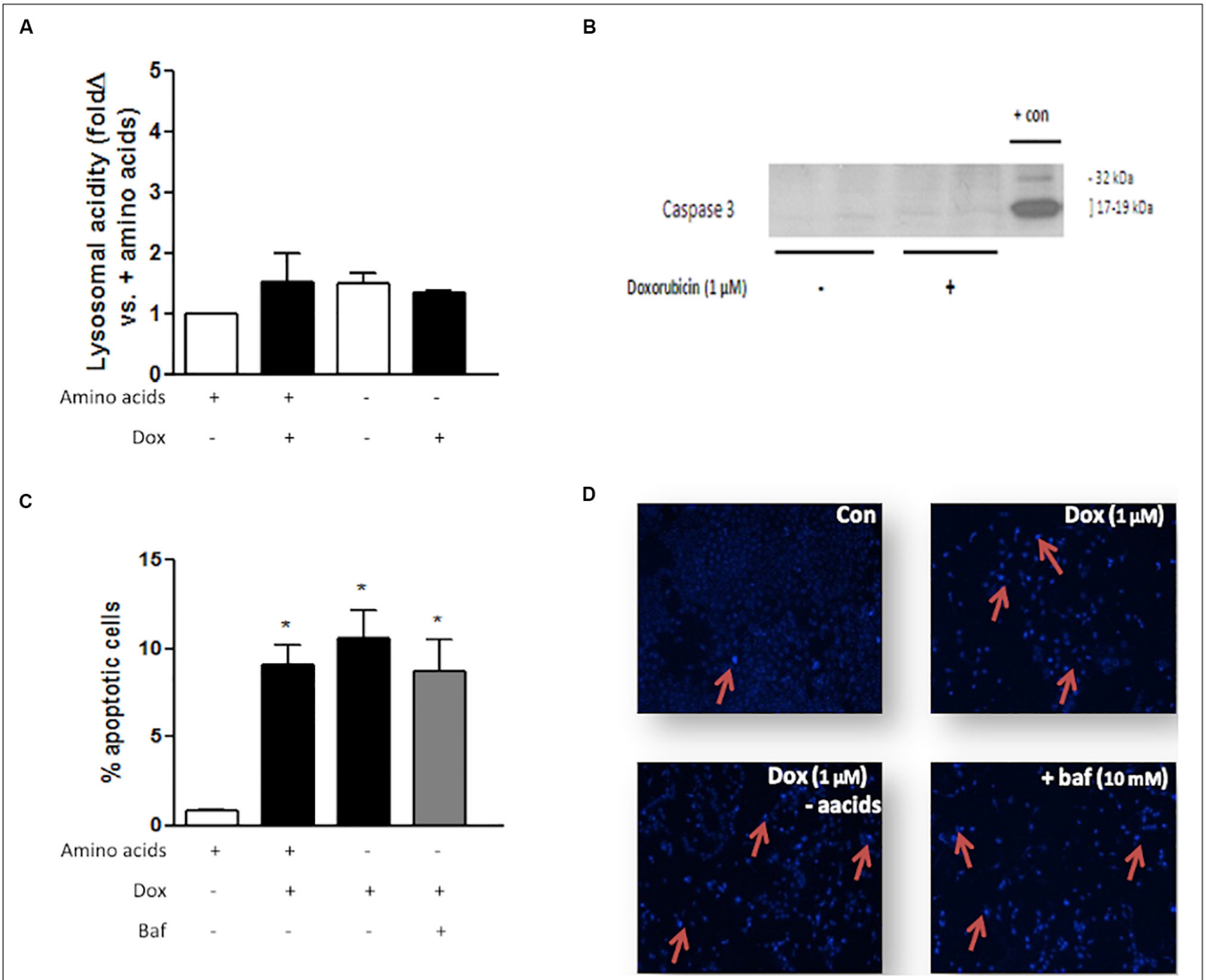

FIGURE 9 | The effect of amino acid starvation, autophagy inhibition and Dox treatment in beclin 1-haploinsufficient MCF7 cells. **(A)** Lysosomal acidity in amino acid-starved MCF7 cells following Dox treatment. **(B)** Representative western blot showing lack of caspase 3 expression in MCF7 cells. **(C)** Bar graph indicating the percentage of nuclei that represents morphological changes characteristic of apoptosis in MCF7 cells following Dox treatment and Baf-mediated autophagy inhibition and **(D)** representative images of MCF7 cells stained with Hoechst. Red arrows demonstrate apoptotic nuclear features. Images were obtained using a 10× objective. Each value represents the mean ± SEM of at least three independent determinations. + con, positive control; con, control; aacids, amino acids; $^{*}p < 0.05$ vs. amino acid control.

