Excessive ER-phagy mediated by the autophagy receptor FAM134B results in ER stress, the unfolded protein response, and cell death in HeLa cells

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ABSTRACT

Autophagy is typically a pro-survival cellular process that promotes the turnover of long-lived proteins and damaged organelles, but it can also induce cell death. We have previously reported that the small molecule Z36 induces autophagy along with autophagic cell death in HeLa cells. In this study, we analyzed differential gene expression in Z36-treated HeLa cells, and found that Z36-induced endoplasmic reticulum (ER)-phagy results in ER stress and the unfolded protein response (UPR). This result is in contrast to the common notion that autophagy is generally activated in response to ER stress and the UPR. We demonstrate that Z36 upregulates the expression levels of FAM134B, LC3, and Atg9, which together mediate excessive ER-phagy, characterized by forming increased numbers of autophagosomes with larger sizes. We noted that the excessive ER-phagy accelerates ER degradation and impairs ER homeostasis, and thereby triggers ER stress and the UPR, as well as ER-phagy-dependent cell death. Interestingly, overexpression of FAM134B alone in HeLa cells is sufficient to impair ER homeostasis and cause ER stress and cell death. These findings suggest a mechanism involving FAM134B activity for ER-phagy to promote cell death.

INTRODUCTION

Autophagy is a highly conserved physiological process, playing important roles in development, differentiation, immune defense, suppression of tumorigenesis and the prevention of neuronal degeneration in multicellular organisms (1-5). It is characterized by the formation of double-membrane autophagosomes which then fuse with lysosomes for the degradation of components inside. During starvation, autophagy is initiated nonselectively to degrade substrates and thus provide nutrients and energy for survival. Meanwhile, autophagy can function selectively to remove damaged organelles or aggregated proteins, as a quality control mechanism (6). A growing number of subcellular components are found to be cleared by selective autophagy, each is named after its specific target such as mitochondria (mitophagy) (7), aggregated proteins (aggrephagy) (8), and peroxisomes (pexophagy) (9), etc. The specificity of autophagy for each target is determined by the target specific autophagy receptors for autophagosome protein LC3. Receptors for mitophagy, such as BNIP3, NIX, and FUNDC1, have been studied most extensively, and they all bind to LC3 in a similar fashion through their short LIR (LC3-interacting region) motifs, but also with subtle differences (10). FAM134B is the first identified receptor for endoplasmic reticulum specific autophagy (ER-phagy) in mammalian cells (11), followed by SEC62, RTN3 and CCPG1 (12). They function in mediating ER turnover while maintain ER homeostasis.

Autophagy is usually considered as an essential pro-survival mechanism for cells during starvation or stress conditions (13,14). Nevertheless, in the past decade, many evidences have suggested that enforced over-activation of autophagy will lead to cell death in certain contexts. Autophagy is involved mechanistically in the death of developmental cells during the salivary gland destruction of Drosophila (15). In human ovarian epithelial cells expressing oncogene H-RAS, high levels of autophagy can lead to caspase-independent autophagic cell death (16). While in cells lacking intact apoptosis pathway, autophagy can also contribute to cell death, in the myeloma cells (17) or the murine embryonic fibroblasts (18). Autosis is a novel described form of autophagy-mediated cell death, which is characteristic by its unique morphological
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features and depends on the cellular Na+/K+-ATPase (19). Although there are many reports showing the regulatory role of autophagy during cell death (20-22), the mechanisms of autophagic cell death remain to be elucidated.

We have previously reported that a small molecule Z36 can induce Beclin1-dependent autophagy and autophagic cell death in HeLa cells (23). Z36-induced cell death showed no characteristic feature of apoptosis or necrotic-like cell death, and it can be inhibited by autophagy inhibitor 3-MA and CQ, or the knockdown of Beclin1, Atg5, and Atg12 genes (23,24). In this study, we show that Z36 treatment significantly upregulates FAM134B and LC3 in HeLa cells, which induces extensive ER-phagy that accelerates ER degradation, and in turn causes ER damage. Consequently, it triggers ER stress and the UPR, which further result in cell death. We also show that the overexpression of FAM134B alone has similar consequences for ER and causes cell death. These findings provide a novel mechanism for autophagy to result in cell death, and establish a new relationship between autophagy and ER stress, in contrast to the common perception of autophagy as the consequence of ER stress.

RESULTS

Z36 upregulates the expression of genes related to autophagy, ER stress and the UPR

To better understand the mechanism for Z36 to induce autophagy and cell death, we firstly compared the morphology of autophagosomes induced by Z36 to those induced by rapamycin (Rapa), which results in canonical protective autophagy (25). Under the fluorescence microscope, GFP-LC3 puncta in Z36-treated HeLa cells appeared to be obviously agglomerated, while the GFP-LC3 puncta were mainly small dots in the cells treated with Rapa (Figure 1A). Using transmission electron microscopy (TEM), we found that there are much more autophagosomes in Z36-treated HeLa cells, compared with those of Rapa. In Z36-treated cells, the estimated average number of autophagosomes per cell was 30, whereas there were 17 and 6 autophagosomes per cell in Rapa or DMSO (as control) treated cells, respectively (Figure 1B). Meanwhile, Z36 treatment led to much larger size for autophagosomes, as the average maximal cross-sectional diameters of autophagosomes in Z36, Rapa and DMSO treated cells were 1.20, 0.74, and 0.70 μm, respectively (Figure 1, C and D). It is reported that Atg8 (homologous of LC3 in yeast) regulates the size of autophagosomes (26), while the level of Atg9 determines the number of autophagosomes (27,28). We have analyzed the expression of these two genes at both mRNA and protein levels. Indeed, the expression levels of both LC3 and Atg9 were significantly higher in Z36-treated cells, than those of Rapa (Figure 1, E and F).

To gain further insights into the regulation of autophagy in cell death, high-throughput RNA-sequencing (RNA-seq) and differential genes expression analysis were performed on HeLa cells treated with DMSO (as control), Z36, and Rapa. The sequencing generated more than 30 million reads for each sample. The majority of the reads (~95% for all samples) were aligned to human genome, and over 80% of all the sequence reads were assembled against human genes (Table 1). Differentially expressed genes (DEGs) analysis showed that there are 3588 DEGs with over 2 folds changes (\(|\log_2\text{fold change}| > 1 & P \text{ value} < 0.05\) in Z36-treated cells versus those of DMSO, with 1654 genes upregulated with the largest log2 scale fold changes of 5.9, and 1934 genes down-regulated with the largest log2 scale fold change of 4.9 (Table 2 & Sheet S1). On the contrary, there were only 58 DEGs for cells treated with Rapa, with the highest log2 scale fold changes less than 3 (Table 2 & Sheet S2).
Noteworthily, expression levels of autophagic genes were significantly changed in Z36-treated cells, and the change pattern was different from Rapa-treated ones (Figure S1A). 8 of the ATG genes were upregulated for more than 2 folds (log2 > 1) after Z36 treatment, while Rapa only caused small changes for the ATG genes, with the highest log2 change of 0.7 (Figure S1B). These data indicate that Z36 treatment leads to significant modulation of a large number of genes at transcriptional level.

