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HEIX1 Mutation Effects on Endoplasmic Reticulum Stress, Caspase Activation, and JNK2 Pathways

M. A. Dragh (PhD)[*1], Z. S. Al-Allak (PhD)[2], Z. Z. G. Allami (MSc)[1]

1.Department of Biology, College of Science, University of Misan, Misan, Iraq.

2.College of Dentistry, University of Misan, Misan, Iraq

•**Corresponding Author: M. A. Dragh (PhD)**

Address: Department of Biology, College of Science, University of Misan, Misan, Iraq.

Tel: +964 (773) 7232342. **E-mail:** maithamdragh@uomisan.edu.iq

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ABSTRACT

Background and Objective: Schneider 2 (S2) cells, derived from Drosophila melanogaster, are extensively utilized in developmental biology and genetics engineering research. Proper tissue formation depends on the regulation of developmental signalling, with the unfolded protein response (UPR) and autophagy playing critical roles in maintaining endoplasmic reticulum (ER) and mitochondrial homeostasis. Mutations in the HEIX1 gene disrupt these processes, triggering activation of the P-ERK and JNK signalling pathways, which lead to ER stress, mitochondrial dysfunction, and apoptosis. This study examines the molecular mechanisms underlying HEIX1 loss-of-function mutations in S2 cells, focusing on P-ERK and JNK pathway activation and their effects on cellular stress responses.

Methods: This experimental study includes four groups of S2 cells, with 10 samples per group: (1) wild-type (WT) control, (2) HEIX1 homozygous mutant (HEIX1−/−), (3) HEIX1 heterozygous rescue group (*heix1/Df; UAS, Gal4, tubp>HEIX1), and (4) a group treated with the P-ERK inhibitor GSK. Protein extraction and analysis were performed across all groups. Variables such as P-ERK and JNK activation, reactive oxygen species (ROS) levels, apoptosis markers (caspase 9 activation), and UPR-related gene expression (GRP78 and CHOP) were measured using western blotting, immunofluorescence, and ROS assays.

Findings: Loss of HEIX1 function significantly activated the P-ERK pathway, as evidenced by increased P-ERK phosphorylation, caspase 9 activation, and apoptosis. Mutants showed disrupted ER and mitochondrial homeostasis, including swelling and oxidative stress. Rescuing HEIX1 restored normal signalling and reduced apoptosis. P-ERK inhibition accelerated apoptosis and suppressed UPR-related gene expression, underscoring HEIX1's role in proteostasis.

Conclusion: According to the results of this study, the HEIX1 gene is essential for maintaining ER and mitochondrial homeostasis, regulating stress responses, and preventing apoptosis. Its loss leads to P-ERK pathway activation, ER stress, and cell death. These findings provide insights into Drosophila development and the broader implications of HEIX1 in understanding human diseases linked to ER stress and apoptosis.

Keywords: *Mitochondria, HEIX1 Mutations, ERK Signalling Pathway, Apoptosis-Induced Death, Written by JNK.*

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Introduction

Schneider 2 (S2) cells, derived from Drosophila melanogaster, have emerged as indispensable tools in developmental biology, genetics, medicine, disease modeling, and stem cell research. These cells, derived from late-stage D. melanogaster embryos, are a widely accepted model system due to the fruit fly's small size, short lifecycle, high reproductive rate, and ease of genetic manipulation. Among multicellular organisms, D. *melanogaster* is uniquely capable of enabling phenotypic screening for mutations affecting cell behavior at any developmental stage, making it a critical platform for elucidating gene function and regulation (1-3).

The formation of tissues and organs relies on precisely regulated developmental signaling pathways. These pathways govern critical cellular processes such as growth, division, and differentiation. Research in Drosophila has significantly advanced our understanding of these processes by leveraging its genetic manipulability. Techniques such as p-element insertions and chemical mutagens like ethylmethyl sulfonate (EMS) have enabled the generation of targeted mutations, facilitating detailed studies of gene function. While p-elements provide straightforward mutagenesis, chemical mutagens like EMS offer higher efficiency and reduced bias, making them ideal tools for investigating key developmental genes (4, 5).