DISCUSSION

Many cancers are known to respond to certain chemotherapeutics or to radiation therapy by increasing autophagic activity. Although the consequences of autophagic activation in these circumstances are still debatable, it appears autophagy acts in its predominant role as survival mediator in many of these cases (Yang and Chen, 2011). Anthracyclins such as Dox have also been shown to increase autophagy levels in cancer cells in some instances. At lower doses, Dox elicits an autophagic response in breast cancer cells (Akar et al., 2008), as well as in sarcoma cell lines (Martinez-Lopez et al., 2016). Additionally, increased levels of ATG2A mRNA have been observed following Dox treatment (Levy and Thorburn, 2011), suggesting increased autophagy activation at the transcriptional level.

It was also reported that 2-deoxy-glucose preserved ATP content and contributed to the cytoprotection of rat cardiomyocytes treated with 1 μM Dox (Chen et al., 2011). This glucose analogue increased markers of autophagy which was posited as a potential reason for protection from cytotoxicity. Conversely, activation of autophagy in cultured cardiomyocytes following 1 μM Dox treatment mediated its cardiotoxic effect (Kobayashi et al., 2010). Here, it was shown that autophagy inhibition resulted in decreased cell death and it was postulated that autophagy directly contributed to Dox-induced toxicity. These exemplify the conflicting nature of studies and reported

