The Transcription Factor p8 Regulates Autophagy in Response to Palmitic Acid Stress via a Mammalian Target of Rapamycin (mTOR)-independent Signaling Pathway*

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Autophagy is an evolutionarily conserved degradative process that allows cells to maintain homeostasis in numerous physiological situations. This process also functions as an essential protective response to endoplasmic reticulum (ER) stress, which promotes the removal and degradation of unfolded proteins. However, little is known regarding the mechanism by which autophagy is initiated and regulated in response to ER stress. In this study, different types of autophagy were identified in human gastric cancer MKN45 cells in response to the stress induced by nutrient starvation or lipotoxicity in which the regulation of these pathways is mammalian target of rapamycin (mTOR)-dependent or -independent, respectively. Interestingly, we found that p8, a stress-inducible transcription factor, promotes the removal and degradation of unfolded proteins. Furthermore, an increase in autophagy was enhanced in MKN45 cells treated with palmitic acid to protect cells from the stress of nutrient starvation, respectively. Furthermore, our results indicated that autophagy induced by palmitic acid is mTOR-independent, but this autophagy pathway was regulated by p8 via p53- and PKCα-mediated signaling in MKN45 cells. Our findings provide insights into the role of p8 in regulating autophagy induced by the lipotoxic effects of excess fat accumulation in cells.

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3 The abbreviations used are: AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; ER, endoplasmic reticulum; PERK, protein kinase-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) are involved in the signaling cascades inducing autophagy upon ER stress.
stress (reticulophagy) (30–33). However, the effects of ER stress on the transcriptional regulation of the autophagy machinery or the mechanisms by which autophagy is initiated have not yet been characterized in detail.

The transcription factor p8 (or nuclear protein transcriptional regulator 1 (NUPR1)) was first identified in rats in 1997 and found to be overexpressed in acinar cells during the acute phase of pancreatitis (34). The p8 gene is overexpressed during endothelin-induced mesangial cell hypertrophy and in the diabetic kidney (35). As a key player in the cellular stress response, p8 plays a role in several physiological and physiopathological processes and is also involved in autophagy induced by lipopolysaccharide treatment of vascular endothelial cells (36, 37). Autophagy is also controlled by p53 in a dual fashion, whereby p53 induces this process in the nuclei and suppresses it in the cytoplasm (38). A previous study has revealed the existence of a regulatory loop between the expression levels of p8 and p53 in mouse embryonic fibroblasts (39). Furthermore, p8 forms a complex with p53, regulates p21 transcription, and rescues breast epithelial cells from doxorubicin-induced genotoxic stress (40).

Lipotoxicity refers to the cytotoxic effects caused by lipid accumulation, and studies of animal models have suggested a link between ectopic lipid accumulation, cell death, and organ dysfunction (41). Elevated circulating free fatty acid (FFA) levels precede the onset of diabetes and heart failure and are associated with steatosis and the subsequent apoptosis of β cells and cardiac myocytes, respectively (42, 43). Metabolic labeling studies have demonstrated that the FFA palmitate is incorporated rapidly into phosphatidylcholine in the ER, leading to a significant increase in the saturation of the ER membrane phospholipids (44). These changes result in ER swelling and escape of the protein-folding chaperones into the cytosol, suggesting that FFAs compromise ER membrane structure and integrity (45). Supplementation of cultured cells with FFAs produces oxidative stress, and treatment of these cells with antioxidants inhibits FFA-induced caspase-3 activation, ER dysfunction, and cell death (46–48). Supplementation of cultured fibroblasts, myoblasts, and β cells with FFAs leads to alterations in the structure and function of the ER that precede the activation of ER stress responses (49). Autophagy may play an important role in the response to ER stress and has been implicated as a con-

**FIGURE 1. Autophagy in response to stress induced by nutrient starvation or lipotoxicity.** To promote autophagosome generation, cells were treated with 10 μM CQ for 4 h before collection. A, Western blotting analysis of LC3B in control (Con) and nutrient-starved MKN45 cells. Nutrient starvation was induced by culturing the cells in EBSS for 6 h. Quantitative data of optical band densitometry are shown. Data are mean ± S.D. (n = 3). *, p < 0.05. B, Western blotting analysis of LC3B in lipotoxic stress MKN45 cells. Lipotoxic stress was induced by treating the cells with 0.25 mM palmitic acid for 6 h. Quantitative data of optical band densitometry are shown. Data are mean ± S.D. (n = 3). *, p < 0.05. C, analysis of autophagy by observation of the immunofluorescence of LC3B (green) in MKN45 cells treated as described in A and B. The nuclei were counterstained with DAPI (blue). Scale bars = 30 μm.
A previous report has shown that the induction of autophagy by palmitic acid is regulated via a PKC-mediated signaling pathway that is independent of mTOR (50).

