The XBP1-MARCH5-MFN2 Axis Confers ER Stress Resistance by Coordinating Mitochondrial Fission and Mitophagy in Melanoma

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Research

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Abstract

Background: Melanoma cells are relatively resistant to ER stress, which contributes to tumor progression under stressful conditions and renders tolerance to ER stress-inducing therapeutic agents. Mitochondria are tightly interconnected with ER. However, whether mitochondria play a role in regulating ER stress resistance in melanoma remains elusive.

Methods: Integrative bioinformatics was employed to figure out the implication of mitochondria in the resistance of melanoma cells to ER stress. A panel of biochemical assays and pre-clinical xenograft mouse model were used to investigate the role of mitochondrial fission and mitophagy in affecting ER stress sensitivity and the underlying mechanisms.

Results: Our integrative bioinformatics analysis revealed that the down-regulation of mitochondrial genes was highly correlated with UPR activation in melanoma. Then we proved that mitochondrial fission and mitophagy were prominently induced in melanoma cells upon ER stress. Pharmacological inhibition of either mitochondrial fission or mitophagy effectively restored the sensitivity of melanoma cells to ER stress both in vitro and in vivo. Mechanistically, the down-regulation of MFN2 was essential for rendering the resistance by promoting mitochondrial fission and mitophagy. XBP1-mediated transcriptional up-regulation of E3 ligase MARCH5 contributed to the ubiquitination and degradation of MFN2 in ER stress-resistant cells, whereas the impaired transduction of this axis indicated the fragile to ER stress. Finally, the relationships among UPR pathway molecules, MARCH5 and mitochondrial genes were confirmed in both publicly accessible databases and tumor specimens.

Conclusions: Together, our findings demonstrate a novel regulatory axis that links mitochondrial fission and mitophagy to the resistance to ER stress. Targeting mitochondrial quality control machinery can be exploited as an approach to reinforce the efficacy of ER stress-inducing agents against cancer.

Background

The overload of misfolded/unfolded proteins in ER lumen can cause ER stress, which would result in the activation of unfolded protein response (UPR) for re-establishing the function of ER [1]. There are three canonical UPR signaling pathway branches initiated by ER transmembrane proteins, ATF6 (activating transcription factor 6), ERN1/IRE1α (endoplasmic reticulum to nucleus signaling 1), and EIF2AK3/PERK (eukaryotic translation initiation factor 2-a kinase 3) [2]. In rapidly-proliferating tumor cells, increased synthesis of proteins in mutated forms that are required for tumor malignancy can induce the accumulation of misfolded proteins [3]. Moreover, since solid tumors often outstrip their nutrient supply, tumor microenvironment is generally characterized by stressful conditions like hypoxia, acidosis and nutrients deprivation [4]. Therefore, tumor cells commonly encounter ER stress from various sources, and they adapt to it for survival, which might otherwise succumb to apoptosis as a consequence. Recent studies have demonstrated that melanoma cells are relatively resistant to ER stress [5], with a number of mechanisms greatly involved in to endow the tumor with growth advantage [5–7]. Moreover, while some
agents triggering ER stress are of great potential for treating multiple cancers including melanoma [8–10], the persistence of a ER stress-tolerant subpopulation of tumor cells can mitigate the therapeutic efficacy [11, 12]. Therefore, it is necessary to forwardly investigate the mechanism underlying the resistance to ER stress, so that to develop druggable target to suppress tumor progression under ER stress-triggering microenvironment, and to reinforce the efficacy of ER stress-inducing agents for melanoma therapy.