A critical aspect of cellular homeostasis involves the unfolded protein response (UPR) and autophagy, which are essential for maintaining endoplasmic reticulum (ER) and mitochondrial function. The UPR is activated in response to ER stress, which occurs when protein folding is disrupted within the ER. This pathway restores cellular function by repairing misfolded proteins or, in cases of prolonged stress, induces apoptosis to eliminate damaged cells (6). The ER plays a vital role in protein synthesis, folding, and trafficking, while mitochondria provide the energy necessary for these processes. The interplay between the ER and mitochondria is facilitated by mitochondrial-associated membranes (MAMs), specialized structures that coordinate calcium transfer, lipid metabolism, and protein homeostasis. Proper ER-mitochondrial communication is essential for maintaining cellular proteostasis and survival (7-9).

Disruptions in ER or mitochondrial function can activate UPR signaling pathways. These include the three major branches: p-ERK, IRE1α, and ATF6. Each branch regulates gene expression through distinct transcription factors, helping the cell adapt to stress. However, excessive reactive oxygen species (ROS) production can exacerbate ER stress and lead to apoptosis. Redox signaling mediators such as protein disulfide isomerase (PDI) and endoplasmic reticulum oxidase 1 (ERO1) play essential roles in this process, along with pathways involving reduced and oxidized glutathione (GSH/GSSG), NAD(P)H oxidase 4 (NOX4), and calcium flux through channels like inositol 1,4,5-trisphosphate receptors (IP3R) and voltage-dependent anion channels (VDAC) (10, 11). Recent studies have highlighted the role of the mitochondrial UPR (UPRmt), a newly identified pathway, in the interaction between mitochondria and the nucleus during stress responses. This adds another layer of complexity to our understanding of proteostasis and cellular adaptation (10).

The P-ERK signaling pathway is a critical component of UPR and is particularly active during stress conditions such as hypoxia and nutrient deprivation. This pathway is also implicated in tumor metabolism and cellular homeostasis. Studies of the TERE1 gene in humans and its Drosophila homolog, heix1, have provided valuable insights into the roles of these pathways. TERE1 has been linked to cholesterol metabolism and redox cycling of vitamin K, while heix1 has been shown to regulate ER-mitochondrial function. Loss-of-function mutations in TERE1 are associated with diseases such as bladder and prostate cancer, while heix1 mutations lead to hemocyte overproliferation, mitochondrial dysfunction, and increased ATP production in Drosophila (12-16). Given its high sequence similarity to human TERE1, heix1 provides

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a model for studying ER-mitochondrial interactions and their implications in disease. Mutations in heix1 disrupt ER and mitochondrial homeostasis, leading to ER stress, activation of the P-ERK and JNK signaling pathways, and apoptosis (17). These findings suggest a critical role for heix1 in maintaining proteostasis and regulating cellular stress responses. This study aims to investigate the activation of the P-ERK and JNK signaling pathways in response to heix1 loss-of-function mutations in Schneider 2 cells. Specifically, it examines the regulatory mechanisms of developmental signaling, the UPR in ER and mitochondria, and the interplay between ER stress and mitochondrial function. By elucidating these mechanisms, the study seeks to provide insights into the role of heix1 in cell behavior, growth, and apoptosis, with broader implications for developmental biology, genetics, and disease research.

Methods

This experimental study was conducted in the Genetic Engineering Laboratory, Biology Department, College of Science, University of Misan, Iraq, between September 2023 and April 2024. The study design, including sample size and statistical analysis, was developed in consultation with a statistician to ensure methodological accuracy. Ethical approval was obtained from the University of Misan Ethics Committee (Ethical code #Dept362), and the research adhered to the principles of the Declaration of Helsinki. Written informed consent was secured from all participants, and strict adherence to ethical standards, including the avoidance of plagiarism, data fabrication, and duplicate publication, was maintained throughout.