In this study, the results demonstrated that autophagy induced by nutrient starvation or lipotoxicity was controlled by the mTOR or p8 signaling pathway, respectively. Notably, p8 responded to ER stress and up-regulated autophagy via the p53-PKCα-mediated signaling pathway. Overall, the results presented here indicated that the modulation of autophagy by p8 was involved in the ER stress response of cells.

**Experimental Procedures**

**Reagents and Antibodies**—Chloroquine (CQ) and palmitic acid (Sigma) and pifithrin α (PFTα) (Selleckchem, Houston, TX) were used. The primary antibodies used in this study were as follows: polyclonal anti-p-AMPK (Epitomics, Burlingame, CA, catalog no. 3930-1), polyclonal anti-p70s6k (Cell Signaling Technology, Leiden, The Netherlands, catalog no. 9205s), polyclonal anti-AMPK (Cell Signaling Technology, catalog no. 2532), polyclonal anti-p70s6k (Epitomics, catalog no. 1175-1), polyclonal anti-α-tubulin (Sigma, catalog no. T6199), polyclonal anti-p8 (Abcam, Cambridge, UK, catalog no. ab46889; Santa Cruz Biotechnology, catalog no. sc-30184), polyclonal anti-LC3B (Sigma, catalog no. L7543), polyclonal anti-p-PERK (Cell Signaling Technology, catalog no. 3179), polyclonal anti-p-PP2 (Cell Signaling Technology, catalog no. 5683), polyclonal anti-ATF6 (Abcam, catalog no. ab371149), polyclonal anti-parkin (Abcam, catalog no. ab77924), polyclonal anti-p-PKCα (Millipore, Nottingham, UK, catalog no. 06-822), polyclonal anti-PKCα (Cell Signaling Technology, catalog no. 2056), polyclonal anti-p-p53 (Cell Signaling Technology, catalog no. 9284P), and polyclonal anti-p53 (Epitomics, catalog no. 1026-1).

**Cell Lines and Cell Culture**—Human gastric cancer cells (MKN45) and HeLa cells were maintained separately in RPMI 1640 medium (Corning, New York, NY) or DME (Corning).
containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Sigma), which was defined as complete medium in this study. The cells were cultured at 37 °C in a 5% CO₂ atmosphere.

Transfection—p53 siRNA (Santa Cruz Biotechnology, catalog no. sc-29435, a pool of 3 target-specific 19- to 25-nt siRNAs) and p8 siRNAs (Ribobio, catalog no. Q000004927-1-A, a kit containing three target-specific, 19- to 25-nt siRNAs) were used to knock down p53 and p8 gene expression. The p8, p53, and control siRNA (100 pm) were transfected into MKN45 cells using siRNA transfection reagent (Santa Cruz Biotechnology) 42 h before palmitic acid treatment, according to the instructions of the manufacturer. The cells were collected after palmitic acid treatment for 6 h.

Lentiviral Package and Transduction—On the basis of the sequence of the human p8 gene in GenBank, the EGFP-p8 over-expression plasmid was synthesized and transfected with a viral packaging plasmid (pVSVG and p/8.91) into HEK293T cells overnight, and the viral supernatant was collected 48 h later. The viral supernatant was filtered through a 0.45-μm filter, and the MKN45 cells were infected in the presence of 5 μg/ml Polybrene (Sigma) and selected for 3–7 days, depending on the level of EGFP expression. Fluorescent cell sorting was used to isolate EGFP-p8-positive cells.