Mitochondria are highly dynamic organelles that are structurally and functionally interconnected with ER [13, 14]. The physical contact and communication between them can act as a signaling hub for the regulation of multiple fundamental biological activities and even the determination of cell fate upon stressful stimuli, especially ER stress [14, 15]. Of note, the morphology of mitochondria can be dynamically regulated by two counteracting processes fusion and fission, and some regulatory elements are capable of sensing and transducing responsive signaling from ER stress. For example, the induction of dynamin-related protein 1 (DRP-1) expression and the resultant mitochondrial fission significantly exacerbates ER stress-induced mitochondrial dysfunction and cell apoptosis in pancreatic β-cell [16]. In addition, potentiated mitochondrial fission and maladaptive UPR induced by the loss of mitofusin 2 (MFN2) expression could profoundly suppress apoptosis and autophagy in mouse embryonic fibroblasts [17]. These reports highlight the critical role of mitochondrial dynamics in regulating the response to ER stress, whereas the actual effect is still controversial in different cells and remains unclear in cancer. What's more, the imbalance of mitochondrial dynamics is highly coupled with mitophagy that is an important process for the elimination of damaged mitochondria and the maintenance of mitochondrial homeostasis [18]. The dysregulation of mitophagy is greatly implicated in the communication between mitochondria and ER, and consequently contributes to the pathogenesis of various ER stress-associated diseases like ischemic brain injury and cancer [19, 20]. Based on these reports, we hypothesized that mitochondrial dynamics and mitophagy might be involved in regulating the resistance to ER stress and therefore participate in melanoma pathogenesis.

In the present study, our integrative bioinformatics analysis first revealed that the down-regulation of mitochondrial genes was highly correlated with UPR activation in melanoma. Through a series of functional and biochemical studies, we then proved that mitochondrial fission and mitophagy were prominently induced to contribute to ER stress resistance both in vitro and in vivo by maintaining mitochondrial function. Subsequent mechanistic study unveiled that the XBP1-MARCH5 axis-mediated degradation of MFN2 linked UPR to mitochondrial fragmentation and mitophagy, thus rendering the tolerance to ER stress. Ultimately, the relationships among UPR pathway molecules, MARCH5 and mitochondrial genes were confirmed in both publicly accessible databases and tumor specimens.

**Materials And Methods**

Further details are available in Supplementary Methods online.

**Statistical analysis**
Data were presented as mean ± SD through at least three independent experiments, and was analyzed using GraphPad Prism (Prism 8.0, USA). Student’s t test was used to compare two groups of independent samples, while one-way ANOVA or two-way ANOVA were used to analyze the differences among multiple groups. Correlations between measured variables were tested by Spearman rank correlation analyses. *P < 0.05; **P < 0.01; ***P < 0.001 considered to be statistically significant.

Results

The down-regulation of mitochondrial genes is related to UPR activation in melanoma

In order to examine the response of melanoma cells to ER stress, we employed two common pharmacological inducers tunicamycin (TM) and thapsigargin (TG) to treat four different melanoma cell lines. The activation of UPR was evidenced by the induction of HSPA5 expression (Fig. S1a). Intriguingly, A2058 and 451Lu cell lines were more refractory to TM or TG -induced cell death compared to WM35 and A375 cell lines (Fig. 1a-b), which was consistent with previous studies that some subpopulations of melanoma cells were relatively resistant to ER stress [5], with the activation of multiple cyto-protective signals implicated in [5–7]. To forwardly understand the molecular mechanism associated with the tolerance to ER stress, we performed gene set enrichment (GSEA) analysis of TCGA Skin Cutaneous Melanoma (SKCM) database to observe the potential biological pathways correlated with UPR. A series of enriched pathways were in positive or negative correlation with UPR pathway molecules XBP1, ERN1 and EIF2AK3, respectively (Fig. S1b). Of note, mitochondrial gene expression and oxidative phosphorylation pathways were in negative correlation with these UPR pathway molecules (Fig. 1c), indicating the potential involvement of mitochondria in regulating the response to ER stress. We forwardly analyzed the relationship between UPR pathway molecules (HSPA5, ATF6, EIF2S1, EIF2AK3 and XBP1) and mitochondrial genes in TCGA SKCM database. UPR pathway was in prominent negative correlation with multiple mitochondrial genes (Fig. 1d). To be specific, the expressions of several mitochondrial genes implicated in oxidative phosphorylation and mitochondrial ribosome were lower in HSPA5-high group compared to HSPA5-low group (Fig. 1e). In consistent, immunohistochemical staining analysis in tumor tissue microarray (TMA) that consists of 84 melanoma cases showed the negative correlation between XBP1 and mitochondria quantity marker COXIV (Fig. 1f). Further, we turned to 88 short-term cultures for analyzing the expression profile of both sensitive A375 cell line and resistant 451Lu cell line. While UPR pathway molecules were prominently up-regulated in 451Lu cell line, mitochondrial genes were generally down-regulated (Fig. 1g). Collectively, the integrated bioinformatics analysis in public datasets and TMA demonstrated that the down-regulation of mitochondrial genes was highly related to UPR activation in melanoma, prompting us to speculate that mitochondria might play a crucial role in mediating the response and resistance to ER stress.

