

BiP Regulates the Unfolded Protein Response and Mitochondrial Respiration in Human Embryonic Stem Cells

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Abstract

Background

Human embryonic stem cells (hESCs) are highly susceptible to dissociation-induced stress, limiting their long-term culture and therapeutic applications. The unfolded protein response (UPR) is essential for maintaining endoplasmic reticulum (ER) proteostasis, which plays a critical role in preserving hESC pluripotency. However, the role of the UPR in coordinating cellular stress adaptation and mitochondrial function in hESCs remains poorly understood. In the present study, we investigated the role of the ER chaperone immunoglobulin heavy chain-binding protein (BiP) in regulating UPR signaling, mitochondrial respiration, and cell survival following dissociation-induced stress in hESCs.

Methods

We generated BiP knockout hESCs using CRISPR/Cas9. Cells were examined both under normal conditions and after dissociation-induced stress. Gene and protein expression were assessed, along with mitochondrial function. Cell survival was evaluated using Annexin V/propidium iodide flow cytometry.

Results

We found that BiP expression is dynamically regulated following dissociation in hESCs. We also showed that BiP knockout did not affect the expression of pluripotency markers but did disrupt coordinated UPR signaling. Moreover, our data revealed that BiP deficiency resulted in susceptibility to dissociation-induced apoptosis, which was not rescued by endoplasmic reticulum stress inhibition. In addition, BiP knockout cells showed significantly reduced basal and maximal mitochondrial respiration and impaired oxidative phosphorylation, while mitochondrial membrane potential remained constant.

Conclusions

BiP is not required for maintenance of pluripotency in hESCs but is essential for coordinating adaptive UPR signaling and sustaining mitochondrial respiratory capacity under stress. Loss of BiP uncouples endoplasmic reticulum stress responses from mitochondrial bioenergetics, resulting in impaired stress resilience and increased apoptosis following dissociation. These findings identify BiP as a key regulator linking proteostasis and mitochondrial function in human embryonic stem cells.

Introduction

Human embryonic stem cells (hESCs) possess the capacity to differentiate into all three germ cell types (1), making them an essential resource for regenerative medicine, disease modeling, and drug screening

(2). However, culture of hESCs remains challenging due to their susceptibility to cell death upon dissociation, a process known as anoikis (3, 4). Our previous study demonstrated that dissociated hESCs undergo iron-dependent cell death, which is characterized by iron accumulation, lipid peroxidation, and disruption of antioxidant defenses (5). Collectively, these findings suggest that hESC survival following dissociation relies on coordinated stress-adaptive mechanisms that are not yet fully identified.

The endoplasmic reticulum (ER) is a crucial organelle that maintains cellular proteostasis, calcium homeostasis, and protein folding (6, 7). Disruption of ER function results in the accumulation of unfolded or misfolded proteins, thereby activating the unfolded protein response (UPR) (8, 9). UPR maintains cell viability and sustains homeostasis under various physiological and pathological conditions (10). The ER chaperone glucose-regulated protein 78 (GRP78), known as immunoglobulin heavy chain binding protein (BiP) (11, 12), serves as a master regulator of the UPR through its interaction with the ER stress sensors serine/threonine protein kinase/endoribonuclease (IRE1), activating transcription factor 6 (ATF6), and protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) (8, 13). Despite its established role in ER proteostasis and stress signaling, the contribution of BiP to stress adaptation and survival in hESCs remains poorly understood.

Mitochondria regulate cellular metabolism, energy production, and cell fate decisions (14). To maintain pluripotency, hESCs exhibit a unique metabolic profile characterized by immature mitochondrial architecture and a predominant reliance on glycolysis (15, 16). Nonetheless, mitochondrial activity remains fundamental for cellular homeostasis, and alterations in mitochondrial function have been implicated in stem cell differentiation and cell death (17, 18). However, whether BiP is required for maintaining mitochondrial bioenergetic capacity and cellular stress resilience in hESCs has not been established. Understanding how BiP coordinates stress responses with mitochondrial homeostasis may therefore provide important insights into the mechanisms that preserve hESC viability under stressful conditions.

In the present study, we investigated the role of BiP in stress adaptation, mitochondrial function, and survival in hESCs using a CRISPR/Cas9-mediated BiP knockout model. We first examined the temporal expression of BiP and UPR-associated genes following dissociation and then assessed the effects of pharmacological modulation of ER stress on stress-responsive transcriptional programs. We also evaluated whether TTNPB, a synthetic retinoid previously shown to improve the survival of dissociated hESCs, modulates BiP expression and ER stress signaling during dissociation-induced stress. Finally, we evaluated mitochondrial bioenergetics, mitochondrial membrane potential, and apoptotic responses in WT and BiP-KO hESCs. We demonstrate that BiP is dispensable for the maintenance of pluripotency under basal conditions but is required for coordinated UPR signaling and mitochondrial respiratory capacity. Furthermore, BiP deficiency increases susceptibility to stress-induced apoptosis despite preserved mitochondrial membrane potential, presenting BiP as a crucial regulator of ES cell stress resilience rather than basal stem cell identity.

Materials and Methods

Cell Culture and Maintenance of hESCs

The hESC line H9 (WiCell Research Institute, USA) was routinely cultured and maintained in chemically defined, feeder-free Laminin-511™ (Nacalai) with mTeSR™1 medium (STEMCELL Technologies) under standard culture conditions (37°C, 5% CO₂). The cells were grown on ECM-coated dishes at $\sim 3 \times 10^4$ cells/cm² in complete mTeSR™1 medium. The cells were centrifuged and resuspended in fresh mTeSR™1 medium containing 10 µM ROCK inhibitor Y-27632 (STEMCELL Technologies). Dissociated hESCs were counted by an automatic cell counter (Thermo Fisher Scientific) and routinely passaged every five days. The medium was replaced daily without Y-27632.