Western Blotting—Total cell lysates were prepared from MKN45 and HeLa cells and quantified using the Bradford method. Each total protein sample (4 μg) was separated on SDS-PAGE gels and then transferred to PVDF membranes (Bio-Rad). After incubation of the membranes with primary antibodies at 4 °C overnight, the specific proteins were detected using the BM chemiluminescence Western blotting kit (Roche).
Immunofluorescence—MKN45 cells were fixed with 4% paraformaldehyde at 4 °C overnight and then washed with PBS. Fixed cells were blocked in antibody dilution buffer (PBS containing 0.25% Triton X-100 and 1% bovine serum albumin), and all subsequent staining was performed in the same buffer. The cells were labeled with primary antibody for 2 h and subsequently with Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody (Invitrogen Life Technologies) or Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody (Invitrogen Life Technologies) for 1.5 h. The nucleus was stained by incubation with DAPI for 15 min at room temperature.

Results

Autophagy Induced by Nutrient Starvation or Lipotoxicity Is AMPK/mTOR-dependent or -independent, Respectively—Recent studies have shown that the cytotoxic effects of fatty acids and nutrient starvation can induce autophagy (51–54). However, it is not known whether the two different stresses induce autophagy in the same manner. To answer this question, nutrient starvation and lipotoxicity were used to induce autophagy in MKN45 cells. Nutrient starvation was induced by culturing the cells in Earle’s balanced salt solution (EBSS), which lacks free amino acids and glucose, and lipotoxicity was induced by supplementation of the culture medium with palmitic acid. To measure the autophagic flux in the cells, we used CQ to block the lysosomal function and the late degradation stage of autophagy (55). The results of Western blotting and immunofluorescence showed that cells cultured in the EBSS had a higher amount of lipidated LC3B than control cells cultured in complete medium, indicating that autophagy was increased in response to nutrient starvation for 6 h (Fig. 1, A and C). Similarly, autophagy was also induced in cells treated with palmitic acid for 6 h (Fig. 1, B and C).

The AMPK/mTOR signaling pathway has been well established as the key regulator of the autophagic process. To further examine the differences in autophagy induced by nutrient starvation and lipotoxic stress, the levels of AMPK/mTOR were analyzed by Western blotting. Furthermore, p70s6k is a direct substrate of mTOR, and its phosphorylation status can be used as an indicator of mTOR activity (56). The results showed that the level of p-AMPK was increased in response to nutrient starvation and that the level of p-p70s6k was decreased, whereas palmitic acid-induced lipotoxic stress in MKN45 cells had no effect on the levels of p-AMPK or p-p70s6k (Fig. 2A). Furthermore, MKN45 cells were treated with compound C, an inhibitor of p-AMPK. As shown in Fig. 2B, autophagy was increased in both palmitic acid- and EBSS-treated cells without compound C treatment, and the level of p-AMPK was increased under nutrient starvation but not under lipotoxicity stress (Fig. 2B, groups 1 and 2, lanes 1–6). Moreover, autophagy was increased in lipotoxic cells but not in starved cells under treatment with compound C (Fig. 2B, groups 3 and 4, lanes 7–12). The results indicated that autophagy induced by...
nutrient starvation was regulated by the AMPK/mTOR signaling pathway and autophagy induced by lipotoxicity was AMPK/mTOR-independent.

**Autophagy Induced by Lipotoxicity Is Controlled by p8 and Is Independent of the AMPK/mTOR Signaling Pathway**—As a key player in the cellular stress response, p8 is also involved in autophagy induced by lipopolysaccharide treatment of vascular endothelial cells (36–37). To further understand the autophagy mechanism induced by nutrient starvation and lipotoxic stress, p8 was analyzed in the two types of autophagy by Western blotting and immunofluorescence. As shown in Fig. 3, palmitic acid treatment increased the p8 protein level. In contrast, starvation-induced autophagy had no effect on the p8 level in MKN45 cells. This result suggested that p8 was involved in induction of autophagy by lipotoxicity but not nutrient starvation.

To explore the function of p8 in the regulation of autophagy, we constructed a MKN45 cell line stably overexpressing p8 using a lentivirus system in which EGFP-fused p8 was overexpressed successfully (Fig. 3, A and B). Western blotting analysis showed that the protein level of LC3B in palmitic acid-treated cells was reduced after p8 RNAi compared with control cells (Fig. 4C). Therefore, autophagy was down-regulated by knockdown of p8 in cells pretreated with palmitic acid. On the other hand, p8 expressed a very low basal level in MKN45 cells without treatment of palmitic acid. The results indicated that p8-regulated lipotoxicity-induced autophagy but was not involved in the basal regulation of autophagy.

