Mitochondrial dysfunction: Characterization of the integrated stress response (ISR), cellular signaling, and homeostasis

A thesis submitted for the degree of

Doctor of Philosophy in Biochemistry

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Certificate

This is to certify that this thesis entitled **"Mitochondrial dysfunction: Characterization of the integrated stress response (ISR), cellular signaling, and homeostasis"** was submitted to the University of Hyderabad by **Mr. Koncha Ramagopal Reddy**, bearing the **Reg. No. 14LBPH05** for the degree of Doctor of Philosophy in Biochemistry is based on the studies carried out by him under my supervision. To the best of my knowledge, this work has not been submitted earlier for the award or diploma from any other University or Institution, including this University.

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Declaration

I, Koncha Ramagopal Reddy, hereby declare that the work presented in this thesis entitled **"Mitochondrial dysfunction: Characterization of the integrated stress response (ISR), cellular signaling, and homeostasis"** is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Prof. Kolluru VA Ramaiah**. I further declare that this work has not been submitted earlier for the award of a degree or diploma from any other University or Institution.

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1. **Ramagopal Reddy Koncha**, Gayatri Ramachandran, Naresh Babu V. Sepuri, and Kolluru V. A. Ramaiah . "CCCP-induced mitochondrial dysfunction-characterization and analysis of integrated stress response to cellular signaling and homeostasis" FEBS J. 2021 Oct;288(19):5737-5754. doi: 10.1111/febs.15868.

The student has attended the following conferences during his Ph.D. program:

1. Presented a poster entitled "**Mitochondrial dysfunction affects translational initiation pathways and cellular homeostasis: Importance of Integrated stress response pathway**" by **Koncha RR,** Naresh Babu V Sepuri, and KVA Ramaiah at a conference "**International Conference on Autophagy and Lysosomes (ICAL)**" held on **January 16-18, 2020** organized by **IISc,** Bangalore.

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3. Presented a poster entitled "**Effect of CCCP induced mitochondrial stress on translation and cellular homeostasis**" by **Koncha RR,** Naresh Babu V Sepuri, and KVA Ramaiah at Bioquest 2018 & 2019 organized by the **University of Hyderabad**, Hyderabad.

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BC802	Research ethics, Data Analysis and Biostatistics	3	Pass
BC803	Lab Seminar and Record	3	Pass

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Dedication

I dedicate my thesis to my chinnanna (younger brother of my father), the late Sri Koncha Sadhasiva Reddy B.Com, who appreciated my intelligence and knowledge and provided me with a financial support to complete my Intermediate education. I owe a debt of gratitude to him, since he was the trigger in transforming my path from farmer to scientist and from the agricultural field to the scientific field. I am grateful for his tremendous support.

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Chapter-I

1. Introduction

Biological systems are very complex. Multiple interactions among various cellular macromolecules, covalent modifications of proteins, inter-organellar communications, and the cross-talk between/among various signaling pathways define changes in gene expression and its regulation which are necessary to maintain day-to-day functions, physiology, metabolism, and homeostasis that include health and disease of cells and cell types. The thesis work here has taken up to analyze primarily whether stressed mitochondria can evoke integrated stress response (ISR), evolutionarily conserved and complex signaling that is mediated through the phosphorylation α-subunit of eukaryotic initiation factor 2 (eIF2α), a heterotrimeric protein that transfers the initiator tRNA on to 40S ribosomal subunits in a GTP-dependent mode during the initiation phase of translation and to determine the eIF2α kinase (s) that is activated. Also, the thesis analyzed the phosphorylation status of eIF4E-BP1 (eukaryotic initiation factor 4E- binding protein 1) that occurs as a consequence of activation of mTORC1, an indicator of active protein synthesis; activation of AMPK, and AKT that are engaged in cellular energy sensing, cell survival, and cell growth respectively; the cross-talk between ISR and these signaling pathways, and the importance of ISR in maintaining cellular homeostasis. Accordingly, the introduction of the thesis is briefly focused on the importance of mitochondria, translation, and its regulation in eukaryotes, ISR, diverse eIF2α kinases and their activation, importance of ISR on health, and disease, and the small molecules that are identified recently targeting various components of ISR.

1.1. Importance of mitochondria

Eukaryotic cells (Human or Mammalian) are equipped with various organelles in the cytosol, viz. mitochondria, lysosomes, nucleus, Golgi apparatus, peroxisomes, and endoplasmic reticulum (ER) which work together as a team to accomplish specific cellular functions such as proliferation, growth, and death. Precise coordination, communication, comprehension, planning, and information sharing to and from amongst these organelles are essential to succeed in specific cellular functions as mentioned above. Among these organelles, mitochondria are unique and unusual in that they contain a genome and are inherited exclusively from the maternal route [1]. Mitochondria are historically referred to as the cell's powerhouse because their principal goal is to manufacture ATP, which is vital to drive various thermodynamic, metabolic, and biochemical reactions in the cell. Pyruvate, an end product of glycolysis, is transported into mitochondria and processed to acetyl-CoA. In mitochondria, acetyl-CoA goes through the Krebs cycle, and following OXPHOS (Oxidative phosphorylation) with respiring O_2 generates ATP as the main product, along with H_2O, CO_2, and heat [2]. In general, mitochondria can produce 36/38 ATP molecules for every glucose molecule processed. Mitochondria convert proton motive force created by sequential activities like pyruvate breakdown, TCA cycle, and electron transport chain (ETC) process into energy (ATP) through the rotating turbine-like apparatus F1-F0 ATP synthase. Thus mitochondria are like cellular dynamos which convert mechanical energy into electrical energy, generally by rotating copper cable coils in a magnetic field. Mitochondria are semi-autonomous and dual-membrane architecture structures with an outer and inner membrane that together create two aqueous regions: the inner membrane space (IMS) and matrix, respectively. The mitochondrial inner membrane is highly convoluted and invaginates into the matrix region to form cristae, which aid in increasing surface area and thus improving function at the inner membrane [3]. The outer membrane functions as a universal or ubiquitous entry point for all types of ions and biomolecules into mitochondria. Additionally, it contains specialized membrane translocases and porin channels that regulate multiple functions like protein import into a matrix, immunological reactions, and apoptosis [4]. There is no membrane potential across the outer membrane because porins allow unrestricted ion diffusion. Certain sections of the outer membrane are engaged in organelle communication [5]. In contrast to the outer membrane, the inner membrane is extremely impermeable to the majority of ions and molecules and necessitates the requirement of respective transporters. Due to its ion selectivity, membrane potential and electrochemical gradient develop around the inner mitochondrial membrane, which acts as a central driving force for ATP synthesis [6]. The ETC apparatus and ATP synthase mega dalton complexes are housed in the inner membrane, which hosts the oxidative phosphorylation process [7]. Additionally, the inner membrane is also involved in protein import into the matrix through respective transporters, transport of metabolites, apoptosis, and others. IMS compartment proteome is implicated in several processes including ETC components assembly and regulation, lipid and protein transport, metal homeostasis, autophagy, and cell death. The matrix of mitochondria contains enzymes that catalyze the TCA cycle, fatty acid oxidation, and heme production [5]. The matrix also contains the mitochondrial genome (circular DNA) and machinery of replication, transcription, and translation. The human mitochondrial genome encodes 37 genes. Two of these genes are dedicated to expressing rRNAs, 22 genes are dedicated to producing mt-tRNAs, and the rest 13 genes encode proteins that are involved in the process of OXPHOS [8]. According to the endosymbiotic hypothesis, mitochondria emerged from α-proteo bacterium around 1.45 billion years ago via the process of phagocytosis, wherein anaerobic nucleus-carrying cell, a primitive eukaryote, ingested the α-proteo bacterium [9-11]. As an offspring of bacteria, mitochondrial lifestyle was akin to that of their ancestral bacteria in certain aspects, such as mtDNA maintenance, replication, transcription, and translation [1, 12, 13]. As symbiotic cells evolved into full-pledged eukaryotic cells, mitochondria lost their independence due to the transfer of their genome to the host. As a consequence, the majority of mitochondrial genes are resided in the host genome [14]. Human mitochondria contain nearly 1500 proteins, but only 13 of them are encoded by mtDNA. The remaining 99 percent are encoded by the nuclear genome and imported from the cytosol after their synthesis. Thus, mitochondria are dependent on the cellular nucleus and cytosol for its transcripts that are translated into proteins [5, 15]. In addition to acting as the cell's powerhouse, during the ATP production process, mitochondria also generate ROS an ETC operation byproduct, which occurs as a result of the leakage of electrons and protons. More than just a powerhouse, mitochondria are signaling hubs due to their involvement in a vast range of cellular functions, by providing metabolites, ROS, and TCA intermediates, and interact with the ER and the nucleus through the membranes [2]. In addition to cellular respiration and production of ATP, mitochondria are also involved in the biosynthesis of the iron-sulfur cluster, heme and porphyrin moieties, nucleotides, carbohydrates, amino acids, proteins, and lipids, and regulate redox metabolism. Mitochondria are fundamentally designed to govern cellular signaling via secondary messengers such as ROS, calcium, and cytochrome C. There are many biological processes that rely on calcium signaling which include mitochondrial function and dynamics and mitochondria-ER communication. Furthermore, mitochondria are involved in apoptosis by releasing pro-apoptotic molecules like cytochrome C, a key event in apoptosis. ROS are known to serve as secondary messenger in cell signaling [5, 16-18]. Due to plethora of responsibilities throughout the range of cell birth to death, mitochondria influence each and every aspect of cellular function. As a reason, maintaining mitochondrial integrity is extremely important for maintaining a healthy metabolism and living a long life [6]. For instance, disrupting the heart of mitochondria, such as the ETC system that deals with ions, causes a flood of ROS released into the cytoplasm. By oxidizing proteins, lipids, carbohydrates, and nucleic acids, ROS is known to cause irreversible damage to them [19]. Mitochondrial fidelity is crucial and the disruption of mitochondrial function and structure, as well as mutations in mitochondrial proteins result in several (approximately 300) metabolic, neurological, muscular, immunological disorders, aging and cancer that finally influences life span [20]. As mentioned earlier, in order to achieve cellular functions under normal as well as stressed conditions, all organelles must collaborate and do teamwork by chatting with one another, which is an inevitable process. To carry out a wide range of functions, mitochondria need to communicate with their neighboring cellular entities via comprehensive network of signaling routes and contact sites, regions of close proximity between membranes, and exchange molecules. This "Mitochatting" is crucial for mitochondria to continuously update their capability, availability, accessibility, functional potential, dysfunction, and their stress, if any, to the rest of the cell. This "chatting" will make the cell to move forward accordingly. The most interactive communication journey, predictably, happens between the nucleus and mitochondria [21]. Mitochondrial stresses frequently lead to a diminution in anabolic activities like the translation to prevent unnecessary energy use and cellular damage [22].

1.2. The importance of translation and its regulation

Protein Synthesis is the final event in the central dogma or information flow in biological systems where the information coded in the deoxy ribonucleotide sequences of genomic DNA is all copied through a process called replication, whereas parts of the functional genome transcribe into different forms of RNA, and the ribonucleotide sequences in messenger RNAs (mRNAs) are decoded to the corresponding amino acids to make proteins through a process called translation [23]. In addition, the central dogma includes the degradation rates of mRNAs and proteins. While replication and repair of damaged DNA are essential to maintain genome integrity through successive generations, gene expression that includes transcription and translation is required for the synthesis of various RNAs and proteins depending on day-to-day functions in different cells and cell types. The central dogma of biology envisaged that transcription and translation are correlated events, and the cellular translational outcome is determined by the corresponding transcript levels, which are influenced by the rate of transcription and/or degradation of mRNAs following a stimulus, thus suggesting gene expression is regulated mainly at transcription [23]. However, this notion has been challenged by numerous studies to show that translational regulation in many cases determines the abundance of proteins and thus translational regulation is a fact and not a fantasy anymore. Recent studies, analyzing the degradation rates of more than 5000 mRNAs and proteins, have shown that only around 40% of the overall protein content in a cell can be attributed to mRNA transcriptional outputs and the respective half-lives, whereas, 41-54% of protein concentration can be attributed to translation rates. The degradation rates of proteins however play a small role in protein turnover [24-27]. These findings, also suggested that abundantly expressed transcripts can sometimes be weakly translated and that conversely, poorly transcribed genes can sometimes be well translated. Transcriptional control of gene expression albeit crucial and compulsory, but it is not adequate to define proteome absolutely [28]. These data also suggest that RT-PCR analysis needs to be combined with the expression of proteins. In general, translation requires a relatively very short time compared to transcription. Thus, translational regulation can be executed relatively at a very short time interval. Several studies suggested that the preferential translation of stress-specific mRNAs and suppression of translation of normal mRNAs in stressed conditions, synthesis of hemoglobin protein in heme-deficient red blood cells or insulin, a secretory protein synthesized in pancreatic β-cells in glucose-deficient cells can be inhibited at the initiation or elongation steps in protein biosynthesis. Translational controls are very predominant in systems such as oocytes, and reticulocytes, and during embryonic development where active transcription is lacking [29-31]. Besides transcription, mRNA processing, polyA tail length, 5' caps, codon frequency, and corresponding tRNA and amino acid pools, specific sequences such as IRES and short uORFs preceding main ORF, secondary structures, and short hairpins in UTRs, lncRNAs, miRNAs, RNA binding proteins also regulate the translation [25, 28, 30]. Cellular proteome abundance is majorly determined by translational regulation rather than protein stability and its half-life [24]. Furthermore, the translation of existing mRNAs into proteins consumes an enormous amount of energy and amino acids. Hence the process requires thorough scrutiny and that's why cells employ stringent regulatory controls. Deregulation of translation has been observed in cancer, neurological diseases, metabolic disorders, aging, disease, and viral infection [8, 30, 32-37]. Collectively, these studies demonstrated the significance and contribution of translational regulation in the process of gene expression and in maintaining cellular homeostasis. Following translation, proteins undergo slight decoration in the form of post-translational modifications (PTMs) like acetylation, glycosylation, methylation, nitrosylation, prenylation, oxidation, ubiquitination, sumoylation, phosphorylation, and others that modulate their activity, function, localization, interactions with other proteins, and turnover. Few of these PTMs are reversible, such as phosphorylation, oxidation, acetylation, and methylation, while others, such as protein processing, and ubiquitination are irreversible [38-40].

1.2.1. Biological synthesis of proteins or translation

Translation starts, grows, and ends in three stages: initiation, elongation, and termination. Recent studies have shown the existence of the fourth stage of protein synthesis that involves the recycling of ribosomes and mRNAs. Each phase is a target of regulation to govern the required proteome in eukaryotes as the process is expensive and uses a large amount of ATP. Regulation at the early stage of protein synthesis makes it rational to be energetically beneficial [41-45]. Thus initiation is a major rate-limiting step in translation and is mediated by about 12 initiation factors in eukaryotes [46-48].

A. Initiation

In eukaryotes, approximately 96 percent of translating mRNAs bear a 5'-methyl guanosine cap and are translated cap-dependently, whereas, the remainder of mRNAs that harbor IRES elements are translated cap-independently [27, 49]. In the first step of initiation, a multifactor complex (MFC) comprising of following eIFs viz. eIF1, eIF1A, eIF3, & eIF5 bind the 40S ribosomal subunit which is then joined by ternary complex (TC), eIF2.GTP.Met-tRNA$_i$ to form 43S initiation complex. In the next step, the 43S complex joins mRNA bound by an eIF4F complex at the 5'end of mRNA. eIF4F is a complex of three proteins: eIF4E, a 5'cap binding protein; 4A, a helicase; and 4G, a scaffolding protein. The 4G protein interacts on one side with eIF3 of the 43S complex and on the other side, it interacts with the PolyA binding protein (PABP) bound by the polyA tail of mRNA to form the 48S initiation complex. Also, eIF4G bound to the 5'end of mRNA interacts with PABP bound at the 3' end of mRNA to form a pseudo circular RNA. Now the ribosome scans the 5'end of mRNA to identify the 'start' AUG codon. This step is aided by eIF1 and 1A. eIF1 inhibits the release of Pi liberated from hydrolysis of GTP bound to eIF2 until an AUG fills the P site in the ribosome. The GTPase activity associated with the γ-subunit of eIF2 is activated by eIF5, a GTPase activating protein

(GAP) that hydrolyses GDP bound to eIF2. Following identification of the 'start' codon AUG, eIF2.GDP and other eIFs exit the 48S preinitiation complex. The joining of the 60S subunit with the 48S initiation complex to form the 80S initiation complex is promoted by eIF5B which hydrolyzes a second GTP. The 80S complex now carrying the initiator tRNA is positioned on the 'start' AUG in mRNA in the P site of the ribosome leaving the A site vacant for the addition of incoming aminoacylated elongator tRNAs. Thus the stage is set for elongation [30, 48, 50, 51]. The binary complex eIF2.GDP liberated by the action of eIF5 and eIF2γ-subunit then exchanges its GDP for GTP to form eIF2.GTP. This guanine nucleotide exchange (GNE) on eIF2 is catalyzed by eIF2B protein, originally identified as hetero pentameric protein but is now known to be hetero decameric protein. Unless the GDP is exchanged for GTP, eIF2 cannot join the initiator tRNA (Met.tRNA$_i$). Since eIF2 unlike prokaryotic IF2 has a higher affinity for GDP than for GTP in physiological conditions where Mg^{2+} is present, the GDP/GTP exchange requires an enzyme, like eIF2B, a guanine nucleotide exchange factor (GEF) [52-54]. Protein synthesis as mentioned above is an energy-expensive process. In the initiation step, 2 molecules of ATP are required for every methionyl tRNA formation and to unwind the mRNA structure respectively. Two GTP molecules are hydrolyzed one that is bound to eIF2 and the second one is hydrolyzed during the joining of the 48S initiation complex to the 60S subunit to form the 80S initiation complex [41].

B. Elongation

The elongation step involves **a)** sequential addition of amino-acylated elongator tRNAs to the A site in ribosome based on the codon sequences in the translating mRNA; **b)** formation of a covalent bond called peptide bond between adjacent amino acids and **c)** then the movement of mRNA by three nucleotides. These steps are mediated by three elongation factors: eEF1A, eEF1B, and eEF2. The first step is catalyzed by eEFIA and eEF1B. The second step that involves peptide bond formation is driven by breaking the high-energy acyl bond that joins the growing polypeptide chain to tRNA and is catalyzed by RNA (28S ribosomal RNA) rather than by protein-like peptidyl transferase as envisaged earlier. The translocation step or movement of mRNA is catalyzed by eEF2. Thus protein synthesis is an example of 'Head Growth' where the energy for the addition of an incoming monomer to the growing polymer is given by the last monomer of the growing polymer which is somewhat different from what is seen in the nucleic acid synthesis that follows ‘tail growth’ where the energy released by the hydrolysis of the incoming dNTP is used to add it to the last dNMP of the growing DNA chain. The elongation factor eEF1A binds with GTP and transports amino acid-charged tRNA to the A site of the 80s ribosome. Following a perfect match between codon and anticodon pair, GTP hydrolyzed to GDP, resulting in the release of eEF1A.GDP from the translation complex. eEF1B, somewhat comparable to eIF2B in the initiation step, is a guanine nucleotide exchange factor that catalyzes the conversion of eEF1A.GDP to eEF1A.GTP which is required for the recycling of eEF1A.GDP to eEF1A.GTP and to bind elongator tRNAs carrying the amino acids. eEF2 acts as the translocase that promotes the ribosome translocation along the mRNA by three bases or one codon [55, 56]. The translocation step is dependent on GTP and involves the movement of the nascent protein chain from the A site to the P-site of the ribosome [57].

C. Termination

The termination phase begins with the entry of non-sense codon or stop codon in the A site of ribosomes. In eukaryotes, termination occurs via two termination factors: eRF1 and eRF3. eRF1 recognizes the stop codon (UAA, UAG, or UGA) on mRNA and stimulates the release of the nascent polypeptide from the ribosomal P site. The amino acid sequence SPF (Serine-Proline-Phenylalanine) in eRF1 recognizes the stop codon, and GGQ (Glycine-Glycine-Glutamine) sequence plays a role in the hydrolysis of the nascent polypeptide. Thus SPF in eRF1 resembles an anticodon in tRNA and it is referred to as ‘peptide anticodon’. eRF3 is a GTPase, that boosts hydrolysis of GTP bound to eRF1 [41, 58, 59]. For each polypeptide synthesis, one GTP molecule is consumed at termination. Termination is almost certainly not viewed as a rate-limiting event in translation [30]. Finally, the ribosome recycling factor (RRF) involves ABCE1, an NTPase in eukaryotes that splits the ribosomes into their subunits: 40S and 60S and releases the mRNA and deacylated tRNA from ribosomes. Recent structural studies of ABCE1, an NTPase suggest that it interacts with initiation and release factors and thus acts at the interface between initiation and termination. It also senses cellular energy levels [60, 61].