Mitochondrial fission and mitophagy are prominently induced under ER stress
Since that mitochondrion is the main organelle responsible for the generation of ATP and the crucial source of intracellular ROS, we examined these two characteristics to see the alteration of mitochondrial function. To our surprise, while TM or TG robustly potentiated mitochondrial ROS generation and suppressed intracellular ATP level in relatively sensitive WM35 and A375 cell lines, ATP level was conversely increased and mitochondrial ROS was unexpectedly reduced in resistant A2058 and 451Lu cell lines (Fig. 2a-b). Moreover, Mito-TEMPO that could specifically eliminate mitochondrial ROS effectively reversed the pro-apoptotic effect of TM and TG in WM35 and A375 cell lines (Fig. 2c-d, Fig. S2a). These results indicated that while mitochondrial function was prominently impaired to trigger cell death in sensitive cells, mitochondrial status was well maintained in resistant cells.

Mitochondrial morphology is dynamically regulated and is tightly coupled with its energy-generating function and quality control process [21]. Therefore, immunofluorescence staining with mitochondrial dye MitoTracker Green was employed to observe the morphological alteration of mitochondria. A2058 and 451Lu cell lines displayed more prominent shift from mitochondrial tubulation to fragmentation under ER stress, while WM35 and A375 cell lines revealed no such alteration (Fig. 2e; Fig. S2b). Since that mitochondrial fission is mainly responsible for the transition from tubulation to fragmentation [22], these results indicated significant mitochondrial fission upon ER stress in resistant cells. Of note, mitochondrial fission can facilitate the elimination of damaged mitochondria by fostering mitophagy [23, 24]. In line with this, immunoblotting analysis showed prominent activation of mitophagy in resistant A2058 cell line, revealed by increased ratio of LC3II/LC3I, decreased expression of p62, and down-regulation of mitochondrial marker TOMM20 and COXIV, whereas no such alterations in sensitive WM35 cell line (Fig. 2f). The co-localization of MitoTracker and LC3 also indicated more mitophagy in A2058 and 451Lu cell lines (Fig. 2g; Fig. S2c). Besides, after the blockage of mitochondrial fission with Mdivi-1 (Fig. S3a), ER stress-activated mitophagy was attenuated in both A2058 and 451Lu cell lines (Fig. S4a-b), confirming that the activation of mitophagy was dependent on mitochondrial fission. Collectively, these results implied that the potentiation of mitochondrial fission and mitophagy was highly related to the resistance to ER stress. While the deficiency of mitochondrial fission and mitophagy contributed to the dysfunction of mitochondria and the fragile of sensitive melanoma cells to ER stress, potentiated mitochondrial fission and mitophagy may help eliminate the damaged mitochondria and render the resistance to ER stress in resistant melanoma cells.

The activation of mitochondrial fission and mitophagy confers the resistance to ER stress

Thereafter, to see whether mitochondrial fission and mitophagy mediated the resistance to ER stress, we employed mdivi-1 and chloroquine (CQ) to block mitochondrial fission and mitophagy respectively. CQ could suppress mitophagy as revealed by the up-regulation of LC3, p62, TOMM20 and COXIV (Fig. 3a). Intriguingly, the suppression of mitochondrial fission or mitophagy in A2058 and 451Lu cell lines led to reduced intracellular ATP level and increased mitochondrial ROS (Fig. 3c-d), indicating attenuated clearance of damaged mitochondria. More importantly, blockage of mitochondrial fission or mitophagy significantly abolished ER stress-induced cell death (Fig. 3e). Concurrent immunoblotting analysis showed up-regulation of pro-apoptotic Bax and cleaved caspase-3, and down-regulation of anti-apoptotic...
Bcl-2 (Fig. 3b). To further prove that targeting mitochondrial fission or mitophagy could sensitize melanoma cells to ER stress inducer in vivo, we employed pre-clinical xenograft model via the subcutaneously injection of resistant A2058 melanoma cells into nude mice. While TM treatment alone led to slight tumor regression, combined inhibition of mitochondrial fission or mitophagy by Mdivi-1 or CQ prominently delayed tumor progression (Fig. 3f). Together, the activation of mitochondrial fission and mitophagy conferred the resistance to ER stress both in vitro and in vivo.