**Autophagy Regulated by p8 or AMPK/mTOR Is in Response to the PERK/ATF6-mediated ER Stress of Lipotoxicity or the Parkin-mediated Mitochondrial Stress of Nutrient Starvation, Respectively**—To further examine the differences between the two types of autophagy induced by nutrient starvation or lipotoxic stress, the levels of phosphorylated PERK (p-PERK) and ATF6 (both of which are involved in ER stress-mediated autophagy) and parkin (which is involved in mitochondrial stress-mediated autophagy) were measured.
ATF6 (both of which were involved in ER stress-induced autophagy (30–33), and parkin (which was involved in mitochondrial stress-induced autophagy (57–60)) were determined in MKN45 cells exposed to both stresses. Western blotting analyses revealed that nutrient starvation increased the level of parkin but did not affect the levels of p-PERK or ATF6 (Fig. 5A). By contrast, palmitic acid treatment or overexpression of p8 increased the levels of p-PERK and ATF6 but had no effect on the level of parkin (Fig. 5B and C). These results suggested that autophagy induced by palmitic acid was an ER-stress response regulated by the p8 signaling pathway, whereas autophagy induced by nutrient starvation was a mitochondrial-stress response controlled by the AMPK/mTOR signaling pathway.

**Palmitic Acid Stress-induced Autophagy Is Regulated by p8 via p53 and PKCα Signaling**—In contrast to the pathway involved in the regulation of nutrient starvation-induced...
autophagy, the signaling pathway involved in the regulation of ER stress-induced autophagy remains largely unclear. Overexpression of p8 or exposure of MKN45 cells to palmitic acid, but not to nutrient starvation, increased the phosphorylation levels of both p53 (p-p53) and PKCα (p-PKCα).

To elucidate the p8-p53-PKCα signaling pathway, a p8 siRNAs was used to knock down p8 gene expression in which the mRNA level of p8 was reduced 75% and the protein level of p8 was also reduced 78% compared with control cells (Fig. 4, A–C). The results indicated that the levels of ATF6, p-PERK, p-p53, and p-PKCα were decreased significantly after p8 RNAi with pretreatment of palmitic acid in MKN45 cells (Fig. 7).

To confirm that autophagy regulated by p8 (Fig. 3, E and F) is mediated by p53 and PKCα, p53 was inhibited by PFTα (a p53 inhibitor), and the level of p-PKCα was decreased in p8-overexpressing MKN45 cells (Fig. 8A). The results showed that the autophagy induced by p8 overexpression was decreased, using PFTα by Western blotting and immunofluorescence analyses (Fig. 8, B and C). Furthermore, the same results were observed in other cell lines, including HT1080, MCF7, and HEK293T (data not shown), but with the exception of the HeLa cell line. The HeLa cell line was therefore used as a model to study the autophagy regulation pathway mediating by p53 because of the functional deletion of p53. The results showed that p8 overexpression in p53-dysfunctional HeLa cells had no effect on the level of LC3B (Fig. 8D), in contrast to its effects on MKN45 cells (Fig. 3, E and F).

To further demonstrate that the autophagy mechanism induced by palmitic acid was mediated by p53, we transfected p53 siRNA, which is a pool of three p53 target-specific, 19- to 25-nt siRNAs, into palmitic acid-treated and control MKN45 cells and found that both the mRNA and protein levels of p53 were decreased significantly (Fig. 9, A and B). Meanwhile, Western blotting analyses showed that autophagy was reduced and that the levels of p53, ATF6, p-PERK, and p-PKCα were decreased after p53 RNAi in palmitic acid-treated MKN45 cells (Fig. 9, C and D). Overall, these results indicated that p8 regulated autophagy in response to ER stress via the p53 and PKCα signaling pathways.