Gene Ontology (GO) enrichment analysis of Z36-resulted DEGs showed that they are mainly involved in pathways associated with ER stress and the unfolded protein responses, as well as lipid biosynthesis and starvation responses, etc (Table 3). Most of these pathways were related to ER stress and the UPR responses, with a majority of ER stress and the UPR genes affected by Z36 treatment (Figure S2A & Table 4). The ER transmembrane proteins, IRE1 (ERN1), PERK (EIF2AK3) and ATF6, acting as ER stress sensors to activate the UPR signaling (29) were upregulated to 4.7, 3.3 and 1.5 folds by Z36, respectively. The genes of some prominent proteins involved in the UPR, including multifunctional transcription factor CHOP (DDIT3) and ER chaperone proteins GRP78 (HSPA5 or Bip) were also highly upregulated in Z36-treated cells (Table 4 & Figure S2, B and C).

All these data indicate that Z36 stimulates strong responses of multiple cellular processes, different from the typical autophagy inducer Rapa. Especially, ER stress and the UPR pathways are highly activated in Z36-treated cells.

**Z36-induced ER stress and the UPR result in cell death, but not autophagy**

To verify ER stress onset, typical markers of ER stress and the UPR, including PERK, IRE1α, eIF2α, and CHOP were examined by western blot. The results showed that the phosphorylation of PERK, IRE1α and eIF2α, and the level of CHOP are increased after Z36 treatment (Figure 2A), confirming that ER stress is indeed activated by Z36 treatment. We then used ER stress inhibitor 4-phenylbutyric acid (4-PBA), a chemical chaperon known to stabilize protein conformation and improve protein folding capacity of ER (30), to inhibit Z36-induced ER stress. Western blot results showed that the levels of ER stress markers are lowered in Z36-treated cells in the presence of 4-PBA, indicating that the degree of ER stress is reduced (Figure 2A). Z36-induced cell death was also reduced from 57% to 37% with 4-PBA treatment (Figure 2B). However, 4-PBA treatment had no obvious effect on the conversion of LC3I to LC3II (Figure 2A). Consistently, there was no significant change for the GFP-LC3 punctate distribution in Z36-treated HeLa cells with or without 4-PBA (Figure 2C). Obviously, attenuating ER stress by 4-PBA reduces Z36-induced cell death, but not affects Z36-induced autophagy.

Among the three UPR pathways, the mRNA expression of IRE1 and PERK were highly affected due to Z36 treatment (Table 4), which promoted us to investigate the roles of these two pathways in Z36-induced autophagy and cell death. GSK2656157, a selective catalytic PERK inhibitor, was used to inhibit PERK pathway of the UPR (31). Western blot analysis showed that the phosphorylation of eIF2α and level of CHOP are reduced by the treatment of GSK2656157 in Z36-treated cells (Figure 3A), and the cell death rate was also decreased from 53% to 32% (Figure 3C). It indicates that the inhibition of PERK pathway with GSK2656157 can inhibit Z36-induced cell death. However, GSK2656157 treatment had no effect on autophagy, as there were no significant change on the conversion of LC3 or GFP-LC3 puncta distribution (Figure 3, A and B). We also used shRNA to knockdown PERK in HeLa cells.
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As expected, the level of p-eIF2α and CHOP were reduced, and the cell death rate was dropped from 61% to 36% in Z36-treated cells with PERK knockdown. Again, there was no change in LC3 conversion and GFP-LC3 puncta distribution (Figure 3, D-F). Meanwhile, re-expression of PERK in PEKR knockdown cells restored the PERK pathway signaling, and significantly increased Z36-induced cell death rate to 86%. Whereas, rescuing PERK still had no effect on LC3 (Figure 3, D-F). CHOP is known to be pro-death in the PERK-mediated eIF2α phosphorylation pathway (32,33), and the mRNA level of CHOP was increased by 10 folds due to Z36 treatment (Table 4). We used RNA interference to reduce the expression of CHOP, and the cell death rate was dropped from 57% to 37% (Figure 3, G and H). These results indicate that PERK-CHOP pathway of the UPR promotes cell death for Z36-induced ER stress.

We next used STF083010, a small-molecule inhibitor of IRE1, to inhibit the endonuclease and mRNA splicing activity of IRE1 (34). Z36 treatment resulted in the upregulation of XBP1 splicing (XBP1s), while the splicing was reduced with the addition of STF083010 (Figure 4, A and B). Interestingly, we found that the Z36-induced cell death rate is increased from 51% to 66% due to the inhibition of IRE1 endonuclease activity (Figure 3D), implying that the IRE1 pathway plays a pro-survival role in Z36-induced ER stress. Meanwhile, western blot showed that the level of CHOP is further upregulated due to STF083010 treatment (Figure 4A), indicating Z36-induced ER stress is diminished when autophagy is inhibited. Meanwhile, the cell death rate was lowered from 63% to 34% (Figure 4B). We also tried to inhibit autophagy with shRNA to knockdown of Atg5, which facilitates the conversion of LC3I to LC3II without affecting PERK-CHOP pathway.

Z36-induced autophagy results in ER stress and cell death

To further study the relationship between Z36-induced autophagy and ER stress, autophagy inhibitor 3-methyladenine (3-MA) was used to pretreat HeLa cells before Z36 treatment. Western blot analysis showed that 3-MA treatment inhibits the LC3 conversion (Figure 5A), and the GFP-LC3 dot areas are also shrank in HeLa cells treated with Z36 (Figure S3A). Interestingly, we found that the phosphorylation of IRE1α, PERK, eIF2α and level of CHOP are also decreased by 3-MA (Figure 5A), indicating Z36-induced ER stress is diminished when autophagy is inhibited. Meanwhile, the cell death rate was lowered from 63% to 34% (Figure 5B). We also tried to inhibit autophagy with shRNA to knockdown of Atg5, which facilitates the conversion of LC3I to LC3II.
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LC3II (37) (Figure 5C). Western blot showed that the conversion of LC3I to LC3II is decreased, consistent with the reduced GFP-LC3 punctate distribution in Atg5 knockdown cells treated with Z36 (Figure 5C & Figure S3B). Meanwhile, the levels of p-IRE1α, p-PERK, p-eIF2α and CHOP were reduced due to Atg5 knockdown (Figure 5C), indicating that Z36-induced ER stress and the UPR are repressed due to the inhibition of autophagy. Consistently, the cell death rate was decreased from 61% to 42%, presumably because of reduced ER stress and the UPR (Figure 5D). When we re-expressed Atg5 in Atg5 knockdown cells, the levels of Z36-induced LC3 conversion and GFP-LC3 dots distribution were restored (Figure 5C & Figure S3B). Recue of Atg5 also enhanced the ER stress-UPR signaling and Z36-induced cell death. (Figure 5, C and D).

Furthermore, we inhibited Z36-induced autophagic flux with chloroquine (CQ), which neutralizes the lysosomal pH and prevents both fusion of autophagosome with lysosome and lysosomal protein degradation. As expected, CQ treatment led to more GFP-LC3 puncta visible in Z36-treated cells, and the level of LC3II was elevated (Figure S3C & Figure 5E). Meanwhile, the inhibition of autophagy flux resulted in reduced levels of p-PERK, p-eIF2α and CHOP in Z36-treated cells (Figure 5E). Unexpectedly, the level of p-IRE1α was increased due to the block of autophagic flux. As a result, the cell death rate was dramatically reduced from 66% to 19% due to CQ treatment (Figure 5F), which is consistent with the above results that the PERK arm promotes cell death and IRE1 pathway is pro-survival for Z36-induced ER stress and the UPR.