Generation of BiP Knockout (KO) hESCs

For CRISPR deletion of BiP from human H9 cells, the pSpCas9(BB)-2A-GFP (pX458) vector (Addgene plasmid #48138) was used. The short guide sequence to target Bip exon 2 was: 5'-CCAATCAGACGTTCCCCTTC-3'. The short guide sequence oligonucleotides were synthesized, annealed, and cloned into the pX458 vector and confirmed by DNA sequencing at the DNA laboratory, University of Calgary. Five µg of DNA plasmid (pX458 containing BiP short guide RNA sequence) were transfected into human H9 cells using jetOPTIMUS (Poluplus) according to the manufacturer's instructions. Four to eight h after transfection, cells were harvested, and genomic DNA was isolated using the KAPA Express Extract Kit (Kapa Biosystems) according to the manufacturer's instructions. Genomic DNA fragments of Bip around the short guide RNA site were amplified by PCR. The primers used to amplify the genomic region of Bip exon 2 were: Forward: 5'-GGAATTCGTAAGTGGGGTTGCGGATG-3', Reverse: 5'-GGGGTACCAAATACTCCCACCACCCACC-3'. PCR products were used for nuclease mutation detection assays using the Alt-R Genome Editing Detection Kit (IDT) according to the manufacturer's protocol. After transfection, GFP-containing cells were sorted into a 96-well plate by Flow Cytometry at the Flow Cytometry Facility, University of Calgary. Single cells were then expanded for further analysis (i.e., Immunocytochemistry and DNA sequencing).

For DNA sequencing confirmation of BiP knockout clones, DNA fragments of the BiP knockout clone around the short guide RNA site were amplified by PCR. The primers used to amplify DNA fragments from the BiP knockout clone were the same as those used for nuclease mutation detection assays. PCR products were subcloned into the pEGFP-C2 vector (Clontech), and plasmid DNA from individual clones was sent for Sanger DNA sequencing at the DNA laboratory, University of Calgary, to confirm all indels.

Immunocytochemistry

Cells were fixed with 4% PFA (Thermo Fisher Scientific) for 15 minutes at room temperature and then washed with PBS (Gibco). In the next step, cells were permeabilized with 0.1% Triton-X 100 (Sigma-

Aldrich) for 15 minutes. After blocking with 10% FBS (Gibco) for 30 minutes, cells were incubated overnight at 4°C with primary BiP antibody (ab21685, 1:500, Abcam), and CHOP antibody (MA1-250, 1:500, Invitrogen). The following day, cells were washed and probed with anti-rabbit Alexa Fluor® 488 secondary antibody (Thermo Fisher Scientific) for 2 h at room temperature. After three PBS washes, DAPI in Slowfade (Thermo Fisher Scientific) was added for nuclear staining. Imaging was performed using an Olympus DP70 camera displayed on an Echo Revolve microscope (Echo)

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was isolated using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific) following the manufacturer’s guidelines and then read using Nanodrop Microvolume Spectrophotometer (Thermo Fisher Scientific). To perform mRNA reverse transcription, the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used. The samples were mixed as follows: 10× RT buffer 5 µL, 25× dNTP Mix 2 µL, 10× RT random primers 5 µL, H2O 10.5 µL, MultiScribe™ Reverse Transcriptase 2.5 µL, and 25 µL of sample. Then, cDNA generation consisted of the following steps: 65°C for 5 min, 42°C for 60 min, and 80°C for 5 min. Lastly, gene expression analysis was conducted using TaqMan Master Mix (Thermo Fisher Scientific) for RT-qPCR on the Applied Biosystems™ Step OnePlus™ Real-Time PCR System. In this study, GAPDH was used as a housekeeping gene to normalize mRNA expressions. TaqMan product information provided in Table 1.

Table 1
TaqMan gene expression assays used for qPCR

Gene	Assay ID	Target	Species
BiP (HSPA5/GRP78)	Hs00607129_gH	UPR signaling	Human
ATF6	Hs00232586_m1	UPR signaling	Human
XBP1u	Hs02856596_m1	UPR signaling	Human
XBP1s	Hs03929085_g1	UPR signaling	Human
ATF4	Hs00909569_g1	UPR signaling	Human
PERK (EIF2AK3)	Hs00984003_m1	UPR signaling	Human
IRE1 (ERN1)	Hs00176385_m1	UPR signaling	Human
GAPDH	Hs02758991_g1	Housekeeping gene	Human
OCT4 (POU5F1)	Hs00999632_g1	Pluripotency marker	Human
SOX2	Hs04234836_s1	Pluripotency marker	Human
NANOG	Hs02387400_g1	Pluripotency marker	Human

Western blotting

Cells were lysed with RIPA buffer containing 100× Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific). Total protein concentration was assessed using a Bradford assay (Biorad), and 50 µg of total protein from each sample was separated by 12% SDS-PAGE before being transferred onto a nitrocellulose membrane. The membranes were blocked in TBS-T containing 5% skim milk at room temperature for 1 h, then incubated with primary antibodies against BiP (ab216851:1000, Abcam) and β-actin (MA515739, 1:2000, Thermo Fisher Scientific) overnight at 4°C. The next day, the process was followed by washing the membrane for 15 min with TBS-T at room temperature and incubation with appropriate secondary antibodies for one h. After three TBS-T washes, a chemiluminescent or colorimetric substrate (PerkinElmer) was applied to generate a detectable signal, which was captured using the Gel Doc system. Protein expression levels were analyzed using ImageJ after background subtraction.

Apoptosis Detection

Apoptotic cells were detected using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences). Cells were washed twice with cold PBS to remove media, then resuspended at a concentration of 1×10^6 cells/mL in 1X Binding Buffer. Five microliters of FITC Annexin V and five microliters of propidium iodide were added to each tube. After gentle vortexing, the cells were incubated in the dark at room temperature for 15 minutes. After incubation, 400 µl of 1X Binding Buffer was added. Flow cytometry analysis was performed within 1h using the Cytex Aurora flow cytometer. Apoptotic cells were identified by Annexin V-PE binding, with 7-AAD distinguishing early apoptotic from late apoptotic/necrotic cells. Controls included unstained cells, cells stained with PE Annexin V only, and cells stained with 7-AAD only.