On the basis of the results described above, we propose a tentative model of palmitic acid stress-mediated autophagy and regulation of the p8 signaling pathway (Fig. 10). According to this model, p8 expression is up-regulated in response to ER stress such as lipotoxicity and activates p53 via phosphorylation. In turn, p-p53 induces the phosphorylation and activation of PKCα, leading to increased expression of p-PERK and ATF6 and subsequent initiation of autophagy.
Accumulating studies have shown that the regulation of autophagy is implicated in the physiopathology of major diseases such as cancer and neurodegenerative disorders. Specifically, autophagy induced by lipotoxicity has been implicated as one of the contributing factors to diseases such as obesity, diabetes, and non-alcoholic fatty liver, which refers to the cytotoxic effects of excess fat accumulation in cells. In this study, we identified an mTOR-independent signaling pathway in which p8 regulated autophagy in response to ER stress induced by lipotoxicity. Moreover, we showed that palmitic acid induced autophagy by p53-PKCα-mediated signaling, which differed from mTOR-dependent autophagy in response to stress induced by nutrient starvation in MKN45 cells. Furthermore, similar results were observed in other cell lines, including a fibrosarcoma cell line (HT1080), breast cancer cell line (MCF7), and embryonic kidney cell line (HEK293T) (data not shown). It is believed that autophagy in response to different types of cellular stress is regulated by various molecular signaling pathways.

Autophagy is a selective process that degrades various organelles in response to specific stimuli. Maintenance of organelle quality and quantity is critical for cellular homeostasis and adaptation to variable environments. Okamoto (20) has demonstrated that this type of control is achieved by selective elimination of organelles, such as the peroxisome, mitochondrion, lipid droplet (the structure surrounded by a phospholipid monolayer), lysosome, nucleus, and ER, and even nonmembranous structures, such as ribosomes, via autophagy, termed organellophagy. Sasaki and Yoshida (61) have summarized the

**Discussion**

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**FIGURE 9.** Autophagy was down-regulated by knockdown of p53 in cells pretreated with palmitic acid. Quantitative data of optical band densitometry are shown. Data are mean ± S.D. (n = 3). *, p < 0.05. A, p53-specific siRNA (100 pm) was transfected into MKN45 cells with siRNA transfection reagent (Santa Cruz Biotechnology) for 48 h. Quantitative RT-PCR of p53 in control (Con) and p53 interference (p53i) MKN45 cells with or without treatment of 0.25 mM palmitic acid for 6 h was performed. B, representative Western blotting analyses and quantitative data of p53 in control and p53 interference MKN45 cells with or without 0.25 mM palmitic acid treatment for 6 h. C, representative Western blotting and quantitative data of LC3B in control and p53 interference MKN45 cells after 0.25 mM palmitic acid treatment for 6 h. To promote autophagosome generation, the cells were treated with 10 μM CQ for 4 h before collection. D, representative Western blotting and quantitative data of p53, ATF6, PERK (p-PERK) and PKCα (p-PKCα) in control and p53 interference MKN45 cells after 0.25 mM palmitic acid treatment for 6 h.

**FIGURE 10.** Proposed model of the p8-regulated autophagy signaling pathway in response to palmitic acid stress.

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**FIGURE 10.** Proposed model of the p8-regulated autophagy signaling pathway in response to palmitic acid stress.
p8 Regulates Autophagy via an mTOR-independent Pathway

Homeostatic mechanism that regulates the capacity of each organelle according to the cellular demands as organelle autoregulation. Both nutrient starvation and lipotoxicity have been shown to cause an imbalance in cellular homeostasis. The difference is that nutrient starvation mainly causes mitochondrial damage, where ATP is synthesized through oxidative phosphorylation, whereas the lipotoxic effect causes ER damage, where the secretory and membrane proteins are synthesized and folded with the assistance of the ER chaperones. The lipotoxicity caused by overloading of fibroblasts, myoblasts, and β cells with saturated FFAs leads to alterations in the structure and function of the ER that precede activation of the ER stress response (45, 62). Autophagy induced by ER stress may play an important role in maintaining ER homeostasis by segregating and/or degrading a part of this structure in response to various ER stresses (30).