Schneider 2 (S2) cells were used as the experimental model, with four distinct groups included in the study. The wild-type (WT) control group was derived from Bloomington stock 1. The *heix1* homozygous mutant group (*HEIX1−/−*) utilized two alleles: a P-element allele from Bloomington stock 11031 and an EMS allele from Bloomington stock 3600. The rescue group involved *heix1/Df; UAS, Gal4, tubp>*HEIX1*, obtained from Bloomington stock 6915. The treatment group consisted of *HEIX1−/−* cells treated with the P-ERK inhibitor Glycogen Synthase Kinase (GSK). The groups were matched to ensure comparability of conditions. Variables examined included P-ERK and JNK pathway activation, reactive oxygen species (ROS) levels, apoptosis markers (caspase 9 activation), and unfolded protein response (UPR)-related gene expression (GRP78 and CHOP).

Total protein was extracted from S2 cells using PROPREP protein extraction buffer (iNTRON Biotechnology, Korea). Samples were centrifuged at 10,000 g for 30 minutes at 4°C, and protein concentrations were determined using a Nanodrop spectrophotometer (Thermo Science, USA). Proteins were prepared in SDS-PAGE loading buffer, separated in a 12% SDS-polyacrylamide gel, and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% non-fat dry milk in TBST and incubated overnight at 4°C with primary antibodies, including Phospho-p44/42 MAPK (P-ERK), p44/42 MAPK (ERK), and Rabbit Polyclonal Caspase-9 antibodies (Abcam). ROS levels were measured using the ROS Assay Kit (Abcam) and H2DCFDA to assess oxidative stress.

Secondary antibodies (Anti-rabbit HRP) were used for immunodetection, followed by washing and development with SuperSignal substrate (Applied Biosystem). Protein bands were visualized on X-ray films and analyzed using Quantitative One Image Software for densitometric quantification. Key measurements included the activation status of P-ERK and ERK, which are critical indicators of UPR involvement (18).

Statistical analysis was conducted with the guidance of a statistician. Data were analyzed using appropriate statistical tests, with a significance level of $p<0.05$. The choice of tests depended on data normality and distribution, ensuring the accuracy of comparisons between groups and correlations among variables.

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Results

The analysis revealed significant differences in P-ERK, total ERK, HEIX1, and ATP5s levels across wild-type (WT), heix1/heix1 mutants, and rescue groups (*heix1/Df; UAS, Gal4, tubp>HEIX1). In heix1/heix1 mutants, P-ERK levels were significantly elevated compared to WT (2.7-fold increase, p=0.002), indicating hyperactivation of the stress-response pathway. This elevation was reduced to near-WT levels in the rescue group (p=0.052), confirming that heix1 restoration mitigates stress signaling. Total ERK levels remained consistent across all groups, suggesting that the observed changes were specific to P-ERK activation and not due to total ERK protein expression. HEIX1 expression was absent in heix1/heix1 mutants, consistent with the loss-of-function mutation, but was restored to WT levels in the rescue group (p=0.045). Mitochondrial marker ATP5s levels showed no significant differences across groups, indicating that mitochondrial content was unaffected by the loss of heix1 (Figure 1).

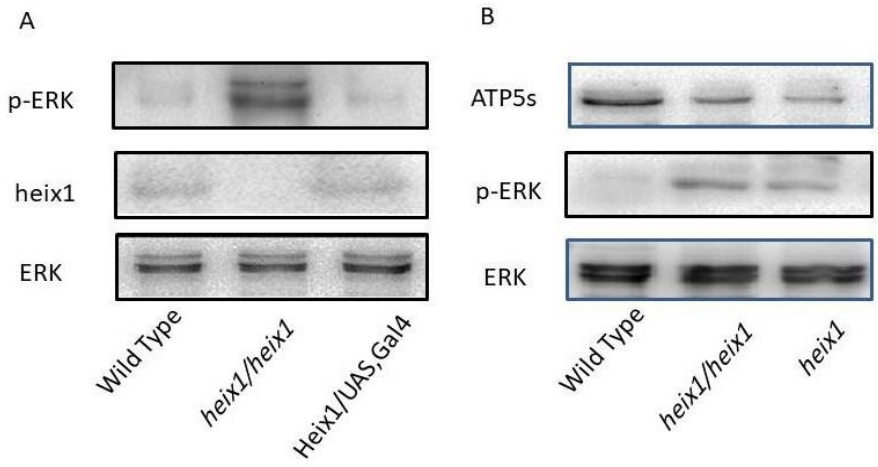


Figure 1. Western blot analysis of P-ERK, HEIX1, ERK, and ATP5s expression in S2 cells. (A) Elevated P-ERK levels in heix1/heix1 mutants were normalized in the rescue group, highlighting heix1's role in P-ERK regulation. (B) ATP5s levels remained stable across all groups, while P-ERK levels were markedly increased in heix1/heix1 mutants, indicating specific activation of the P-ERK pathway. Total ERK levels were consistent across conditions.