Measurement of Mitochondrial Respiration

Mitochondrial respiration was assessed using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies) and the Seahorse XF Cell Mito Stress Test following the manufacturer's guidelines. Cells were plated at a density of 8×10^4 cells per well in XF24 microplates and maintained for 48 h at 37°C in a humidified incubator with 5% CO₂. Prior to measurement, the culture medium was replaced with Seahorse assay medium supplemented with 25 mM D-glucose, 2 mM sodium pyruvate, and 4 mM L-glutamine. Oxygen consumption rate (OCR) was recorded at baseline and after sequential injections of oligomycin (1 µg/mL; Enzo Life Sciences, BML-CM111), FCCP (1 µM; Enzo Life Sciences, BML-CM120), and antimycin A (1 µM; Sigma-Aldrich, A8674). Following completion of the assay, cellular protein content in each well was quantified using a BCA protein assay kit (Thermo Fisher Scientific, 23225), and OCR values were normalized to total protein levels.

Tetramethylrhodamine, ethyl ester (TMRE) Staining

Mitochondrial membrane potential (MMP) in hESCs was assessed using TMRE (87917; Sigma-Aldrich). First, cells were incubated with TMRE (150nM) at 37°C for 20 minutes. As a positive control for mitochondrial depolarization, 10 μ M of FCCP was added 10 minutes before the end of TMRE staining incubation. Then, cells were dissociated using accutase and centrifuged at 1000 RPM for 5 minutes. Finally, the cell pellet was resuspended in PBS at a concentration of 1×10^6 cells/mL and analyzed by Attune flow cytometry, with TMRE excitation at ~ 549 nm and emission at ~ 574 nm. Mitochondrial membrane potential was reported as mean fluorescence intensity values of live, single cells analyzed using FlowJo analysis software.

Statistical Analysis

Unless otherwise specified, all experiments were performed in triplicate, and data was presented as mean $\pm$ S.E.M. A one-way ANOVA followed by Tukey's post hoc test was used to analyze differences between groups. When comparing two independent groups, a student's t-test was used. A p-value ≤ 0.05 was considered statistically significant.

Results

BiP expression is dynamically regulated following dissociation and pharmacological modulation in hESCs

Low level of BiP in hESCs may lead to a decreased protective effect against ER stress, making them more vulnerable to ER stress (19). To understand the pattern of BiP following dissociation, BiP mRNA and protein levels were measured at different time points in hESCs. As shown in Fig. 1A, BiP mRNA expression was significantly reduced at 2, 4, and 24 h post-dissociation compared with baseline levels. Despite the decrease in mRNA expression, BiP protein levels exhibited a distinct temporal pattern (Fig. 1B). Western blot analysis demonstrated a gradual increase in BiP protein levels from 0 to 4 h, with the highest expression observed at 4 h post-dissociation. By contrast, BiP protein expression decreased significantly at 24 h. Thus, while BiP mRNA expression remained reduced after dissociation, BiP protein levels showed a transient increase at early time points before declining by 24 h, suggesting a dynamic regulation of BiP expression during the cellular response to dissociation-induced stress.

To further examine whether pharmacological modulation could influence BiP expression, hESCs were treated with TTNPB alone or in combination with Y-27632. As shown in Fig. 1D, combined treatment of hESCs with TTNPB and Y-27632 significantly increased BiP expression compared to individual treatments. We also found TTNPB and/or Y-27632 decrease dissociation-induced CHOP expression in hESCs (Fig. 1F).

BiP deficiency does not affect hESCs pluripotency

Given the dissociation-induced downregulation of BiP in hESCs, we next generated BiP knockout cells using CRISPR/Cas9 system to investigate the role of BiP in hESC homeostasis (Fig. 2A). Successful deletion of BiP was confirmed by DNA sequencing of the targeted locus (Fig. 2B). Additionally, immunocytochemical analysis further verified the absence of BiP protein expression in BiP knockout (KO) hESCs, whereas wild type (WT) hESCs exhibited strong BiP expression (Fig. 2C).

We next asked whether BiP is functionally required for maintaining pluripotency under basal conditions. To determine whether BiP deletion affects pluripotency, the expression of the pluripotency-associated genes OCT4, NANOG, and SOX2 was evaluated by RT-qPCR. No significant differences in the mRNA expression levels of pluripotent marker were observed between WT and BiP-KO hESCs (Fig. 2D-F). These results indicate that BiP deletion does not alter the expression of key pluripotency markers and suggest that BiP is not required for maintaining the pluripotent state of hESCs under the conditions examined.

BiP deficiency disrupts coordinated transcriptional regulation of UPR-associated genes following dissociation in hESCs

Given the role of BiP as a central regulator of the UPR (12), we first asked whether BiP deletion alters the expression dynamics of genes associated with the IRE1-XBP1 signaling pathway. Significant differences in the relative mRNA expression of IRE1, XBP1u, and XBP1s were observed between WT and BiP-KO cells over time (Fig. 3A-C). As shown in Fig. 3A, IRE1 expression remained unchanged over time in WT cells, whereas it increased at 4 and 24 h post-dissociation in BiP-KO hESCs. Moreover, IRE1 expression was significantly higher in BiP-KO cells than in WT cells at 24 h post-dissociation. By contrast, XBP1u expression remained relatively stable in WT cells, however decreased significantly at 4 and 24 h in BiP-KO hESCs (Fig. 3B). Accordingly, XBP1u expression was significantly lower in BiP-KO cells than in WT cells at both time points. We also found that XBP1s expression increased significantly at 24 h post-dissociation in BiP-KO hESCs; however, it remained unchanged in WT over time (Fig. 3C). Together, these findings indicate that BiP deficiency disrupts the upstream transcriptional homeostasis and basal XBP1 transcript pool, while preserving the scale of XBP1 splicing associated with mRNA output of the IRE1-XBP1 pathway following dissociation-induced stress in hESCs; a response that remains coordinated in WT cells but is transcriptionally dysregulated in BiP-KO cells.