Taken together, our data clearly demonstrate that Z36-induced autophagy results in ER stress and the UPR, in contrast to the fact that autophagy is commonly perceived as a consequence of ER stress and the UPR (38,39).

**FAM134B mediated ER-phagy is the pre-requisite of ER stress and cell death induced by Z36**

In our RNA-seq data, FAM134B was significantly upregulated (6 folds) after Z36 treatment (Table 4), which was confirmed from real-time PCR analysis (Figure S4). FAM134B is a receptor for ER-phagy, and it has been demonstrated that overexpression of FAM134B or its yeast counterpart Atg40 directly promotes ER-phagy and causes ER fragmentation (11,40). We examined the ER morphology of Z36-treated HeLa cells using TEM, and found that there are indeed pronounced morphological changes and fragmentation of ER (Figure 6A). ER whorls were also observed inside autolysosomes (Figure 6B). These are the characteristic morphology reported for FAM134B mediated ER-phagy (11).

We used shRNA to mediate the knockdown of FAM134B in HeLa cells (Figure 6C), and found that it led to attenuated LC3 conversion and reduced GFP-LC3 puncta in Z36-treated cells, indicating that Z36-induced autophagy is suppressed (Figure 6, C and D). Western blot analysis also showed that the levels of ER stress and the UPR markers are reduced, suggesting that the Z36-induced ER stress is also attenuated due to the knockdown of FAM134B (Figure 6C). Consistently, Z36-induced cell death rate was dropped as well, from 57% to 39% (Figure 6E). It is noticed that FAM134B protein is reduced by 35% due to shRNA knockdown, while it is only elevated by ~20% due to Z36 treatment for HeLa cells with or without transfection of shRNA, significantly lower than the elevation at mRNA level.

Next, we performed transient overexpression of mCherry-FAM134B in HeLa cells, together with GFP-LC3, and it was observed that both mCherry and GFP puncta are formed and they are mostly co-localized.
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together, consistent with the previous report that the overexpression of FAM134B alone can promote ER-phagy. Z36 treatment led to further accumulation larger GFP puncta which are predominantly co-located with mCherry puncta, along with the appearance of mCherry only puncta (Figure 7A). As mCherry-FAM134B resides on ER, it is possible that the mCherry only puncta are engulfed ER fragments from autophagosomes bound by the endogenous LC3, or resulted from the extinction of GFP fluorescence owing to the acidic milieu inside autolysosomes. In addition, Z36 treatment promoted the colocalization of LC3 positive autophagosomal puncta with Rtn4 positive puncta from tubular ER or Climp-63 positive puncta from sheet-like cisternal ER, based on immunofluorescence analysis (Figure S5). These are consistent with the previous report that the overexpression of FAM134B initiates ER-phagy, and it affects both Climp-63 positive sheets and Rtn4 enriched tubules/edges of sheets in MEF cells (11). Taken together, it appears that Z36-induced autophagy is predominant ER-phagy in nature.

In order to study the effect of FAM134B mediated ER-phagy on ER stress and cell death, we constructed FAM134B LIR-motif mutant (FAM134B-mut) in which the LIR sequence “FELL” was replaced with “GEGG”, so that it can no longer bind to LC3. It was observed that there are almost no mCherry and GFP dots in HeLa cells overexpressing mCherry-FAM134B-mut and GFP-LC3, while only GFP puncta can be observed with Z36 treatment (Figure 7A), indicating that the ability of FAM134B to induce ER-phagy was abolished by the mutation. Western blot analysis showed that the overexpression of FAM134B further increases the conversion of LC3I to LC3II in Z36-treated cells, while the levels of p-IRE1α, p-PERK, p-eIF2α and CHOP are increased as well. On the contrary, no obvious differences were observed for the LC3 conversion and ER stress-UPR markers in Z36-treated cells, with or without the overexpression of FAM134B-mut (Figure 7, B and C). Cell death analysis showed that the overexpression of FAM134B boosted Z36-induced cell death rate from 65% to 85%, while the mutant protein took no change in cell death (Figure 7D).

It is interesting to notice that the overexpression of FAM134B alone in HeLa cells can result in enhanced ER stress and cell death, as well as LC3 conversion and GFP-LC3 puncta distribution (Figure 7, B-D). We further investigated the effects of the overexpression of FAM134B and its mutant on cells without Z36 treatment. Overexpression of FAM134B alone indeed increased the cell death rate from 15% to 29%, which could be suppressed by 3-MA and 4-PBA, while the overexpression of FAM134B-mut had no obvious effect on cell death (Figure 8A). Similar to that of Z36 treatment, the overexpression of FAM134B alone upregulated the mRNA levels of LC3 and Atg9, which are genes regulating the size and number of autophagosomes, respectively. The mRNA levels of the UPR genes XBP1s, IRE1α and CHOP were also upregulated (Figure 8B), in line with the data of Z36-treated cells (Figure 1F & Table 4). As expected, the expression of these genes were not affected by the overexpression of FAM134B-mut, except that the mRNA of CHOP was slightly higher (Figure 8B). Although the mRNA of PERK was not affected by the overexpression of wily-type FAM134B (Figure 8B), western blot analysis revealed that the phosphorylated PERK is upregulated, along with the UPR markers p-IRE1α, p-eIF2α, and CHOP, and the conversion of LC3I to LC3II is enhanced as well (Figure 8C). Again, no significant changes were observed for these proteins in the FAM134B-mut transfected cells (Figure 8C). In accordance with the results of Z36 treatment, the upregulation of ER stress and the UPR markers due to FAM134B overexpression could be inhibited by autophagy
inhibitor 3-MA or ER stress inhibitor 4-PBA, while 4-PBA did not affect the conversion of LC3 (Figure 8C). All these data further support that Z36-induced autophagy should be principally FAM134B mediated ER-phagy, which is directly involved in Z36-induced ER stress, the UPR and cell death.

Furthermore, we investigated the ER turnover by monitoring the degradation of ER structural proteins Rtn4 and Climp-63. The results showed that Z36 treatment leads to progressive degradation of Rtn4 and Climp-63 with time, while they are relative unchanged for control cells. Inhibition of autophagy by 3-MA significantly suppressed the degradation rates, while ER stress inhibitor 4-PBA had no effect (Figure 8D). Similarly, increasing degradation of Rtn4 and Climp-63 were also observed in cells overexpressing wild-type FAM134B without Z36 treatment, but not for FAM134B-mut. Autophagy inhibitor 3-MA could inhibit the degradation of Rtn4 and Climp-63, while ER stress inhibitor 4-PBA failed (Figure 8E), in cells overexpressing FAM134B.

Taken together, it is apparent that Z36 treatment upregulates of FAM134B expression and the conversion of LC3I to LC3II, which results in massive selective ER-phagy. The excessive ER-phagy degrades ER and impairs ER homeostasis, then causes ER stress and the unfolded protein responses and cell death. Therefore, it is ER-phagy mediated by FAM134B that is central to Z36-induced ER stress, the UPR and cell death.

**DISCUSSION**

In this study, we demonstrate that Z36 upregulates the expression levels of ER-phagy receptor protein FAM134B, along with key autophagic proteins LC3 and Atg9, which are responsible for the size and number of autophagosomes, respectively. Z36 also increases the conversion of LC3I to LC3II. As a result, Z36 treatment induces excessive ER-phagy in HeLa cells, which further leads to ER stress and the UPR. This is in contrary to the common perception that generally regards autophagy, including ER-phagy, as the consequence of ER stress and the UPR. (41,42).