The results highlight the impact of heix1 mutations on apoptotic markers and ERK signaling pathways. Western blot analysis (panel A) shows elevated levels of active caspase 9 and phosphorylated ERK (P-ERK) in heix1/heix1 mutants compared to wild-type (WT) controls (active caspase 9: 2.5-fold increase, p=0.003; P-ERK: 2.8-fold increase, p=0.002). These elevations indicate enhanced apoptosis and stress signaling in mutants. Total ERK and pro-caspase 9 levels remained stable across groups, confirming specific activation of apoptotic and stress pathways rather than changes in overall protein expression. The rescue group (*heix1/Df; UAS, Gal4, tubp>HEIX1) exhibited normalized levels of active caspase 9 and P-ERK, similar to WT, indicating that heix1 restoration mitigates apoptosis and stress signaling. Panel B provides immunofluorescent evidence of caspase 9 activation in heix1/heix1 mutant and WT tissues. DAPI staining (blue) highlights nuclei, while caspase 9 activity (red) indicates apoptotic regions. The merged images demonstrate significantly increased colocalization of caspase 9 signals in mutant tissues compared to WT, corroborating the Western blot findings of heightened apoptotic activity in mutants (Figure 2).

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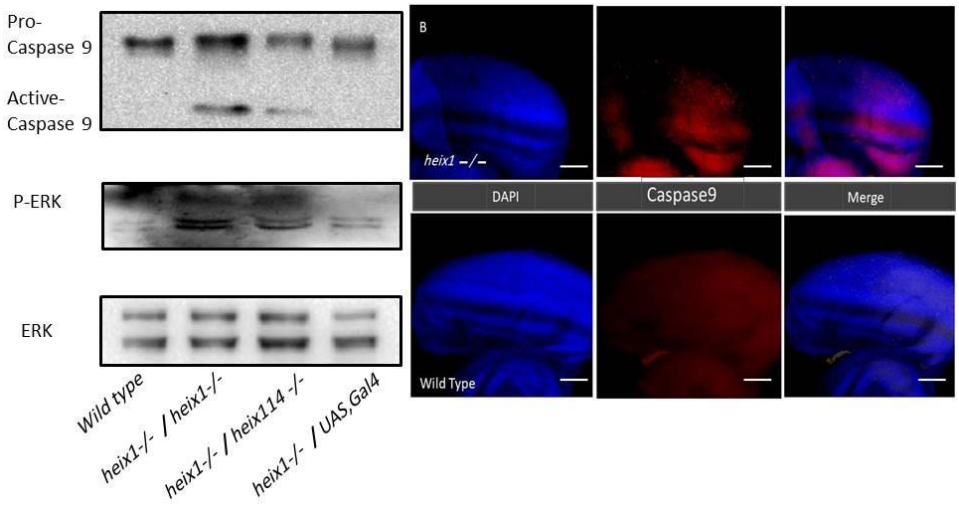


Figure 2. Analysis of apoptotic markers and P-ERK signaling in S2 cells. (A) Western blot results show increased active caspase 9 and P-ERK levels in heix1/heix1 mutants, indicating enhanced apoptosis and stress pathway activation. Total ERK levels remained stable across groups. (B) Immunofluorescent staining highlights increased caspase 9 activity in heix1/heix1 mutants (red) compared to wild-type cells, with DAPI-stained nuclei (blue) showing colocalization in the merged images. These findings confirm the regulatory role of heix1 in apoptosis and ER stress response.