Our results from the PERK branch of the UPR showed a decreased expression of eIF2 α at 24 h post-dissociation in BiP-KO compared to WT (Fig. 3D). In line with this, ATF4 expression decreased at 4 and 24 h following dissociation compared to WT (Fig. 3E). In contrast, both eIF2 α and ATF4 remained stable in WT over time. We next examined ATF6 expression in WT and BiP-KO hESCs following dissociation. As presented in Fig. 3F, BiP-KO hESCs exhibited lower ATF6 expression at 24 h post-dissociation compared with WT cells, while ATF6 expression remained constant in WT over time. Overall, these results indicate that BiP is required for maintaining transcriptional homeostasis and coordinated UPR gene expression dynamics following dissociation-induced stress in hESCs.

BiP deficiency compromises cell survival following dissociation and is not rescued by TUDCA treatment

To determine whether BiP deficiency affects cell survival following dissociation and to assess the effects of ER stress inhibition and ROCK inhibition, apoptosis was analyzed in WT and BiP-KO hESCs treated with TUDCA and/or Y-27632. Under untreated conditions, BiP-KO hESCs exhibited a higher proportion of early apoptotic cells (29.61%) and a lower percentage of viable cells (69.30%) compared with WT hESCs (19.63% and 79.46%, respectively). Treatment with Y-27632 increased early apoptosis to 24.90% in WT cells and 30.94% in BiP-KO cells, while reducing the proportion of viable cells to 73.56% and 63.13%, respectively. TUDCA treatment decreased early apoptosis in WT cells from 19.63% to 15.56% and increased cell viability from 79.46% to 81.89%. In contrast, TUDCA treatment did not improve cell survival in BiP-KO hESCs, as early apoptosis increased from 29.61% to 34.94% and the percentage of viable cells decreased from 69.30% to 61.22%. Combined treatment with TUDCA and Y-27632 further reduced cell viability in both groups, with WT and BiP-KO cells exhibiting 67.73% and 58.31% viable cells, respectively. Notably, BiP-KO hESCs treated with TUDCA and Y-27632 showed the highest proportion of early apoptotic cells (40.76%). These findings indicate that BiP deficiency compromises cell survival following dissociation and that TUDCA is insufficient to rescue the apoptosis-associated phenotype in BiP-KO hESCs.

BiP deficiency impairs mitochondrial respiration without affecting membrane potential in hESCs

BiP loss significantly impaired mitochondrial respiratory function in hESCs. Measurement of oxygen consumption rate (OCR) revealed that BiP-KO cells exhibited markedly reduced basal and maximal respiration compared to WT controls (Fig. 5A and B), indicating compromised mitochondrial respiratory capacity. This reduction was accompanied by decreased ATP-linked respiration, suggesting impaired oxidative phosphorylation and diminished ATP production (Fig. 5C). Furthermore, proton leak was significantly reduced in BiP-KO hESCs, consistent with altered mitochondrial membrane dynamics and reduced electron transport chain activity (Fig. 5C). Moreover, mitochondrial membrane potential was assessed in WT and BiP-KO hESCs under basal conditions using TMRE staining. As shown in Fig. 5D, no significant differences in TMRE fluorescence intensity were observed between WT and BiP-KO cells. These results suggest that BiP deficiency affects mitochondrial respiration while preserving mitochondrial membrane potential in hESCs.

Discussion

There is growing evidence that BiP is crucial for UPR regulation, cell survival, and metabolic control (20). Previous studies have shown that BiP expression is relatively low in hPSCs, rendering them vulnerable to stress-induced cell death (19). In the present study, BiP protein expression exhibited dynamic changes following dissociation-induced stress, with a transient increase at early time points followed by a decline

at 24 h, suggesting that the adaptive capacity of hESCs to cope with dissociation-induced stress becomes compromised over time. In our previous study, we demonstrated that the retinoid analogue TTNPB reduces apoptosis through decreasing Caspase-3 in hPSCs (21). Here, we showed that TTNPB attenuates ER stress, as evidenced by downregulation of CHOP expression. Interestingly, BiP is expressed during mouse embryogenesis, and its promoter is activated by retinoic acid (RA) signaling (22). Our finding that TTNPB treatment elevated BiP expression is consistent with the involvement of RA-associated signaling, suggesting that TTNPB may alleviate dissociation-induced ER stress at least partially through the upregulation of BiP and subsequent suppression of pro-apoptotic UPR signaling.

These observations led us to hypothesize that BiP plays an important role in maintaining hESC homeostasis during dissociation-induced stress. Therefore, to directly investigate the functional significance of BiP in hESCs, we generated BiP-knockout cells and first examined whether loss of BiP affects the maintenance of pluripotency under basal conditions. While BiP has been reported to be involved in the maintenance of pluripotency and stem cell survival (23), we found that BiP deficiency did not alter pluripotency in hESCs under basal conditions. Although BiP has been implicated in protein synthesis, proper protein folding, and maintenance of ER proteostasis (24), all of which are crucial for hPSC pluripotency and self-renewal (25), our findings suggest that BiP may be more important for sustaining cellular homeostasis and stress resilience than for maintaining pluripotency itself.

Under normal conditions, BiP binds to PERK, IRE1, and ATF6 and maintains these ER stress sensors in an inactive state. However, BiP is released from these proteins upon ER stress, leading to activation of the UPR (26). The complete loss of BiP has been reported to result in embryonic lethality due to massive apoptotic cell death during early development (27), highlighting its essential role in developmental viability. In contrast, our findings showed that in hESC culture conditions, BiP deficiency does not induce obvious cell death under basal conditions but instead leads to impaired stress adaptation and increased susceptibility to dissociation-induced apoptosis. Additionally, suppression of BiP via siRNA knockdown has been shown to reverse this resistance and increase susceptibility (28). Our results revealed that BiP loss led to activation of the IRE1-XBP1 pathway, characterized by increased IRE1 and XBP1s expression and decreased XBP1u expression.