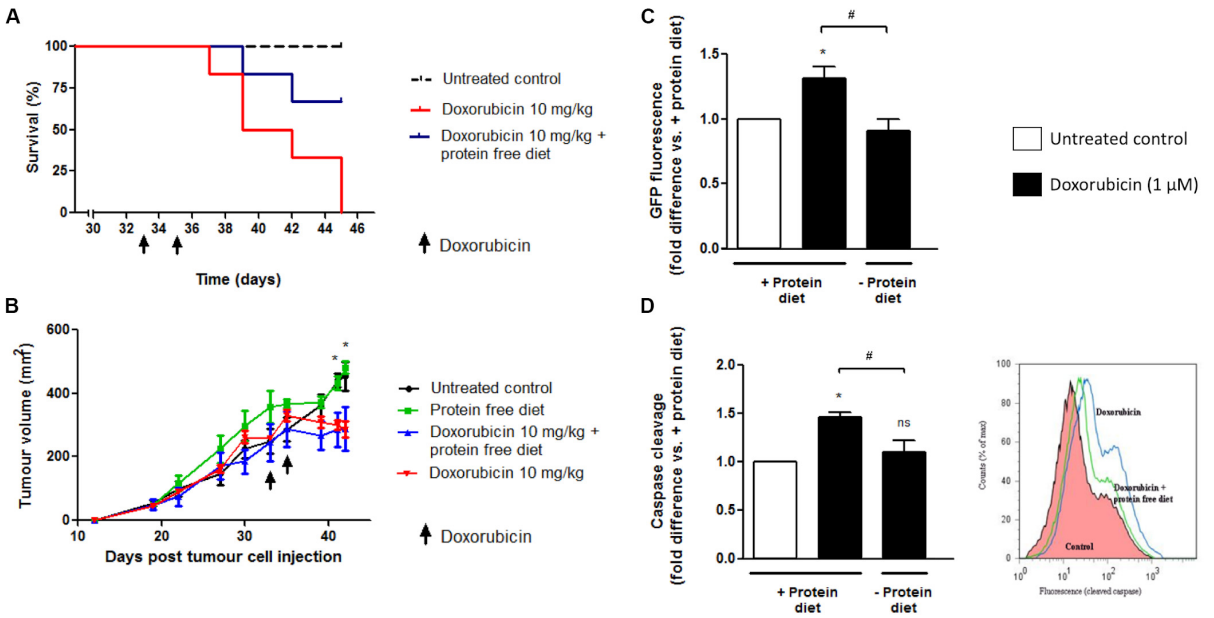

FIGURE 10 | The effect of 24 h protein starvation during high dose doxorubicin (10 mg/kg) treatment on survival and tumor growth of E0771 tumor-bearing mice. Mice received two i.p. injections of doxorubicin (10 mg/kg) over a period of 3 days and were placed on either a isocaloric diet or a diet free of protein for a total 24 h immediately following each doxorubicin administration. **(A)** Survival of tumor-bearing mice following doxorubicin treatment with or without protein starvation, $N = 6$. **(B)** Changes in tumor volumes of tumor-bearing mice following doxorubicin treatment with or without protein starvation, $N = 6$. Values are expressed as mean ± SEM. $^{*}p < 0.05$ untreated control vs. doxorubicin and vs. doxorubicin + protein free diet. **(C)** Intratumour autophagy flux in GFP-LC3 mice following doxorubicin treatment with or without protein starvation. A decrease in GFP signal indicates increased autophagy flux. $N = 3$. Values are expressed as mean ± SEM. $^{*}p < 0.05$ vs. untreated + protein diet. $^{\#}p < 0.05$. **(D)** Intratumour caspase cleavage in GFP-LC3 mice following doxorubicin treatment with or without protein starvation. FLIVO™ caspase dye was used to assess caspase cleavage, with increased fluorescence indicating cleavage. $N = 3$. Values are expressed as mean ± SEM. $^{*}p < 0.05$ vs. + protein diet. $^{*}p < 0.05$; $^{\#}p < 0.05$; ns, no significance.

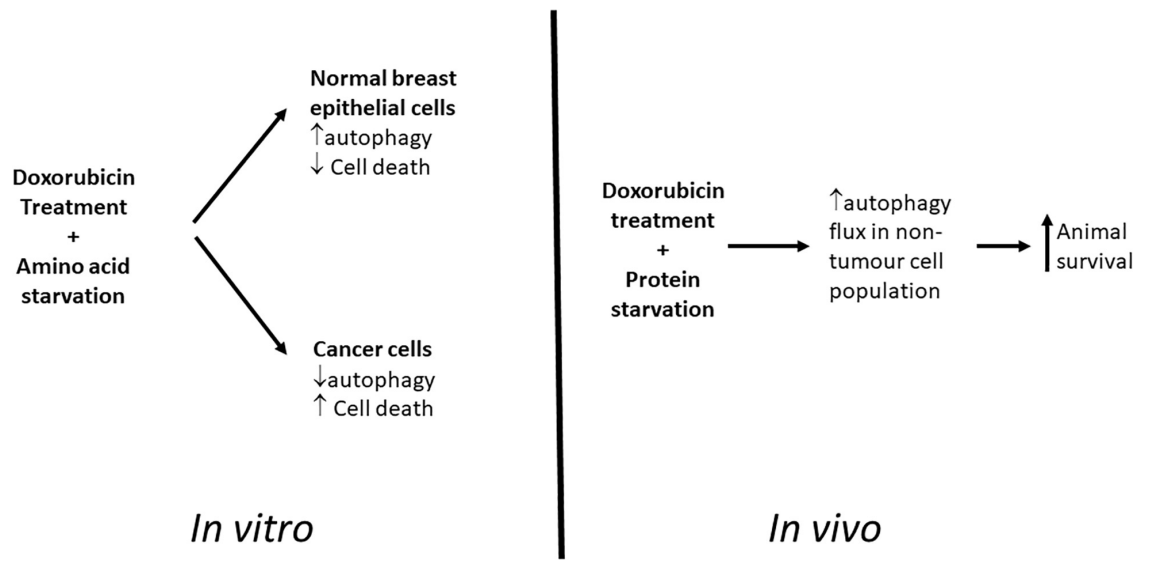

FIGURE 11 | Graphical Summary. Proposed model for differential toxicity in normal breast epithelial cells and breast cancer cells.

findings relating autophagy to drug toxicity and illustrate the need for further research into this important topic.

Autophagy activity is increased in response to stress, where it functions as an important mechanism whereby damaged organelles and aggregated proteins are degraded in lysosomes. However, there is still no clear mechanism whereby induction of autophagy could lead to tolerance against chemotherapy agents. Lysosomal fusion and throughput of autophagy depends

on the low pH of lysosomal compartments (Klionsky et al., 2008). Increasing the lysosomal pH with pharmacological agents is an effective method of autophagy inhibition, as disruption of the fusion event between autophagosomes and lysosomes prevents progression of autophagy. Some drug resistant cell lines are able to tolerate alkaline chemotherapy drugs such as the anthracyclines by sequestering and deactivating these agents in the acidic compartments of lysosomes (Hurwitz et al., 1997). Exposure of these compounds to environments of low pH renders them inactive and unable to access sites of chemotoxicity such as the nucleus.