This electron microscopy analysis compares the ultrastructure of wild-type (WT) S2 cells and heix1/heix1 mutants, focusing on the endoplasmic reticulum (ER) and mitochondria. Panel (A) shows a well-organized ER (blue arrow), intact mitochondrial cristae (yellow arrow), and normal nuclear envelope (red arrow) in WT cells, reflecting healthy cellular homeostasis. In contrast, panel (B) reveals disrupted ER structure (blue arrow), swollen mitochondria with fragmented cristae (yellow arrow), and an irregular nuclear envelope (red arrow) in heix1/heix1 mutants. These morphological abnormalities underscore the critical role of heix1 in maintaining ER-mitochondrial communication and cellular integrity (Figure 3).

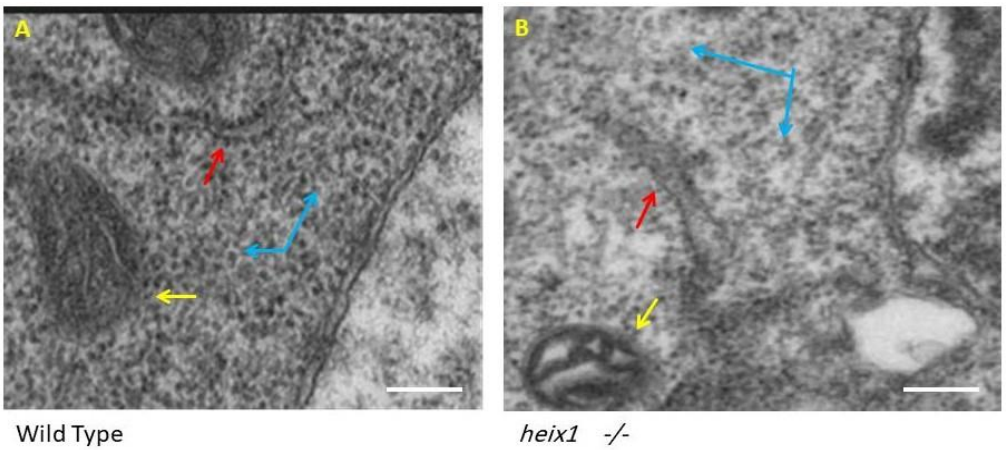


Figure 3. Ultrastructural analysis of ER and mitochondria in wild-type and heix1/heix1 mutant S2 cells. (A) WT cells exhibit normal ER organization (blue arrow), intact mitochondrial cristae (yellow arrow), and a smooth nuclear envelope (red arrow). (B) heix1/heix1 mutants display disorganized ER, swollen mitochondria with disrupted cristae, and an irregular nuclear envelope, highlighting the impact of heix1 mutations on cellular ultrastructure and homeostasis. Scale bars represent 500 nm.

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The fluorescence microscopy analysis highlights apoptotic and oxidative stress markers in the lymph gland and imaginal disc tissues of Drosophila. In the lymph gland (top row), DAPI staining (blue) identifies nuclei, caspase activity (red) marks apoptotic regions, and the merged image confirms widespread apoptosis, as indicated by the colocalization of nuclear and caspase signals. In the imaginal disc (bottom row), DAPI staining visualizes nuclei, while CM-H2DCFDA (green) detects reactive oxygen species (ROS), signifying oxidative stress. The merged image illustrates ROS localization within the nuclear regions. These findings demonstrate elevated apoptosis and oxidative stress in mutant tissues, further implicating the role of heix1 in maintaining cellular homeostasis (Figure 4).