The down-regulation of MFN2 induces mitochondrial fission and mitophagy under ER stress

Previous studies have revealed that mitochondrial dynamics is regulated by a series of regulatory elements, including DRP1, fission 1 (Fis1), mitochondrial fission factor (MFF), MFN1, MFN2 and optic atrophy 1 (OPA1) [13]. Our immunoblotting analysis showed that the expressions of DRP1, Fis1, MFF, MFN1 and OPA1 were not signicantly altered as the concentrations of TM or TG increased, or the duration prolonged (Fig. 4a; Fig. S5a). However, MFN2 expression was down-regulated in both time and dose-dependent manners (Fig. 4a; Fig. S5a). MFN2 is a GTPase protein localized in the outer mitochondrial membrane as a crucial facilitator of mitochondrial fusion [25], and the reduction of MFN2 can be a trigger of mitophagy [26]. Therefore, we proposed that MFN2 down-regulation may be responsible for ER stress-induced mitochondrial fission and mitophagy. Of note, the phosphorylation of DRP1 was capable of sensing exogenous signal to regulate mitochondrial fission [27–29], with phosphorylation at S616 promoting fission whereas phosphorylation at S637 counteracting it [30]. The expression of phosphor-DRP1 at S637 displayed marginal alteration and the phosphor-DRP1 at S616 was significantly reduced (Fig. 4a; Fig. S5a). Considering that ER stress indeed induced mitochondrial fission, down-regulation of phosphor-DRP1 at S616 appeared to be the compensated feedback to avoid excessive fission.

We then obtained the overexpression of MFN2 in A2058 cell line, which could profoundly reverse ER stress-induced mitochondrial fission (Fig. 4b). In consistent, mitophagy was also repressed as revealed by immunofluorescence staining and immunoblotting analysis (Fig. 4c-d). After the overexpression of MFN2, intracellular ATP level was decreased and mitochondrial ROS was increased under ER stress (Fig. 4e-f), indicating that the suppression of mitochondrial fission and mitophagy exacerbated mitochondrial dysfunction under ER stress. More importantly, overexpression of MFN2 led to more cell death upon ER stress (Fig. 4g, Fig. S5b), with the expressions of pro-apoptotic caspase-3 and Bax increased, and the expression of anti-apoptotic Bcl-2 down-regulated (Fig. S5c). Together, MFN2 down-regulation was responsible for the potentiation of mitochondrial fission and mitophagy, so as to render the resistance to ER stress.

March5 Facilitates The Degradation Of Mfn2 Under Er Stress
We then investigated the mechanism underlying the down-regulation of MFN2 under ER stress. TM or TG treatment had minimal effect on MFN2 mRNA level (Fig. 5a, Fig. S6a), indicating that post-translational regulation rather than transcriptional regulation accounted for MFN2 down-regulation. It has been reported that MFN2 can be regulated by ubiquitination modification and proteasomal degradation [31]. After the treatment with proteasome inhibitor MG132, ER stress-induced down-regulation of MFN2 was reversed (Fig. 5b). Moreover, cycloheximide (CHX) pulse-chase assay showed that the turnover of MFN2 protein was increased upon ER stress (Fig. 5c). Immunoprecipitation assay revealed that more K48-linked ubiquitin chain bonded to MFN2 (Fig. 5d), suggesting that ubiquitination modification and proteasomal pathway were required for the down-regulation of MFN2.

Parkin has been documented as the canonical E3 ubiquitin ligase of MFN2 [26]. However, the loss of parkin frequently occurred in melanoma [32]. Therefore, it was of low possibility that parkin mediated the down-regulation of MFN2. Huwe1, Mul1 and MARCH5 were also candidate E3 ubiquitin ligases of MFN2 [31, 33, 34]. The mRNA levels of these three ligases were all increased upon ER stress (Fig. 5e, Fig. S6b). While neither the deficiency of Huwe1 nor Mul1 reversed the down-regulation of MFN2 (Fig. S6c-d), the knockdown of MARCH5 could partially recover MFN2 expression (Fig. 5f). Moreover, the deficiency of MARCH5 attenuated the interaction between K48-linked ubiquitin chain and MFN2 (Fig. 5g). Together, it was MARCH5, rather than Huwe1 or Mul1, mediated the ubiquitination and degradation of MFN2.