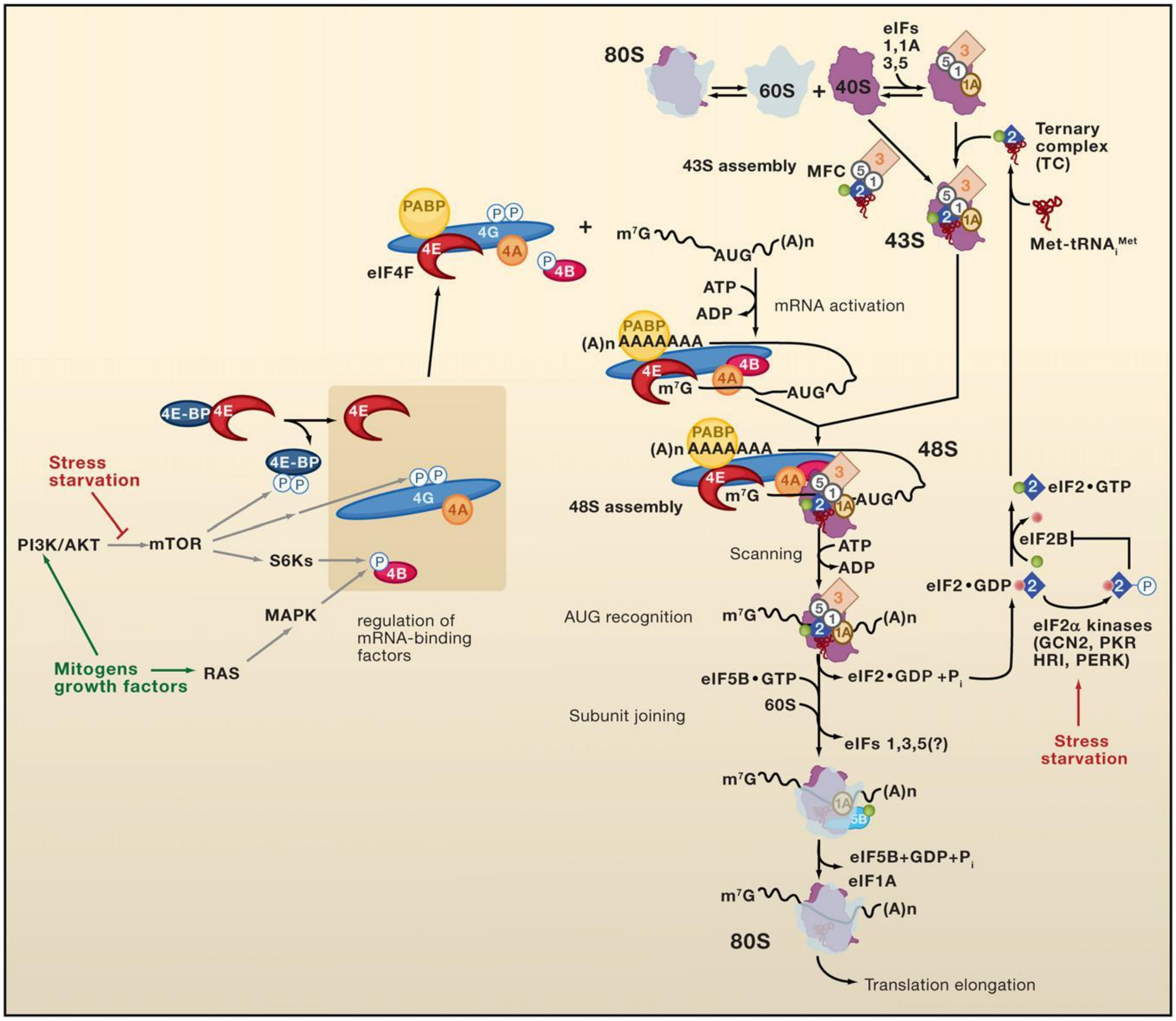


Fig. 1. Initiation of translation in eukaryotes. In **step1**, eIF1, eIF1A, and eIF3 dissociate 80S ribosomes into 40S and 60S through their binding to 40S. In **step 2,** the 40S complex bound by the multifactor complex (MFC) consisting of the above eIFs along with eIF5 joins the ternary complex, eIF2.GTP.Met-tRNA$_i$ to form 43S preinitiation complex (PIC). In **step3,** activated mRNA that is bound by eIF4F complex (eIF4E, 4G, and 4A) at the 5'end of mRNA where the eIF4G, interacts on one side with PABP bound at the 3'end of mRNA that promotes pseudo circularization of the mRNA and interacts also with eIF3 of the 43S complex and thus facilitates the formation of 48S initiation complex. In **step4**, the 48S preinitiation complex bound at the 5'cap scans the mRNA at the 5'end to identify the "start site". ATP hydrolysis promotes eIF4A or other helicases to unwind any 2$^{\mathbf{o}}$ structure in mRNA before the start site with partial hydrolysis of the eIF2 bound GTP in the ternary complex to eIF2.GDP.Pi. Start AUG codon recognition promotes dissociation of eIF1 from the 40S, and release of Pi and eIF2.GDP. In **step 5**, the joining of 60S occurs, soon after the release of eIFs bound by the 40S subunit, in the presence of eIF5B.GTP and the GTP hydrolysis trigger release of eIF5B.GDP and eIF1A, to yield the final 80S initiation complex where the tRNA is positioned in the P site of the ribosome. eIF2.GDP is recycled to eIF2.GTP by eIF2B, a guanine nucleotide exchange factor. In response to divergent stresses, activated eIF2α kinases phosphorylate the α-subunit of heterotrimeric eIF2,

which inhibits eIF2B activity and decreases the formation of ternary complex and eIF4F assembly which is blocked by eIF4E- BP1 interaction. Phosphorylation of eIF4E-BP1 by mTOR by mitogens and growth factors dissociates eIF4E-BP1 from eIF4E that relieves the translational inhibition. (Picture source: [30, 32])

1.2.2. Translational control

Regulation of protein synthesis is important as the process consumes a significant amount of energy and raw materials. Rate limiting steps exist in all anabolic and catabolic pathways, and these steps generally occur in the early phase of the pathway to ensure proper regulation. The rate-limiting step in translation is the initiation phase, which is regulated chiefly by phosphorylation-dephosphorylation of initiation factors like eIF2 and eIF4E-BP or eEF2 in eukaryotes [42, 62]. In addition, structural features, and regulatory elements in mRNA at the 5' and 3' ends like Poly A tail, IRES elements, short uORFs etc., are also involved in the regulation. Phosphorylation of eIF2, eIF4F comprising eIF4E, eIF4A, B and eIF4G and eIF4E-BP1 proteins regulate global rates of translation. Phosphorylation of the small or α- subunit of eIF2 inhibits general protein synthesis and up-regulates the translation of gene-specific mRNAs. In contrast, hyperphosphorylation of eIF4-BP1stimulates global translation at the initiation phase. Phosphorylation of elongation factor2 (eEF2) in eukaryotes at Thr-56 residue by Ca^{2+}calmodulin-dependent kinase (eEF2K) inhibits translational elongation. Studies linking cellular signaling pathways to translation regulation suggested that eIF2α phosphorylation which is induced in response to various stressors evokes an integrated stress response (ISR) pathway, whereas the mammalian target of rapamycin (mTOR) pathway is evoked by growth factors, mitogens, nutrient signals, etc., stimulate phosphorylation of eIF4E BP-1 and inhibits eEF2 phosphorylation through the inactivation of its kinase, eEF2K [63, 64]. Keeping in view of the work, the introduction is limited to discussing eIF2 subunit structure, function, phosphorylation, and its importance in the regulation of translational initiation, ISR, and mTOR pathways.

A. eIF2 subunit composition, function, phosphorylation, and interactions

It is a three-subunit protein with different molecular masses: α (36 kDa), β (38 kDa), and γ (52 kDa). However, their migration on SDS-PAGE revealed that the β-subunit migrates close to the γ-subunit displaying a molecular mass of 50 kDa and it is probably due to a large number of lysine residues in the β-subunit [65]. The eIF2 protein delivers initiator tRNA ($Met\text{-}tRNA_i$) to the 40S ribosomal subunit in a GTP-dependent manner in the first step of initiation of translation. Phosphorylation of the conserved 51 serine residue in eIF2α by different kinases inhibits protein synthesis. While α-subunit is implicated in the regulation of protein synthesis, γ-subunit is implicated in various functions such as Met-tRNA$_i$, GTP and GDP binding, and GTP hydrolysis. All these functions of the γ-subunit are aided by the β-subunit. In addition, the β-subunit is a hub for protein-protein interactions and also interacts with mRNA. It is shown that in yeast the γ-subunit is central and it is bound by α- and β-subunits on either side [66, 67]. It is suggested that these two subunits, β, and α do not interact with each other. However, our studies suggest that human α- and β-subunits interact with each other [68]. Both β- and γ-subunits are phosphorylated by different kinases, like CKII, PKA, and PKC in vitro. However, the physiological function of these phosphorylations is not well understood [65, 69-71]. eIF2 γ-subunit is in fact a GTPase. Its GTPase activity is stimulated by eIF5 (a GTPase activating protein) and the hydrolysis of GTP bound to eIF2 occurs after the formation of the 48S complex [51]. Phosphorylation of eIF2α is stress, survival, and suicidal signal [72] and regulates translational initiation. Phosphorylation of the conserved serine-51 residue in eIF2α inhibits the guanine nucleotide exchange activity of eIF2B, and general translation [73-75]. However phosphorylation of eIF2α also up-regulates the translation of gene-specific mRNAs having multiple uORFs like ATF4 (Activating transcription factor 4), GCN4 (General control nonderepressible 4), CHOP (C/EBP homologous protein), etc., and many of them code for transcriptional factors which in turn facilitate the expression of genes involved in redox metabolism, amino acid metabolism, chaperones, autophagy and cell death [51, 76-78].

B. mTOR mediated translational regulation

Another critical and rate-limiting step in initiation is activating the mRNA and converting it into a translation-competent state. This function is carried out by the eIF4F complex, which consists of eIF4E, eIF4G, and eIF4A. Additionally, the eIF4F complex also facilitates the recruitment of 43S PIC on mRNA through interactions between eIF3 and eIF4G. The cap-binding protein eIF4E attaches to the 5'-cap of messenger RNA and then allows additional subunits such as eIF4A and eIF4G to join. When amino acids, energy, nutrients, and growth factors are exhausted, eIF4BP1 becomes hypophosphorylated, forms a strong bond with eIF4E, thereby hindering the assembly of eIF4A and eIF4G, resulting in the inhibition of translation initiation.

This inhibition is released when eIF4BP1 is phosphorylated by mTORC1 [79, 80]. A wide variety of stresses reduces the phosphorylation of eIF4E-BP1 and stimulates the phosphorylation of eIF2α. Thus one finds an inverse correlation between phosphorylation of eIF2α and eIF4E-BP1 on protein synthesis. mTORC1 also phosphorylates the scaffold protein eIF4G in the eIF4F complex in response to growth factor stimuli, and it is assumed that eIF4G phosphorylation stimulates translation initiation [79]. Further, eIF4A an RNA helicase that is part of the eIF4F complex unwinds the secondary structures in mRNAs at the 5' UTR and enhances the translation of mRNAs like Myc, cyclin D1, and insulin-like growth factor (IGF) that encode proteins participated in the cell cycle, proliferation, and growth [42]. Additionally, signal transduction pathways alter eIF4A's helicase activity in response to numerous environmental inputs. For example, when eIF4A is coupled with the regulatory element eIF4B, its unwinding capacity is augmented. eIF4B is phosphorylated by S6K which is activated in turn by mTORC1. In addition RSK (Ribosomal S6 kinase) which is activated by ERK (extracellular-signal-regulated kinase) also phosphorylates eIF4B. These conditions result in increased interaction between eIF4A and eIF4B [81, 82]. mTORC1 is a protein kinase (PIKK family) that is activated by the PI3-AKT (Phospho ionositide-AKT or Protein kinase B), Ras (Rat sarcoma virus)-ERK, and Wnt signaling pathways, all of which are reported to lower the activity of its negative regulators, TSC1 (Tumor suppressor complex 1) and TSC2 [79, 83]. The cytokine TNFα (Tumor necrosis factor α) also stimulates mTORC1 by activating IKK (IkappaB kinase), which inhibits TSC1 and TSC2 [84]. Upon energy scarcity, AMPK (AMP-activated protein kinase) can be activated, switching on ATP generating pathways while limiting ATP consuming operations, such as protein synthesis, to conserve energy. Activated AMPK inhibits mTORC1 by regulating TCS2 and raptor and thus reduces translation [85, 86]. Hypoxia, which lowers energy levels, activates the HIF1-REDD1 (Regulated in DNA damage and development 1, also known as DDIT4) axis, which prevents mTORC1 function [87]. Because amino acids are building blocks of a protein, when these are not enough, SESN2 (Sestrin2) shuts down mTROC1 by negatively regulating RAGA and RAGB (Ras small GTPase protein A and B) proteins, a family of small GTPases [88]. Translational control during the initiation phase is achieved through modulating eIF2 and eIF4EBP1 phosphorylation/dephosphorylation as a result of external and internal inputs. eIF4BP1 mediated regulation is limited to nutrition, growth factors, energy, and amino acids-related issues, whereas eIF2-mediated regulation is more widespread. Thus, accumulating pieces of evidence indicate that the occurrence of translation inhibition is a consequence of either reduced eIF4EBP1 phosphorylation or increased eIF2α phosphorylation, or a combination of the two, depending on the circumstances.

In addition to the initiation, translation control also occurs during the elongation phase by phosphorylation/dephosphorylation of eEF2, eEF1A, and eEF1B under the conditions of stress insults [64, 89]. Phosphorylation of eEF2 by eEF2K inhibits the translocation of the ribosome during elongation. mTORC1 mediated S6K improves eEF2 performance by inhibiting eEF2 kinase via phosphorylation at Ser 366 and encouraging protein phosphatase-2A [64, 89]. Kinetics of initiation, codon frequency in the mRNA, and matching tRNA pool in the cells also influence elongation [26].

1.3. Integrated stress response (ISR)

Cells face a plethora of stressful situations which drive them to respond in ways that are either protective or cause cell death. To deal with these circumstances, cells are equipped with a myriad of stress response systems. Among these, the ISR pathway has received the most significant attention [90]. The ISR is a cellular signaling cascade that is evolutionarily conserved, stress adaptive, and cell defensive found in eukaryotes. ISR can be activated in response to a wide variety of external stresses, including nutrient deficiency, energy shortage, hypoxia, bacterial and viral infections, pathological conditions, aging, ROS, physical stresses such as UV irradiation, osmotic shock, cold shock, and heat shock, and chemical stresses such as metal poisoning, arsenate exposure, etc. Also, physiological stresses like oncogene activation and ER stress, heme, amino acid deficiency, etc., can elicit the ISR [91, 92]. ISR typically involves stress-induced activation of eIF2α kinases that integrate into a uniform downstream signaling cascade manifested as elevation of eIF2α phosphorylation on its Ser-51 residue, inhibition of eIF2B activity, repression of general translation, and preferential translation of a team of mRNAs like ATF4 and CHOP that code the respective transcriptional factors and GADD-34, a cofactor of eIF2α phosphatase. The term “integrated stress response” is first coined by Harding and Ron in 2002. The beauty of ISR is that simply adding a single phosphate moiety to eIF2α, greatly influences the cellular transcriptome, translatome (mRNAs engaged with ribosomes or translational machinery), and proteome. Because of its universal/impartial feature of activation in response to nearly all stimuli, ISR is regarded as the "biological alarm" of the cell. The primary purpose of ISR activation is to protect the cell by activating signaling machinery capable of repairing damage, resolving stress, and restoring cellular homeostasis. However, if stress is high, either in terms of persistence or intensity that will surpass the ISR's adaptive response-ability, ISR then will switch its focus towards the implementation of cell death, most likely via apoptosis by launching the expression of pro-apoptotic components by CHOP [78, 93, 94]. Since stress-induced eIF2α phosphorylation is affected by one or more of the eIF2α kinases, the next section reviews the various eIF2α kinases that are well characterized and their mechanism of activation.

1.3.1. eIF2α kinases and their activation

ISR activation is commenced by the activation of the eIF2α kinases by different stressors. eIF2α kinases serve as stress sensors and relay the stress signal to initiate downstream signaling pathways; thus, eIF2α kinases are described as "sentinels against stress". They belong to the family of serine-threonine kinases. The numbers of eIF2α kinases vary across the eukaryotic evolutionary tree. However, mammals are known to have only four, each of which acts as a cellular sentry and is tuned to a specific stimulus [90, 91]. These are GCN2 (General control nonderepressible2), PERK (PKR-like ER kinase), HRI (Heme-regulated inhibitor), and PKR (Protein kinase RNA-activated), and these are activated in response to amino acid deficiency, ER stress, viral infection or double-stranded RNA, and heme scarcity respectively, and phosphorylate eIF2α at ser-51. All these eIF2α kinases share significant homology in their kinase domain sequences, which explains their common function in phosphorylating eIF2α. Additional distinguishable sequences govern the selective activation in response to corresponding stress. GCN2, HRI, and PKR are located in the cytosol, whereas PERK is located within the ER membrane [77, 95]. The homodimerization and subsequent autophosphorylation of eIF2α kinases are essential for them to become activated, and this is a common characteristic of all of the eIF2α kinases.

A. GCN2 (or EIF2AK4) is the principal responder to amino acid and nutrient availability and the only eIF2α kinase which is conserved throughout nearly all eukaryotes. The canonical or conventional stressor that drives GCN2 activation is amino acid starvation [90]. GCN2 is activated through the binding of uncharged tRNAs to GCN2. The His-RS (Histidyl-tRNA synthetase) domain of GCN2 binds uncharged tRNAs that have accumulated as a consequence of essential amino acid deprivation or a drop in non-essential amino acid synthesis [96]. Binding of uncharged tRNAs to GCN2 causes a conformational change which helps to relieve inhibitory interactions of the kinase domain, culminating in dimerization of GCN2 and autophosphorylation at Thr-882 and Thr-887 amino acids located in the kinase activation loop, both of which are necessary for productive kinase activity [97, 98]. Besides being activated in response to a shortage of amino acids, GCN2 is also activated in response to UV irradiation, low glucose, oxidative stress, viral infection, high salt, and anti-cancer agents that diminish the activity of histone deacetylases and proteasomes [95, 99-104]. However, it is still unclear how these cues stimulate GCN2 and whether or not uncharged tRNAs are involved in the process. To date, eIF2α has been identified as the only known substrate of GCN2.

B. PERK (aka PEK, EIF2AK3) is a transmembrane protein located across the ER membrane. The N-terminal regulatory portion is situated in the ER lumen, while the C-terminal portion is located in the cytosol and comprises a kinase domain and autophosphorylation sites. PERK is primarily responsible for maintaining ER homeostasis and is activated in response to the buildup of unfolded or misfolded proteins in the ER during the event of ER stress. It is first identified in the pancreas and named as PEK. It happens to be an ER transmembrane protein that behaves like PKR kinase, it is also called PERK [105, 106]. Excessive demand for secretory protein synthesis beyond the folding capacity, a decline in oxidative conditions to promote disulfide bonds in proteins, disruption in ER calcium homeostasis, improper posttranslational modifications, and protein folding and assembly defects all result in the deposition of misfolded proteins, which can elicit ER stress. Under these circumstances, ER stress elicits an adaptive signaling pathway known as the unfolded protein response [107]. Three ER transmembrane proteins or stress sensors, PERK, IRE1α, and ATF6, sense the unfolded protein population in the ER and are activated during UPR. UPR mitigates ER stress by diminishing nascent protein load and boosting ER folding capability. PERK is in charge of the first strand, whereas IRE1α and ATF6 are in charge of the second [90, 108]. In terms of oligomerization and trans-autophosphorylation, the activation mechanism of PERK is quite similar to that of GCN2. During normal cell functioning, PERK (also IRE1 and ATF6) is in an incompetent state due to its N-terminal regulatory region associated with BiP/GRP78 in ER lumen. When unfolded and misfolded proteins build in the ER lumen, the regulatory region of PERK becomes freed from the inhibitory binding of BiP/GRP78

[109]. This leads to oligomerization of PERK and autophosphorylation at Thr-980 residue in the cytosolic region of the kinase domain, which is vital for PERK activation and to phosphorylate eIF2α [77, 95]. PERK-mediated ISR, one of three arms of UPR activation, is an integral part of ER stress. During ER stress, PERK activation takes place in parallel with the other two UPR arms, however, the timing of each arm might vary. Activation of PERK occurs immediately in response to ER stress when compared to the other two sensors as it results in translational attenuation of newly synthesized polypeptides mediated by eIF2α phosphorylation that reduces the burden on folding machinery [110, 111]. In addition to phosphorylating eIF2α, PERK also phosphorylates NRF2, another transcription factor that stimulates the expression of various antioxidant enzymes to restore redox balance during ER stress [112]. Other stimuli that are known to promote PERK activity include oxidative stress, glucose deprivation, hypoxia, and a high-fat diet [99, 113-115]. ER, unlike cytosol, is rich in the oxidative environment with high GSSG/GSH which is essential for the formation of S-S bonds (Disulfide bonds) from sulfhydryl groups in secretory proteins synthesized on ER.