We have clearly showed that inhibitions of ER stress and the UPR have no effect on Z36-induced ER-phagy, while perturbations of autophagy machinery significantly impact ER stress and the UPR. Inhibitions of autophagy at early stage by 3-MA or Atg5 knockdown, can suppress both IRE1 and PERK pathways of the UPR (Figure 5, A and C), while inhibition of autophagic flux with CQ only attenuates the PERK pathway, with IRE1 pathway further enhanced (Figure 5E). This may suggest that the intact autophagic flux machinery of Z36-induced ER-phagy should have multiple roles in regulating ER stress and the UPR.

Interestingly, we found that the upregulation of PERK pathway of the UPR promotes Z36-induced cell death (Figure 3), while the upregulation of IRE1 arm of the UPR is pro-survival (Figure 4). Therefore, the PERK pathway and IRE1 pathway plays opposite roles in regulating Z36-induced cell death. When ER stress and the UPR or autophagy are suppressed with some shRNAs or inhibitors, both PERK and IRE1 pathways are normally downregulated, thus only modest effects on cell death are observed. Consistently, the overexpression of FAM134B results in a moderate increase in cell death, as both PERK and IRE1 pathways are upregulated (Figure 7, C and D). However, when IRE1 is knockdown with shRNA or its endonuclease activity is inhibited with STF083010, it is found that the expression of CHOP in the PERK pathway is upregulated (Figure 4, B and E), and the cell death rates are increased (Figure 4, D and G). On the other hand, when the autophagic flux is inhibited with CQ, the pro-death PERK pathway is suppressed, but
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the pro-survival IRE1 pathway is enhanced (Figure 5E). As a result, the Z36-induced cell death rate is reduced most dramatically (Figure 5F). This is different from the effects of inhibiting autophagy with 3-MA or Atg5 shRNA, which moderately reduces the cell death rates, as both PERK and IRE1 pathways are downregulated (Figure 5, A-D).

Selective ER-phagy is suggested to play key roles in maintaining ER homeostasis to limit stress-induced ER expansion by delivering ER fragments to lysosome for degradation (11,41). However, for Z36-induced ER-phagy, we found accumulation of autophagosomes characterized by much larger size and number than those due to rapamycin treatment. These are consistent with the upregulation of FAM134B, LC3, and Atg9 expression (Figure S4 & Figure 1F). Meanwhile, the rate for ER degradation is very much accelerated as indicated by the progressive degradation of ER marker proteins Rtn4 and Climp-63 (Figure 8D). As a result, the overly activated ER-phagy indiscriminately degrades ER till it can no longer function properly and impair the ER homeostasis. Apparently, Z36-induced ER stress and the UPR are originated from the “over-eat” of Z36-induced excessive ER-phagy, and further causes cell death. Therefore, the cell death induced by Z36 is not only resulted from ER stress and the UPR, but also a direct consequence of ER-phagy, and thus it is an ER-phagy-dependent cell death according to the recommended by Klionsky et al. (25) and the Nomenclature Committee on Cell Death (NCCD) (42), because that FAM134B mediated ER-phagy is the pre-requisite for ER stress and the UPR.

It is previously reported that the overexpression of FAM134B in U2OS cells or its functional counterpart Atg40 in yeast can directly activated ER-phagy (11,40). With Z36 treatment, we observed a dramatic increase (~4 fold) of FAM134B expression at mRNA level (Figure S4). However, Z36 treatment only increased FAM134B protein expression by ~20% from western blot analysis (Figure 6C). But this should not reflect the real situation, since FAM134B is key to fragment and sequester ER into autophagosomes, and it is subjected to continuous degradation due to Z36-induced ER-phagy. Interestingly, we find that ER-phagy can be triggered by the overexpression of FAM134B alone in HeLa cells, which also displays similar effects as Z36-induced ER-phagy does, e.g. it can the upregulate the mRNA of LC3 and Atg9, and enhance the conversion of LC3, as well as impair ER homeostasis through the degradation of ER, and activate the ER stress-UPR response (Figure 8, B-E). Meanwhile, the overexpression of FAM134B also promotes the death of HeLa cells, although the cell death rate is lower than that of Z36 treatment (Figure 8A). Considering the fact that Z36 treatment regulates the mRNA expression of 3588 genes from our RNA-seq results (Table 2), the mechanism underline Z36-induced cell death should be more complicated than just the overexpression of FAM134B, even though FAM134B should play a critical role in this process.

Previous reported clinical observations indicate that FAM134B exhibits a tumor suppressive function, as lower expression of FAM134B is associated with worse pathological outcomes, such as larger tumor size, more advanced stages of cancer, higher rates of cancer recurrence, and lower survival rates (43). More future studies are needed to reveal the detail mechanisms for Z36-induced cell death and the expression regulation of FAM134B in cancer cells, which may provide new strategies for antitumor therapy development.

EXPERIMENTAL PROCEDURES

Cell culture conditions and antibodies. HeLa cells were cultured in DMEM (Solarbio, Cat. No. 12100-500) supplemented with 10% fetal
bovine serum (HyClone, Cat. No. SV30087) under 5% CO2 in a humidified incubator at 37°C. To induce autophagy, cells were treated with Rapamycin (Sigma, Cat. No. R0395) or Z36 (Sigma, Cat. No. SML0176) at the indicated concentrations for the indicated time. To inhibit ER stress, cells were pretreated with 2 mM 4-Phenylbutyric acid (4-PBA) (Sigma, Cat. No. P21005) for 1 hour. To induce autophagy, cells were treated with Rapamycin (Sigma, Cat. No. R0395) or Z36 (Sigma, Cat. No. SML0176) at the indicated concentrations for the indicated time. To inhibit ER stress, cells were pretreated with 2 mM 4-Phenylbutyric acid (4-PBA) (Sigma, Cat. No. P21005) for 1 hour. To inhibit PERK and IRE1 activation, cells were pre-treated with 1 μM GSK2656157 (Santa Cruz, Cat. No. sc-490341) or 80 μM STF080010 (Sigma, Cat. No. SML0409) for 1 hour. To inhibit autophagy, cells were pretreated with 1 mM 3-Methyladenine (3-MA) (Sigma, Cat. No. M9281) for 2 hours, or 20 μM chloroquine (CQ) (Sigma, Cat. No. C6628) for 8 hours. All stock solutions for Z36, 4-PBA, GSK2656157, STF080010, 3-MA were prepared using DMSO as solvent, and CQ stock solution was in PBS. The following antibodies were used for western blot and immunofluorescence: mouse anti-LC3B (MBL, Cat. No. M152-3), rabbit anti-LC3B (Sigma, Cat. No. L7543), anti-ATG9 (Abcam, Cat. No. ab117591), anti-ATG5 (Abcam, Cat. No. ab108327), anti-FAM134B (ProteinTech, Cat. No. 21537-1-AP), anti-CHOP (Santa Cruz, Cat. No. sc-7351), anti-EIF2α (pSer51) (Abcam, Cat. No. ab32157), anti-EIF2α (Cell Signaling Technology, Cat. No. 5324), anti-IRE1α (pSer724) (Abcam, Cat. No. ab124945), anti-IRE1α (Cell Signaling Technology, Cat. No. 3294), anti-PERK (pThr980) (Cell Signaling Technology, Cat. No. 3179), anti-PERK (Cell Signaling Technology, Cat. No. 5683), anti-XBP1s (Biolegend, Cat. No. 647501), anti-NogoA+B (Abcam, Cat. No. ab47085), anti-CKAP4 (ProteinTech, Cat. No. 16686-1-AP), anti-β-actin (Cwbio, Cat. No. CW0096A), anti-BFP (Abbkine, Cat. No. ABM40180). The following secondary antibodies were used: Goat anti-rabbit IgG-HRP (Santa Cruz, Cat. No. sc-2004), Alexa Fluor™ 594 goat anti-rabbit antibody (Invitrogen, Cat. No. R37117), Alexa Fluor™ 488 goat anti-mouse IgG (H+L) (Invitrogen, Cat. No. A11001).