In agreement with our observation, Suzuki et al. showed that BiP knockdown leads to UPR activation and apoptosis through activation of IRE1-XBP1 pathway in HeLa cells (29). Growing evidence suggests that enhanced BiP expression attenuates UPR activation and protects cells from ER stress-mediated cell death (30, 31), conversely, reduced BiP levels make the cells more vulnerable to ER stress with increased UPR induction (32, 33). Interestingly, BiP deficiency did not uniformly affect all branches of the UPR in our model. We found that BiP deletion resulted in decreased expression of eIF2 α , ATF4, and ATF6, despite activation of the IRE1-XBP1 axis, suggesting that BiP deficiency leads to dysregulated rather than globally activated UPR signaling. Given the critical role of the UPR in determining cell fate under stress conditions, such uncoordinated signaling may contribute to the increased susceptibility of BiP-KO hESCs to dissociation-induced apoptosis. Consistently, TUDCA was insufficient to rescue the apoptosis-associated phenotype in BiP-KO hESCs, further supporting the notion that BiP functions as a central

regulator of stress resilience in hESCs. However, the molecular mechanisms by which BiP deficiency leads to dysregulated UPR signaling remain to be elucidated, and further investigations are needed to define the regulatory networks involved.

Considering the well-established interplay between ER stress signaling and mitochondrial bioenergetics, we examined whether BiP deficiency influences mitochondrial function in hESCs. BiP-KO cells showed a significant reduction in both basal and maximal oxygen consumption rates, along with lower ATP-linked respiration, revealing compromised oxidative phosphorylation capacity and reduced mitochondrial respiratory efficiency. These results are consistent with previous reports demonstrating that ER stress and UPR signaling are tightly coupled to mitochondrial metabolic regulation and can directly influence oxidative phosphorylation capacity (34). Consistent with our observation that BiP deficiency impairs mitochondrial respiration, functional suppression of GRP78 in porcine embryos has been shown to induce mitochondrial dysfunction and compromise embryonic development, further supporting a role for BiP in maintaining mitochondrial fitness during early developmental stages (35).

Conclusion

In summary, our study demonstrates that BiP is a critical regulator of stress adaptation in human ESCs. Although BiP deficiency does not affect the maintenance of pluripotency under basal conditions, it profoundly disrupts the coordinated regulation of UPR signaling, leading to selective activation of the IRE1-XBP1 axis, suppression of PERK and ATF6 pathways, and increased susceptibility to dissociation-induced apoptosis. Furthermore, BiP loss impairs mitochondrial respiratory function without altering mitochondrial membrane potential, indicating a specific defect in bioenergetic capacity rather than structural mitochondrial integrity. Together, these findings identify BiP as an essential factor linking ER proteostasis to mitochondrial metabolism and cell survival, thereby maintaining cellular homeostasis under stress conditions in human pluripotent stem cells.

Abbreviations

ATF

Activating transcription factor 6

ANOVA

Analysis of variance

BiP

Immunoglobulin heavy chain-binding protein

CHOP

C/EBP homologous protein

ER

Endoplasmic reticulum

GAPDH

Glyceraldehyde 3-phosphate dehydrogenase

GRP78
Glucose-regulated protein 78
hESCs
Human embryonic stem cells
IRE1
Inositol-requiring enzyme 1
KO
Knockout
OCR
Oxygen consumption rate
OCT4
Octamer-binding transcription factor 4
NANOG
Homeobox protein NANOG
SOX2
SRY-box transcription factor 2
PERK
Protein kinase R (PKR)-like endoplasmic reticulum kinase
qPCR
Quantitative polymerase chain reaction
ROCK
Rho-associated protein kinase
ROS
Reactive oxygen species
TMRE
Tetramethylrhodamine ethyl ester
TUDCA
Tauroursodeoxycholic acid
UPR
Unfolded protein response
WT
Wild type
XBP1
X-box binding protein 1
XBP1s
Spliced X-box binding protein 1
XBP1u
Unspliced X-box binding protein 1
Y-27632
Rho-associated protein kinase inhibitor Y-27632.

Declarations

Ethics approval and consent to participate

The human embryonic stem cell line H9 (WA09) was obtained from WiCell Research Institute (Madison, WI, USA) under a Material Transfer Agreement. The H9 cell line was originally derived from human blastocysts with appropriate ethical approval and informed consent from the donors, as reported in the original publication describing the derivation of human embryonic stem cell lines (36). The authors did not collect human biological samples or recruit human participants for this study.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Author Contribution

SBA and AG conceived and designed the research; SBA performed the formal analysis and investigation and wrote the original draft of the manuscript. SBA, AG and IAK performed the research and acquired the data. SBA and DER wrote the final draft of the manuscript. TES contributed to mitochondrial experiments, and IAK contributed to data acquisition. DER supervised the project and provided funding. All authors read and approved the final manuscript.

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Data Availability

Most data generated or analyzed during this study are included in this published article. The original gel pictures from western blot can be found in Supplementary Figs. 1 and 2.