C. PKR (also called EIF2AK2) is an important component of the interferon-mediated antiviral mechanism and is localized in both the cytosol and nucleus. Double-stranded RNA, a byproduct of RNA virus replication, is the canonical stressor of PKR activation. The activation of PKR by double-stranded RNA is a critical step in the antiviral response, and this feature defines PKR as a pattern recognition receptor (PRR) that identifies pathogen-associated molecular patterns (PAMPs) found in pathogens and thus plays a vital role in innate immunity [95, 116-120]. PKR is comprised of an N-terminal portion that contributes to regulatory function and a C-terminal portion responsible for catalytic function. The N-terminal region contains two tandem and contiguous repeats of dsRNA binding motifs named dsRBM1 and dsRBM2, to which dsRNA binds. The C-terminus catalytic domain has a kinase activity as well as a dimerization interface where dimerization occurs, which is crucial for PKR activation and functioning [116, 121]. During viral infection, when dsRNA molecules bind to PKR's dsRBMs, conformational changes in the PKR occur, resulting in sequential events such as homodimerization via the C-terminal kinase domain, autophosphorylation at Thr-446 residue, and eventually functional activation of PKR [95, 118, 120]. As part of an antiviral defense program, PKR is activated by a viral infection and phosphorylates eIF2α. In the fight between virus and host, the host evokes eIF2α phosphorylation and cell death to contain virus replication, whereas, viruses produce proteins that down-regulate eIF2α phosphorylation that promotes virus survival. However persistent eIF2α phosphorylation results in host cell death and will limit the virus propagation [72, 122-124]. It has also shown that the PKR is activated in response to a variety of stressors, like endoplasmic reticulum and oxidative stress, ribotoxic stress, bacterial infection, TNF-α, growth factor deficit, heparin, IL-1, ceramides, heat shock proteins, calcium, synthetic double-stranded RNA poly IC, fatty acids, lipopolysaccharides, etc. However, all of these cues induce PKR activation in a dsRNA-independent fashion [125-127]. Cellular proteins, PACT (Protein activator of PKR), and its murine ortholog RAX (PKR-associated protein X) can bind double-stranded RNA and activate PKR [128, 129]. Besides eIF2, PKR stimulates p53 activity and also modulates the transcriptional factors like MAPK, STATs, NF-κB, etc. [118, 130]. In response to diverse stresses, PKR is autophosphorylated at various serine, tyrosine, and threonine residues. However, phosphorylation at Thr-446 and Thr-451 residues are crucial for homo dimerization and catalytic kinase activity to phosphorylate eIF2α [95, 125]. Moreover, recent research from our laboratory has observed that PKR is phosphorylated at two different residues (such as Threonine and Tyrosine) in response to insulin treatment and has demonstrated the importance of PKR in insulin signaling. Acute insulin treatment enhances Tyrosine residue phosphorylation in PKR and decreases its capacity to phosphorylate eIF2α, resulting in increased protein synthesis, whereas, chronic insulin treatment results in Threonine phosphorylation of PKR that activates PKR's ability to phosphorylate eIF2α. Acute insulin exposure enhances the interaction of IRβ with PKR, resulting in the Tyrosine phosphorylation of PKR and aiding resistance to polyIC-induced Threonine phosphorylation of PKR [125].

D. HRI (also known as EIF2AK1) is a cytosolic eIF2α kinase that is predominantly expressed in the erythroid lineage and bone marrow. Very early studies in red blood cells and their lysates prepared from rabbits have indicated that the expression of HRI is related to heme-sufficiency. Reticulocyte lysates prepared from red blood cells of phenylhydrazine-treated rabbits, displayed a reduction in globin synthesis that is accompanied by phosphorylation of eIF2α and addition of hemin and iron protoporphyrin, inhibits eIF2α phosphorylation and promotes globin synthesis. Further studies of protein synthesis in vitro in reticulocyte lysates in the presence of various agents such as GSSG, heavy metals and also denatured proteins, etc., have shown that phosphorylation of eIF2α is a consequence of activation of HRI [131-136]. The canonical stressor that triggers the HRI to become aware and active is heme inadequacy. Because of the heme-binding property, it was previously believed that HRI expression was exclusive to reticulocytes and bone marrow but no other tissues. Recent studies however have shown that HRI activation occurs in mouse embryonic fibroblasts, C2C12 myoblast cells, the CA1 region of the dorsal hippocampus, and neurons in response to diffusible gasses and small chemical molecules [137-141]. Analysis of HRI protein levels, in non-erythroid cells, is varied, exhibiting rich in the spleen and liver and low amounts in the brain, kidney, and lung [142]. Interestingly, when compared to the other three eIF2α kinases, HRI mRNA levels in neuronal/brain tissue are very high. However, the levels of HRI protein in these tissues are extremely low, which explains the importance of translational control in addition to transcriptional regulation in these tissues [140]. In a recent study, Alvarez-Castelao et al. inhibited the ubiquitin-proteasome system (UPS) in neuronal cells and provided a convincing explanation of how and why the HRI protein levels in neurons are maintained in very low quantities when compared to cells of the erythroid lineage. According to this study, HRI protein levels are kept low in neurons due to its short half-life and constant degradation as a UPS substrate. Although HRI mRNA levels were plentiful in comparison to the other three eIF2α kinases, HRI mRNA translation was extremely poor due to the presence of a large number of rare codons in its mRNA, which is known to hinder translational efficiency. Additionally, it was shown that heme levels in neurons are extremely low or undetectable, which is known to favor HRI activation [140]. The fascinating aspect of HRI is the fact that HRI's expression is translationally controlled and that same HRI regulates the translational control of the transcriptome in response to stress stimuli by phosphorylating eIF2α. HRI monitors heme levels in erythroid cells and ensures the α- and β- globin subunits synthesis is commensurate with the heme availability in the cell. Functional hemoglobin comprises heme, α-, and β- globin subunits, and these are assembled in a ratio of 4:2:2. Proper stoichiometry is critical for erythrocytes, as an excess of any one component is lethal to erythrocytes and their progenitors [143].The HRI kinase is composed of five domains: an N-terminal domain, a kinase I domain, a kinase insert domain, a kinase II domain, and a C-terminal domain. [95]. In contrast to other eIF2α kinases, which undergo dimerization or oligomerization in response to stress, HRI is typically available in dimer form in resting conditions. Under normal conditions, when heme levels are excessive, each monomer is bound by two heme moieties, one at the N-terminus and the other at the kinase insert region, which causes the HRI dimer to become inactive. By stimulating the formation of inter disulfide bonds between HRI monomers, heme suppresses the HRI's eIF2α kinase activity [142]. The heme molecule that interacts with the kinase insertion domain is dynamic and reversible, and it is responsible for regulating HRI kinase activity, thus modulating HRI activity in response to cellular heme levels [144]. HRI is autophosphorylated at various Ser, Tyr, and Thr residues in response to heme deficit; however, autophosphorylation at Thr-483 and Thr-485 residues that are situated in the activation loop of the kinase II domain is critical for the HRI to attain the eIF2α kinase activity [145]. Apart from heme deprivation, HRI also gets activated by arsenate, heat-shock, proteasome inhibition, heavy metals, sulfhydryl reagents, glucose deficiency, ethanol, nitric oxide (NO), denatured proteins, iron deficiency, osmotic shock and oxidative stress via heme-independent mechanisms [135, 146, 147]. Iron deficiency is shown to activate HRI and lowers the translation of both in mitochondria and cytosol. Lack of HRI leads to cytosolic unfolded protein response and affects mitochondrial respiration in murine primary erythroblasts in response to iron deficiency [147]. Two HSPs, viz. Hsp90 and Hsc70, play a significant role in the regulation of HRI. By interacting with nascent HRI, Hsp90 promotes the maturation of HRI and its transition into a stable, active heme-regulated kinase. Hsc70 appears to play an unfavorable function in the regulation of HRI activation by blocking auto phosphorylation [135, 148, 149]. Recent studies reported that HRI activity is regulated by HSPB8 gene that produces a heat shock protein which regulates inflammatory cytokine production [150]. HRI bound by HSPB8 is inactive and its release promotes HRI kinase activation and evokes ISR in response to intracellular bacterial infection. This is somewhat similar to PERK activation in ER stress. PERK is inactive when it is bound by BiP encoded by HSPA5 gene and its release activates PERK [151]. Recent studies have also suggested that both PKR and GCN2 are regulated by HSPs [152]. These reports suggest that all eIF2α kinases are regulated by heat shock proteins and that ISR activation can be also a consequence of accumulation of unfolded proteins in various cellular locations. As of now, like GCN2, HRI currently has only one substrate called eIF2α.

The redundancy in eIF2α kinases is important. Although as mentioned earlier each of them is tailored for a specific stress stimulus in sensing and transmitting the stress, the mapping however may not always be a one-to-one manner [91]. For instance, GCN2 is activated and triggers eIF2α phosphorylation in PERK$^{-/-}$MEF cells under the conditions of ER stress [153].

Furthermore, in mice models of Alzheimer's disease and soft tissue sarcoma in which GCN2 has been genetically ablated, PERK can compensate for the GCN2 absence. Additionally, functional redundancy in between PERK and GCN2 kinases was found in HeLa cells challenged by hypothermia and excess of unfolded proteins, where deletion of GCN2 or PERK was balanced by activation of either of the kinase [78]. During some stress conditions, two kinases are activated, although the timing of their activation varies. ER stress causes PERK activation in HeLa and HEK293A cells at the start of the stress course and also induces the expression of PACT, an activator of PKR at a later time point in the stress course. While the activation of PERK promotes survival signaling, the activation of PKR promotes apoptotic signaling [154]. Nucloefection, an electroporation transfection method, and treatment of H_2O_2 cause the activation of GCN2 and PERK [99, 155]. Heat stress, for example, activates PERK in human endothelium cells but PKR in MEFs suggests that the same stress in some cases may activate more than one eIF2α kinase. This may be due to a regulation of the kinase by cell-type-specific protein expression and protein interactions with kinase [156, 157]. Rather than classical stresses that stimulate eIF2α phosphorylation, there are a plethora of varied stresses that also induce eIF2α phosphorylation. However, some of these stressors cannot be neatly assigned to a specific kinase and it was not clear which eIF2α kinase was triggered. Various studies have demonstrated that different kinases are activated in response to glucose limitation and oxidative stress. Certain stresses, as can be analyzed theoretically, would be capable of inducing more than one kinase among the four kinases. For example, ubiquitin proteasomal degradation inhibition can activate GCN2 and HRI. Proteasomal degradation inhibition leads to limitation of amino acid pool in the cytosol which results in activation of GCN2. Since HRI is a known substrate for proteasomal degradation, its inhibition leads to the accumulation of HRI. The activation of accumulated HRI occurs as heme levels do not match the levels of abundance of HRI under those conditions [91, 140].

1.3.2. ISR inhibits eIF2B activity, TC formation, and translation and promotes gene-specific expression

ISR refers to the gene expression reprogramming in response to inhibition in the GDP/GTP exchange or GNE activity of eIF2B and translational inhibition of general mRNAs mediated by stress-induced eIF2α phosphorylation. Unphosphorylated eIF2 as mentioned above delivers initiator tRNA (Met.tRNA$_i$) to 40S ribosomes in a GTP-dependent fashion**.** At the final event of initiation, eIF2 is exit the initiation complex as eIF2.GDP. For this to reenter the initiation cycle, the binary complex, eIF2.GDP is converted to eIF2.GTP. However, eIF2 has more affinity for GDP than that of GTP, the exchange of guanine nucleotides on eIF2 is accomplished by eIF2B, a dedicated guanine nucleotide exchange factor which was described earlier as a hetero pentameric protein consisting of five subunits: α, β, γ, δ and ε. Also, it is a rate-limiting factor and represents 15-20% of total cellular eIF2. Hence, a small amount of phospho-eIF2α (Phosphorylated on Ser-51) can sequester all the available eIF2B into a tight and non-productive complex where the guanine nucleotide exchange (GNE) activity of eIF2B is nonfunctional [73-75, 158, 159]. Further studies have shown that ε- and γ-subunits of eIF2B form a catalytic complex that contributes to the catalytic exchange of GDP for GTP, whereas, α-, β-, and δ- subunits form a regulatory complex that binds phospho-eIF2α more tightly [160]. Recent studies, using a combination of mass spectrometry and overexpression of eIF2B subunits in vivo, have shown that eIF2B is a large two-fold symmetric heterodecameric protein assembled from two subcomplexes: consisting of two tetramers (β, γ, δ, and ε) and a homodimer comprising of two α–subunits [161]. Structural studies reveal the ε-subunit carrying nucleotide exchange is found associated at the opposing ends of the decamer. The interaction between eIF2γ and the two domains of eIF2Bε stabilizes the guanine nucleotide-binding pocket of eIF2γ and thus facilitates the release of GDP and loading of eIF2 with fresh GTP from the cytosol. Recent modeling studies suggest that the exchange of GDP on eIF2 for GTP by eIF2B is the substituted mechanism involving the transfer of GTP from eIF2Bγ to the vacant site on eIF2γ which then induces further conformational changes and dissociation of eIF2 and eIF2B rather than a sequential mechanism that assumes release of GDP from eIF2.GDP.eIF2B before binding GTP and dissociation of eIF2B [162]. Also, recent structural studies from Dr. Venki Ramakrishnan's laboratory, using the cryo-EM structure of yeast eIF2B complexed with phosphorylated eIF2α, at a very low resolution of 4.2 A^0, have determined the subunit regions in eIF2B involved in the binding of eIF2 (phosphorylated and unphosphorylated eIF2α [163]. These studies have shown that two eIF2 molecules bind to the opposite sides of the heterodecameric eIF2B complex. The interaction between eIF2B and eIF2 is carried out by the N-terminal domain 1 in eIF2α and also by γ- and β-subunits of eIF2. However, the eIF2α domain 1 which is inserted into the N-terminal helix bundles of α- and δ- subunits of eIF2B, part of the regulatory complex has the strongest contact compared to β- and γ-subunits of eIF2 interacting with the catalytic subunits (ε) of eIF2B. Biochemical and genetic studies have suggested the interaction between domain 1 in eIF2α with the α- and δ-subunits of eIF2B is further stabilized upon phosphorylation of Ser-51 in eIF2α. This is due to slight alterations in the conformation of the eIF2α helix 58-63 region. Phosphorylated eIF2α induces significant structural rearrangement in eIF2α promoting the formation of a patch of hydrophobic surface on eIF2α that interacts at a different site on eIF2B, thus sterically intervenes in the eIF2 binding to the ε-subunit or catalytic domain in eIF2B. Since the helix 58-63 structure in eIF2α adopts a slightly different conformation and also interacts with the acyl acceptor stem region of initiator tRNA (Met-$tRNA_i$), these findings suggest Met-$tRNA_i$ and eIF2B compete for the same binding site [163, 164]. Due to the lack of functional eIF2B activity, the formation of ternary complexes, eIF2.GTP.Met.$tRNA_i$, is limited and thus inhibits translational initiation. Earlier studies suggested the involvement of a type1 phosphatase in the dephosphorylation of eIF2α in quasi-physiological conditions or using translating reticulocyte lysates [158]. Subsequent studies have identified two regulators of the type1 phosphatase that dephosphorylates eIF2α: CReP which is expressed constitutively [165] and GADD 34 (Growth arrest DNA damage 34) which is expressed on induction when eIF2α is phosphorylated in response to a various several biological or physical stresses, as a part of ISR [166]. G-Actin is also found to activate both these regulator proteins on their association with protein phosphatase1 suggesting that the ISR feedback loop is linked to the polymerization of actin [167, 168]. ISR induced repression in general translation and selective translation reprograms gene expression. ISR is usually initiated by the activation or autophosphorylation of one or more members of the eIF2α kinase family (described earlier) which are activated in response to different stressors but they all phosphorylate the same Serine-51 of eIF2α. The central event of ISR is the phosphorylation of eIF2α, which results in a transient attenuation or cessation of protein synthesis and an acceleration in the translation of a cadre of mRNAs that contain uORFs, such as GCN4, ATF4, CHOP ATF5, C/EBPα & β that play a role in transcription, EPRS involved in tRNA charging, and CDKN1A involved in the cell cycle [77]. In addition, translation of the following mRNAs such as IBTKα, BACE1, and BiP [169] involved in maintaining cellular protein homeostasis, SLC35A4 and CAT1 [170] involved in nutrient transport, and PKCη involved in cellular signaling are also stimulated by eIF2α phosphorylation [77]. Translational shutdown mediated by eIF2α phosphorylation and expression of GCN4 in yeast and ATF4 in metazoans is considered hallmarks of ISR [78]. Although it is true, that ISR enhances the translation of mRNAs featuring short uORFs like ATF4, GADD34, CHOP, etc., but on a genome-wide basis, 50% of eukaryotic mRNAs contain uORFs. However, as of date, only a few such mRNAs are found translated efficiently when eIF2α is phosphorylated. It is not clear whether the preferential translation of these mRNAs with uORFs differ in response to different stressors that activate different eIF2α kinases. In addition, post translational modifications of ISR components like subunits of eIF2B can also affect the GNE activity of eIF2B and can modify the formation TC complexes and translation independent of eIF2α phosphorylation [171]. Also the rates of dephosphorylation of eIF2α may vary depending on the expression of cofactors of phosphatase viz. GADD-34 in response to ISR activation or CReP/ PPP1r15B (constitutive reverser of eIF2α phosphorylation) which is expressed constitutively in different cell types.