**XBP1 promotes the transcription of MARCH5 to mediate MFN2 down-regulation and mitochondrial fission and mitophagy**

Therefore, we went on to identify the signaling pathway(s) of the UPR responsible for transcriptional up-regulation of MARCH5. The expressions of ATF6, IRE1α and their downstream spliced XBP1 was significantly induced (Fig. S7a-b) [5]. In addition, the phosphorylation of both PERK and its downstream eIF2α were increased (Fig. S7a-b). While the deficiency of eIF2α had marginal effect on ER stress-induced MFN2 down-regulation (Fig. S7c), the knockdown of either IRE1α or ATF6 reversed MFN2 reduction, so did the knockdown of their downstream XBP1 (Fig. 6a-c) [35]. More importantly, the knockdown of ATF6, IRE1α or their downstream XBP1 significantly reversed ER stress-induced mitochondrial fission (Fig. 6d-f), whereas the knockdown of eIF2α had no impact (Fig. S7d-e). Therefore, it was IRE1α/ATF6-XBP1 branches of UPR mediated the crosstalk between ER and mitochondria. Forwardly, bioinformatics analysis showed the potential binding site at the promoter of MARCH5 to XBP1 (Fig. S8a). The knockdown of XBP1 abolished ER stress-induced MARCH5 up-regulation at both mRNA and protein levels (Fig. 6g-h). Moreover, chromatin immunoprecipitation (ChIP) assay revealed more enrichment of XBP1 on the MARCH5 promoter upon ER stress (Fig. 6i). Therefore, XBP1 acted as a transcriptional factor to promote MARCH5 expression and thereby induce MFN2 down-regulation and mitochondrial fission.

**The XBP1-MARCH5-MFN2 axis links UPR to mitochondria in vivo**

To further confirm the regulatory axis linking UPR to mitochondria, we first turned to publicly available melanoma GSE datasets. As was shown, the expressions of UPR pathway markers HSPA5, XBP1 and ATF6 were in positive correlation with MARCH5 (GSE19234, GDS3966 and GSE7553). The expression of
MARCH5 was in negative correlation with mitochondrial marker COX4I1 (GSE7553 and GDS1375) (Fig. 7a). Forwardly, we employed immunohistochemical staining analysis in TMA. In consistent with previous results, the staining intensity of XBP1 was positively correlated with MARCH5, and the staining intensity of MFN2 was in negative correlation with MARCH5 and LC3 respectively (Fig. 7b). Moreover, immunofluorescence staining analysis of implanted A2058 tumors showed that the expression of XBP1 was significantly increased after TM treatment, so was the staining intensity of MARCH5 (Fig. S9). Besides, the expressions of both MFN2 and COXIV were decreased upon ER stress (Fig. S9). After the co-treatment with either CQ or mdivi-1, the expressions of COXIV and MFN2 were prominently recovered (Fig. S9). Together, our analysis in public datasets, TMA and implanted tumors confirmed that the XBP1-MARCH5-MNF2 axis linked UPR to mitochondria in vivo.

Discussion

Tumor microenvironment is typically characterized by nutrient deprivation, hypoxia, glucose shortage and the accumulation of toxic metabolites, which are all triggers of ER stress [4, 36]. In addition, increased synthesis of proteins in rapidly-proliferating tumor cells can induce the overload of misfolded proteins in ER [3]. In response, various adaptive alterations can act as the driver to enable cell survival and tumor malignancy, once the pro-apoptotic UPR outputs are successfully limited [3, 37]. For example, the capacity of detoxifying cytotoxic ROS could be potentiated by the activation of PERK to facilitate tumor cell proliferation [38]. In addition, sub-lethal ER stress was capable of inducing autophagy for eliminating toxic cytosolic protein aggregates and damaged organelles to ensure tumor cell survival [37]. Of note, mitochondrion is the crucial organelle of which the structure and function are highly connected with ER, and is relatively sensitive to pathological stimuli that induce ER stress. The communication between ER and mitochondria is greatly implicated in metabolic diseases like diabetes and obesity with unsolved ER stress and mitochondrial dysfunction [39–42]. Extending to this, our present study unveiled the role of mitochondrial dynamics and mitophagy in mediating the resistance to ER stress, highlighting the importance of ER-mitochondria crosstalk in cancer pathogenesis. Tumor cells with relative stronger capacity to preserve the quality of mitochondria were more resist to ER stress, owing the advantage to survive the stressful circumstances. Besides, given that some ER stress inducers have been documented of great potential for the treatment of melanoma [10], therapeutic approaches triggering severe ER stress may be more useful for the subgroup of tumors with the deficiency of mitochondrial quality control machinery, in particular with defect mitochondrial fission and mitophagy.