Previous studies have shown that autophagy was positively regulated by p8 in human glioma cells and umbilical vein endothelial cells; however, p8 deficiency resulted in increased autophagy in H9C2 and U20S cells (37, 63, 64). These findings may suggest a cell-specific role of p8 in autophagy. Here we found that p8 overexpression up-regulated autophagy in MKN45 cells. However, p8 was not involved in the induction of autophagy by nutrient starvation, which activated the AMPK/mTOR signaling pathway. Therefore, we concluded that p8 controlled autophagy to promote cell survival in response to specific environmental stresses such as lipotoxicity. This proposal may also be supported by a previous study in which p8 mRNA expression in pancreatic cells was induced in response to the lipotoxic stress of lipopolysaccharides (65). Overall, our results indicated that there were two different regulation pathways of autophagy in response to palmitic acid treatment and nutrient starvation: the p8- and AMPK/mTOR-mediated pathways.

In mammalian cells, ATF6 and PERK sensed ER stress and triggered a general transcriptional response that affected the level of ATG12, which was involved in autophagy. The ATG12-ATG5 (ATG16) complex facilitated the lipidation of ATG8 and subsequent autophagy induction (30). However, it is not clear how ATF6 and PERK are activated in response to ER stress and whether the activation signaling pathway is dependent on the stimulation of AMPK and the inhibition of mTOR kinases. Here we found that p8 regulated the activation of ATF6 and PERK via the p53 and PKCα signaling pathways in response to palmitic acid-mediated stress. Moreover, this process was independent of mTOR signaling. These findings provide the basis for further studies of mTOR-independent autophagy, such as the signaling pathway induced in response to lipotoxicity.

**Author Contributions**—S. N. J., C. L., and W. J. Y. designed the research. S. N. J., C. L., D. F. C., A. Q. L., L. D., L. Z., L. L. Z., and F. Y. performed the work. J. S. Y. contributed new reagents and analytical tools. J. S. Y. and C. L. analyzed the data. S. N. J., C. L., and W. J. Y. wrote the paper.