1.3.3. ISR regulation

ISR enables cells to retain energy, resources, and assets that will become scarce during a stress regime [92]. The majority of stress stimuli, except UV irradiation, that cause eIF2α phosphorylation are transient, reversible, and biphasic thus, culminating in a translational suppression is dynamic and manifests as an on/off pattern [100]. Additionally, despite the fact that eIF2α phosphorylation inhibits global protein synthesis, a fraction of mRNAs engaged in stress recovery, treatment, repair, and restoration pathways are immune to this inhibition and are translated more efficiently in the presence of eIF2α phosphorylation. Under circumstances of stress, eIF2α phosphorylation swaps and redirects the initiation complex machinery towards selective translation of these stress-specific mRNAs. These transcripts, including ATF4, ATF5, GADD34, and CHOP that belong to a family of CCAAT/enhancer-binding proteins have uORFs preceding the main ORF, in their 5’ UTRs and function as barcodes for scanning by the 43S PIC, which allows for selective or choosy translation under only stressful situations [77, 172-175]. Preferential translation of ATF4-like mRNAs containing short uORFs in the 5’ leader preceding the main ORF involves phospho-eIF2 mediated delay in the ribosomal reinitiation, aiding the bypass of the inhibitory uORFs, results in enhanced ATF4 coding region translation [172, 176]. In addition, eIF2α phosphorylation also promotes the translation of some of the rare mRNAs or mRNAs with internal ribosome entry sites (IRES) such as BiP and CAT-1 [77, 108, 169, 170, 172] and this may be due to a decline in the translation of competing mRNAs as the translation of general mRNAs are inhibited. ATF4 is the most widely investigated transcription factor of ISR in the cell's arsenal to fight off stress. ATF4, a bZIP transcription factor, is a member of the ATF/CREB transcriptional factors family. ATF4 protein synthesis is a direct instant result of eIF2α phosphorylation-mediated translational control [172], which in turn drives the transcription of ATF3, ATF5, CHOP, GADD34, and others that will also participate in the ISR [78]. Regardless of the underlying stimulus, ATF4 commonly promotes the transcription of genes that are involved in redox metabolism, amino acid metabolism, autophagy, chaperones, organelle quality control, and apoptosis [76, 93, 100, 177, 178]. ATF4, as a homodimer or heterodimer with other transcription factors such as CHOP and ATF3, drives the transcription of multiple genes as well as its own to reinforce and strengthen the ISR. Indeed, ATF4 plays a crucial role in governing the cells' fate in response to ISR activation [178]. ATF4-driven transcriptional program is primarily involved in cellular survival, stress, adaptation, and homeostasis restoration via activating autophagy and antioxidant defenses [177, 179]. During persistent stress, the ATF4-induced transcriptional program shifts towards cell death through induction of CHOP, another transcription factor that in turn induces genes involved in cell death to eliminate stress-injured or damaged cells [76, 78, 93]. As mentioned earlier, phosphorylation of eIF2α is biphasic and reversible, and dephosphorylation of eIF2α is necessary for the resumption in general protein synthesis. ISR-induced ATF4 triggers the transcription of GADD-34, a cofactor of PP1 that promotes dephosphorylation of eIF2α. It acts as a negative feedback mechanism [158, 166, 180]. Also, expression of GADD-34 can be a death signal if that happens earlier to the stress-induced cellular recovery as the resumption of protein synthesis generates reactive oxygen species and enhances the cellular burden of protein folding. A wide variety of stimuli have been shown to elicit ISR, however, this does not mean that the eIF2α-P/ATF4 cascade and its molecular targets are activated in the same way under all conditions. The amount and persistence of eIF2α-P in concert with ATF4, and its dimerization partners govern the ultimate outcome of ISR [78]. Although ATF4 is a common mediator of ISR, it delivers distinct tailored responses to specific cellular stress stimuli to achieve the best possible relief of stress-induced cell damage. This may be due to differential regulation of ATF4 at the translational and transcriptional levels, interaction with diverse transcriptional factors, and subsequent induction of various target genes of ATF4, all of which are reliant on the stress intensity, cell type, cellular context, activated eIF2α kinase, and ultimately underlying trigger [78, 100]. For instance, UV irradiation and mTOR inhibition elicited ISR is atypical from traditional ISR signaling in terms of ATF4 regulation. In the majority of the stress conditions, phosphorylated eIF2α favors the translation of ATF4 to counter the stress damage [106]. However, ATF4 expression is regulated differentially in response to UV exposure and mTOCRC1 inhibition by Torin1. Synthesis of ATF4 was impeded in UV irradiation even though robust eIF2α phosphorylation occurs. UV exposure, although results in hyperphosphorylation of eIF2α, but represses the transcription of ATF4 unlike in ER stress. Thus no new ATF4 transcripts are transcribed. Therefore during UV-irradiation, due to the lack of availability of ATF4 transcripts, preferential translation was hampered despite high levels of eIF2α phosphorylation [100]. In addition to UV-irradiation, mTORC1 also regulates ATF4 expression by modulating its translation and mRNA stability. mTORC1 inhibitor Torin 1 inhibits ATF4 expression by reducing the rate of translation and stability of its mRNA through the 4E-BPs [181]. Many recent studies have also revealed that the ISR pathway communicates with other signaling pathways, including mTORC1, AKT, and AMPK [99, 182, 183].The cross-talk between ISR and these signaling pathways can occur directly through eIF2α phosphorylation or indirectly through ATF4 mediated transcriptional program. As an example, in MEFs, eIF2α phosphorylation mediated by GCN2 and PERK induces AKT activation, which in turn provides protection against H_2O_2-induced oxidative stress [99]. During amino acid deprivation, GCN2-mediated eIF2α phosphorylation directly inhibits mTORC1 signaling and is independent of ATF4 involvement. Other investigations have also demonstrated that ATF4-driven transcriptional induction of mTOC1 negative regulators such as SESN2, REDD1, 4EBP1, ASNS, GRB10 and TRIB3 inhibit mTORC1 as well [147, 184-189].

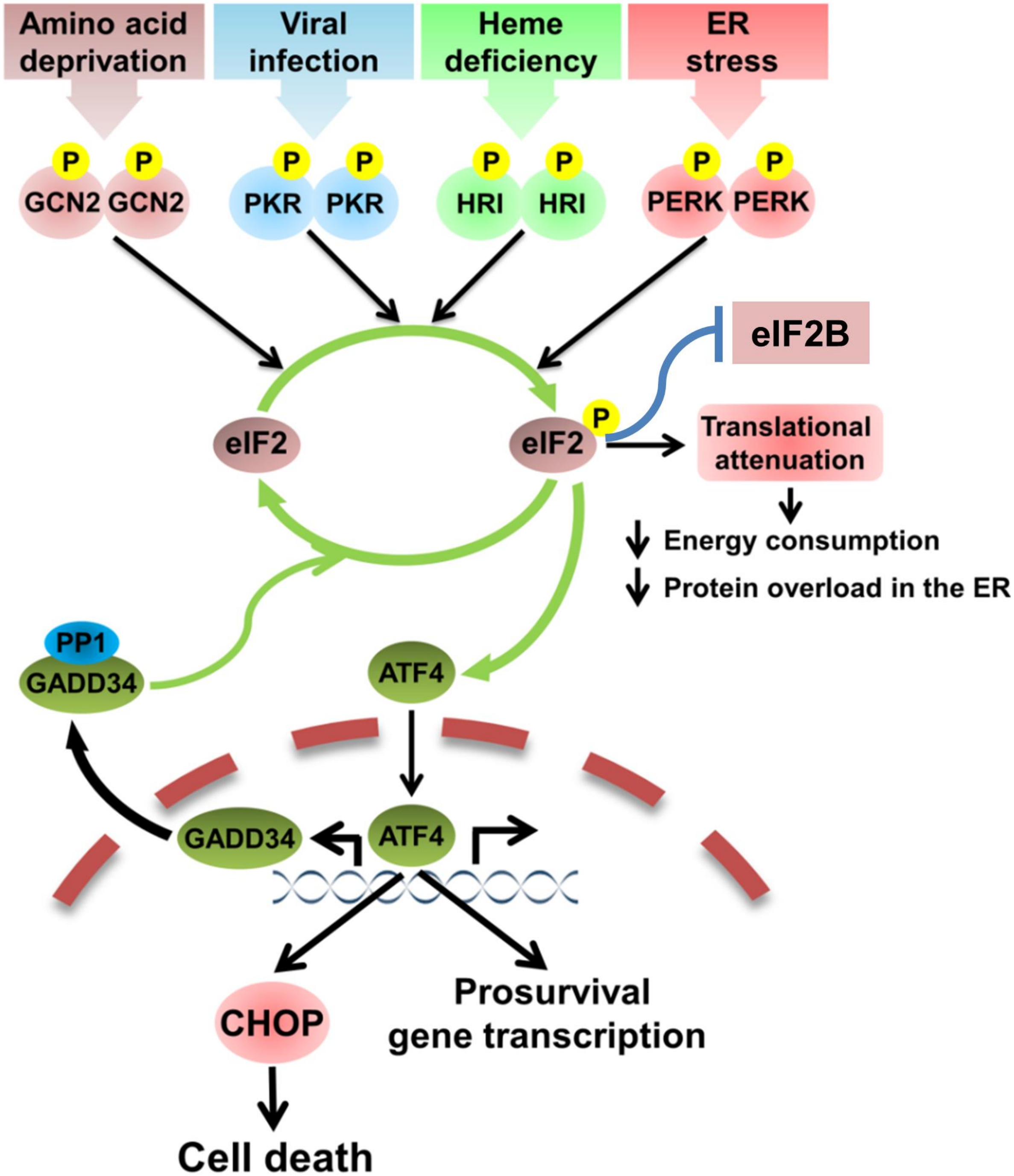


Fig. 2. Integrated stress response pathway. The amino acid deficit, virus infection, heme depletion, ER stress, and various other stresses stimulate GCN2, PKR, HRI, and PERK kinases that phosphorylate eIF2α, a key event of ISR signaling. eIF2α phosphorylation signals the suppression in the translation of general mRNAs and preferential translation of stress-related mRNAs like ATF4. ATF4 is the key transcription factor, and further induces the transcription of multiple genes that participated in stress recovery, redox metabolism, and cellular and protein homeostasis. ATF4 also stimulates the expression of GADD-34, a cofactor of type1 protein

phosphatase that dephosphorylates eIF2α. Phosphorylated eIF2α binds to eIF2B and inhibits its GNE activity thus it reduces the active eIF2.GTP pool in the cytosol and thereby the formation of TC. Persistent stress promotes the expression of CHOP which in turn stimulates genes involved in cell death.

1.3.4. Importance of ISR in Health and Disease

Recent studies highlighted the importance of ISR in health and disease. ISR regulates memory, virus infection, immunity, cell cycle regulation, metabolism, aging, pulmonary and neurological disorders, tumorigenesis, and protein and cellular homeostasis which explains the versatility of ISR [54, 77, 177, 190-193]. The pancreas and brain are the two most affected organs by ISR dysfunction. The importance of ISR in normal physiology and development is well substantiated by knocking out phosphorylation (replacing Ser-51 with Ala) in both the copies of eIF2 and ablation of PERK. Such mice suffer from post-natal lethality [108, 194]. In contrast, persistent activation of eIF2α phosphorylation that occurs in GADD-34 knock-out embryos promotes the death of embryos suggesting the vital role of ISR during the process of embryonic development [195]. Weak or milder ISR is associated with diseases. Improper ISR signaling affects intellectual activities, promotes neurodegeneration, impairs glucose homeostasis and myelination, and is the cause of metabolic disorders. Homozygous mutations in CReP or its gene PPP1R15B are associated with a significantly smaller brain and body size due to a defect in development, diabetes, and cognitive dysfunction [196-198]. Mutations in the γ-subunit of eIF2 cause an intellectual disability called MEHMO, an X-linked syndrome. It causes hypogenitalism, microcephaly, mental deficiency, and obesity [199, 200]. Since β- and γ-subunits interactions define the formation of ternary complexes, eIF2.GTP.Met $tRNA_i$, mutations in either of these subunits will lower the formation of ternary complexes [201] which causes different pathologies in different tissues. Reduced ternary complex formation lowers translation as mentioned above. Mutations in eIF2B cause VWM, vanishing white matter disease, a rare autosomal disorder that is associated with a reduction in the eIF2B GNE activity and the ability of eIF2 to join the initiator tRNA [202, 203]. VWM patients suffer from myelin loss, ataxia, reduced cognition, and death [204]. PERK mutations in humans cause Wolcott-Rallison syndrome (WRS), childhood diabetes in humans, and epiphyseal dysplasia or skeletal disorders. WRS is characterized by enhanced misfolded secretory proteins in the ER [205, 206]. Loss of GCN2 kinase function or mutations in GCN2 causes pulmonary arterial hypertension [207]. This disease arises due to the disruption of GCN2 that promotes angiogenesis during amino acid starvation [208].

ISR plays a role in the brain functions like long-term memory formation. Inhibition of ISR either by genetic or pharmacological means enhances long-term memory formation in birds and rodents. Activation of ISR by enhancing the levels of eIF2α-P either by decreasing the phosphatase activity using salubrinal, a small molecule, or enhancing the activation of PKR kinase in hippocampus or mutations in the γ-subunit of eIF2, inhibits long-term memory formation and causes intellectual disability [209-213]. Negative regulation of ISR or reduced levels of eIF2α-P in the hippocampus affects memory [214]. ISR is also involved in synaptic plasticity and the physical fitness of synapses. Two main types of synaptic plasticity: long-term potentiation, LTP, which strengthens synapses, and long-term depression, LTD, which weakens synapses are dependent on ISR-mediated changes in protein synthesis [215]. This is accompanied by two regulators: OPHN1, rho GAP implicated in the inhibition of AMPA receptor, and ATF4, which regulates CREB-driven gene expression in LTP [216, 217]. ISR activation is associated with age-related loss in protein homeostasis, cognitive disorders or mental disabilities, or neurological disorders like Alzheimer's. eIF2α kinases play an important role in ISR-mediated disorders. One of the aspects of ISR activation is an accumulation of misfolded proteins that occurs during aging. Activation of ISR as mentioned above is also a consequence of cognitive defects caused by traumatic brain injury in mouse models [209]. Many age-related neurological and metabolic disorders like Alzheimer's, amyotrophic lateral sclerosis, Huntington's disease, Charcot-Marie-Tooth disease, traumatic brain injury, and diabetes displayed an alteration in protein synthesis, folding, and degradation. Phospho-eIF2α and auto phosphorylated or activated eIF2α kinases like PERK, PKR and GCN2 were detected in postmortem brains of people and animals suffering from cognitive and neurodegenerative disorders [54]. Pharmacological inhibition of PKR mitigates defects in translation and long-term memory in Down syndrome-carrying mouse models [218]. Restoration of ISR-mediated translational inhibition promotes also neuroprotection. Overexpression of the phosphatase cofactor, GADD-34 that dephosphorylates eIF2α or agents that inhibit PKR or PERK, or anti-depressant like trazodone that increases the eIF2 ability to form eIF2.GTP.Met.tRNAi, ternary complexes, promotes neuronal survival and also reduce the loss of neurons that are affected by prion disease [219-221]. In insect and mouse models of Alzheimer's and Parkinson's diseases, inhibition of PERK and thereby ISR is found neuroprotective and prevents neurodegeneration. The mechanism appears to be through inhibition of ISR-mediated translational reprogramming [222-225]. The results from various experiments suggest that fine-tuning of ISR is important and required to carry out the proper functioning of pancreatic β-cells, involved in secreting insulin and regulating glucose homeostasis. Normal functioning of pancreatic β-cell requires ISR-mediated eIF2α-P in a narrow range and depending on the necessity it has to be turned on and off dynamically [54].

Also, ISR which is evoked in response to increased protein synthesis and decreased protein folding, shuts down general protein synthesis as a consequence of eIF2α phosphorylation and promotes the induction of CHOP and ATF4 that regulate the balance between survival and death has been exploited by tumor cells [226]. Protein synthesis is increased in many cancer cells, because of mutations in PTEN or activation of oncogenes like MYC that can cause a burden on the protein folding enzymes and thus activate PERK-mediated ISR to limit the protein synthesis [227, 228]. GCN2 kinase that regulates amino acid metabolism is also needed for the maintenance of tumor cells. Deletion of GCN2 or ATF4 reduces the growth of tumor cells [229, 230]. In fact treating cancer cells with L-asparaginase and inhibitors of GCN2 leads to apoptosis [231]. Hence pharmacological targeting of GCN2-mediated ISR in cancer cells suffering from amino acid starvation may be advantageous to suppress the tumor propagation. Also, activation of ISR occurs as part of innate immunity. Various cytokines like IL-1β and IL-6 are secreted in response to ISR activation [232]. Hence, inhibition of ISR may be beneficial to promote anti-inflammatory responses. ISR is found to stimulate NF-kB, an inflammatory transcription factor that is regulated in turn by IkB, a short-lived protein. IkB binding with NF-kB represses NF-kB activity. ISR-induced translational inhibition promotes a reduction in IkB steady-state concentration levels and thus activates NF-kB [102, 233]. Also, HRI kinase activation mediated ISR play a key role in NF-kB activation in response to intracellular pathogens recently and ATF4 induced by ISR activation upregulates HSPB8 chaperone expression. Association of HSPB8 with NOD1/2, the pattern recognition receptors, promotes large inflammasome complexes which further contributes to IkB degradation through ubiquitinylation and promotes the NF-kB activation [150]. ISR-driven ATF4 directly induces the IL-6 expression, an inflammatory cytokine [234]. PKR, can also bind to NOD-like receptors on its activation and activate inflammasome [119]. Also, eIF2α phosphorylation mediated ATF4 can induce the transcription of NLRP1, one of the NOD-like receptors suggesting that not only activated PKR assembles with NOD-like receptors but also ISR activation is crucial for activation of inflammasome [235]. Saturated fatty acids promote cardiovascular diseases and atherosclerosis by inducing ER stress, ISR activation through a PERK branch of UPR, caspase 1 activation, and IL-1β secretion in macrophages [191]. In mouse models, ISRIB prevents fatty acid-induced inflammation and the progression of atherosclerosis.

1.3.5. Pharmacological modulation of ISR

ISR signaling is like a double-edged sword [76]. It can be either beneficial or detrimental and it is all depending on the context of the biological situation. Deregulation of ISR has been associated with multiple pathological situations as mentioned above. Thus, external modulation of ISR by pharmaceuticals has emerged as a promising therapeutic approach. In the past few years, various drugs were developed that specifically target the various ISR components to augment or suppress this signaling pathway. Interestingly, drugs that either repress or activate ISR signaling cascade have been shown to diminish cancer cell proliferation in vivo, indicating that ISR's dual role in supporting cell survival as well as cell death [141, 229]. ISR signaling can be activated by pharmacological drugs in two ways: either by activating eIF2α kinases, which promote phosphorylation of eIF2α, or by inhibiting the phosphatases that dephosphorylate eIF2α, as phosphorylation of any substrate is dependent on the respective kinase and phosphatase activities. N,N'-dialylureas, and BTdCPU activate HRI, BEPP monohydrochloride activates PKR, CCT020312 specifically activates PERK without inducing a general UPR and halofuginone, arginine deiminase, histidinol, and asparaginase activate GCN2. Salubrinal, guanabenz, and its derivative Sephin1 prevent eIF2α dephosphorylation via disrupting the interaction of PP1 and its cofactor GADD34. Multiple inhibitors of eIF2α kinases have also been developed to minimize ISR signaling. Indirubin-3'-monoxime and SP600125 suppress GCN2, whereas, GSK2606414 and GSK2656157 suppress PERK. C16 and aminopyrazolindane inhibit PKR and HRI kinases, respectively [78]. In addition to the above small molecules that affect the activation or inhibit eIF2a kinases, other agents such as chemical chaperones, like TUDCA, a hydrophilic bile salt, and 4-PBA (4-Phenyl butyric acid) relieve protein aggregation and reduce ER stress-induced ISR activation, expression of BiP and cell death. In contrast, TUDCA also mitigates UV irradiation-induced ISR activation and cell death [236, 237]. ISRIB, a small molecule inhibitor discovered recently, unlike the above molecules, activates the guanine nucleotide exchange activity of eIF2B and attenuates ISR signaling [54, 209]. Since ISRIB is used in the present thesis to characterize CCCP-induced ISR activation, the information about ISRIB is presented here.

A. ISRIB

2-(4-Chlorophenoxy)-N-[(1r,4r)-4-[2-(4-chlorophenoxy) acetamido] cyclohexyl] acetamide, is a small molecule inhibitor of the ISR pathway that blocks the eIF2α phosphorylation consequences viz. inhibition of eIF2B activity and general translation, stress granule formation, expression of ATF4 and its downstream signaling. ISRIB was discovered by Peter Walter's lab at UCSF (University of California, San Francisco) in 2013 [209]. ISRIB is highly potent, easily permeable, reaches the systemic circulation, blood-brain barrier permeable, and has good pharmacokinetic properties. ISR-mediated loss in protein synthesis in the hippocampus, synaptic plasticity, and memory is rescued by ISRIB in Alzheimer's disease mouse models [54, 209, 238]. In 2013, Sidrauski et al, have shown that ISRIB blocks the synthesis of ATF4 and CHOP which are controlled by the PERK arm of UPR without affecting eIF2α phosphorylation in ER-stressed cells. By increasing eIF2B activity, ISRIB enhances translation and mitigates the expression of ATF4. Since it does not overcome ER stress, translational resumption further results in unfolded protein accumulation and deposition in ER and promotes cell death. It reverses the memory decline in old-aged mice by improving dendritic spine density and increasing intrinsic neuron excitability [209]. In addition, by resetting ISR, ISRIB is shown to reduce T-cell and IFN-γ mediated immune responses in aged mice [239, 240]. Recent studies also reported that ISRIB prevents neuronal injury in mouse models of Down's syndrome, prion disease, traumatic brain injury, and cellular stress [218, 241-243]. ISRIB not only rescues eIF2-P mediated ISR disorders, but it also restores the demyelination in Vanishing White Matter Disease models, a neurological disorder that is caused by mutations in various subunits of eIF2B. VWMD mutations in eIF2B hamper its GEF activity by blocking the assembly of productive heterodecameric complex, while ISRIB restores a broad spectrum of mutations by stabilizing an effective or functional heterodecameric complex [244].