The alteration of mitochondria in response to stressful stimuli and the resultant influence is still controversial. On one hand, pharmacological stress like doxorubicin treatment could induce mitochondrial fragmentation via the phosphorylation and degradation of MFN2, leading to cell apoptosis [43, 44]. On the other, ER stress and nutrient stress were also capable of promoting the assembly of respiratory chain super-complexes to increase mitochondrial respiration and cell proliferation [45]. Our present data revealed that the potentiation of mitochondrial fission caused by the loss of MFN2 played a protective role in response to ER stress. In contrast to a previous report that the genetic ablation of MFN2 in fibroblasts amplified ER stress and exacerbated ER stress-induced apoptosis [17], our data proved that
the loss of MFN2 expression ameliorated the lethal effect of ER stress in melanoma, raising the notion that the role of mitochondrial dynamics in response to ER stress is cell-type specific. To synergically intervene mitochondrial function should take cell context into consideration so that the proper effect can be obtained. Besides, accumulative evidence has revealed that MFN2 was required for the induction of mitophagy by promoting depolarization-induced translocation of Parkin to the mitochondria [46, 47]. However, some studies otherwise documented that MFN2 played a suppressive role in the activation of mitophagy by maintaining mitochondria-ER contacts and preventing the dissociation of mitochondria from the ER [25, 26]. Our data showed that the down-regulation of MFN2 facilitated mitophagy via the induction of mitochondrial fission under ER stress, supporting the suppressive effect of MFN2 on mitophagy.

In melanoma, the hyper-activation of MAPK signaling pathway was responsible for the increased mitochondrial fission, the inhibition of which was capable of suppressing the progression of \textit{BRAF}-mutant melanoma [48, 49]. In addition, the deficiency of mitochondrial fusion facilitator MFN2 augmented the migratory capacities of melanoma cells [50, 51]. These findings proved the oncogenic role of mitochondrial fission in melanoma under normal conditions. Beyond this, our data highlighted that mitochondrial fission also contributed to melanoma progression under stressful conditions, indicating that the inhibition of mitochondrial fission was a useful therapeutic strategy in a broad spectrum of pathogenic backgrounds. According to our results, increased mitochondrial fission was responsible for the activation of mitophagy that helped eliminate damaged mitochondria and maintain mitochondrial function, which was in line with previous studies [52, 53]. In this regard, mitochondrial fission can act as a crucial precursor of mitochondrial quality control to confer the resistance to ER stress and other stressful stimuli in multiple diseases.

As a commonly-used therapeutic agent for melanomas harboring \textit{BRAF} mutation, Vemurafenib has been documented to exert its effect via the induction of ER stress [54]. In addition, a compound recently called HA15 had strong anti-tumor effect on melanoma by specifically targeting HSPA5 and triggering severe ER stress [10]. These reports unveiled the great potential of pharmacological ER stress inducers for the treatment of melanoma. Given that tumor cells with the deficiency of mitochondrial fission and mitophagy were more sensitive to ER stress, to identify melanomas with defect mitochondrial quality control machinery might help improve the treatment outcome. More importantly, concurrent suppression of either mitochondrial fission or mitophagy could synergize with ER stress inducers like HA15 or Vemurafenib for the treatment of melanomas. Additional investigations in pre-clinical mice models or clinical trials are needed to further verify the effect of the combined therapy.