Data from the current study clearly indicated that the non-tumourigenic cell line MCF12A is more susceptible to programmed cell death during treatment with a moderate dose of Dox (1 μM) than the metastatic breast cancer cell line MDAMB231. MCF12A cells had a significantly increased appearance of morphological changes characteristic of apoptosis (**Figure 1A**), as well as significantly enhanced caspase 3/7 activity (**Figure 3A**). On the other hand, MDAMB231 cells showed few signs of late stage apoptosis and a more modest increase in activation of caspase 3/7 (**Figures 1B**, **3D**). Interestingly, depletion of amino acids from the culture medium of MCF12A cells during Dox treatment resulted in a significant protection from loss of membrane integrity (a sign of late stage cell damage), as assessed by the trypan blue assay (**Figure 5E**). Previous experimental data revealed that amino acid starvation resulted in an enhanced autophagy response (unpublished from this group). It is suggested here that this increase in autophagy could be responsible, at least in part, for the tolerance to Dox treatment seen here. On the other hand, MDAMB231 cells did not experience any protection if starved of amino acids during treatment (**Figure 5F**).

Both MCF12A and MDAMB231 cells responded to the presence of Dox (1 μM) by increasing LC3 II protein levels (**Figure 2**), indicating an autophagic response during treatment. A slight increase in beclin 1 protein expression was also observed, however, these changes were not statistically significant. While beclin 1 plays an important role in the regulation of autophagy, it is also cleaved by caspase 3 during apoptosis (Wirawan et al., 2010). Since apoptosis was induced under these conditions, it may have resulted in varying levels of detectable beclin 1 protein expression and thus the lack of a significant effect. Inhibition of autophagy with ATG5 siRNA lead to a significant increase in caspase 3/7 activity only in MDAMB231 cells (**Figure 3E**), suggesting that autophagy has a protective role during chemotherapy of these cells. Importantly, the addition of Baf, 6 h prior to analysis, greatly increased caspase 3/7 activity during Dox treatment in both cell lines (**Figures 3C,F**), but had a particularly pronounced influence on the resistant cancer cell line. The differential effects displayed between ATG5 siRNA and Baf treatment in these cell lines might be due to the fact that Bafilomycin represents a mechanism of late-stage autophagy inhibition, while ATG5 knockdown facilitates autophagy inhibition at an earlier stage. Agents such as Baf, a specific inhibitor of vacuolar H^+ATPase (V-ATPase) (Klionsky et al., 2008), are known to rapidly and reversibly inhibit fusion between autophagosomes and lysosomes, if administered for short time periods (Chen et al., 2011), through the mechanism of inhibiting lysosomal acidification (Hurwitz et al., 1997). Furthermore, many drug resistant cancer cells are thought to increase sequestration and deactivation of chemotherapy drugs within lysosomes (Kobayashi et al., 2010). The proposed mechanism is through increased uptake of these compounds into endosomes, which eventually fuse with lysosomes to deliver these drugs into the acidic internal environments where they become deactivated (Wirawan et al., 2010). Baf and other agents that raise the pH of lysosomes can increase cytotoxicity by preventing fusion and inactivation of drugs in this way. Therefore, the increased caspase activity observed in the current study, following Baf administration, suggested that Baf prevented the sequestration and deactivation of Dox, therefore allowing Dox to exert its cytotoxic effects on the cell. As autophagy inhibition also resulted in increased caspase activity here, it is possible that mass engulfment of cytoplasmic material (which includes Dox) could facilitate delivery of this drug into lysosomal compartments in this cell line and thereby have a protective influence. As MDAMB231 cells have high basal autophagy levels (unpublished data), internalized Dox could be rapidly delivered to lysosomes and deactivated, conferring a partial resistance.

These assertions are further strengthened by qualitative evidence gained from fluorescence microscopy of MDAMB231 cells receiving Dox treatment. Intracellular localization of Dox can be tracked by exploiting the autofluorescence of Dox, a technique that has been utilized successfully in MDAMB231 and other cell lines in the past (Yoshimori et al., 1991). Treatment of MDAMB231 cells resulted in the clear accumulation of Dox at perinuclear regions (**Figure 4B**). Identification of the localization of lysosomes at the same time, by fluorescently tagging the lysosomal associated membrane protein LAMP-2A, demonstrates lysosomal accumulation in regions associated with Dox accumulation in these cells. This implies that Dox is located in association with lysosomes during these conditions. MCF12A cells show little accumulation of Dox after a 24 h treatment and have no discernible accumulation of LAMP-2A signal (**Figure 4A**). Furthermore, the addition of Baf 6 h prior to imaging, led to a vast and pronounced accumulation of Dox within the cytoplasmic compartment of MDAMB231 cells. Interestingly, accumulation of the LAMP-2A signal is mostly dissipated and little association between LAMP-2A and Dox was evident after the addition of Baf. Therefore, it appears as though Dox is not associated with lysosomes when administered in the presence of the vacuolar H^+ATPase inhibitor Baf, but rather elsewhere within the cell. As Baf inhibits the fusion of autophagosomes and endosomes with lysosomes (Hurwitz et al., 1997; Chen et al., 2011), it is possible that the observed accumulations of Dox is due to increased cytoplasmic accumulation of autophagosomes and endosomes containing Dox, but which are unable to fuse with lysosomes. Further investigation of this phenomenon is required.