Genetic and biochemical investigations have revealed that the molecular target of ISRIB is eIF2B. eIF2B is a hetero decameric complex composed of 2 copies of 5 different subunits: α, β, γ, δ, and ε. eIF2B complex consists of two βδγε tetramers and one α-subunit dimer. eIF2Bε along with eIF2Bγ constitutes the catalytic core that catalyzes the reaction of GDP/GTP exchange in eIF2 [245]. The remaining three subunits such as α, β, and δ build a regulatory sub-complex, which provides a binding site to phosphorylated eIF2α [246]. Recent structural studies suggest that ISRIB binds eIF2B and facilitates the formation and stabilization of active eIF2B hetero decameric complex (two copies of five subunits) from its smaller sub-complexes: two hetero tetramers comprising β, γ, δ, and ε and a dimer of α. [242, 247]. Active and denovo eIF2B complex having GNE activity exchanges the GDP to GTP on eIF2. Thus, it stops the signal flow from phosphorylated eIF2α on global translation initiation. ISRIB binds at the interface between δ- and β-subunits of eIF2B tetramers (β,δ,γ, and ε) and enhances the octamer formation. The resulted octamer serves as a platform for the joining of the α-subunit dimer of eIF2B. Thus ISRIB staples the eIF2B subunits across a twofold symmetry axis stabilizing the eIF2B decamer and enhancing its GNE activity. Reducing the synthesis of decameric eIF2B by genetic approaches, rendered cells resistant to ISRIB suggesting that ISRIB does not increase the total levels of eIF2B, but rather enhances the GNE activity of eIF2B when the tetramer assembles into a decameric complex [54, 248, 249]. Hence ISRIB enhanced GNE activity relieves the suppression of translation mediated by eIF2α phosphorylation. During stress conditions phosphorylated eIF2α binds to another site in the regulatory sub-complex of eIF2B (δ, β, α) and acts as a non-competitive inhibitor rather than as a substrate anymore. The addition of ISRIB to the cells makes denovo complex assembly of active decameric eIF2B from the pool of sub-units or mini subunit complexes and reinitiates translation even in the presence of eIF2α phosphorylation. ISRIB mitigates only low levels of the ISR signaling and the efficacy of ISRIB is determined by levels of phospho-eIF2α and availability of the eIF2B subunit population in the cells. When eIF2α phosphorylation levels surpass more than critical threshold values, more eIF2B is captured by phosphorylated eIF2α and forms a stable eIF2B-eIF2-P inactive complex that depletes free eIF2B levels. Thus excessive eIF2α phosphorylation lowers the efficiency of ISRIB to promote an active eIF2B decameric complex from the existing subunits. Under these circumstances, ISRIB cannot attenuate the ISR activation. Hence, ISRIB is only efficient or operational within a small range of intracellular P-eIF2 concentrations [241, 250].

Chapter-II

2. Aim of the study

The ISR is an evolutionarily conserved complex signaling pathway in metazoans and it is triggered in response to nearly all stresses that disrupt cellular functions. Previous studies from this laboratory have shown that stressors that affect protein synthesis in cytosol, and protein folding in ER or damage DNA in the nucleus have been shown to evoke the ISR pathway by activating one of the four eIF2α kinases like PERK, GCN2, HRI, or PKR. While HRI, GCN2, and PKR are cytosolic kinases, PERK is localized to ER. To enter into an area of inter-organellar communication, here we want to determine whether dysfunctional or stressed mitochondria can activate ISR mediated by cytosolic or ER eIF2α kinases. Keeping in view of the various mitochondrial functions such as heme biosynthesis, ATP production, amino acid, nucleotide, and lipid metabolism and its connection with the ER membrane, it is likely the disruption of mitochondrial function can evoke the activation of all four eIF2α kinases. It is likely the stressors that impair the mitochondrial function may also result in heme and amino acid depletion, which are known canonical signals for HRI and GCN2 activation, respectively. Since mitochondria are linked with ER through MAMS (Mitochondria-associated ER membranes) mitochondrial damage can also disturb ER homeostasis and the disturbed ER can activate PERK. Furthermore, mitochondria have their own genome that encodes 37 genes. The mitochondrial RNA transcripts are foreign to the cytosol. In response to mitochondrial stress, if the RNAs are released into the cytosol, there will be a chance of these RNAs activating PKR kinase. Additionally, mitochondria act as a powerhouse and manufacture ATP through the OXPHOS process, which involves both electrons and protons. Hence mitochondrial damage can result in reduced ATP and increased ROS. Earlier reports have shown that ATP deficit causes activation of PERK through suppression of the sarcoplasmic/ER Ca^{2+}-ATPase pump (SERCA). There have been multiple findings demonstrating that energy deprivation and oxidative stress induced by various stressors (other than mito stressors) such as 2-DG (deoxy glucose), UV-irradiation, H_2O_2, arsenate, etc., can activate distinct kinases in diverse ways. When we started the thesis work, there are not many studies indicating that dysfunctional mitochondria can activate an eIF2α kinase and induce ISR. Hence we planned to study the activation of ISR and characterize the eIF2α kinase involved in mitostress. In the current investigation, we employed CCCP to elicit a mitochondrial dysfunction. CCCP is an uncoupler of oxidative phosphorylation and is a protonophore that has been shown to impair ATP synthesis by leaking protons accumulated during the ETC reaction.

As a result, CCCP generates a lot of ROS and deprives the ATP pool. Furthermore, it has the potential to affect multiple mitochondrial activities. According to theoretical predictions, CCCP-induced mitochondrial distress has the potential or likelihood of activating all four eIF2α kinases as mentioned above; however, activation of four kinases in the same stress has not been observed so far. It was generally observed that ISR was triggered by the activation of a single kinase or, in a few cases, two kinases. Based on this information we addressed the following questions.

1) Does CCCP-induced mitochondrial dysfunction evokes ISR activation?
2) Which one of the four well-known eIF2α kinases is accountable to trigger ISR activation during CCCP treatment?
3) What could be the possible cause (ATP depletion or enhanced ROS) for ISR activation in CCCP-treated cells?
4) Does CCCP affect general translation and regulation?
5) Does CCCP affect other signaling pathways that include activation of AMPK, mTOR, and AKT?
6) Is there a cross-talk between ISR and these signaling pathways?
7) Does CCCP-induced mitochondrial dysfunction affect cellular homeostasis and the role of ISR in maintaining cellular homeostasis?

These questions are addressed using HepG2 cells, ISRIB, the small molecule ISR inhibitor, and N-acetyl Cysteine, an antioxidant.

Chapter-III

3. Materials and methods

3.1. Chemical reagents and antibodies

CCCP, thapsigargin, 17-AAG, ISRIB, caspase-3 substrate AC-DEVD-AFC, N-acetyl cysteine (NAC), Propidium iodide, protease, and phosphatase inhibitors, and mouse monoclonal anti-actin antibody (Cat# A5441) were obtained from Sigma, Bangalore, India. Tunicamycin was purchased from Calbiochem, Bangalore, India. Pan-caspase inhibitor z-VAD-fmk (Cat# FMK001) was obtained from R&D Systems, Minneapolis, MN, USA. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from HiMedia, Mumbai, India. 100X Antibiotic-Antimycotic was obtained from GIBCO, Mumbai, India. Rabbit polyclonal anti-phospho eIF2α-ser51 antibody (Cat# NSB728) was imported from Novus Biologicals, Centennial, CO, USA. Rabbit polyclonal anti-BiP antibody (Cat# Sc-13698) was purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Rabbit polyclonal anti-eIF2α antibody (Cat# 9722S), Rabbit monoclonal anti-PARP antibody (Cat# 9532S), Rabbit monoclonal anti-ATF4 antibody (Cat# 11815S), Rabbit monoclonal anti-PERK antibody (Cat# 5683S), Mouse monoclonal anti-CHOP antibody (Cat# 2895S), Rabbit polyclonal anti-LC3A/B antibody (Cat# 4108S and 2775S), Rabbit polyclonal anti-GCN2 Antibody (Cat# 3302S), Rabbit monoclonal anti-phospho AMPKα-Thr172 antibody (Cat# 2535S), Rabbit monoclonal anti-AMPKα antibody (Cat# 5831S), Rabbit monoclonal anti-4E-BP1 antibody (Cat# 9644S), Rabbit polyclonal anti-p70S6 kinase antibody (Cat# 9202S), Rabbit monoclonal anti-phospho 4E-BP1 (Thr37/46) antibody (Cat# 2855S), Rabbit polyclonal anti-phospho p70 S6 kinase (Thr389) antibody (Cat# 9205S), Rabbit monoclonal anti-phospho AKT-Ser473 antibody (Cat# 4060S), Rabbit monoclonal anti-AKT (PAN) antibody (Cat# 4691S), and Rabbit monoclonal anti-PKR antibody (CST Cat# 12297) were obtained from Cell Signaling Technology, Danvers, MA, USA.

3.2. Cell culture and transfection

HepG2 and HeLa cell lines were purchased from the Center for Cellular and Molecular Biology (CCMB) Hyderabad, India. DR-Wild-type (ATCC CRL2977) and GCN2-KO-DR (ATCC CRL2978) MEF cell lines were a kind gift from Dr. Noorudhin Khan, Department of Biotechnology & Bioinformatics, University of Hyderabad, India. HepG2, WT MEF, and GCN2 KO MEF cells were grown in DMEM containing 10% (v/v) fetal bovine serum, sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. Equal numbers of cells were seeded in 60 mM culture dishes. After 24 h of seeding, unless otherwise indicated, cells were insulted with different stress inducers viz. CCCP (25 μM), tunicamycin (12 μM), or thapsigargin (1 μM) for different periods as indicated in the figures. Pretreatment with 250 nM ISRIB, 5 mM NAC, 20 μM z-VAD-fmk, or with 2 μM 17-AAG for 1 hr was carried out before exposing them to different stress regimens wherever indicated. For depletion of HRI kinase, 2 x 10^5 HepG2 cells per well were seeded in a 2 mL antibiotic-free normal growth medium which was supplemented with FBS and incubated in the CO_2 incubator till the cells attained 60–70% confluence. Cells were transfected with 50 nM HRI siRNA duplex (Cat# sc-39053) or 50 nM scrambled siRNA duplex (Cat# sc- 37007) using lipofectamine RNAimax (Cat# 3778030) from Invitrogen, (Waltham, MA, USA) and were incubated in serum and antibiotic-free DMEM medium. After 6 hrs of transfection, serum and antibiotic-free DMEM was replaced with complete DMEM. Following 48 hrs, the medium was replaced with fresh DMEM and exposed to CCCP for different periods as mentioned in the legends of figures.

3.3. Preparation of cell extracts and western blot analysis

HepG2, HeLa, or MEF cells, exposed to different stress conditions or no stress, were washed once with phosphate-buffered saline solution (PBS), scraped to dissociate adhered cells in PBS buffer, and then centrifuged at 4000 rpm. Cell pellets were lysed with RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 mM NaF, 50 mM β-Glycerophosphate, 1% NP-40) supplemented with 1 mM PMSF and phosphatase and protease inhibitor cocktails. An equal amount of protein samples were separated on SDS/PAGE (8–14%), and proteins were transferred to a nitrocellulose membrane and then probed by the respective antibodies as described previously [236]. The blots were developed using G-Biosciences chemiluminescence reagents by Versadoc obtained from Bio-Rad, Hercules, CA, USA.

3.4. Translation assay

HepG2 cells were cultured until 70% confluency was attained. Initially, cells were washed twice with PBS, once with DMEM without L-methionine, and incubated at 37 °C for 1 hr in DMEM without L-methionine (HiMedia Cat# AL813A) containing 2% dialyzed FBS (GIBCO Cat#

A3382001). Then, cells were treated with 25 μM CCCP alone or in the presence of 5 mM NAC or 250 nM ISRIB as indicated. Post 1 hr incubation, ^{35}S methionine (ARC Cat# ARS0119-specific activity 1175 Ci/mmol) was added to a final concentration of 6 μCi/1 mL, and then, the cells were incubated for one more hour. After radiolabeling, cells were washed once with PBS, scraped to dissociate the adhered cells in PBS buffer, and then centrifuged at 4000 rpm. Cell pellets were lysed with RIPA buffer. An equal amount of protein samples were separated on 12% SDS/PAGE and coomassie stained. Radioactivity in SDS-PAGE gels was detected using a Typhoon TRIO + Variable Mode Imager (GE Healthcare, Chicago, IL, USA).

3.5. Caspase 3 activity assay

Caspase 3 activity was measured by monitoring the hydrolysis of fluorogenic substrate, N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (AC-DEVD-AFC). Samples carrying 150 μg protein in a final volume of 40 μL were incubated with 200 μL of caspase assay buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM DTT, 0.1% CHAPS, 10% Sucrose, 1 mM EDTA) containing 20 μM caspase 3 substrate, AC-DEVD-AFC at 30 °C for 30 min. After incubation, fluorescence intensity was measured by Molecular Devices SpectraMax M2 Spectrofluorometer (λ ex = 400 nm and λ em = 510 nm).

3.6. Semi-quantitative-PCR and real-time PCR analysis

Following various treatments, cells were washed with PBS and harvested in TRIZOL (Invitrogen) reagent. Total RNA was isolated from treated and untreated cells according to the manufacturer's instructions. An equal amount of RNA from different samples was treated with DNase 1 (Thermo Fisher Scientific-EN0521) before proceeding to cDNA synthesis to avoid DNA contamination. RNA integrity was checked by 2% agarose gel electrophoresis, and purity was verified spectrophotometrically by taking the absorbance ratio at A260/280 using NanoDrop 2000 (Thermo Scientific). Post-Dnase 1 treatment, 1 μg of RNA was used in a 20 μL reaction mixture to synthesize cDNA using Verso cDNA synthesis kit (Catalogue No. AB1453B Thermo Scientific). 1 μL cDNA was used as a template for the semiquantitative PCR (25 cycles) using respective primers. After completion of the PCR reaction, PCR products were resolved on 3% agarose gel electrophoresis and analyzed by ethidium bromide staining. Quantitative PCR (qPCR) was performed using an Applied Biosystems QuantStudio 3.0. The expression of different genes was measured using the SYBR Green master mix (DyNAmoColorFlash SYBR Green qPCR Kit Thermo scientific-F416L). mRNA levels were quantified or measured using the ΔΔCt method. The following primers are used:

ATF4 (FP-TCCAACAACAGCAAGGAGGATG, RP-TCCAACGTGGTCAGAAGGTCATC); CHOP (FP-CCTGGAAATGAAGAGGAAGAATC, RP-ACTGGAATCTGGAGAGTGAGG; GAPDH (FP-GAGAAGGCTGGGGCTCA, RP-TGCAGGAGGCATTGCTGA); HRI (FP-ACACCAACACATACGTCCAG, RP-GCTCCATTTCTGTTCCAAACG); LC3 (FP-GTGATAATAGAACGATACAAGGT, RP-CTGAGATTGGTGTGGAGACG) p62 (FP-GACTACGACTTGTGTAGCGTC, RP-AGTGTCCGTGTTTCACCTTCC); XBP-1 (FP-GAGAACCAGGAGTTAAGACAGCGC, RP-TCCTTCTGGGTAGACCTCTGGGA).

3.7. Cell death analysis by FACS (Propidium Iodide staining)

To quantify the percentage of dead and live cells, cells were stained with PI and analyzed by FACS. Cells were exposed to 25 µM CCCP and 1 µM thapsigargin for 36 hrs in the presence and absence of 250 nM ISRIB or 20 µM z-VAD-fmk or 5 mM NAC. Following stress exposure, both live and dead cells were collected and spun at 1400 rpm for 4 min. The cell pellet was then washed with 200 µL of PBS and then centrifuged again at 1400 rpm for 4 min. Finally, cells were incubated with 2 µg/mL PI in 200 µL of PBS for 15 min in dark. After incubation, the percentages of PI-positive cells were quantified by a flow cytometer, BD/LSR Fortessa.

3.8. Statistical analysis

Each experiment has been repeated a minimum of three times, and data are expressed as mean ± standard deviation (SD) unless mentioned otherwise. Data were analyzed for statistical significance using the Student's t-test. The statistical analyses were performed using GraphPad-Prism 7 (SAN DIEGO, CA, USA). $P < 0.05$ was considered to be statistically significant.

Chapter-IV

4. Results

4.1. CCCP-induced mitochondrial stress triggers the ISR pathway and it is independent of PERK, GCN2, or PKR activation

CCCP, a chemical mitochondrial uncoupler, induces leakage of protons that were accumulated during the electron transport chain, from the mitochondrial intermembrane space into the mitochondrial matrix and reduces the development of a proton electrochemical gradient. It thus increases ROS levels and reduces ATP production [251]. Since ISR is activated in response to diverse stresses in eukaryotes, we sought to investigate the ISR activation and respective activated ISR regulator (EIF2AK) in response to CCCP-induced mitochondrial dysfunction. Analysis of 25 μM CCCP-treated HepG2 cells at various time periods (0.5, 1.0, 2, 3, 6, & 12 hrs) displayed typical ISR markers: i) induction of eIF2α phosphorylation (**Fig. 3A**, rows 3 & 4 eIF2α-P vs Total eIF2); ii) expression of ATF4 and iv) CHOP transcriptional factors (rows 5 & 6). Results indicate the expression of ATF4 appears at 3 hrs and CHOP at 6 hrs after CCCP treatment and is consistent with the idea that these transcriptional factors are produced as part of an altered gene expression program that is a direct and instant result of eIF2α phosphorylation. The ATF4 expression signals the CHOP upregulation [90, 172, 193]. To determine whether CCCP-induced mitochondrial stress is relayed to ER, we analyzed here initially the ISR branch of UPR [110, 192]. ER is rich in an oxidative environment to promote disulfide bonds in the newly made secretory proteins, covalent modifications, and proper folding of these newly synthesized polypeptides [252]. UPR or unfolded protein response, an adaptive signaling pathway evoked by stressed ER and executes its action by three stress sensors/transmembrane proteins which are located in the ER membrane. These are PERK, IRE1α, and ATF6 which are inactive in normal conditions and are bound by ER-localized chaperone BiP/GRP78 [109]. In response to ER stress, BiP/GRP78 is dissociated from three UPR sensors and makes ER stress sensors active. Activation of PERK, the ER-resident eIF2α kinase occurs immediately when compared to the other stress sensors, IRE-1α and ATF6, as phosphorylation of eIF2α shuts down the ongoing translation and reduces a burden on folding machinery. In fact, ER is stressed whenever accumulation or deposition of unfolded proteins in ER lumen, may arise when increased protein synthesis exceeds the capability of protein folding, covalent modification of nascent proteins and their degradation are impaired, and reduced calcium levels in ER which are essential for the proper functioning of various ER chaperones. While eIF2α phosphorylation inhibits the translation of general mRNAs, it enhances the translation of genes carrying short uORFs such as ATF4 and CHOP that code transcriptional factors and GADD34, a cofactor of eIF2α phosphatase. IRE1α, the second ER stress sensor, on activation, it catalyzes unusual splicing of XBP1 transcripts in the cytosol that yields the functional transcription factor XBP1. Activation of ATF6 results in its transportation to the Golgi apparatus where it is cleaved by proteases and transformed into a functional transcription factor. Overall ER stress-induced UPR activation promotes translational attenuation of general mRNAs and synthesis of ATF4, XBP1, and ATF6 transcriptional factors which in turn activate the transcription of the genes participating in protein folding, redox metabolism, amino acid biosynthesis, ERAD pathway, and autophagy to maintain protein and cellular homeostasis [77, 110, 253].

HepG2 cells, treated with 25 µM CCCP or 12 µM tunicamycin a typical ER stressor (positive control for UPR) for different periods were analyzed for the ISR markers such as phosphorylation of eIF2α, expression of ATF4, and CHOP (**Fig. 3A**, rows 2-5), as described above and also for UPR specific markers: BiP/GRP78 expression (Fi**g. 3A**, row 2) and PERK activation (Row 1) by immune blot analysis with respective antibodies. Another UPR marker, XBP-1 mRNA splicing was also analyzed by semi-quantitative PCR using respective primers (**Fig. 3D**). PERK activation was assessed by differential migration on 8% SDS-PAGE. ER stress induces PERK activation or its autophosphorylation. Autophosphorylated PERK migrates slowly compared to native or total PERK and thus it appears at a high molecular weight [236] in SDS-PAGE. Both stressors, CCCP or tunicamycin-induced eIF2α phosphorylation occurred within 30 minutes and continued up to 3 hrs where it reaches a maximum level (**Fig. 3A**, row 3, lane 1 vs 2–7 representing CCCP or lanes 8–12 representing tunicamycin). Relative levels of phosphorylated eIF2α normalized with total eIF2α (row 4) and actin (row 7) are shown in the bar diagram (**Fig. 3B** Bar diagram). However, eIF2α phosphorylation levels declined after 3 hrs and it may be due to the expression of GADD34 that directs the type 1 phosphatase to dephosphorylate eIF2α [158, 180]. GADD34 is also an ISR-induced factor and is expressed downstream to ATF4 and CHOP [193, 254]. Resumption of protein synthesis that occurs due to dephosphorylation of eIF2α by GADD34 however can promote cell death if that occurs before UPR is resolved. Both CCCP and tunicamycin treatments displayed enhanced expression levels of ATF4 **(Fig. 3A,** row 5, lanes 2–7 & 8–12**)** and CHOP **(**row 6, lanes 2–7 & 8–12) transcriptional factors in HepG2 cells compared to untreated control cells (lane 1).