**Conclusions**

In summary, we elucidated a protective role of mitochondrial fission and mitophagy in melanoma cells upon ER stress. Mechanistically, IRE1α/ATF6-XBP1 branches of UPR promoted the expression of E3 ligase MARCH5, which facilitated MFN2 degradation to trigger mitochondrial fission and subsequent
mitophagy. Targeting mitochondrial quality control machinery like mitochondrial fission and mitophagy can be exploited as an approach to reinforce the efficacy of ER stress-inducing agents against cancer.

**Abbreviations**

ATF6, activating transcription factor 6; EIF2AK3/PERK, eukaryotic translation initiation factor 2-a kinase 3; ER, endoplasmic reticulum; ERN1/IRE1, endoplasmic reticulum to nucleus signaling 1; SQSTM1/p62, sequestosome 1; TG, thapsigargin; TM, tunicamycin; UPR, unfolded protein response; XBP1, x-box binding protein 1; ACTB, actin beta; BAX, BCL2 associated X, apoptosis regulator; DMEM: dulbecco’s modified Eagle’s medium; EM: electron microscopy; FBS: fetal bovine serum; H&E: hematoxylin and eosin; HMGB1: high mobility group box 1; MAP1LC3/LC3: microtubule associated protein 1 light chain 3; NS: not significant; cleaved PARP1 cleaved poly (ADP-ribose) polymerase 1; qRT-PCR quantitative real-time reverse transcription PCR

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animal by International Committees.

**Consent for publication**

Not applicable.

**Availability of data and material**

Please contact the corresponding author for all data requests.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

WNG and CYL conceived of the study and carried out its design. HNW, XLY, SG, SJW, JYM and TZ performed the experiments. HNW, XLY, SG, QS, YZT and HW conducted the statistical analyses. WNG
applied the ethical documents. LTJ and TWG provided suggestions for experimental design. WNG and CYL wrote and revised the paper. All authors read and approved the final manuscript.

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Figures
Mitochondria is related to the sensitivity to ER stress in melanoma. (a-b) The percentage of cell death in four distinct melanoma cell lines in response to different concentrations of TM or TG. (c) GSEA analysis of the correlation between UPR pathway molecules and mitochondrial gene expression/mitochondrial oxidative phosphorylation in TCGA SKCM database. (d) Heatmap of representative genes in UPR pathway and mitochondrial function in TCGA SKCM database. (e) The differences of the expressions of
indicated mitochondrial genes between HSPA5-low and HSPA5-high group in TCGA SKCM database. (f) Representative immunohistochemical staining images of XBP1 and COXIV in TMA that consists of 84 melanoma cases. The correlation between XBP1 and COXIV expression was displayed on the right. Scale bars = 100 μm. (g) Heatmap of representative genes in UPR pathway, mitochondrial oxidative phosphorylation and ribosomal proteins in 451Lu and A375 cell lines in short-term cultures dataset. Data represent the mean ± SD of triplicates. P value was calculated by two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2
Figure 2

Mitochondrial fission and mitophagy indicate the sensitivity to ER stress. (a) The alteration of intracellular ATP level after the treatment with TM or TG in four different melanoma cell lines. (b) The alteration of mitochondrial ROS after the treatment with TM or TG in four different melanoma cell lines. (c) The alteration of mitochondrial ROS in TM or TG-stimulated WM35 and A375 cell lines after the co-treatment with mito-TEMPO. (d) The percentage of cell death in TM or TG-stimulated WM35 and A375 cell lines after the co-treatment with mito-TEMPO. (e) Representative confocal microscope images of the mitochondrial network in A2058 and WM35 cell lines with treatment as indicated. Scale bars = 20 μm. The proportion of melanoma cells (n=100 cells for each sample) with tubulated, intermediate and fragmented mitochondria was quantified. (f) The protein expressions of LC3, p62, COXIV, TOMM20 in A2058 and WM35 cell lines with treatment as indicated. (g) Immunofluorescence staining analysis of LC3 and MitoTracker, and their co-localization in A2058 and WM35 cell lines with treatment as indicated. Scale bars = 20 μm. Data represent the mean ± SD of triplicates. P value was calculated by two-tailed Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 3