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Figures

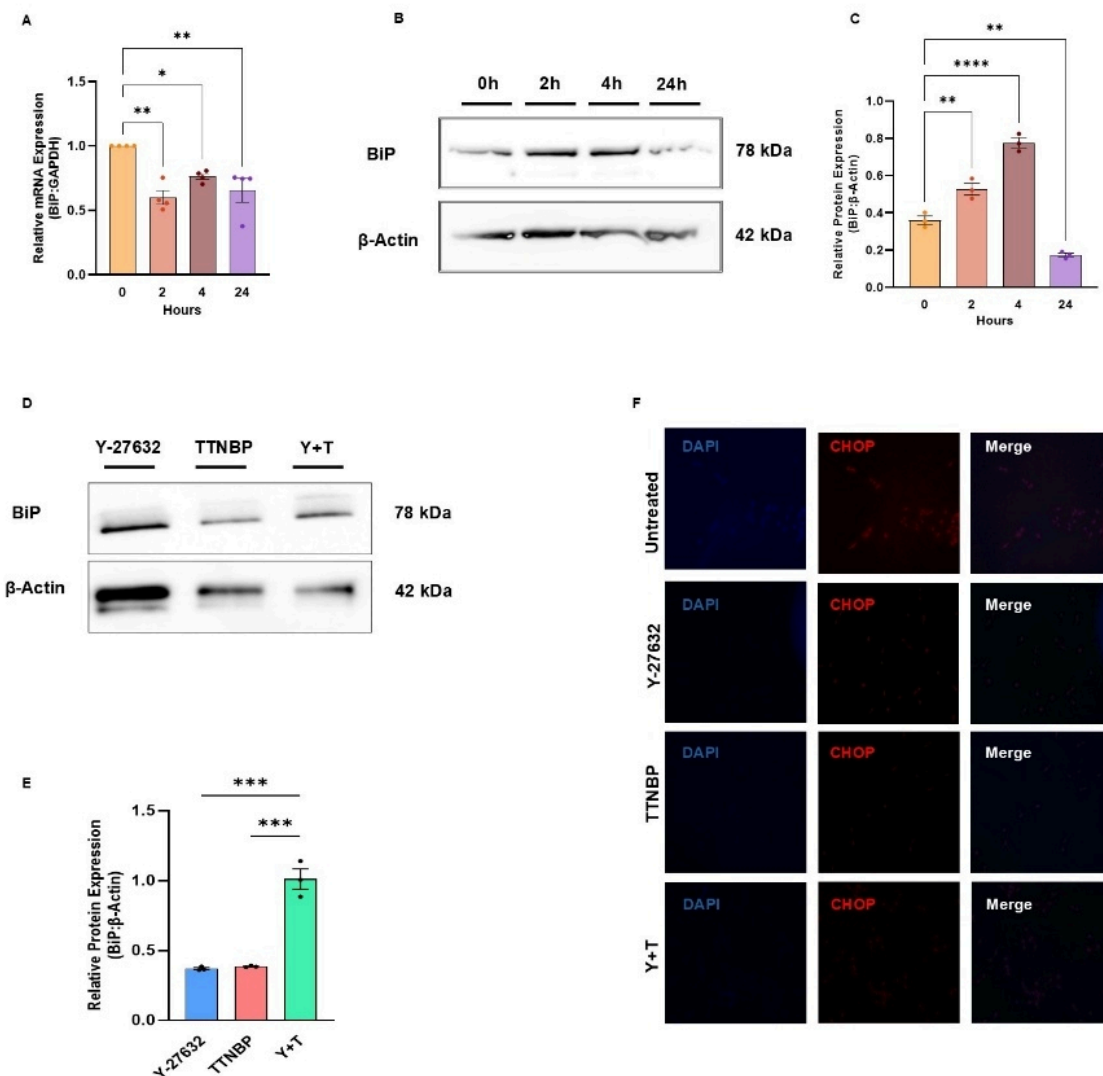


Figure 1

Dynamic regulation of BiP expression following dissociation and pharmacological modulation in hESCs. (A) Real time qPCR analysis of BiP expression at 0, 2, 4, and 24 h post-dissociation in WT hESCs. GAPDH was used to normalize gene expressions. (B) Representative Western blot analysis of BiP protein expression at 0, 2, 4, and 24 h after dissociation. (C) Densitometric quantification of BiP protein levels following Western blot analysis at the indicated time points. (D) Western blot analysis of BiP expression in hESCs treated with TTNBP (0.5μM) in combination with Y-27632. All the experiments were performed 24 h after the dissociation of hESCs. (E) densitometric analysis of BiP protein levels; immunoblotting was performed 24 h after dissociation. β-actin was used as an internal control. (F) Immunostaining of hESCs for the proapoptotic marker CHOP following treatment with TTNBP (0.5μM) and Y-27632. All data are shown as mean ± SEM (n = 3). Statistical significance is indicated as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, ns (p > 0.05).