Tunicamycin-treated cells however have shown PERK activation/autophosphorylation within 30 minutes, which was monitored by the detection of a slower migrating band on 8% SDS-PAGE, and at 12 hrs of tunicamycin treatment, a strong band was observed at high molecular weight compared to untreated or control cells (**Fig. 3A**, row 1, lane 1 vs 8–12). But CCCP-treatment failed to induce PERK activation even at 12 hours also though CCCP treatment promoted eIF2α phosphorylation significantly **(Fig. 3A,** row 1, lane 1 vs 2-7**).** Also, the expression of ER chaperone, BiP was observed only in cells that were exposed to **t**unicamycin but not in CCCP treated cells (**Fig. 3A,** row 2, lane 1 vs 2–7 vs lanes 8–12 & **Fig. 3C,** bar diagram**,** represents relative levels of BiP expression) suggesting BiP is typical ER stress-induced UPR marker. We further examined the IRE1α branch signaling of UPR by monitoring XBP1 splicing by semi-quantitative PCR in cells treated either with CCCP for 6 and 12 hrs or with tunicamycin for 8hrs. XBP1 splicing occurred in tunicamycin-treated cells whereas splicing was not observed in CCCP-treated cells (**Fig. 3D**, lane 1 vs 4 & lanes 1 vs 2 & 3 respectively). Thus by comparing the activation of PERK, expression of BiP and IRE1α- mediated XBP1 splicing in tunicamycin and CCCP-treated cells, we conclude that CCCP-induced mitochondrial dysfunction leading to enhanced phosphorylation of eIF2α is independent of PERK activation, and thus CCCP-induced ISR activation is not a result of activation of UPR signaling that occurs in the presence of tunicamycin. To determine the eIF2α kinase that is activated in CCCP-treated cells, further studies are undertaken to find out whether it is a result of the activation of other stress-activated eIF2α kinases viz. PKR, GCN2, or HRI. These kinases are well characterized and are located in the cytoplasm.

GCN2, a highly conserved eIF2α kinase and it is activated by the accumulation of uncharged tRNAs as a consequence of amino acid/nutrient deprivation. In addition to amino acid deprivation, GCN2 activation also occurs in response to UV-B irradiation, oxidative stress, and proteasome inhibition [101, 102, 255]. Upon amino acid starvation, uncharged tRNAs bind to the HisRS region of GCN2 which promotes its dimerization and autophosphorylation leading to GCN2 activation [256, 257]. Since mitochondria carry many of those enzymes involved in amino acid biosynthesis, we tested whether CCCP treatment affects amino acid metabolism and thereby activates GCN2 kinase. To determine GCN2 activation, CCCP-induced eIF2α phosphorylation was analyzed in experiments with WT and GCN2 Knock out (KO) MEFs (**Fig. 3E**). Initially, we analyzed eIF2α phosphorylation in cells irradiated with UV-B for 60 seconds and incubated for 0.5, 1.5, and 3 hrs. Consistent with previous findings [101], it is observed here that UV-B irradiation induces robust eIF2α phosphorylation in Wt MEFs **(Fig. 3E** row 2, lane 1 vs 2-4**)** but does not stimulate the same in GCN2 KO MEFs (lane 5 vs 6-8) suggesting that UV-induced eIF2α phosphorylation is mediated by GCN2 kinase. Next, we treated WT and GCN2 KO MEFs with 25 µM CCCP for 1, 2, and 3 hrs, 12 µM tunicamycin for 3 hrs, or with KRB (which mimics amino acid starvation) for 3hrs, and monitored the levels of eIF2α phosphorylation by western blot. As expected KRB insult induces more eIF2α phosphorylation in WT MEFs compared to GCN2 KO MEFs **(Fig. 3F**, row 2, lane 6 vs 12**)**. In the absence of GCN2, KRB still stimulated the eIF2α phosphorylation in GCN2 KO MEFs although it is 65-70% low compared to WT MEFs. For this, we reasoned that it may be due to the activation of other eIF2α kinases in GCN2 KO MEFs exposed to KRB. In contrast, CCCP and tunicamycin stimulated almost the same levels of eIF2α phosphorylation in both WT MEFs and GCN2 KO MEFs **(Fig. 3F,** row 2, lanes 2–4 vs 7–9 represents CCCP, and lane 5 vs 11 represents tunicamycin and **Fig. 3G** bar diagram indicating relative levels of phospho-eIF2α**)**. Since tunicamycin is known to activate PERK, the lack of GCN2 in GCN2 KO MEFs did not affect the tunicamycin-induced eIF2α phosphorylation. Therefore, these data suggest that eIF2α phosphorylation in UV-B irradiated and KRB-treated HepG2 cells is mediated by GCN2 activation, whereas, CCCP-induced eIF2α phosphorylation in GCN2 knockdown cells is independent of GCN2 activation.

Next, we tested whether CCCP-induced mitochondrial dysfunction activates PKR, one of the well-characterized eIF2α kinases that is activated by double-stranded RNA species or virus infection. PKR is an interferon-inducible dsRNA-dependent kinase and is activated by dsRNA virus infection to counteract the virus propagation. Along with virus infection, PKR is also activated by numerous stress stimuli such as synthetic dsRNA analog polyI:C, bacterial lipopolysaccharide, cytokines, ROS, etc.,[91, 117, 258]. The latest study demonstrated the PKR activation in response to ionic and hyperosmotic stress caused by 500 mM NaCl [91]. To determine PKR's role in the CCCP-induced ISR pathway, HepG2 cells were treated with 25 µM CCCP for various time points or with 400 or 500 mM NaCl for 1 hr and then analyzed the PKR activation by monitoring its differential migration and phospho-eIF2α levels by probing with respective antibodies on immunoblot. Our results indicate that both CCCP and NaCl- treated cells displayed eIF2α phosphorylation (**Fig. 3H,** row 2, lane 1 vs 2-7 & Fig. 3I bar diagram).

However, PKR activation, analyzed by its mobility shift/slower migration on SDS-PAGE is observed in salt-treated cells only but not in CCCP-treated cells (row 1, lanes 6 & 7 vs lanes 2-5). This finding, therefore, rules out the activation of PKR also in CCCP-induced ISR. However, NaCl induces much stronger eIF2α phosphorylation compared to CCCP- treatment. So overall our findings indicate that CCCP-induced eIF2α phosphorylation is not mediated by activation of PERK, GCN2, or PKR kinases.

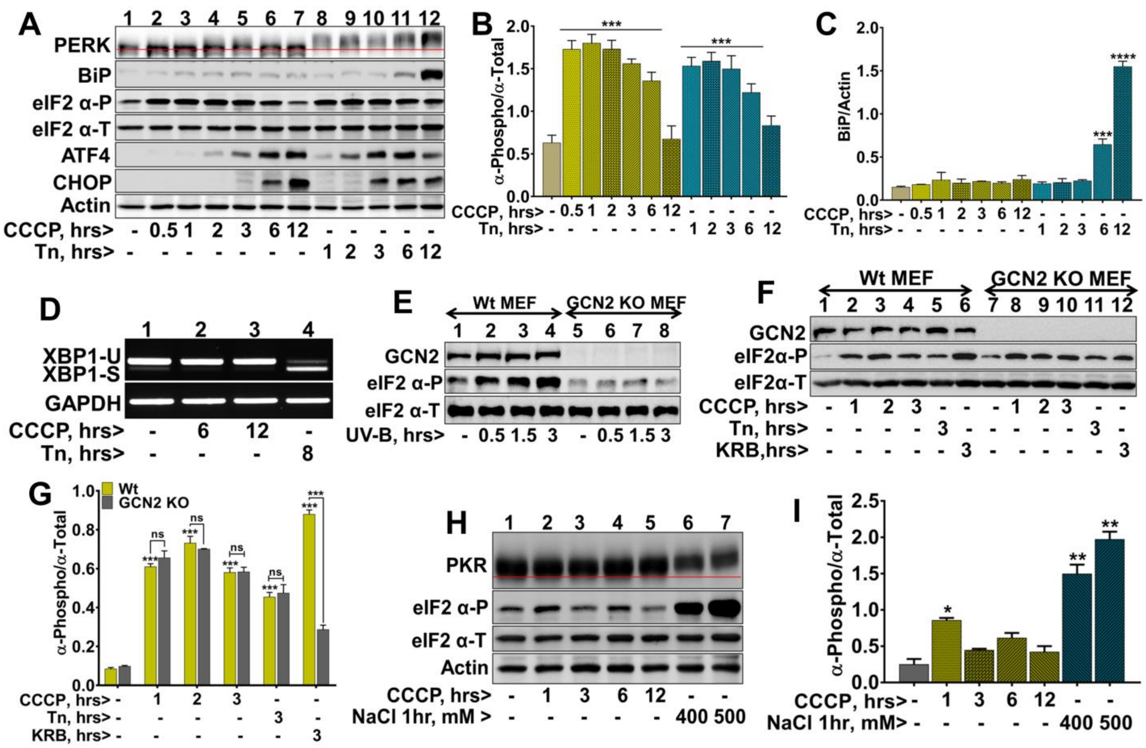


Fig. 3. CCCP evokes an ISR pathway but not UPR, GCN2, or PKR activation. Panel (A) analysis of 25 μM CCCP or 12 μM tunicamycin-induced phospho and un phospho-forms of PERK by differential migration on 8% SDS/PAGE, expression of BiP, phosphorylation of eIF2α, total eIF2α, expression of ATF4 and CHOP, and actin (rows 1–7) at different time points as indicated in the figure in HepG2 cell lysates by their respective antibodies in a western blot as described in 'Materials and methods'. Panels (B) and (C) quantification of band intensities of phosphorylated eIF2α and expression of BiP from Panel (A). Panel (D) semi-quantitative PCR analysis of unspliced and spliced XBP-1 mRNA (XBP1-U and XBP1-S) in HepG2 cells treated with CCCP or tunicamycin for indicated time points. Panels (E) and (F) western blots indicating eIF2α phosphorylation and GCN2 kinase levels at different time points in Wt and GCN2 KO MEFs that were treated with UV-B for 60 seconds or also with CCCP, tunicamycin, or KRB. Panel (H) western blot analysis of PKR activation and eIF2α phosphorylation in HepG2 cells that were treated with CCCP or different concentrations of NaCl at indicated time points. Panels (G) and (I) quantification of band intensities of phosphorylated eIF2α from Panels (F) and (H) respectively by ImageJ software (NIH, Bethesda, MD, USA) analysis. Data represented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 (Student's t-test). Data represented in Panel (I) from two technical replicates.

4.2. CCCP-induced mitochondrial stress activates ISR through heme-regulated eIF2α kinase (HRI)

According to earlier studies, four kinases viz. PERK, GCN2, PKR, and HRI have been identified so far and assumed the occurrence of extra other eIF2α kinases. A recent study that conducted experiments using MEF cells devoid of all currently known four eIF2α kinases to 14 various stress stimuli, concluded that vertebrates have four eIF2α kinases only and probably no more additional kinases [91]. Since our studies above (**Fig. 3**) ruled out the activation of three of these kinases (PERK, GCN2, and PKR) in CCCP-treated cells, we further moved to investigate the role of HRI in the CCCP stress regimen. HRI, a heme-regulated eIF2α kinase, was found earlier in reticulocytes and erythroid precursors that senses intracellular heme levels [259]. The absence of heme results in the activation of HRI through its autophosphorylation and dimerization. It phosphorylates eIF2α and shuts down the synthesis of the globin protein in reticulocytes or their lysates [135, 259]. To examine the possibility of HRI kinase activation, initially, we treated HepG2 cells with 25 μM CCCP for 1 and 2 hrs in the presence of 2 μM 17-AAG, a geldanamycin derivative that is known to inhibit HSP90 and HRI interaction which is indispensable and contributes HRI activation [149], and estimated the levels of phospho-eIF2α by western blot. Consistent with these reports, we noticed here that CCCP-induced eIF2α phosphorylation is significantly prevented by 17-AAG pretreatment (**Fig. 4A**, row 1, lanes 2 & 3 vs 5 & 6), suggesting that HRI may play a role in CCCP-induced ISR activation. The relative levels of eIF2α phosphorylation were quantified by normalizing with total eIF2α and actin and shown in the bar diagram (**Fig. 4B**). To further confirm the involvement of HRI and its activation under the conditions of CCCP insult, we depleted HRI transcripts by using siRNA in CCCP-treated cells. HepG2 cells were transfected with either 50 nM scrambled siRNA or HRI siRNA by using lipofectamine RNAimax. Post 48 hrs of transfection, cells were exposed to CCCP for 1.5 hrs to monitor the levels of eIF2α phosphorylation or 8 hrs to monitor the expression of ATF4 and CHOP. HRI knockdown was confirmed by estimating the HRI mRNA levels by semi-quantitative PCR and RT-PCR as shown in **Figs. 4 C** and **D** respectively. We tried to assess HRI protein levels by western blot, but the two HRI antibodies which we purchased did not recognize the HRI protein in our conditions. HRI siRNA transfected cells displayed a 90% reduction in mRNA levels compared with scramble siRNA transfected counterparts (**Fig. 4C**, row 1, lanes 3 & 4 vs lanes 1 & 2 & **Fig. 4D**). CCCP treatment didn't alter the HRI transcript levels either in scrambled siRNA (**Fig. 4C** lane 1 vs 2 & **Fig. 4D**), or in HRI siRNA transfected cells (lane 3 vs 4 & **4D**). Consistent with the reduction in HRI mRNA levels, eIF2α phosphorylation was impaired in CCCP-treated HRI siRNA transfected at 1.5 hrs (**Figs. 4E** & **F**), and accordingly, the expression of ATF4 and CHOP mRNA levels (**Fig. 4I** & **J**) and protein levels (**Fig. 4G** & **H**) were diminished at 8 hrs. In addition to HepG2 cells, HRI-mediated eIF2α phosphorylation, ATF4, and CHOP expression were also observed in CCCP-treated **HeLa cells** (**Fig. 4K, L** & **M**). Collectively, these findings suggest that the HRI kinase is important for relaying the CCCP-induced mitochondrial stress to ISR activation (HRI-eIF2α-P-AFT4 axis) rather than PERK, GCN2, or PKR.

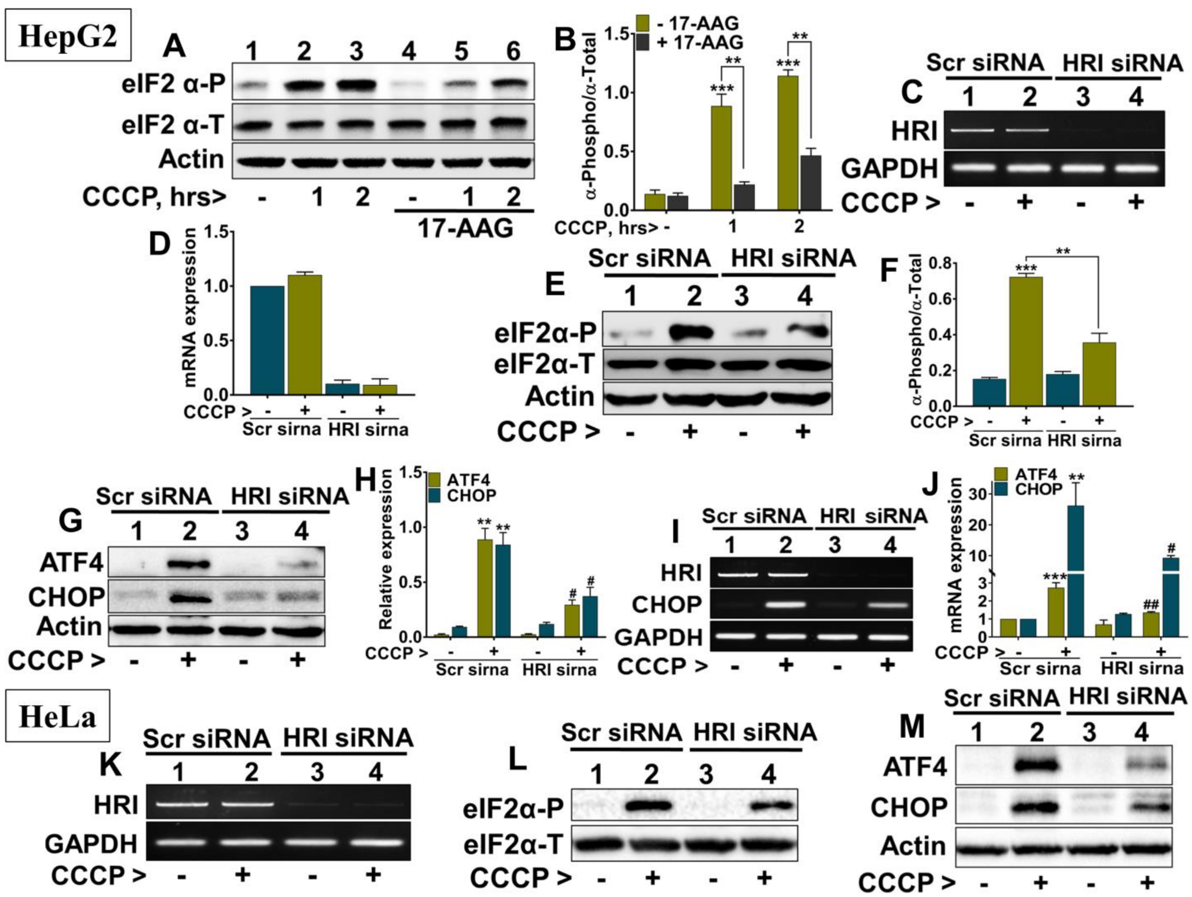


Fig. 4. CCCP activates HRI kinase. Panel (A) Western blot representing CCCP-induced eIF2α phosphorylation at 1 and 2 hrs in HepG2 cells that were pretreated for 1 hr with or without 2 μM 17-AAG. Panel (B) Quantification of band intensities of phospho-eIF2α from Panel (A). Panels (C) and (D) Analysis of HRI mRNA levels by semi-quantitative PCR and RT-PCR, respectively, in HepG2 cells, after 48 hrs of transfection with scrambled siRNA (scr siRNA) or HRI siRNA which were then treated with CCCP for 1.5 hr. GAPDH was used as a loading control. Panels (E) and (G) represent levels of CCCP-induced eIF2α phosphorylation at 1.5 hrs and expression of ATF4 and CHOP at 8 hrs in HepG2 cells transfected with scr siRNA or HRI siRNA, respectively. The figure is a western blot. Panels (F) and (H) Represent the quantification of band intensities of eIF2α phosphorylation in Panel (E) or ATF4 and CHOP levels in Panel (G), respectively. Panel (I), HRI mRNA levels were determined by semi-quantitative PCR in CCCP-treated HeLa cells that were transfected with either HRI siRNA or scr siRNA and analyzed after 48 h of transfection. Panels (J) and (K) western blot analysis of eIF2a phosphorylation and expression of ATF4 and CHOP in HRI depleted CCCP-treated HeLa cells. Data represented as mean $\pm$ SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's t-test). In Panel (H), * indicates the significance compared between Scr untreated vs Scr CCCP treatment, # indicates the significance compared between Scr CCCP vs HRI CCCP treatment.