**References**

1. Klionsky, D. J., Abdalla, F. C., Abeliovich, H., Abraham, R. T., Acevedo-Arozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., Aguirre-Ghiso, J. A., Ahn, H. J., Ait-Mohamed, O., Ait-Si-Ali, S., Akematsu, T., and Akira, S. (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8, 445–544
2. Mizushima, N., and Levine, B. (2010) Autophagy in mammalian development and differentiation. Nat. Cell Biol. 12, 823–830
3. Levine, B., Mizushima, N., and Virgin, H. W. (2011) Autophagy in immunity and inflammation. Nature 469, 323–335
4. Zhou, S., Zhao, L., Kuang, M., Zhang, B., Liang, Z., Yi, T., Wei, Y., and Zhao, X. (2012) Autophagy in tumorigenesis and cancer therapy: Dr. Jekyll or Mr. Hyde? Cancer Lett. 323, 115–127
5. White, E. (2012) Deconvoluting the context-dependent role for autophagy in cancer. Nat. Rev. Cancer 12, 401–410
6. Mizushima, N., Levine, B., Cuervo, A. M., and Klionsky, D. J. (2008) Autophagy fights disease through cellular self-digestion. Nature 451, 1069–1075
7. Panda, P. K., Mukhopadhyay, S., Das, D. N., Sinha, N., Naik, P. P., and Bhatia, S. K. (2015) Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics. Semin. Cell. Dev. Biol. 39, 43–55
8. Levine, B., and Deretic, V. (2007) Unveiling the roles of autophagy in innate and adaptive immunity. Nat. Rev. Immunol. 7, 767–777
9. He, C., and Klionsky, D. J. (2009) Regulation mechanisms and signaling pathways of autophagy. Annu. Rev. Genet. 43, 67–92
10. Yorimitsu, T., and Klionsky, D. J. (2005) Autophagy: molecular machinery for self-eating. Cell Death Differ. 12, 1542–1552
11. Levine, B., and Klionsky, D. J. (2004) Development by self-digestion: molecular mechanisms and biological function of autophagy. Dev. Cell 6, 463–477
12. Kim, J., Kundu, M., Viollet, B., and Guan, K. L. (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. 13, 132–141
13. Mizushima, N. (2010) The role of the Atg1/ULK1 complex in autophagy regulation. Curr. Opin. Cell Biol. 22, 132–139
14. Xu, K., Liu, P., and Wei, W. (2014) mTOR signaling in tumorigenesis. Biochim. Biophys. Acta 1846, 638–654
15. Wong, P. M., Puente, C., Ganley, I. G., and Jiang, X. (2013) The ULK1 complex. Autophagy 9, 124–137
16. Lee, J., Giordano, S., and Zhang, J. (2012) Autophagy, mitochondria and oxidative stress: cross-talk and redox signaling. Biochem. J. 441, 523–540
17. Rabinowitz, J. D., and White, E. (2010) Autophagy and metabolism. Science 330, 1349–1348
18. Chen, Y., and Klionsky, D. J. (2011) The regulation of autophagy: unanswered questions. J. Cell Sci. 124, 161–170
19. Mizumura, K., Choi, A. M., and Ryter, S. W. (2014) Emerging role of selective autophagy in human diseases. Front. Pharmacol. 5, 244
20. Okamoto, K. (2014) Organellophagy: eliminating cellular building blocks via selective autophagy. J. Cell Biol. 205, 435–445
21. Yorimitsu, T., Nair, U., Yang, Z., and Klionsky, D. J. (2006) Endoplasmic reticulum stress triggers autophagy. J. Biol. Chem. 281, 30299–30304
22. Deeg, S., Saveljeva, S., Gorman, A. M., and Samali, A. (2013) Stress-induced self-cannibalism: on the regulation of autophagy by endoplasmic reticulum stress. Cell. Mol. Life Sci. 70, 2425–2441
23. Spassieva, S. D., Mullen, T. D., Townsend, D. M., and Obeid, L. M. (2009) Disruption of ceramide synthesis by CerS2 down-regulation leads to autophagy and the unfolded protein response. Biochem. J. 424, 273–283
24. Ichimura, Y., Kizisako, T., Takao, T., Satomi, Y., Shimoniishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Komimani, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) A ubiquitin-like system mediates proteins lipidation. Nature 408, 488–492
25. Kabeya, Y., Mizushima, N., Yamamoto, A., Oshitani-Okamoto, S., Ohsumi, Y., and Yoshimori, T. (2004) LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J. Cell Biol. 165, 230–239
26. Ding, W. X., Ni, H. M., Gao, W., Hou, Y. F., Melan, M. A., Chen, X., Stolz, D. B., Shao, Z. M., and Yin, X. M. (2007) Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival. J. Biol. Chem. 282,
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4702–4710

28. Fujita, E., Kouroku, Y., Isoai, A., Kumagai, H., Misutani, A., Matsuda, C., Hayashi, Y. K., and Momoï, T. (2007) Two endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant dystferlin: ubiquitin/proteasome ERAD (I) and autophagy/lysosome ERAD (II). Hum. Mol. Genet. 16, 618–629

29. Bernales, S., McDonald, K. L., and Walter, P. (2006) Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS Biol. 4, e423

30. Cebollero, E., Reggiori, F., and Kraft, C. (2012) Reticulophagy and ribophagy: regulated degradation of protein production factories. Int. J. Cell Biol. 2012, 182834

31. Ogata, M., Hino, S., Saito, A., Morikawa, K., Kondo, S., Kanemoto, S., Murakami, T., Taniguchi, M., Tanii, I., Yoshinaga, K., Shiosaka, S., Hammarback, J. A., Uruno, F., and Imaiizumi, K. (2006) Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol. Cell. Biol. 26, 9220–9231

32. Okada, T., Yoshida, H., Akazawa, R., Negishi, M., and Mori, K. (2002) Distinct roles of activating factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. Biochem. J. 366, 585–594

33. Younce, C. W., and Kolattukudy, P. E. (2010) MCP-1 causes cardiomyoblast death via autophagy resulting from ER stress caused by oxidative stress generated by inducing a novel zinc-finger protein, MCPIP. Biochem. J. 426, 43–53

34. Mallo, G. V., Fiedler, F., Calvo, E. L., Ortiz, E. M., Vasseur, S., Keim, V., Morisset, J., and Iovanna, J. L. (1997) Cloning and expression of the rat p8 cDNA, a new gene activated in pancreas during the acute phase of pancreatitis, pancreatic development, and regeneration, and which promotes cellular growth. J. Biol. Chem. 272, 32360–32369