**Plasmids, RNA interference and transfection.**

Plasmid encoding GFP-LC3 was constructed by subcloning GFP and LC3 ORFs into vector pcDNA 3.1(+) using BspEI and XbaI cloning sites. Plasmid encoding mCherry-FAM134B was cloned into vector pmCherry-C1 (YouBio, Cat. No. G105780) using BglII and BamHI cloning sites. Plasmid mCherry-FAM134B-mut (FELL LIR substituted by GEGG) was generated with site-directed mutagenesis. RNA interference plasmid GV298 (purchased from Genechem) was used for gene knockdown. The corresponding target sequences for RNA interference are listed below:

- **Atg5:** 5’-TTCATGGGAATTGGACCAAT;  
- **CHOP:** 5’-GGAAAGGTCTCAGCTTGTA;  
- **FAM134B:** 5’-AGCTATCAAAGACCAGTTA;  
- **PERK:** 5’-TTTGGAATCTGTCACTAAT  
- **IRE1:** 5’-AATACTCTACCACGGCT  
- **CON207:** 5’-TTCTCGAAGGTGTACAGT.  

Vector GV219 (purchased from Genechem) was used to constructed expression plasmids of Atg5, PERK and IRE1 for rescuing gene knockdown, with BFP encoded for co-expression. The corresponding mutated sequences for shRNA resistant are listed below:

- **Atg5:** 5’-TCCACGGGTATCGACCAAT;  
- **IRE1:** 5’-AGTATTCCACTTCCCTTTA  
- **PERK:** 5’-CTTAGAGTCCGTAACGAAC  

Transient transfections of plasmids or shRNA were performed with X-tremeGENE HP DNA transfection reagent (Roche, Cat. No. 06366546001) as the manufacturer’s instruction.
**RNA extraction and cDNA synthesis.** HeLa cells were collected and resuspended with PBS into a 1.5-ml microfuge tube. Samples were centrifuged at 1000 rpm for 3 mins to pellet the cells at room temperature. Total RNAs were extracted with the RNeasy Mini kit (QIAGEN, Cat. No. 74104). The complementary DNA was reverse transcribed with 1 μg of total RNA and oligo dT using GoScript™ Reverse Transcription System (Promega, Cat. No. 0000202447).

**RNA-seq and data analysis.** cDNA library preparation and Illumina high-throughput sequencing (Illumina Hiseq2000) was performed by BIOPIC at Peking University. Each sample had two repeats. Demultiplexed and quality filtered mRNA-seq reads were aligned to the GRCh37/hg19 human genome using Subjunc program (http://bioinf.wehi.edu.au/subread/), then Ensembl gene annotation v75 was used for gene-level quantification. The raw counts data of the expressed genes were normalized for RNA composition using TMM method (http://www.ncbi.nlm.nih.gov/pubmed/20196867) from EdgeR package (https://bioconductor.org/packages/release/bioc/html/edgeR.html), then transformed to log2CPM values using voom method (http://www.ncbi.nlm.nih.gov/pubmed/24485249) from the R Limma package (https://bioconductor.org/packages/release/bioc/html/limma.html). Next linear model was built for each comparison using Limma package, and statistics for differential expression analysis were computed. To filter for differential expression, two-fold change with FDR ≤ 0.05 were used as the cutoff.

**Real-time PCR analysis.** Real-time PCR was carried out using FastStart Universal SYBR Green Master (Roche, Cat. No. 04913850001) and a StepOnePlus™ real-time PCR instrument (Applied Biosystems). Relative expression was evaluated with ΔΔCT method and GAPDH was used as the internal reference to normalize gene expression. Primers for real-time PCR were as follows:

- **GAPDH**
  - F: 5’-GGAGCGAGATCCCTCCAAAAT-3’,
  - R: 5’-GGCTGTGTCTACATTTCTCATGG-3’;
- **LC3**
  - F: 5’-GATGTCCGACTTATTTGAGACG-3’,
  - R: 5’-TTGAGCTGTAAGCCGCTTCTA-3’;
- **Atg9**
  - F: 5’-CTGGGGCGAGTGACAAAG-3’,
  - R: 5’-CTGGGCATTGTCAGGAAATGGA-3’;
- **CHOP**
  - F: 5’-GGAAACAGAGTGGTCATTCCC-3’,
  - R: 5’-CTGCTTGAGCTCATTCTCTC-3’;
- **XBP1s**
  - F: 5’-GGTCGAGCCAAGGAGTTAAGACAG-3’,
  - R: 5’-CAGAGGGATCTCTCAAGACTAGG-3’;
- **PERK**
  - F: 5’-GAACCAGACATGAGACGAG-3’,
  - R: 5’-GGATGACACCAAGGAACCG-3’;
- **IRE1**
  - F: 5’-CAGAGGGATCTCTCAAGACTAGG-3’;
  - R: 5’-GCCATCTATTAGGATCTGGGAGA-3’.

**Western blotting.** HeLa cells were lysed with lysis buffer (Beyotime, Cat. No. P0013). Proteins were separated with 4-20% gradient SDS-PAGE, and transferred to PVDF membranes. The membranes were first blocked with 5% low-fat milk in TBS buffer (20 mM Tris, 150 mM NaCl, 0.02% Tween-20, pH 7.4), and then incubated with the indicated primary antibodies overnight at 4°C. Then HRP-conjugated secondary antibodies were used. Bands detection was performed with a Chemiluminescent Substrate kit (Thermo, Cat. No. 34077) and analyzed with Tanon 5200.

**Electron microscopy assay.** For transmission electron microscopy (TEM), wild-type HeLa
cells were initially fixed using 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (PH 7.4) for 1 hour at 37 °C, then post-fixed in 2% OsO₄ for 1 hour at room temperature. After being dehydrated in a graded series of ethanol, cells were embedded into Spurr’s resin. Then the samples were sliced into 70 nm sections using an ultramicrotome (Leica Microsystem). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope Tecnai G² 20 TWIN (FEI) at 120kV. The size of autophagosomes was calculated from the diameters of the largest 1-3 autophagosomes cross-sections in each cell in TEM images. The diameter values were using the formula: diameter = 2 × radius. Radius of each autophagosomes was measured as previously described (26).

Immunofluorescence microscopy assay. HeLa cells were grown to 60% confluence on a coverslip. After treatments, cells were washed three times with PBS, and fixed with freshly prepared methyl alcohol at -20 °C for 20 min. Cells were incubated with primary antibodies overnight at 4°C and, after washing with PBS, stained with fluorescent secondary antibodies for 1 hour at room temperature. After rinsed with PBS, cells were stained with DAPI for 15 min at room temperature. The cells were then further washed for three times with PBS. Cell images were captured using Nikon A1RSi confocal microscope.