4.3. CCCP-induced mitochondrial stress promotes AMPK and AKT activation, suppresses mTOR1, and induces autophagy

We also analyzed the effect of mitochondrial dysfunction on pro-survival cellular signaling pathways such as AMPK, mTORC1, AKT, and autophagy that can typically respond to oxidative insults and/or energy depletion to reorient cellular metabolism and homeostasis to aid cell survival. Previous reports have shown that AMPK, an intracellular energy sensor, the activation occurs in response to ATP depletion and oxidative stress, and down-regulates translation/protein biosynthesis by inhibiting the mTORC1 to minimize the energy consumption [42] and activates autophagy to recycle the amino acids [260]. AKT activation is another key event employed by cells to protect against oxidative and ER stress [261, 262]. Since multiple studies have shown that CCCP-treatment results in both ATP depletion and ROS production, we investigated the response of the AMPK and AKT activation in CCCP-exposed HepG2 cells by western blot analysis at various time points. We observed that CCCP-treated HepG2 cells were associated with an increase in AMPK phosphorylation at Thr-172 (**Fig. 5A**, row 1, lanes 2–5 vs 1) and a decrease in phosphorylation of S6K at Thr-389 (row 3, lanes 2–5 vs 1) and eIF4E-BP1 at Thr-37/46 (row 5, lanes 2–5 vs 1) in a time-dependent manner when compared to untreated controls. Since CCCP-induced AMPK activation and ATF4 expression, are known to stimulate autophagy, we also assessed autophagy in cells treated with CCCP. HepG2 cells treated with CCCP for 3, 6, and 12 hrs, displayed elevated levels of LC3I/II protein (**Fig. 5B**, lane 1 vs 2–4), a well-known autophagy marker. Phosphorylation of eIF4E-BP1 and S6K assist the recruitment of 43S complex at 5’ cap structure and RNA-helicase activity of eIF4A respectively which favors the fruitful translation initiation [42]. Hence, hypophosphorylated eIF4E-BP1 and S6K in CCCP-treated cells may be inhibitory to translation. CCCP-induced eIF2α phosphorylation occurred within 30 minutes and was found increasing with time up to 3 hrs (**Fig. 5C**, row 1, lanes 2–4 vs 1). After 3 hrs, a reduction in eIF2α phosphorylation was observed (row 1, lanes 5-7). The reduction may be because of a negative feedback regulation mediated by GADD34 [180], a cofactor of eIF2α phosphatase. In addition to eIF2α phosphorylation, we observed CCCP also promoting AKT phosphorylation at Serrine-473 in a time dependent fashion (**Fig. 5C**, row 3, lanes 4-7 vs 1). But at early time points such as at 1 and 2 hrs, CCCP-treated HepG2 cells displayed low levels of AKT phosphorylation compared to untreated cells (**Fig. 5C,** row 3, lanes 2 & 3 vs 1). The initial reduction in AKT phosphorylation may be as a consequence of the activation of the pentose phosphate pathway (PPP) that generates NADPH and a reductive environment that may be stimulating the activity of tyrosine phosphatase PTEN, a negative regulator of PI3K-AKT/PKB [263, 264]. Further, we also assessed the protein synthesis rates that are known to be downregulated by high eIF2α phosphorylation and hypophosphorylated eIF4E-BP1. Treatment with CCCP exhibited a gradual reduction in protein synthesis as measured by radioactive S^{35} methionine with time in HepG2 cells (**Fig. 5D**, lanes 2 & 3 vs 1), which is consistent with the phosphorylation levels of eIF2α, and eIF4E-BP1 (**Fig. 5A** & **B**). Collectively, these data suggest that CCCP treatment promotes autophagy or pro-survival cell signaling such as AKT activation and inhibition in the mTOR pathway that are known to be regulated by ISR in various stresses [99, 177, 182, 184, 262, 265]. CCCP-treated cells displayed an inverse correlation in phosphorylation of eIF2α and eIF4E-BP1 which is consistent with previous findings in diverse stress conditions such as amino acid starvation, hypoxia, ER stress, energy deficit, etc. [184, 266]. Activation/inhibition of these cell signaling pathways in CCCP-treated cells is a consequence of possibly oxidative stress or energy depletion or both.

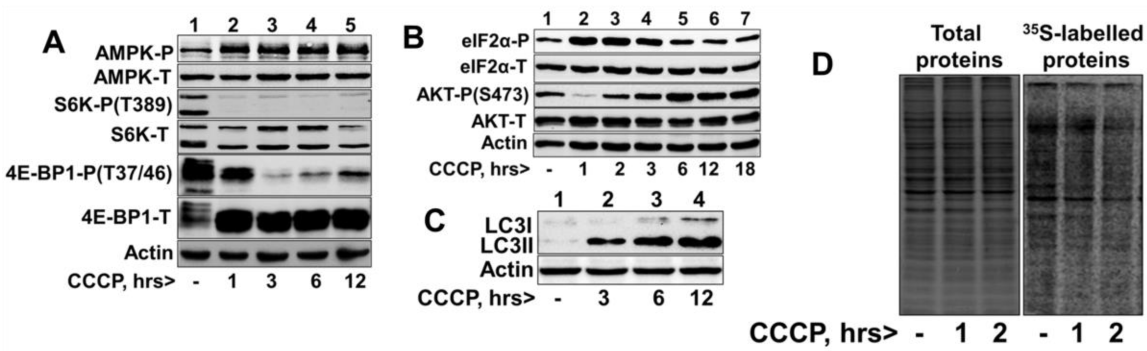


Fig. 5. CCCP induces activation of AMPK, AKT, and the expression of LC3I/II and inhibits S-6K and eIF4E-BP1 phosphorylation and translation. Panel (A) HepG2 cells were treated with 25 µM CCCP for 1, 3, 6, and 12 hrs and analyzed for phosphorylation of AMPKα, S6K, and eIF4E-BP1 by western blot using their respective phosphor-specific antibodies: phospho-thr172 AMPKα, phospho-thr389 S6-kinase, phospho-thr37/46 eIF4E-BP1, and compared to their total levels and β-actin. Panel (B) Immunoblot indicating phospho-eIF2α, eIF2α-total, phospho-ser473 AKT, AKT-total, and β-actin in cells treated with CCCP at different time points as indicated in the figure. Panel (C) CCCP-induced autophagy marker LC3-1/LC3-II levels at different time points determined by a western blot. The figure is a representation of two independent experiments. Panel (D) represents the translation rates by [S^{35}] incorporation into newly synthesized proteins in CCCP-treated HepG2 cells.

4.4. CCCP-induced oxidative stress drives the ISR activation

Several studies have shown that oxidative stress evokes the ISR pathway mediated by the activation of any of the eIF2α kinases to protect cells. Oxidative stress triggers the activation of PERK and GCN2 in MEF cells [99], HRI in Schizosaccharomyces pombe, and primary erythroid precursors [267, 268], and PKR in SH-SY5Y neuroblastoma cells [269]. Because CCCP is known to promote oxidative stress [251], we examined the activation of ISR and cellular signaling pathways in the presence of NAC, a well-known ROS scavenger. In addition, we also compared the effects of NAC, an antioxidant, on mitochondrial stress with a recently discovered ISR inhibitor ISRIB, to find out any cross-talk between ISR and the above-mentioned signaling pathways. NAC is reported to suppress ROS due to its ability to scavenge free radicals and augment glutathione levels in cells [270]. ISRIB lowres the ISR outputs and signaling by blunting the effects of eIF2α phosphorylation [238]. To this end, HepG2 cells were treated with 5 mM NAC or 250 nM ISRIB for 1 hr before being exposed to CCCP and incubated for various periods, and concomitantly monitored the levels of eIF2α-P and ISR genes like ATF4 and CHOP (**Fig. 6**). We observed that NAC appreciably reduces the CCCP-induced eIF2α phosphorylation (**Fig. 6A**, lanes 6–8 vs 2–4 & **Fig. 6B** bar diagram) and its downstream transcription factors

ATF4 and CHOP expression (**Fig. 6C**, row 1 & 2, lanes 6–8 vs 2–4 & **Fig. 6D** & **E**) at various time points, suggesting that oxidative stress or high ROS is primarily root cause for the ISR activation in CCCP-incubated cells. In contrast, ISRIB, unlike NAC, fails to reduce eIF2α phosphorylation more efficiently than NAC, but it slows down to some extent at initial time points (0.5, 1.0. & 1.5 hrs) and later it enhances further, albeit slowly relative to the CCCP treated cells (**Fig. 6F**, lanes 8–12 vs2–6 & **Fig. 6G**). Further ISRIB, reduces the expression of ATF4 and CHOP protein (**Fig. 6H**, rows 1 & 2, lanes 6–8 vs 2–4 & **Fig. 6I** & **J**) as well as their mRNA levels (**Fig. 6K** & **L**), despite of eIF2α phosphorylation levels maintained in ISRIB and CCCP-treated cells. The reduction in ISR-induced expression of ATF4 and CHOP mRNA and proteins suggest that ISRIB reverses the effect of CCCP-induced alteration in the gene expression program mediated by eIF2α phosphorylation. This may be due to ISRIB's ability to increase the formation of heterodecameric eIF2B complex from its sub-complexes [54] resulting in enhanced eIF2B’s GNE activity. The initial reduction in CCCP-stimulated phospho-eIF2α levels by ISRIB might be because of its ability to enhance the rate of eIF2.GDP recycling to eIF2.GTP by active functional decameric eIF2B. This interpretation is consistent with a very early study conducted a long time ago that showed free eIF2 was phosphorylated much more efficiently than eIF2 in the eIF2.eIF2B complex [74]. The inability of ISRIB to inhibit eIF2α phosphorylation in CCCP-treated cells (2 & 3 hrs) may also be due to the inhibition in the expression of ATF4 and CHOP which enhances GADD-34’s expression, an eIF2α phosphatase cofactor, and ISRIB may not affect the activity of eIF2α kinase. Therefore these data suggest that activation of ISR in CCCP-treated cells was specific to high levels of ROS or oxidative stress. Both NAC and ISRIB reduce CCCP-induced ATF4 and CHOP expression, but the underlying mechanisms of their actions appear to be different.

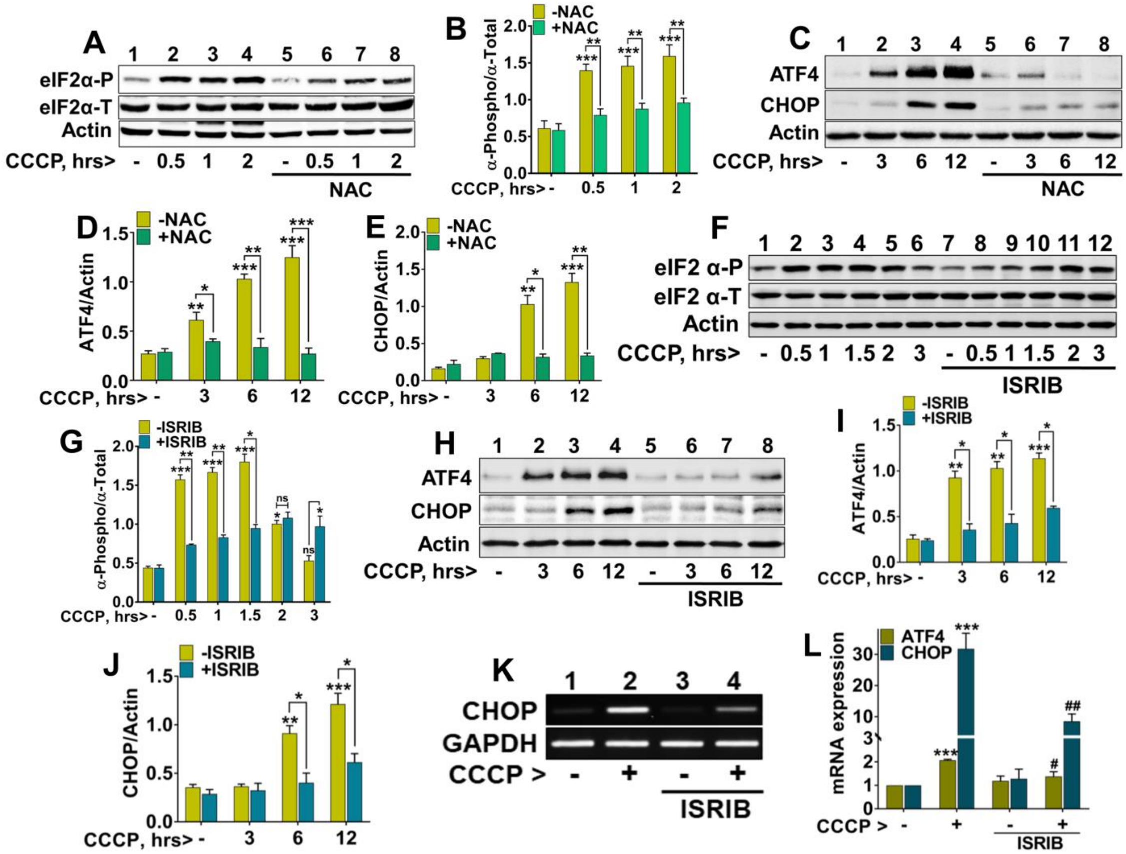


Fig. 6. NAC and ISRIB reduce CCCP-induced eIF2α phosphorylation and ATF4 and CHOP expression. HepG2 cells were incubated for 1 hr with 5 mM NAC or with 250 nM ISRIB, then supplemented with CCCP and incubated for different periods as indicated in the figure. Western blots indicating the levels of phospho-eIF2α, ATF4, and CHOP proteins in HepG2 cells treated with CCCP in the presence of NAC panels (A) and (C); in the presence of ISRIB panels (H) and (F). Panels (B) and (G) Bar diagrams represent relative levels of eIF2α-P, and panels (D), (E), (I), and (J) bar diagrams represent relative levels of ATF4 and CHOP proteins from corresponding panels in (A), (C), (H) and (F). Panel (K) and (H) Quantification of ATF4 and CHOP mRNA levels by semi-quantitative and RT-PCR respectively in cells treated with CCCP or ISRIB and CCCP for 6 hrs. Data represented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t-test).

4.5. NAC efficiently restores CCCP-suppressed eIF4E-BP1phosphorylation and translation than ISRIB

Further, we studied the effect of CCCP-induced high ROS and ISR activation on the mTORC1 pathway by monitoring the phosphorylation levels of eIF4E-BP1, a well-known mTORC1 substrate. Previous studies have indicated that mTORC1 is inhibited by AMPK [260, 271], and is been shown recently that phosphorylation of eIF4E-BP1 is linked to eIF2α phosphorylation driven ISR also [182, 184, 272] or its downstream target ATF4, that up-regulates the transcription of various upstream inhibitors of mTORC1, such as TRIB3 [188], REDD1 [185], SESN2 [88] or GRB10 [147]. Particularly during amino acid starvation, an inverse correlation occurred between eIF2α and eIF4E-BP1 phosphorylation levels without ATF4 involvement (Nikonorova et al., 2018). While phosphorylation of eIF2α attenuates translation, eIF4E-BP1 phosphorylation enhances it. To determine the role of oxidative stress and its induced ISR in this process, HepG2 cells were treated with CCCP for 3, 6, and 12 hrs in the presence of NAC or ISRIB and then analyzed phospho-eIF4E-BP1 levels by western blot. NAC completely reversed the CCCP-suppressed eIF4E-BP1 phosphorylation (**Fig. 7A**, lanes 6–8 vs 2–4 & **Fig. 7B**), whereas, ISRIB reverses partly (**Fig. 7C**, lanes 6–8 vs 2–4 & **Fig. 7D**). The restored phospho-eIF4E-BP1 levels were inversely correlated with the reduced phospho-eIF2α levels in the presence of NAC or ISRIB (**Fig. 6A & F**). Mitochondrial stress, induced by ETC inhibitors such as myxothiazol and piericidin-A promotes the expression of SESN2 via ISR-mediated ATF4-dependent mode [272] and is known to inhibit mTORC1 [88]. Hence, we further assessed the SESN2 expression in CCCP-treated cells and whether it plays any role in mTORC1inhibition. Surprisingly SESN2 expression was observed at post 6 hrs of CCCP treatment and it was inhibited by ISRIB as expected (**Fig. 7E** lanes 6-7 vs 2-4). The current scenario supports the established evidence that eIF2α phosphorylation directly regulates the mTORC1 instead of through ATF4 [184]. Because in our studies we observed that expression of SESN2 occurs at 6 hrs in CCCP treated cells, whereas the inhibition of eIF4E-BP1 and S6K phosphorylation occur between 0.5-1.0 hr after CCCP treatment, it is unlikely ATF4 induced genes may be regulating the eIF4E-BP1 phosphorylation in CCCP treated cells. In contrast, the decline in eIF4E-BP1 phosphorylation is coinciding with increasing or enhanced eIF2α phosphorylation, it is likely the phosphorylation of eIF4E-BP1 is regulated by eIF2αphosphorylation as has been suggested in amino acid deficiency [184]. Consistent with this suggestion, we observed here NAC, which reverses the CCCP-induced eIF2α phosphorylation completely and restores phosphorylation of eIF4E-BP1, whereas, ISRIB which slows down the rate of eIF2α phosphorylation in CCCP treated cells, restores partly eIF4E-BP1 phosphorylation. To relate these observations, we also monitored protein synthesis analyzed by the incorporation of radiolabeled [S^{35}] methionine into nascent proteins in cells treated with CCCP, CCCP + NAC, or ISRIB for one hour (**Fig. 7F**). An equal amount of protein was separated on SDS-PAGE and autoradiographed (Panel B vs Panel

A). CCCP inhibits protein synthesis as expected (**Fig. 7F** lane 2 vs 1). As expected NAC recovered completely CCCP-suppressed protein synthesis (lane 4 vs 2), whereas ISRIB restores protein synthesis partially in CCCP-treated cells (lane 6 vs 2) and is less efficient compared to NAC (lane 4 vs 6). Thus these results on protein synthesis are consistent with their differential capacities of inhibition and restoration of eIF2α and eIF4E-BP1 phosphorylation levels respectively under the conditions of CCCP insults.

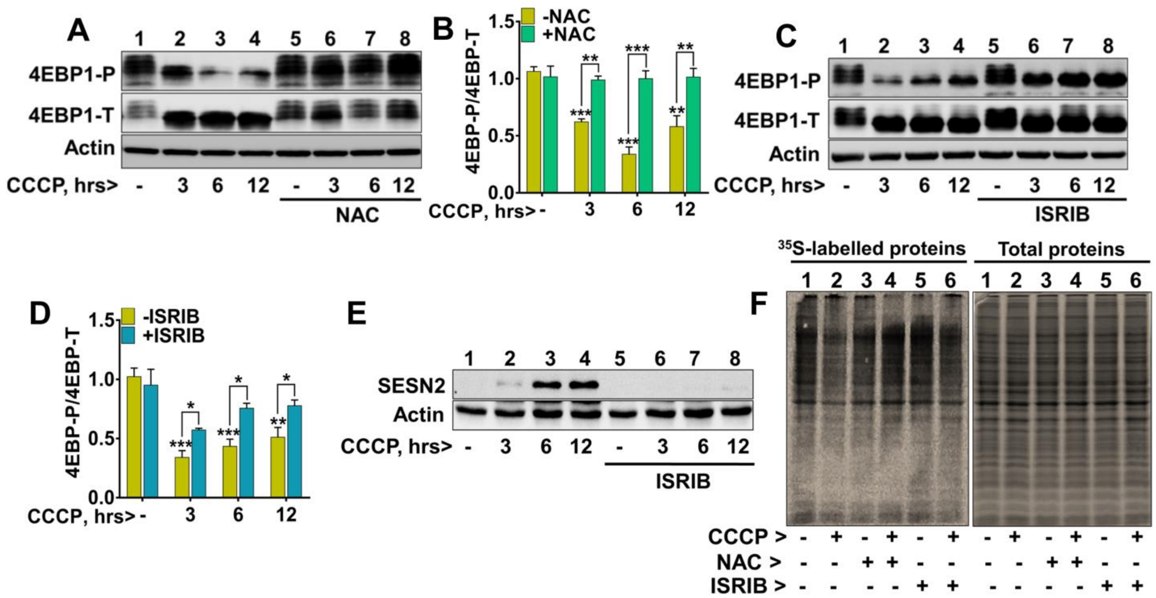


Fig. 7. CCCP-induced inhibition in eIF4E-BP1 phosphorylation and protein synthesis in NAC or ISRIB-pretreated cells. Panels (A) and (C) Western blots indicating eIF4E-BP1 phosphorylation in cells that were treated with CCCP in the presence and absence of NAC or ISRIB at different time points as indicated in the figure. Panels (B) and (D) Quantification of eIF4E-BP1 phosphorylation in Panels (A) and (C), respectively. Panel (E) Analysis of SESN2 protein levels in cells treated with CCCP in the presence of ISRIB by western blot. Panel (F) Protein synthesis in HepG2 cells. Cells were treated with CCCP for 2 hrs in the presence and absence of NAC or ISRIB and then pulse-labeled with 6 µCi/mL of S^{35} methionine for 1 hr. Total cell extracts were separated by SDS/PAGE, coomassie stained (right panel), and analyzed by autoradiography (left panel). The figure is representative of two independent experiments. Data represented as mean $\pm$ SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t-test).