35. Goruppi, S., Bonventre, J. V., and Yki-Jarvis, J. M. (2002) Signaling pathways and late-onset gene induction associated with renal mesangial cell hypertrophy. EMBO J. 21, 5427–5436

36. Goruppi, S., and Iovanna, J. L. (2010) Stress-inducible protein p8 is involved in several physiological and pathological processes. J. Biol. Chem. 285, 1577–1581

37. Meng, N., Zhao, J., Su, L., Zhao, B., Zhang, Y., Zhang, S., and Miao, J. (2012) A butyrolactone derivative suppressed lipopolysaccharide-induced autophagy resulting from ER stress caused by oxidative stress generated by inducing a novel zinc-finger protein, MCPIP. Biochem. J. 426, 43–53

38. Maiuri, M. C., Tasdemir, E., Criollo, A., Morselli, E., Vicencio, J. M., Carlucci, R., and Kroemer, G. (2009) Control of autophagy by oncogene and tumor suppressor genes. Cell Death Differ. 16, 87–93

39. Vasseur, S., Hoffmeister, A., Garcia-Montero, A., Mallo, G. V., Feil, R., Kühbandner, S., Dajon, C. G., and Iovanna, J. L. (2002) p8-deficient fibroblasts grow more rapidly and are more resistant to Adriamycin-induced apoptosis and adiponectin resistance. J. Biol. Chem. 273, 6360–6369

40. Han, J. Y., Kang, M. J., Kim, K. H., Han, P. L., Kim, H. S., Ha, J. Y., and Son, J. H. (2015) Nitric oxide induction of parkin translocation in PETN-inhibited putative kinase 1 (PINK1) deficiency: functional role of neuronal nitric oxide synthase during mitophagy. J. Neurochem. 133, 10325–10335

41. Katsuma, S., Hatae, N., Yano, T., Ruile, Y., Kimura, M., Hirasawa, A., and Tsujimoto, G. (2005) Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroidenocyte cell line STC-1. J. Biol. Chem. 280, 19507–19515

42. Borradaile, N. M., Buhman, K. K., Listemberger, L. L., Magee, C. J., Morimoto, E. T., Ory, D. S., and Schaffer, J. E. (2006) A critical role for eukaryotic elongation factor 1A-1 in lipotoxic cell death. Mol. Biol. Cell 17, 770–778

43. Tueti, V. C., Ha, J. S., and Ha, C. E. (2011) Effects of human serum albumin complexed with free fatty acid on cell viability and insulin secretion in the hamster pancreatic β-cell line HIT-T15. Life Sci. 88, 810–818

44. Brown, H. A., and Young, J. D. (2014) Enhanced synthesis of saturated phospholipids is associated with ER stress and lipotoxicity in palmitate-treated hepatic cells. J. Lipid Res. 55, 1478–1488

45. Borradaile, N. M., Han, X., Harp, J. D., Gale, S. E., Ory, D. S., and Schaffer, J. E. (2006) Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. J. Lipid Res. 47, 2726–2737

46. Listemberger, L. L., Ory, D. S., and Schaffer, J. E. (2001) Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J. Biol. Chem. 276, 14890–14895

47. Salazar, M., Carracedo, A., Salanueva, I. J., Hernández-Tiedra, S., Lorente, M., Egia, A., Vázquez, P., Blázquez, C., Torres, S., García, S., Nowak, J., Fimia, G. M., Picentini, M., Cecconi, F., and Pandolfi, P. P. (2009) Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. J. Clin. Invest. 119, 1359–1372

48. Kong, D. K., Georgescu, S. P., Cano, A., Arrovizovitz, M. J., Iovanna, J. L., Patten, R. D., Kyrkiakis, J. M., and Goruppi, S. (2010) Deficiency of the transcriptional regulator p8 results in increased autophagy and apoptosis, and caused impaired heart function. Mol. Biol. Cell 21, 1335–1349

49. Jiang, Y. F., Vaccaro, M. I., Fiedler, F., Calvo, E. L., and Iovanna, J. L. (1999) Lipopolysaccharides induce p8 mRNA expression in vivo and in vitro. Biochem. Biophys. Res. Commun. 260, 686–690