The activation of mitochondrial fission and mitophagy confers the resistance to ER stress. (a) The expressions of LC3, p62, COXIV, TOMM20 in A2058 and 451Lu cell lines with treatment as indicated. (b) The expressions of caspase 3, cleaved-caspase 3, Bax and Bcl2 in A2058 and 451Lu cell lines with treatment as indicated. (c-e) The alteration of intracellular ATP level, mitochondrial ROS and cell viability in A2058 and 451Lu cell lines with treatment as indicated. (f) Images of isolated tumors from xenograft
mice that received indicated treatment. Tumor volumes in each group were calculated and displayed on the right. Data represent the mean ± SD of triplicates. P value was calculated by two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 4**

The down-regulation of MFN2 mediates mitochondrial fission and mitophagy under ER stress. (a) The expressions of mitochondrial dynamics-related regulators in A2058 and 451Lu cell lines after the
treatment with TM or TG. (b) Representative confocal microscope images of the mitochondrial network in A2058 cell line with treatment as indicated. Scale bars = 10 μm. The proportion of melanoma cells (n=100 cells for each sample) with tubulated, intermediate and fragmented mitochondria was quantified. (c) Immunofluorescence staining analysis of LC3 and MitoTracker, and their co-localization in A2058 and WM35 cell lines with treatment as indicated. Scale bars = 10 μm. (d) The expressions of MFN2, COXIV and TOMM20 in A2058 cell line with treatment as indicated. (e-g) The alteration of intracellular ATP level, mitochondrial ROS and cell death in A2058 cell line with treatment as indicated. Data represent the mean ± SD of triplicates. P value was calculated by two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001.
MARCH5 promotes the degradation of MFN2 under ER stress. (a) The mRNA level of MFN2 in A2058 cell line with the treatment of TM or TG. (b) The protein level of MFN2 in TM or TG-stimulated A2058 cell line after the co-treatment with MG132. (c) The protein level of MFN2 in TM or TG-stimulated A2058 cell line after the co-treatment with CHX. (d) Co-immunoprecipitation analysis of the interaction between K48-linked ubiquitin chain and MFN2 after the treatment with TM or TG. (e) The mRNA level of MARCH5 in
A2058 cell line after the treatment with TM or TG. (f) The protein level of MFN2 and MARCH5 in TM or TG-stimulated A2058 cell line after the knockdown of MARCH5. (g) Co-immunoprecipitation analysis of the interaction between K48-linked ubiquitin chain and MFN2 in TM or TG-stimulated A2058 cell line with or without the knockdown of MARCH5. Data represent the mean ± SD of triplicates. P value was calculated by two-tailed Student’s t-test. ***P < 0.001.

Figure 6
XBP1 promotes the transcription of MARCH5 to mediate MFN2 down-regulation. (a-c) The protein level of MFN2 in TM or TG-stimulated A2058 cell line with or without the knockdown of ATF6, IRE1α or XBP1. (d-f) Representative confocal microscope images of the mitochondrial network in TM or TG-stimulated A2058 cell line with or without the knockdown of ATF6, IRE1α or XBP1. Scale bars = 10 μm. The proportion of melanoma cells (n=100 cells for each sample) with tubulated, intermediate and fragmented mitochondria was quantified. (g) The mRNA level of MARCH5 in TM or TG-stimulated A2058 cell line with or without the knockdown of XBP1. (h) The protein level of MARCH5 in TM or TG-stimulated A2058 cell line with or without the knockdown of XBP1. (i) ChIP assay shows the enrichment of XBP1 on the MARCH5 promoter after the treatment with TM. Data represent the mean ± SD of triplicates. P value was calculated by two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 7

(a) The correlation among UPR pathway molecules, MARCH5 and mitochondrial marker COXIV in multiple publicly available melanoma GSE datasets. (b) The correlation between XBP1 and MARCH5, MARCH5 and MFN2, MFN2 and LC3 in melanoma TMA consisting of 84 cases. Correlations between measured variables were tested by Spearman rank correlation analyses. Scale bars = 20 μm.

The XBP1-MARCH5-MNF2 axis links UPR to mitochondria in vivo. (a) The correlation among UPR pathway molecules, MARCH5 and mitochondrial marker COXIV in multiple publicly available melanoma GSE datasets. (b) The correlation between XBP1 and MARCH5, MARCH5 and MFN2, MFN2 and LC3 in melanoma TMA consisting of 84 cases. Correlations between measured variables were tested by Spearman rank correlation analyses. Scale bars = 20 μm.
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