Cell viability assay. Cell viability was measured using Trypan-blue dye exclusion assay as described previously (23). After treatment for 30 hours, HeLa cells were trypsinized and suspended with DMEM, then stained with trypan blue (Solarbio, Cat. No. C0040) for 3 minutes. The cell death rate was then counted using Countess II Automated Cell Counter (Thermo Fisher Scientific Inc.).

Statistical Analysis. For quantitative analysis, values were obtained from three independent experiments, and data were presented as points and standard deviation. Statistical analyses were performed using the Student’s t test or two-way ANOVA analysis, with p value < 0.05 being considered significant.
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FOOTNOTES

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The RNA-seq data have been deposited in NCBI Gene Expression Omnibus (GEO) under the accession number GSE130006.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.
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Table 1. Statistics of RNA-sequencing results.

|                  | DMSO      | Rapa      | Z36       |
|------------------|-----------|-----------|-----------|
| Total Reads      | 37919718  | 35026128  | 31670837  |
| Assigned         | 31452808  | 28920709  | 26046972  |
| Percentage Mapped| 96.50%    | 95.80%    | 95.20%    |
| Percentage Assigned| 82.90%   | 82.60%    | 82.20%    |

Table 2. Summary of differentially expressed genes.

|                  | Rapa vs DMSO | Z36 vs DMSO |
|------------------|--------------|-------------|
| Up-regulation    | 33           | 1654        |
| Down-regulation  | 25           | 1934        |
| Total            | 58           | 3588        |
Table 3. Biological process (GO) enrichment analysis results of differentially expressed genes (|fold change| ≥ 1.0 & FDR < 0.05) in Z36-treated HeLa cells.

| GO biological process                                             | Count | P-value      |
|-------------------------------------------------------------------|-------|--------------|
| response to endoplasmic reticulum stress                         | 35    | 1.15E-16     |
| cellular response to external stimulus                           | 38    | 2.19E-13     |
| endoplasmic reticulum unfolded protein response                  | 25    | 1.34E-12     |
| cellular response to unfolded protein                            | 25    | 2.52E-12     |
| cellular response to extracellular stimulus                      | 32    | 2.79E-12     |
| response to unfolded protein                                     | 28    | 6.90E-12     |
| cellular response to topologically incorrect protein             | 25    | 1.48E-11     |
| response to topologically incorrect protein                      | 28    | 3.87E-11     |
| cellular response to nutrient levels                             | 27    | 1.12E-09     |
| response to starvation                                           | 27    | 2.18E-09     |
| cellular response to starvation                                  | 24    | 6.29E-09     |
| alcohol biosynthetic process                                     | 18    | 8.63E-06     |
| PERK-mediated unfolded protein response                          | 8     | 1.03E-05     |
| sterol biosynthetic process                                      | 12    | 1.52E-05     |
| ER-nucleus signaling pathway                                     | 11    | 2.14E-05     |
| cholesterol biosynthetic process                                 | 11    | 4.93E-05     |
| circadian rhythm                                                 | 18    | 2.09E-04     |
| regulation of transcription from RNA polymerase II promoter in response to stress | 12  | 2.69E-04 |
| circadian regulation of gene expression                          | 12    | 3.92E-04     |
| regulation of DNA-templated transcription in response to stress  | 12    | 5.62E-04     |
| intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress | 9    | 6.56E-04     |
| positive regulation of transcription from RNA polymerase II promoter in response to stress | 8    | 1.05E-03     |
| IRE1-mediated unfolded protein response                           | 11    | 1.96E-03     |
| regulation of endoplasmic reticulum stress-induced intrinsic apoptotic signaling pathway | 8    | 2.01E-03     |
| steroid biosynthetic process                                     | 15    | 2.92E-03     |
| cellular response to decreased oxygen levels                     | 15    | 2.92E-03     |
| ERAD pathway                                                      | 11    | 3.87E-03     |
| cellular response to glucose starvation                          | 8     | 4.83E-03     |
| positive regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress | 6    | 6.39E-03     |
| cholesterol metabolic process                                    | 14    | 1.04E-02     |
| regulation of fat cell differentiation                           | 13    | 1.76E-02     |
| ER-associated ubiquitin-dependent protein catabolic process       | 10    | 2.16E-02     |
| serine family amino acid biosynthetic process                    | 6     | 2.28E-02     |
| macroautophagy                                                    | 10    | 2.92E-02     |
| negative regulation of response to endoplasmic reticulum stress  | 7     | 3.51E-02     |
Table 4. ER stress and the UPR associated genes upregulated in Z36-treated cells.

| Symbol   | Description                                                                 | Fold change | P-value         |
|----------|------------------------------------------------------------------------------|-------------|-----------------|
| DDIT3    | DNA-damage-inducible transcript 3                                           | 10.0        | 9.67E-18        |
| CREBRCF  | CREB3 regulatory factor                                                      | 8.9         | 5.37E-15        |
| CHAC1    | ChaC, cation transport regulator homolog 1                                   | 8.7         | 1.62E-15        |
| DNAJB9   | DnaJ (Hsp40) homolog, subfamily B, member 9                                 | 8.5         | 7.95E-17        |
| CDKN1A   | cyclin-dependent kinase inhibitor 1A (p21, Cip1)                            | 8.5         | 4.22E-18        |
| TRIB3    | tribbles pseudokinase 3                                                      | 8.0         | 8.43E-19        |
| BBC3     | BCL2 binding component 3                                                    | 7.2         | 4.19E-14        |
| ASNS     | asparagine synthetase (glutamine-hydrolyzing)                               | 7.1         | 2.68E-17        |
| HERPUD1  | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 | 6.3         | 1.09E-18        |
| FAM134B  | family with sequence similarity 134, member B                               | 6.0         | 3.05E-16        |
| PPP1R15A | protein phosphatase 1, regulatory subunit 15A                               | 6.0         | 1.55E-17        |
| NUPR1    | nuclear protein, transcriptional regulator, 1                               | 5.7         | 1.23E-16        |
| HMOX1    | heme oxygenase (decycling) 1                                                | 5.3         | 1.96E-16        |
| INSIG1   | insulin induced gene 1                                                       | 5.1         | 4.04E-18        |
| ATG2A    | autophagy related 2A                                                        | 4.8         | 3.75E-15        |
| ERN1     | endoplasmic reticulum to nucleus signaling 1                                | 4.7         | 9.22E-14        |
| WIP1     | WD repeat domain, phosphoinositide interacting 1                            | 4.4         | 2.56E-15        |
| ULK1     | unc-51 like autophagy activating kinase 1                                   | 3.8         | 8.08E-16        |
| CEBPB    | CCAAT/enhancer binding protein (C/EBP), β                                    | 3.5         | 7.41E-15        |
| MAP1LC3B | microtubule-associated protein 1 light chain 3 β                             | 3.5         | 1.25E-16        |
| MAP1LC3B2| microtubule-associated protein 1 light chain 3 β 2                           | 3.4         | 1.75E-14        |
| HSPA5    | heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)               | 3.3         | 5.89E-18        |
| EIF2AK3  | eukaryotic translation initiation factor 2-α kinase 3                        | 3.3         | 6.60E-14        |
| ATF4     | activating transcription factor 4                                           | 3.0         | 7.75E-16        |
| XBP1     | X-box binding protein 1                                                      | 3.0         | 1.63E-14        |
**Figure 1. Comparison of autophagosomes in Z36-treated and Rapa-treated HeLa cells**