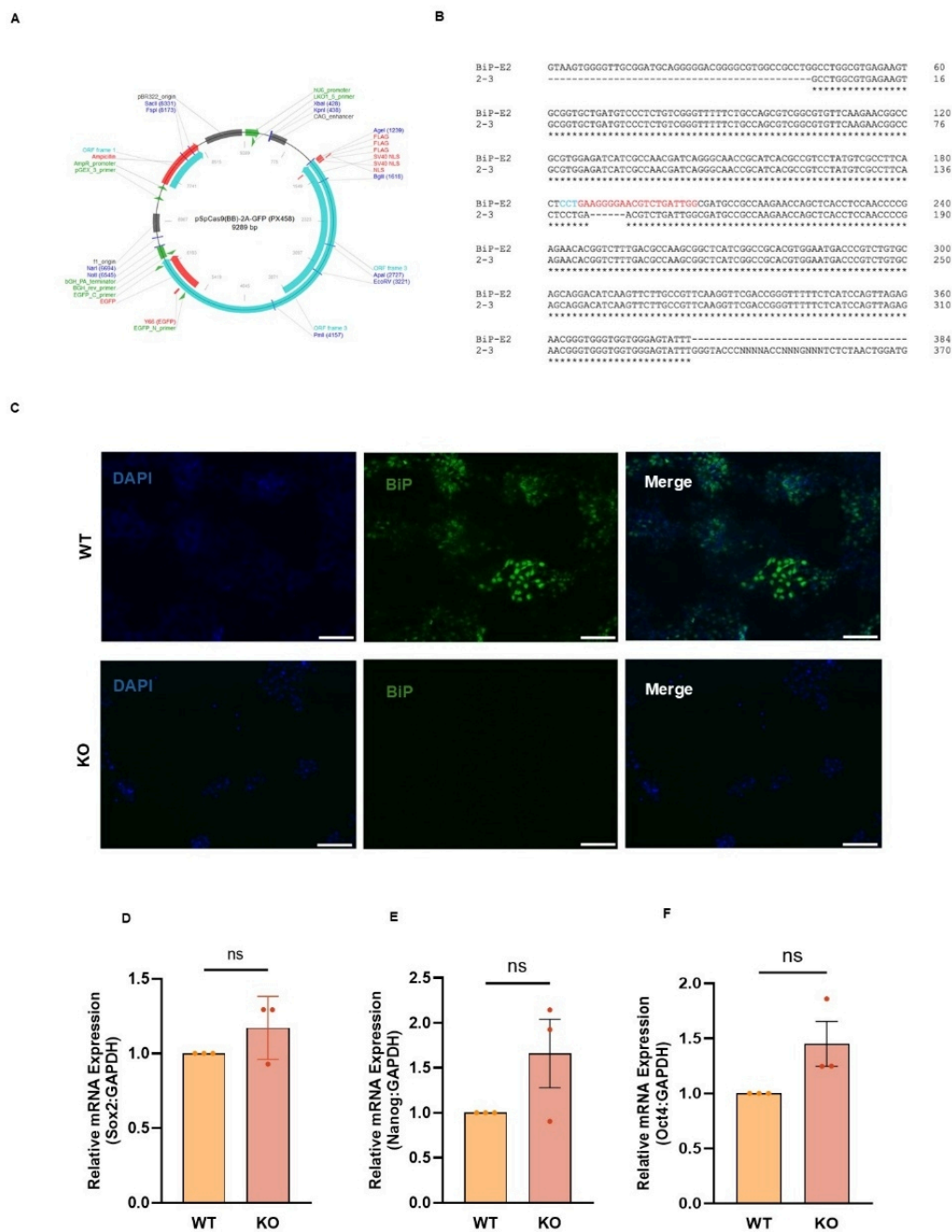


Figure 2

Generation and characterization of BiP knockout hESCs and assessment of pluripotency. (A) Plasmid map of the pSpCas9(BB)-2A-GFP (pX458) vector used for ligation of the BiP sgRNA targeting exons 2, 5, and 7. (B) DNA sequencing analysis of clone 2–3 showing a six-nucleotide deletion, confirming successful BiP knockout. (C) Immunocytochemical analysis of BiP expression in WT and BiP-KO hESCs. Cells were counterstained with DAPI for nuclei and FITC for BiP expression. The merged images show cellular localization. (D-F) Expression of pluripotency markers OCT4, NANOG, and SOX2 quantified by RT-qPCR. GAPDH was used for normalization. NS indicates no statistically significant differences.

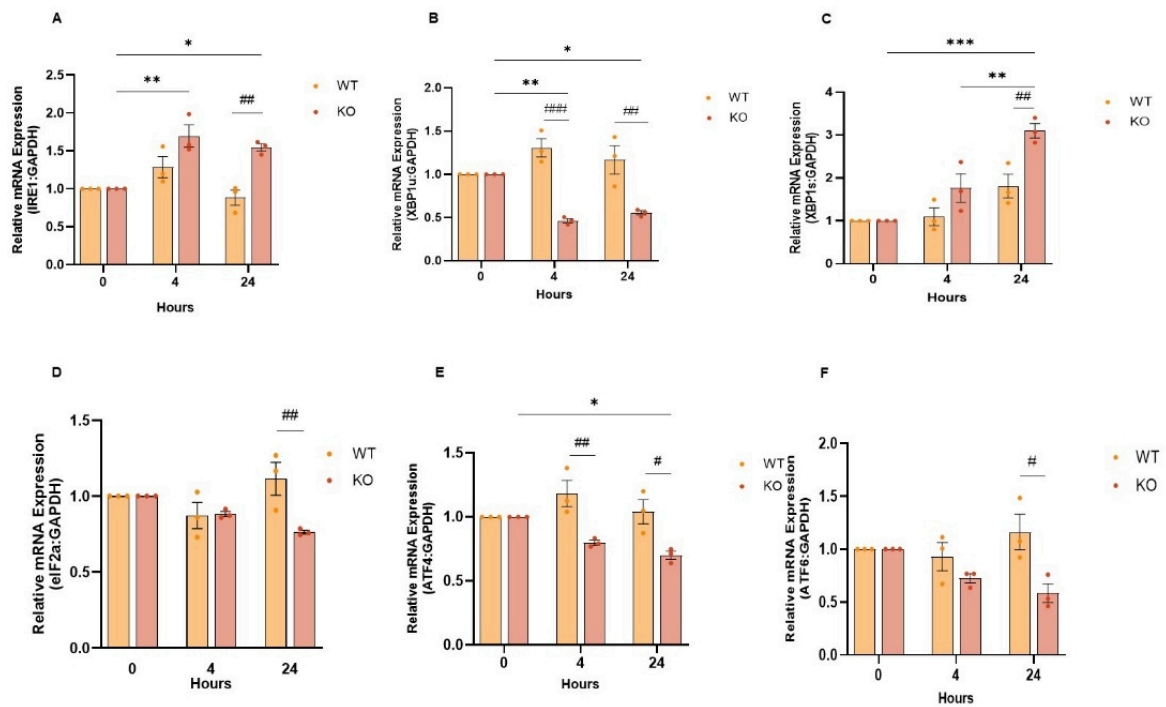


Figure 3

BiP deficiency disrupts UPR signaling dynamics following dissociation in hESCs. Time-course expression Relative mRNA expression levels of unfolded protein response pathway IRE1 (A), XBP1u (B), spliced XBP1 (XBP1s) (C), eIF2α (D), ATF4 (E), and ATF6 (F) in WT and BiP-KO hESCs determined by RT-qPCR. GAPDH was used to normalize gene expressions. All data are presented as mean ± SEM (n = 3). Statistical significance is indicated as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ns (p > 0.05) for within-group comparisons. #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001 indicate significant differences between WT and BiP-KO.