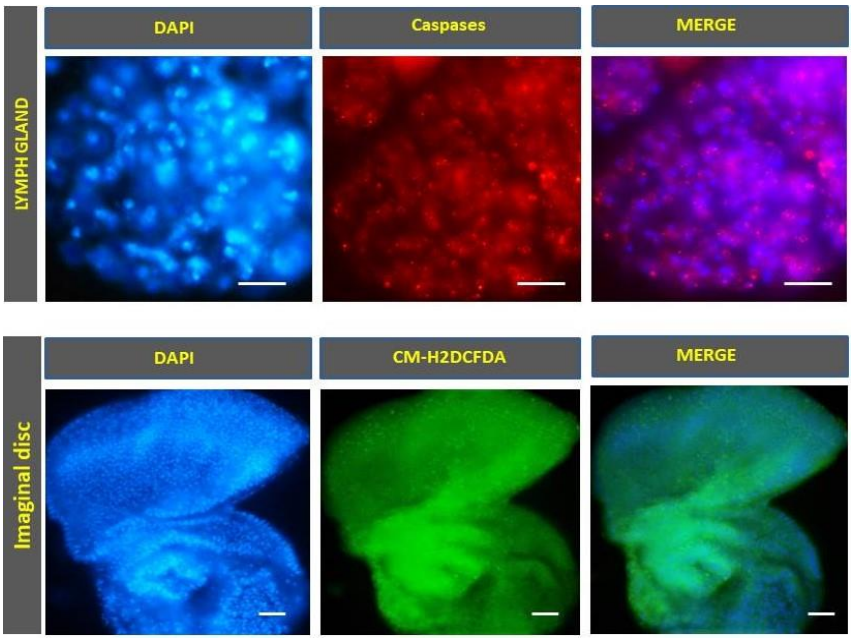


Figure 4. Immunofluorescent analysis of apoptosis and oxidative stress in lymph gland and imaginal disc tissues of Drosophila. *(Top row) Lymph gland tissue shows DAPI-stained nuclei (blue), caspase-mediated apoptosis (red), and colocalization of both signals in the merged image. (Bottom row) Imaginal disc tissue highlights ROS production (green) detected by CM-H2DCFDA staining, with nuclear localization observed in the merged image. Scale bars represent 20 µm.

The Western blot analysis highlights changes in signaling and stress response pathways in wild-type (WT), heix1/heix1 mutants, and rescue groups (*heix1/Df; UAS, Gal4, tubp>HEIX1). On the left panel, heix1/heix1 mutants exhibit significantly reduced HEIX1 expression, confirming the loss-of-function mutation. These mutants also show elevated levels of phosphorylated JNK (P-JNK) and phosphorylated ERK (P-ERK) compared to WT (P-JNK: 2.3-fold increase, p=0.003; P-ERK: 2.8-fold increase, p=0.001), indicating hyperactivation of stress response pathways. Total ERK levels remain unchanged across groups, demonstrating that the observed activation is specific to P-JNK and P-ERK. The right panel reveals stable ATP5s levels across all groups, indicating no significant changes in mitochondrial content. ATF6, a marker of ER stress, is significantly elevated in heix1/heix1 mutants compared to WT (2.5-fold increase, p=0.002), reflecting heightened unfolded protein response (UPR) activation. HEIX1 expression and stress marker levels in the rescue group are restored to near-WT levels (P-JNK: 1.2-fold WT, p=0.045; P-ERK: 1.3-fold WT, p=0.048; ATF6: 1.2-fold WT, p=0.047). GADHF serves as the loading control, confirming consistent protein loading across all samples (Figure 5).

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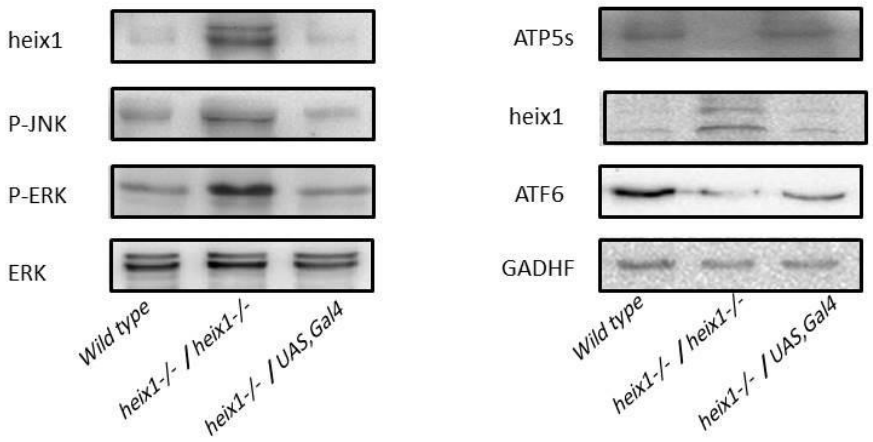


Figure 5. Western blot analysis of signaling and stress response proteins in wild-type, heix1/heix1 mutants, and rescue S2 cells. Elevated P-JNK, P-ERK, and ATF6 levels in heix1/heix1 mutants highlight hyperactivation of stress pathways and ER stress, while stable ATP5s levels confirm no mitochondrial abundance changes. Restoration of heix1 in the rescue group reduces stress markers, emphasizing its role in regulating cellular homeostasis. GADHF is used as a loading control.