(A) Comparison of GFP-LC3 punctate distribution in HeLa cells treated with Rapa and Z36. Cells were transfected with GFP-LC3 plasmid for 24 hours, and then treated with DMSO, 1 μM Rapa or 13 μM Z36 for 10 hours. Scale bars, 20 μm. (B) Estimated number of autophagosomes per cell in HeLa cells after 10 hours treatment with DMSO, 1 μM Rapa, or 13 μM Z36. The numbers of autophagosomes in each cell were determined based on analysis of TEM images (n = 30 cells for each sample from three replicate experiments). ***P < 0.001. (C) Representative TEM images of autophagosomes in HeLa cells treated with DMSO, 1 μM Rapa, or 13 μM Z36 for 10 hours, showing the size of autophagosomes. Scale bar, 500 nm. (D) Estimation of maximal autophagosome diameters from TEM images. (n = 30 for each sample of three replicate experimental sets). ns, not significant, ***P < 0.001. (E) Western blot and (F) quantitative real-time PCR analysis of LC3 and Atg9 expression levels in HeLa cells treated with DMSO, 1 μM Rapa, or 13 μM Z36 for 10 hours. The intensity of respective protein band in (E) were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. ns, not significant, **P < 0.01, ***P < 0.001.
Figure 2. Inhibition of ER stress reduces cell death, but does not affect autophagy

(A) Western blot analysis of the effects of ER stress inhibitor 4-PBA on ER stress and the UPR markers and LC3 conversion in Z36-treated HeLa cells. HeLa cells were treated with 13 μM Z36 for 10 hours, with or without the pretreatment of 2 mM 4-PBA, and DMSO was used as control. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. ns, not significant, *P < 0.05, **P < 0.01, ***P < 0.001. (B) Effect of 4-PBA on Z36-induced cell death. HeLa cells were treated with 13 μM Z36 for 30 hours, with or without the pretreatment of 2 mM 4-PBA, and DMSO was used as control. ns, not significant, ***P < 0.001. (C) Punctate distribution of GFP-LC3 in Z36-treated HeLa cells with or without 4-PBA. Cells were transfected with GFP-LC3 plasmid for 24 hours, and then treated with DMSO or 13 μM Z36 for 10 hours. Scale bars, 20 μm. The GFP-LC3 aggregates area present in cells were quantified using ImageJ, and the LC3 dots area per cell was calculated (n = 50 cells for each sample of three replicate experiments). ns, not significant.
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Figure 3. Inhibitions of PERK arm of the UPR reduces cell death, but not autophagy

(A) Western blot analysis of the effects of PERK inhibitor GSK2656157 on the UPR markers and LC3 conversion in Z36-treated HeLa cells. HeLa cells were treated with 13 μM Z36 for 10 hours, with or without the pretreatment of 1 μM GSK2656157, DMSO was used as control. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. ns, not significant, **P < 0.01, ***P < 0.001. (B) Punctate distribution of GFP-LC3 in Z36 or DMSO treated HeLa cells with or without GSK2656157. Cells were transfected with GFP-LC3 plasmid for 24 hours, and then treated with DMSO or 13 μM Z36 for 10 hours. Scale bars, 20 μm. The GFP-LC3 aggregates area present in cells were quantified using ImageJ, and the LC3 dots area per cell was calculated (n = 50 cells for each sample of three replicate experiments). ns, not significant. (C) Effect of GSK2656157 on Z36-induced cell death. HeLa cells were treated with 13 μM Z36 for 30 hours, with or without the pretreatment of 1 μM GSK2656157, and DMSO was used as control. ns, not significant, ***P < 0.001. (D) Western blot analysis of the effects of PERK knockdown on the UPR markers and LC3 conversion in HeLa cells treated with Z36. Cells were transfected with CON207, shPERK or both shPERK and PERK plasmids for 24 hours, and then treated with DMSO or 13 μM Z36 for 10 hours. CON207 was used as control plasmid. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. ns, not significant, *P < 0.05, **P < 0.01, ***P < 0.001. (E) Punctate distribution of GFP-LC3 in Z36-treated
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HeLa cells treated as in (D). Scale bars, 20 μm. The areas of green fluorescent puncta present in cells were quantified using ImageJ, and the LC3 dots area per cell was calculated (n = 50 cells for each sample of three replicate experiments). ns, not significant. (F) Effect of PERK knockdown on Z36-induced cell death. HeLa cells were transfected with respective plasmid as in (D) for 24 hours, and then treated with DMSO or 13 μM Z36 for 30 hours, CON207 was a control plasmid. ns, not significant, ***P < 0.001. (G) Efficiency for shRNA knockdown of CHOP determined with western blot. The intensity of CHOP band was quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. **P < 0.01. (H) Effect of CHOP knockdown on Z36-induced cell death. HeLa cells were transfected with shRNA plasmid for 24 hours, and then treated with DMSO or 13 μM Z36 for 30 hours, CON207 was a control plasmid. ns, not significant, ***P < 0.001.
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Figure 4. Inhibitions of IRE1 pathway of the UPR increases cell death, but does not affect autophagy.

(A) IRE1 inhibitor STF083010 inhibits endogenous XBP1 mRNA splicing. HeLa cells were treated with DMSO and 13 μM Z36 for 10 hours, or co-incubated with Z36 and 80 μM STF083010. XBP1 splicing (XBP1s) was determined with quantitative real-time PCR. *P < 0.05, **P < 0.01. (B) Western blot analysis of the effects STF083010 treatment on the UPR markers and LC3 conversion in HeLa cells treated with Z36. HeLa cells were treated with 13 μM Z36 for 10 hours, with or without the pretreatment of 80 μM STF083010. DMSO was used as control. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. ns, not significant, *P < 0.05, **P < 0.01, ***P < 0.001. (C) Punctate distribution of GFP-LC3 in Z36 or DMSO treated HeLa cells with or without STF083010. Scale bars, 20 μm. The GFP-LC3 aggregates area present in cells were quantified using ImageJ, and the LC3 dots area per cell was calculated (n = 50 cells for each sample of three replicate experiments). ns, not significant. (D) Effect of STF083010 on Z36-induced cell death. HeLa cells were treated with 13 μM Z36 for 30 hours, with or without the pretreatment 80 μM STF083010, DMSO was used as control. ns, not significant, ***P < 0.001. (E) Western blot analysis of the effects of IRE1 knockdown on the UPR markers and LC3 conversion in HeLa cells treated with Z36. Cells were transfected with CON207, shIRE1α or both shIRE1α and IRE1α encoding plasmids for 24 hours, and then treated with DMSO or 13 μM Z36 for 10 hours, CON207 was used as control plasmid. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. ns, not significant, *P < 0.05,
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**P < 0.01, ***P < 0.001. (F) Punctate distribution of GFP-LC3 in HeLa cells treated as in (E). Scale bars, 20 μm. The areas of green fluorescent puncta present in cells were quantified using ImageJ, and the LC3 dots area per cell was calculated (n = 50 cells for each sample of three replicate experiments). ns, not significant. (G) Effect of IRE1α knockdown on Z36-induced cell death. HeLa cells were transfected with respective plasmid as in (E) for 24 hours, and then treated with DMSO or 13 μM Z36 for 30 hours, CON207 was a control plasmid. ns, not significant, ***P < 0.001.
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Figure 5. Inhibitions of autophagy machinery suppress both ER stress and the UPR, as well as cell death