Autophagic flux is clearly increased in MDAMB231 cells in response to Dox treatment (**Figure 6**), and lysosomal acidity greatly increased as a result of this treatment in both MCF12A cells (**Figure 5C**) and in MDAMB231 cells (**Figure 5D**). Interestingly, when these cell lines were treated with Dox in

culture medium deprived of amino acids, lysosomal acidity significantly decreased in MDAMB231 cells, but remained elevated in MCF12A cells. Notably, alterations in lysosomal acidity were associated with corresponding changes in caspase 3/7 activity in both cell lines (**Figures 5A,D**). MDAMB231 cells experienced significantly greater levels of caspase 3/7 activity during conditions of depressed lysosomal acidity, while MCF12A cells were granted a relative protection from the cytotoxic impact of drug treatment. Strikingly, if MDAMB231 cells were analyzed after 12 h of combined Dox/amino acid starvation treatment, these increases in apoptosis activation were not evident. Importantly, at this time point of amino acid starvation lysosomal acidity was confirmed to be elevated compared to baseline (unpublished data), further supporting the premise of lysosomal acidity deprivation facilitated increases in caspase activity in this model.

If decreased lysosomal acidity levels during amino acid starvation prevent fusion of autophagosomes with lysosomes then the probability of drug access to sites of cytotoxic action would be increased. This would result in the increased apoptosis activation demonstrated here. Dox treatment is associated with genotoxic stress and prolonged g2/m cell cycle arrest (Yamamoto et al., 1998), due to intercalation of this agent with DNA and activation of the g2/m DNA damage checkpoint. Therefore, decreased abundance of Dox within lysosomes during amino acid deprivation would result in an exacerbated accumulation of cells in the g2/m phase of the cell cycle. Our experimental data supports this with an approximately 6% increase in the percentage of MDAMB231 cells in the g2/m phase of the cell cycle if Dox treated cells are simultaneously starved of amino acids (**Figure 8B**). Additionally, the relative protection of MCF12A cells can be attributed to increased autophagy levels and decreased access of Dox to the nucleus, as amino acid starvation during drug treatment resulted in a cell cycle profile similar to that of untreated cells (**Figure 8A**). Also, qualitative experimental evidence shows that Dox treatment during amino acid starvation causes decreased Dox and LAMP-2A accumulation and an increased Dox signal in the nuclear regions of MDAMB231 cells (**Figure 7B**), whereas the opposite is observed in MCF12A cells during similar treatment (**Figure 7A**).

Data suggested that alterations in lysosomal acidity are linked to increased apoptosis induction or protection in MDAMB231 cells and MCF12A cells, respectively. However, the role for autophagy should not be overlooked. Inhibition of autophagy with ATG5 siRNA resulted in a prominent increase in levels of Dox in the nucleus and at the perinuclear zone in MDAMB231 cells (**Figure 7B**), while MCF12A cells exposed to ATG5 siRNA prior to treatment showed signs of increased cytoplasmic Dox accumulation (**Figure 7A**). Interestingly, a cell line with decreased autophagy (MCF7), due to haploinsufficiency in the gene coding for beclin 1, displayed no alterations in lysosomal acidity during Dox treatment, in the presence or absence of amino acids (**Figure 9A**). While this cell line does not possess active caspase 3 (**Figure 9B**), it did show signs of significantly increased apoptosis during Dox treatment. These levels of apoptosis did not increase if treatment occurred in the absence of amino acids or the presence of Baf (**Figures 9C,D**). Together, these results indicate that active autophagic machinery is required for the accumulation of Dox within lysosomes and demonstrated an important role for autophagy in these processes. As interest in modulation of autophagy during cancer treatment increases and novel strategies such as fasting therapy during high-dose cancer treatment are explored, it is imperative that studies be undertaken to understand the underlying mechanisms driving these beneficial effects before use begins in a clinical setting.