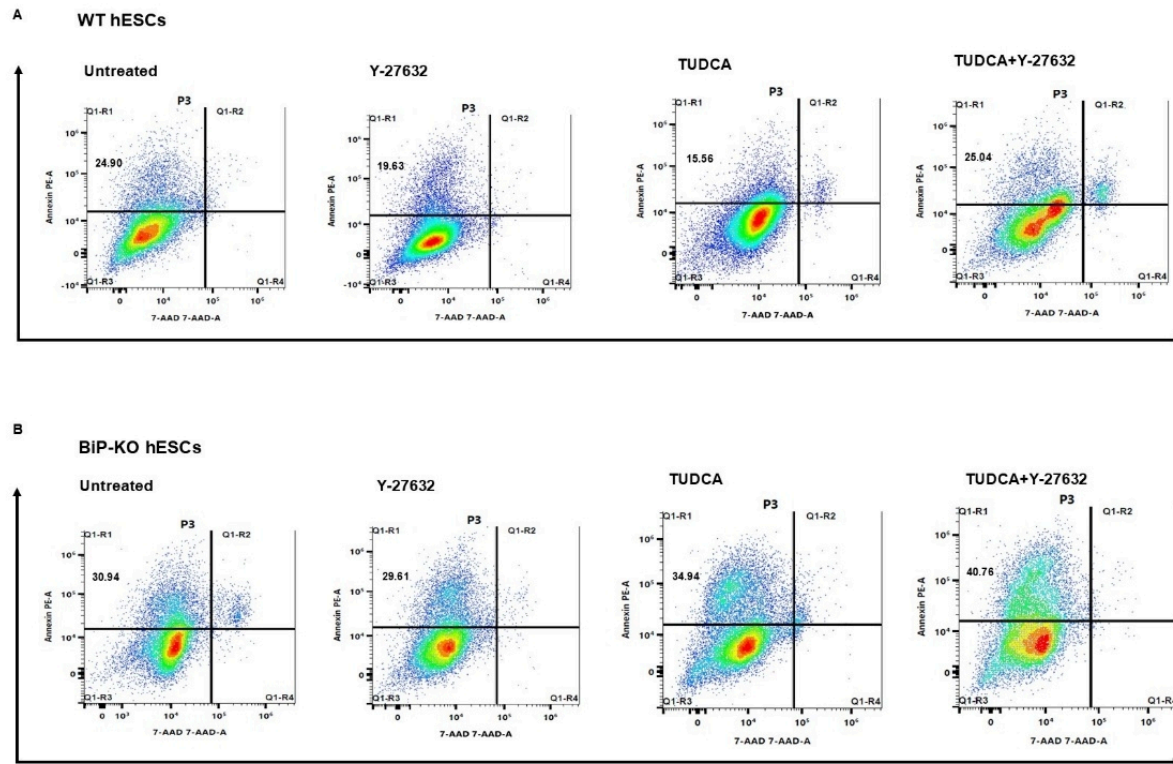


Figure 4

BiP deficiency compromises hESC survival and is not rescued by ER stress inhibition. (A) Representative flow cytometry plots showing Annexin V/PI staining in WT hESCs under different treatments. (B) Representative flow cytometry plots showing Annexin V/PI staining in BiP-KO hESCs under different treatments. Quantification of apoptotic cell populations is shown in the corresponding plots. Data are presented as mean $\pm$ SEM ($n = 3$).

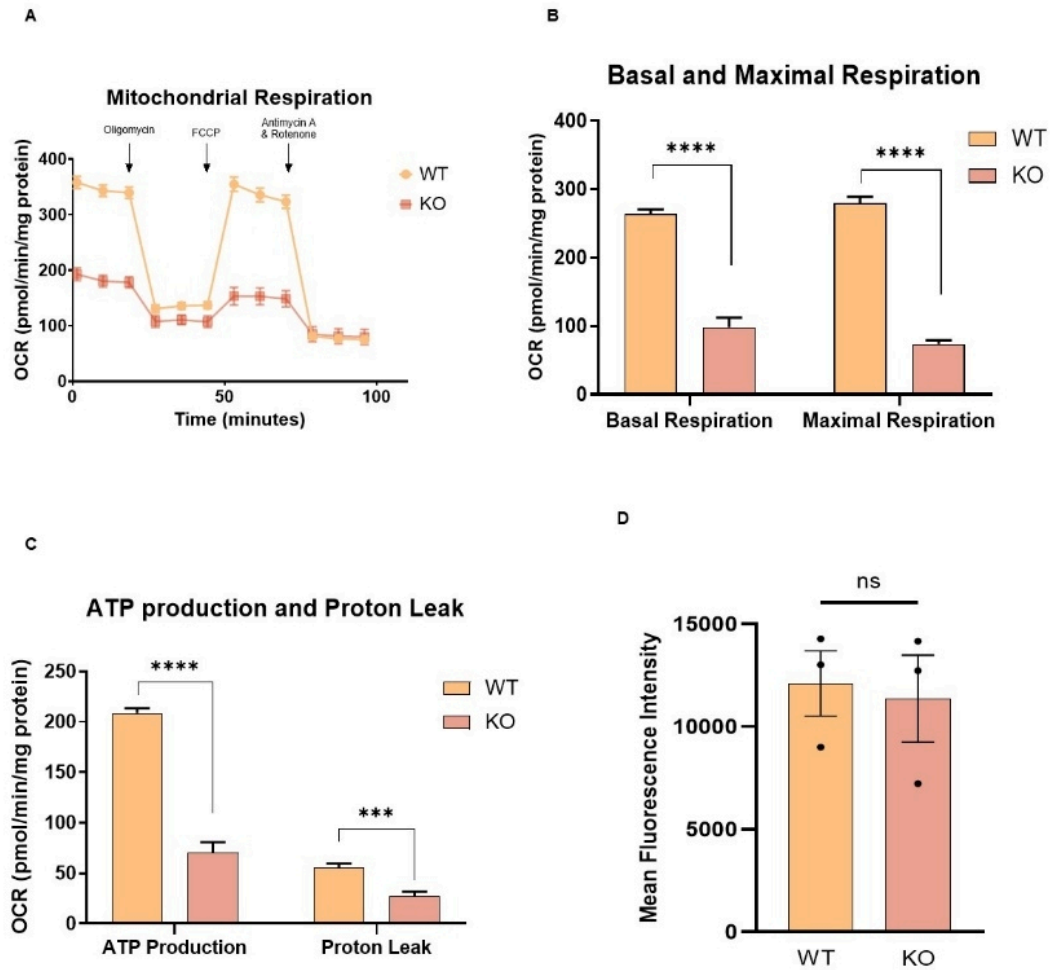


Figure 5

BiP deficiency impairs mitochondrial respiration but not mitochondrial membrane potential in hESCs.

(A) Basal oxygen consumption rate (OCR) in WT and BiP-KO hESCs measured using Seahorse XF analysis. (B) Maximal respiration capacity in WT and BiP-KO cells following FCCP treatment. (C) Quantification of ATP-linked respiration and proton leak in WT and BiP-KO hESCs. (D) Mitochondrial membrane potential is reported as mean fluorescence intensity (MFI) of TMRE signal in live cells measured by flow cytometry in WT and BiP-KO hESCs. All data are presented as mean $\pm$ SEM (n = 3). Statistical significance is indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns ($p > 0.05$).

Supplementary Files

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