(A) Western blot analysis of the effects of 3-MA treatment on ER stress and the UPR markers and LC3 conversion in Z36-treated HeLa cells. HeLa cells were treated with 13 μM Z36 for 10 hours, with or without the pretreatment of 1 mM 3-MA, DMSO was used as control. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. *P < 0.05, ***P < 0.001. (B) Effect of 3-MA on Z36-induced cell death. HeLa cells were treated with 13 μM Z36 for 30 hours, with or without the pretreatment of 1 mM 3-MA, DMSO was used as control. ns, not significant, ***P < 0.001. (C) Western blot analysis of the effect of Atg5 knockdown on ER stress and the UPR markers and LC3 conversion. Cells were transfected with CON207, shAtg5 or both shAtg5 and Atg5 encoding plasmids for 24 hours, and then treated with DMSO or 13 μM Z36 for 10 hours, CON207 was used as control plasmid. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. ns, not significant, *P < 0.05, **P < 0.01, ***P < 0.001. (D) Effect of knockdown or rescue of Atg5 on Z36-induced cell death. HeLa cells were transfected with respective plasmids as in (C) for 24 hours, and then treated with DMSO or 13 μM Z36 for 30 hours, CON207 was a control plasmid. ns, not
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significant, ***P < 0.001. (E) Western blot analysis of the effects of CQ treatment on ER stress and the UPR markers and LC3 conversion in Z36-treated HeLa cells. HeLa cells were treated 13 μM Z36 for 10 hours, with or without the pretreatment of 20 μM CQ, DMSO was used as control. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (F) Effect of CQ treatment on Z36-induced cell death. HeLa cells were treated with 13 μM Z36 for 30 hours, with or without pretreatment with 20 μM CQ, DMSO was used as control. ns, not significant, ***P < 0.001.
Figure 6. Z36 upregulates FAM134B expression and induces ER-phagy in HeLa cells

(A) Transmission electron microscopy images showing ER fragmentation in Z36-treated cells. ER is indicated by arrows. Scale bar, 1 μm. (B) Transmission electron microscopy images showing ER whorls and autophagosomes in HeLa cells treated with Z36 for 10 hours. Note the ring-shaped ER whorls (left) and engulfment of ER whorls inside the autolysosome (right). Autolysosome is indicated by black arrow, ER whorls are indicated by white arrowheads. Scale bars, 500 nm. (C) Western blot analysis of ER stress and the UPR markers and LC3 conversion in FAM134B knockdown HeLa cells treated with or without Z36. 24 hours after shRNA plasmids transfection, cells were treated with DMSO and 13 μM Z36 for 10 hours, CON207 was used as control plasmid. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (D) Punctate distribution of GFP-LC3 in control and FAM134B knockdown HeLa cells treated with DMSO or 13 μM Z36. Scale bars, 20 μm. The areas of green fluorescent puncta present in cells were quantified using ImageJ, and the LC3 dots area per cell was calculated (n = 50 cells for each sample of three replicate experiments). ns, not significant, ***P < 0.001. (E) Effect of FAM134B knockdown on Z36-induced cell death. HeLa cells were transfected with shRNA plasmids for 24 hours, and then treated with DMSO or 13 μM Z36 for 30 hours, CON207 was used as control plasmid. ns, not significant, ***P < 0.001.
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Figure 7. Z36-induced FAM134B mediated ER-phagy triggers ER stress, the UPR and cell death

(A) Co-localization of LC3 with wild-type mCherry-FAM134B and its LIR mutant FAM134B-mut in HeLa cells. Cells were first co-transfected with GFP-LC3 and mCherry-FAM134B or mCherry-FAM134B-mut plasmids for 24 hours, and then treated with DMSO or 13 μM Z36 for 10 hours. Autophagosomes represented by green fluorescent puncta are indicated by arrows. Scale bars, 10 μm. 

(B) Quantification of GFP-LC3 puncta areas in control and FAM134B or FAM134B-mut overexpression HeLa cells treated with DMSO or 13 μM Z36. The areas of green fluorescent puncta present in cells were quantified using ImageJ, and the LC3 dots area per cell was calculated (n = 50 cells for each sample of three replicate experiments). ns, not significant, ***P < 0.001. 

(C) Western blot analysis of the effect of FAM134B and its LIR mutant FAM134B-mut overexpression on ER stress and the UPR markers and LC3 conversion in HeLa cells treated with or without Z36. The cells were first transfected with mCherry-FAM134B or mCherry-FAM134B-mut for 24 hours, then treated with DMSO or 13 μM Z36 for 10 hours, Vector was used as control. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data
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represents values of three independent experiments. ns, not significant, *P < 0.05, **P < 0.01, ***P < 0.001. (D) Effect of FAM134B wild type and mutant overexpression on Z36-induced cell death. The cells were first transfected with mCherry-FAM134B or mCherry-FAM134B-mut for 24 hours, then treated with DMSO or 13 μM Z36 for 30 hours. ns, not significant, ***P < 0.001.
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Figure 8. ER-phagy mediated by FAM134B results in ER stress and cell death

(A) Effect of FAM134B and its LIR motif mutant FAM134B-mut overexpression on cell death. HeLa cells were first transfected with empty vector, mCherry-FAM134B and mCherry-FAM134B-mut plasmids for 24 hours. The mCherry-FAM134B overexpressing cells were also treated with 1 mM 3-MA or 2 mM 4-PBA. Cell death was assayed after additional 30 hours. ns, not significant, **P < 0.01, ***P < 0.001. (B) Quantitative real-time PCR analysis of mRNA expression levels for LC3, Atg9, XBP1s, PERK, IRE1, and CHOP in FAM134B and FAM134B-mut overexpressing HeLa cells. HeLa cells were first transfected with respective plasmid for 24 hours, and the cells were collected for analysis after additional 10 hours. ns, not significant; **P < 0.01; ***P < 0.001. (C) Western blot analysis of ER stress and the UPR markers and LC3 conversion in FAM134B and FAM134B-mut overexpressing HeLa cells. The FAM134B overexpression cells were also treated with 1 mM 3-MA or 2 mM 4-PBA. HeLa cells were first transfected with empty vector, mCherry-FAM134B and mCherry-FAM134B-mut plasmid for 24 hours, and the cells were collected for analysis after additional 10 hours. The intensity of respective protein band were quantified using ImageJ, relative to β-actin, then normalized to control. Data represents values of three independent experiments. ns, not significant, *P < 0.05, **P < 0.01, ***P < 0.001. (D) and (E) Western blot analysis of ER structural proteins Rtn4 and Climp-63 degradation in HeLa cells. (D) Cells were treated with 13 μM Z36, or together with 1 mM 3-MA or 2 mM 4-PBA, DMSO was used as control. Time 0 is the time for adding Z36 to the cells. (E) Cells were transfected with mCherry-FAM134B, or mCherry-FAM134B-mut, and empty vector as control. The effects of 1 mM 3-MA or 2 mM 4-PBA treatments
on mCherry-FAM134B overexpression cells were also assayed. Time 0 is set to be 24 hours after transfection. The intensity of Rtn4 and Climp-63 were quantified using ImageJ, relative to β-actin, then normalized to 0h. Data represents mean ± S.D. of three independent experiments. ns, not significant, **P < 0.01, ***P < 0.001.
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