Therefore, in this part of the study we aimed to determine the effect of short-term protein starvation in mice receiving a high cumulative dose of doxorubicin treatment. Using a mammary tumor model, it was shown that high-dose doxorubicin treatment (10 mg/kg for a cumulative dose of 20 mg/kg over 3 days) resulted in low survival rates of rodents possessing large, aggressive tumors (**Figure 10A**). Remarkably, survival was significantly improved if mice were placed on protein free diets immediately after drug administration (**Figure 10A**). Although evidently extremely toxic to the tumor-bearing mice in this model, the dose of doxorubicin used here was sufficient to significantly reduce tumor size after only 8 days. However, these reductions in tumor sizes were not diminished in those mice starved of proteins during treatment (**Figure 10B**).

Since our *in vitro* experimental data has shown that while a non-cancer cell line experienced protection if starved of amino acids during doxorubicin treatment, a cancer cell line with high basal autophagy activity had increased cell death. We have therefore utilized our GFP-LC3 tumor-bearing mouse model to assess the impact of protein starvation on autophagy flux in the host derived stromal subpopulation within the mammary tumors of mice treated with doxorubicin. We have shown an increase in intratumour autophagic flux in the non-cancer cell population of these tumors (**Figure 10C**). Interestingly, this increase in autophagy flux in the tumor stromal subpopulation correlated with a decrease in intratumour apoptosis (**Figure 10D**).

Cancers are extremely heterogenous by nature and much of their volume can be comprised of non-cancer cells that are vital for their continued growth and development (Lee and Tannock, 2006). As solid tumors rely on non-cancer cells for survival, any protection of these cells from cytotoxicity during short term starvation could indirectly result in prolonged tumor cell survival during chemotherapy. Also, the benefits of fasting in cellular protection from cytotoxicity may rely partly on altered circulating hormone levels (Li et al., 2010), which necessitates further investigation using *in vivo* models. In fact, reduced circulating IGF-I levels have been implicated in the differential protection of normal cells and cancer cells in response to fasting and improved chemotherapeutic index during doxorubicin treatment (Koutsilieris et al., 1999).

Our cell culture based studies demonstrated a promising differential protection of non-cancer cells and increased signs of apoptosis in cancer cells during chemotherapy when these cell lines were starved of amino acids (**Figure 11**). However, translation of this model in vivo has shown that although protein deprivation appears to increase survival rates without impacting on reductions in tumor volumes during high dose doxorubicin treatment, a potentially increased protection may be occurring in cells within these tumors.

The model established here, and the related findings, have presented a novel and unique platform for further research into this remarkable phenomenon, and particularly the role of autophagy therein.

This data represents a caveat to researchers aiming to utilize fasting or starvation diets in combination with conventional chemotherapy, and illustrates that additional mechanistic data is required in order to better understand the indirect consequences of such treatment strategies. Together with the *in vitro* experimental data, these promising findings imply a role for autophagy in the differential protection during doxorubicin treatment.

CONCLUSION

The results of this study suggest that the inherent resistance to the alkaline chemotherapeutic agent Dox exhibit by the MDAMB231 cells can be attenuated by elevating the lysosomal pH in a pharmacological manner. Furthermore, short term amino acid starvation was shown to be a realistic avenue for adjuvant therapy since it strengthened the cytotoxic effect of Dox in the MDAMB231 cancer cells but not in the normal MCF12 cells. Non-cancerous cells were potentially protected from the anthracycline due to the ability to increase autophagic activity in response to short term amino acid starvation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee Stellenbosch University.

AUTHOR CONTRIBUTIONS

MT contributed to the conception and design of the study, and collected and analyzed data. TD drafted the initial manuscript. TN contributed to critical revision and intellectual input of the manuscript. BS participated in the animal study and contributed to critical revision. A-ME contributed to the conceptualization and design of the entire study, and supervised and contributed to critical revision and intellectual input of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.565915/full#supplementary-material

Supplementary Figure 1 | The effects of ATG5 siRNA on MCF12 and MDAMB231 cells. Transfection of **(A)** MCF12A and **(B)** MDAMB231 cells with ATG5-targetting siRNA does not have any effect on caspase 3/7 activity under control conditions. Results represent the fold change in luminescence compared to untransfected cells and are proportionate to caspase activity. Each value represents the mean ± SEM of at least three independent determinations.

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