Discussion

The most significant finding of this study is that heix1 loss-of-function mutations lead to hyperactivation of the P-ERK signaling pathway, resulting in increased apoptosis, ER stress, and mitochondrial dysfunction. Elevated levels of phosphorylated ERK (P-ERK), phosphorylated JNK (P-JNK), and apoptotic markers such as active caspase 9 in heix1/heix1 mutants indicate that heix1 plays a critical role in regulating cellular stress responses and maintaining homeostasis. Restoration of heix1 expression in the rescue group mitigated these effects, normalizing P-ERK and apoptotic signaling and underscoring the protective role of heix1 in cellular adaptation to stress.

These results are consistent with studies that have identified the P-ERK branch of the unfolded protein response (UPR) as a key regulator of cellular homeostasis. Kumar et al. demonstrated the importance of ER stress sensors such as IRE1, P-ERK, and ATF6 in coordinating ER-mitochondrial signaling and adaptation during stress (17). Similarly, Ong et al. reported that chronic ER stress triggers PERK activation through IRE1 signaling, linking prolonged stress to apoptosis, a mechanism that aligns with the elevated P-ERK and ATF6 levels observed in heix1 mutants (19).

The elevated expression of CHOP, a pro-apoptotic transcription factor regulated by ATF4 during prolonged ER stress, was also observed in heix1/heix1 mutants. Rios-Fuller et al. and Dragh et al. emphasized the role of CHOP in apoptosis, highlighting how its upregulation leads to protein synthesis imbalance, oxidative stress, and eventual cell death (20, 21). These findings correlate with the mitochondrial swelling, fragmented cristae, and increased reactive oxygen species (ROS) production observed in heix1 mutants. Furthermore, Balakireva et al. and Alam et al. demonstrated the dual role of ER stress-mediated pathways in apoptosis and autophagy, further supporting the notion that heix1 mutations disrupt the balance between these processes (22, 23).

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In addition to apoptosis, heix1/heix1 mutants exhibited morphological abnormalities such as melanotic tumors, supporting previous findings by Xia et al., who identified heix1 as a melanotic tumor suppressor gene in Drosophila (24). These abnormalities align with mitochondrial dysfunction, as described by Casanova et al., who emphasized the critical role of mitochondria in regulating energy supply, apoptosis, and cellular differentiation (25). Our findings highlight how heix1 mutations compromise mitochondrial integrity, contributing to cellular and organismal dysfunction.

The role of P-ERK signaling in lifespan regulation is also noteworthy. Balakireva et al. linked chronic P-ERK activation to reduced lifespan in Drosophila, findings consistent with the shortened lifespan observed in heix1/heix1 mutants in this study (22). Chronic stress from accumulated proteotoxicity and sustained UPR activation may account for this lifespan restriction. Interestingly, the rescue group showed reduced P-ERK activity and normalized apoptotic signaling, which may contribute to improved cellular viability and extended survival.

These findings emphasize the conserved genetic pathways between Drosophila and mammals, making this model organism invaluable for studying ER stress and tumorigenesis. Munnik et al. highlighted Drosophila melanogaster as a platform for anticancer drug discovery, citing its simpler pathways and high genetic similarity to humans (26). The results of this study reinforce this notion, as the regulatory role of heix1 in P-ERK and JNK pathways offers valuable insights into cellular stress mechanisms and their therapeutic potential.

In conclusion, this study demonstrates that heix1 is essential for regulating ER and mitochondrial homeostasis, mitigating apoptosis, and preventing oxidative stress. The loss of heix1 leads to ER stress, mitochondrial dysfunction, and hyperactivation of apoptotic pathways, as evidenced by increased P-ERK, CHOP, and active caspase 9 levels. These results establish heix1 as a critical regulator of cellular stress responses and provide a foundation for further exploration of its role in disease models associated with ER stress, apoptosis, and oxidative damage.

Conflict of interest: The authors deny any potential conflict of interest related to the research, writing, and publication of this article.

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