4.6. CCCP induces autophagy in an ATF4-dependent manner

Autophagy is a highly conserved intracellular degradation process carried out by lysosomes [273]. It is a housekeeping mechanism involved in the clearing of damaged cellular organelles, degradation of misfolded proteins, and removing intracellular pathogens to maintain cell health [274]. Autophagy is considered as a cell survival process activated during situations of ER stress, nutrient shortage, oxidative stress, hypoxia, and amino acid starvation [177, 179, 265, 275]. AMPK, a metabolic fuel gauze is known to activate autophagy in response to nutrition or energy depletion conditions. AMPK is activated by low levels of ATP and mtROS [276]. Activated AMPK stimulates autophagy by inhibiting the mTORC1 or by activating the ULK1, a subsequent target of mTORC1, that is known to play a crucial role in the initiation of autophagy [260, 271]. Autophagy is also regulated by ISR-mediated ATF4 in addition to AMPK. [177]. To distinguish between ATF4 and AMPK-mediated autophagy, HepG2 cells were incubated for 3, 6, and 12 hrs in the presence of NAC or ISRIB and monitored the AMPK phosphorylation and LC3I/II levels by western blot. We found that CCCP-induced AMPK phosphorylation was almost completely mitigated by NAC (**Fig. 8A**, row 1, lanes 6–8 vs 2–4 & **Fig. 8B**) but not by ISRIB (**Fig. 8D**, row 1, lanes 6–8 vs 2–4 & **Fig. 8E**). However, both NAC and ISRIB significantly lower the CCCP-induced LC3I/II expression on a similar scale (**Fig. 8A** & **D**, row 3, lanes 6-8 vs 2-4, & **Fig. 8C** & **F**). RT-PCR data also reveal that the expression of autophagy genes such as p62 and LC3B was reduced by ISRIB (**Fig. 8G** & **H**), which suggests that the eIF2α phosphorylation-ATF4 axis of ISR may be regulating autophagy. Consistent with this notion, further we assessed the mRNA levels of p62 and LC3B and LC3II protein levels by RT-PCR and western blot respectively in CCCP-challenged cells in which HRI was deleted. We observed that CCCP-treated HRI depleted cells were associated with low levels of p62 and LC3B transcripts (**Fig. 8I** & **J**) and LC3I/II protein (**Fig. 8K**, lane 4 vs 2) compared to their respective counterparts. Collectively these data suggest that CCCP-impacted AMPK activation is a consequence of oxidative stress, which is consistent with previous studies stating that ROS can also activate the AMPK despite ATP levels [277]. In the case of ISRIB, CCCP-induced AMPK activation is not inhibited, possibly due to a lack of antioxidant activity. Even though CCCP-induced phospho-AMPK levels were not altered by ISRIB, but it reduces the LC3I/II expression as has been observed in CCCP and NAC treated cells. In addition to ISRIB, genetic loss of HRI also suppressed autophagy under the conditions of CCCP insults, suggesting that CCCP-induced autophagy is not mediated by phospho-AMPK, as reported [278] and it seems to be regulated by the ISR pathway. This result is in line with concrete evidence that eIF2AK-eIF2αP-ATF4 plays a crucial role in autophagy regulation [177].

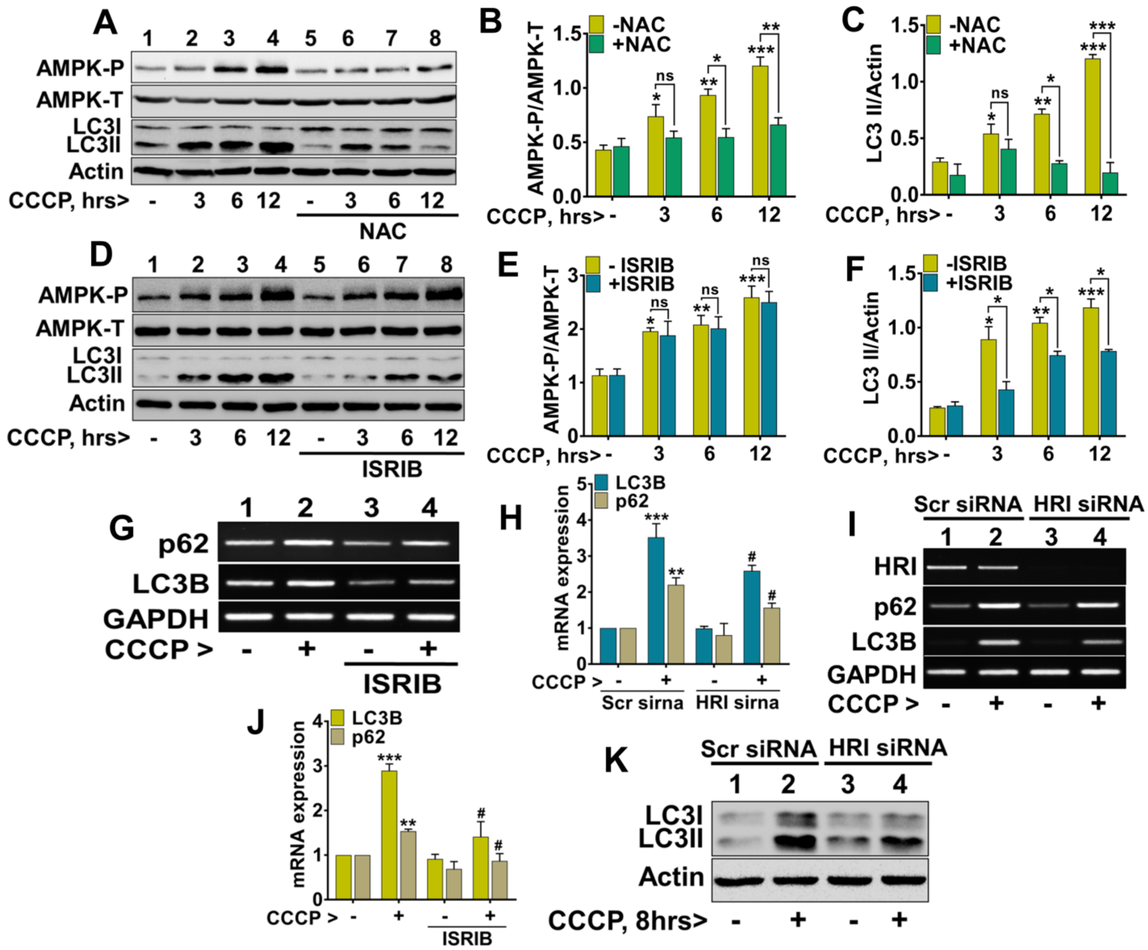


Fig. 8. CCCP-induced AMPK phosphorylation and LC3I/II expression in NAC or ISRIB-pretreated cells. Panels (A) and (D) western blots represent total and phospho-AMPK levels and levels of LC3 I/II in CCCP-treated cells in the presence of NAC or ISRIB respectively. Panels (B), (C), (E), and (F) quantification of AMPK phosphorylation and LC3 I/II levels from panels (A) and (D). Semi-quantitative PCR and RT-PCR representing the p62 and LC3B mRNA levels in HepG2 cells that were treated with CCCP in the presence of ISRIB panels (G) and (H) and HRI deletion panels (I) and (J). Panel (K) western blot representing the LC3I/II levels in CCCP-treated HRI knockdown cells. Data represented as mean $\pm$ SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t-test).

4.7. NAC, ISRIB, and HRI depletion compromises CCCP-induced AKT activation

We then investigated CCCP-induced oxidative stress and the resulting ISR on AKT activation. AKT activation is a key cellular signaling mechanism employed by cells to counter oxidative stress, which promotes cell survival [261]. Previous reports have shown that ER stress and oxidative stress-induced eIF2α phosphorylation accelerates AKT activation as a means of defending against stress [99, 262]. We found that CCCP-induced AKT phosphorylation was significantly mitigated by NAC (**Fig. 9A** lanes 5 and 6 vs 2 and 3 & **Fig. 7B**). Since ATF4 is shown to stimulate genes associated with redox metabolism [76, 77], we monitored CCCP-induced AKT phosphorylation levels in the presence of ISRIB. We observed that ISRIB inhibits ATF4 expression, and impaired AKT activation in CCCP-treated cells, hinting that ISR likely contributes to AKT activation (**Fig. 9C** lanes 6-8 vs 2-4 & **Fig. 9D**). Since CCCP-induced ISR is mediated by HRI kinase, we sought to investigate AKT activity in HRI kinase depleted cells. We found that AKT Ser-473 phosphorylation was appreciably reduced by HRI knockdown in HepG2 cells after exposure to CCCP for 8 hrs (**Fig. 9E** lane 4 vs 2 & **Fig. 9F**). We further examined the relevance of AKT activation under the conditions of cell death or cellular homeostasis in the CCCP stress regimen. HepG2 cells were treated with CCCP in the presence of AKT inhibitors LY294002 or wortmannin and monitored the PARP cleavage, an apoptosis marker by western blot analysis. As expected CCCP-induced AKT phosphorylation was significantly inhibited by both LY294002 and wortmannin. Inhibition of AKT phosphorylation in CCCP-treated cells displayed elevated PARP cleavage (**Fig. 9G** & **H**) which is consistent with AKT's pro-survival role under the conditions of oxidative stress.

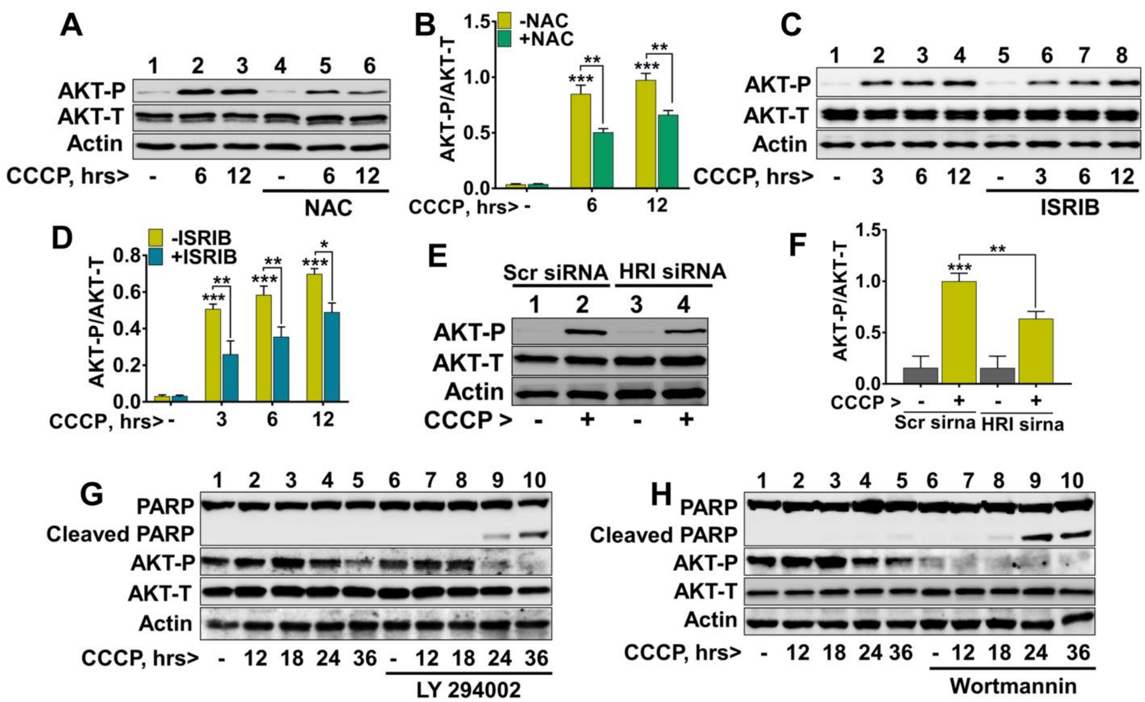
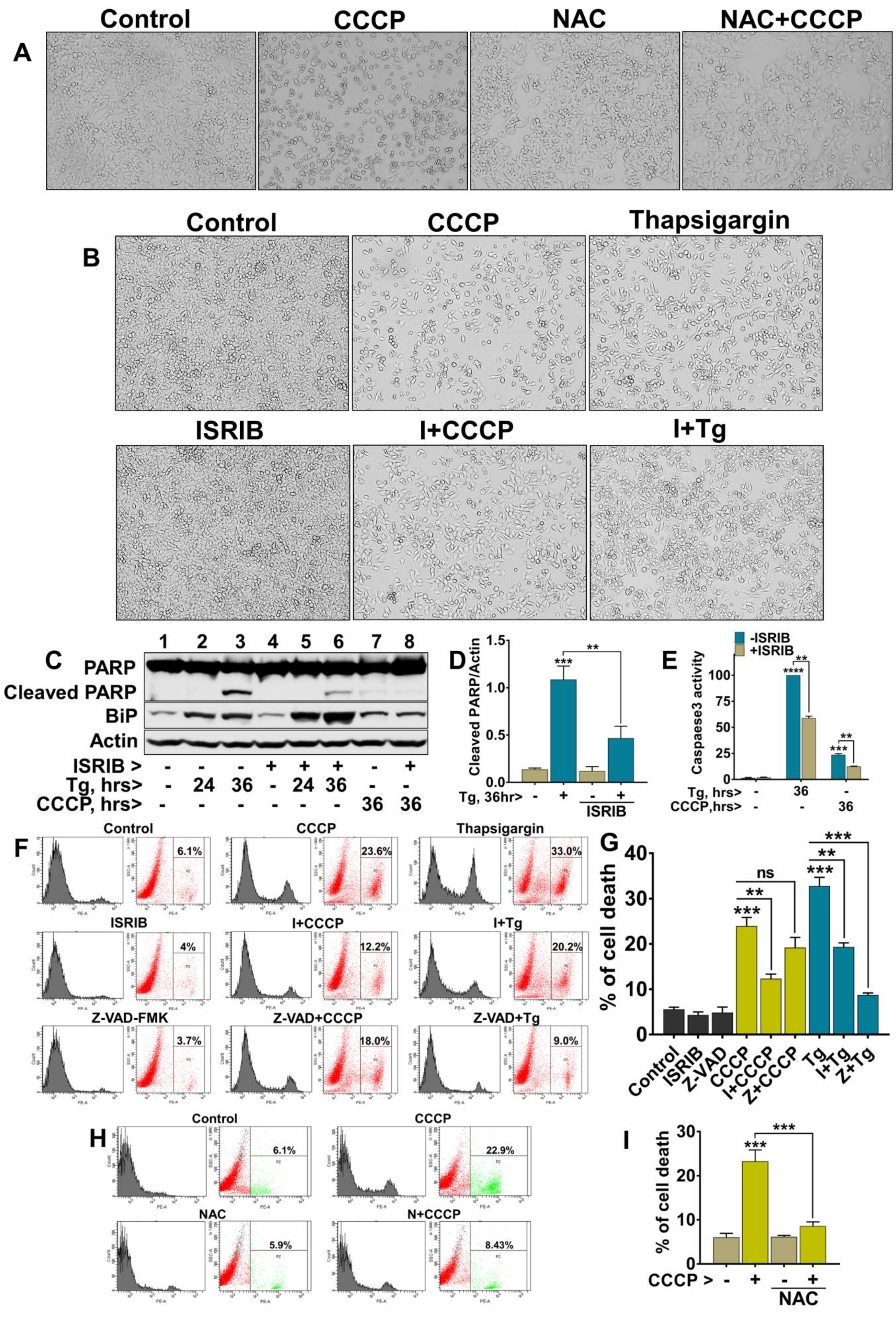


Fig. 9. CCCP-induced AKT activation in the presence of ISRIB, NAC, or AKT inhibitors. HepG2 cells were treated with NAC, ISRIB, LY294002, or Wortmannin for one hour, exposed to CCCP for different time points as indicated in the figure, and analyzed for the levels of phospho-AKT and PARP cleavage by western blot analysis. Panels (A) and (C) western blots indicate the total and phosphorylated AKT in HepG2 cells treated with CCCP in the presence of NAC or ISRIB respectively. Panels (B) and (D) bar diagrams represent relative levels of AKT phosphorylation from corresponding panels in (A) and (C), respectively. Panels (G) and (H) western blots indicating the PARP cleavage and phosphorylated AKT in HepG2 cells treated with CCCP in the presence of LY294002, or Wortmannin.

4.8. CCCP induces non caspase-mediated cell death unlike thapsigargin and is mitigated by NAC and ISRIB

ER and oxidative stress evoked ISR signaling considered as a prosurvival mechanism that can initially repair the stress damage and assure cytoprotection. However, if stress is persistent and severe, it will give up the adaptive response and orchestrate the pro-apoptotic machinery to execute cell death [93, 94]. To determine the effect of increased ROS and activation of ISR on cellular homeostasis, HepG2 cells were treated with CCCP in the presence of NAC or ISRIB and analyzed cell death by their morphology, FACS, PARP cleavage, and caspase activity (**Fig. 10**). CCCP-induced cell death was also compared with thapsigargin, which induces caspase-mediated cell death, in the presence of ISRIB or z-VAD-fmk, a pan-caspase inhibitor. We observed that CCCP treatment for 36 hrs significantly induces cell death as analyzed by cells under an inverted microscope and it was reduced by both NAC and ISRIB (**Fig. 10A** & **B**). However, NAC has efficiently inhibited cell death compared to ISRIB, indicating that oxidative stress is triggering cell death in CCCP-treated cells. ISRIB also reduces the amount of cell death caused by thapsigargin or ER stress, as evaluated by morphology and PARP cleavage (**Fig. 10B** & **C**). Cells treated with thapsigargin displayed an increase in BiP expression at 24 hrs and 36 hrs (**Fig. 10C**, row 2, lanes 2 and 3 vs 1) and PARP cleavage at 36 hrs (row 1, lane 3 vs 1 & **Fig. 10D**). But cells treated with CCCP for 36 hrs fail to induce BiP expression and PARP cleavage (**Fig. 10C,** lane 7) like thapsigargin even though cell death was noticed morphologically. Consistent with PARP cleavage, thapsigargin-treated cells were associated with a much higher caspase-3 activity which is determined here by Ac-DEVD-AFC hydrolysis when compared to CCCP-treated cells and it was mitigated by ISRIB (**Fig. 10E**). PI-positive cells (dead cells) quantification by FACS or flowcytometry also revealed that NAC effectively decreased the CCCP-induced cell death from 23% to 8% (**Fig. 10H** & **I**). ISRIB also decreases the dead cell density from 23% to 12% in CCCP and 33% to 20% in thapsigargin treatment. However, z-VAD-fmk, a pan-caspase inhibitor efficiently prevented thapsigargin-induced cell death (9%) which is almost close to control cells (6%) but fail to prevent CCCP-induced cell death significantly (**Fig. 10F** & **G**). Therefore, overall these data indicate that CCCP stimulates mostly non caspase-mediated cell death and was driven by oxidative stress, whereas thapsigargin induces caspase-mediated cell death.


A
Control
CCCP
NAC
NAC+CCCP
B
Control
CCCP
Thapsigargin
ISRIB
I+CCCP
I+Tg
C
1 2 3 4 5 6 7 8
PARP
Cleaved PARP
BiP
Actin
ISRIB >
Tg, hrs>
CCCP, hrs>
D
Cleaved PARP/Actin
Tg, 36hr>
ISRIB
E
-ISRIB
+ISRIB
Caspaese3 activity
Tg, hrs>
CCCP,hrs>
F
Control 6.1%
CCCP 23.6%
Thapsigargin 33.0%
ISRIB 4%
I+CCCP 12.2%
I+Tg 20.2%
Z-VAD-FMK 3.7%
Z-VAD+CCCP 18.0%
Z-VAD+Tg 9.0%
G
% of cell death
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Control
ISRIB
Z-VAD
CCCP
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H
Control 6.1%
CCCP 22.9%
NAC 5.9%
N+CCCP 8.43%
I
% of cell death
CCCP >
NAC

Fig. 10. CCCP-induced cell survival and death, in the presence of ISRIB or NAC. Panels (A) and (B) morphological analyses of HepG2 cells were pretreated with 250 nm ISRIB or NAC for 1 hr and then supplemented with 25 μM CCCP or 1 μM thapsigargin for 36 hrs. Panel (C) western blot indicates the expression of BiP, PARP, and β-actin. Panel (D) quantification of cleaved PARP in Panel (C) by ImageJ software from three independent experiments. Panel (E) caspase-3 activity, estimated by Ac-DEVD-AFC hydrolysis fluorimetric assay in HepG2 cell extracts that were exposed as mentioned in Panel (A). Panel (F) represents FACS analysis of PI-positive cells obtained after the treatment of cells with CCCP or thapsigargin in the presence and absence of ISRIB or z-VAD-fmk, a pan-caspase inhibitor for 36 hrs. Panel (G) quantification of PI-positive cells in Panel (D) from three independent experiments. Panel (H) represents FACS analysis of PI-positive cells that were pretreated with NAC and then supplemented with CCCP for 36 hrs, and the quantification of PI-positive cells is shown in Panel (I) bar diagram. Data represented as mean $\pm$ SD of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Student's t-test).

Chapter-V

5. Discussion

Our findings suggest that mitochondrial dysfunction caused by CCCP evokes an ISR pathway that is mediated by activation of eIF2α kinase, HRI (**Fig. 4**), but not by PERK, GCN2, or PKR kinases (**Fig. 3**). Our findings are consistent with some of the very recent observations where mitochondrial dysfunction caused by CCCP or oligomycin treatments are shown to promote the ISR pathway through the activation of HRI kinase [279, 280]. Other studies have shown that mitochondrial dysfunction can evoke the ISR pathway but have not characterized the eIF2α kinase [22] or observed that it is not one of the four well-characterized kinases [20], and others have implicated GCN2 [281] or PERK [282]. HRI is an important hemoprotein in erythroid precursors that senses heme levels and is activated in the absence of heme, denatured proteins, oxidative stress, and regulated by molecular chaperones Hsp90 and Hsc70 [135, 149]. While Hsp90 is required for newly synthesized HRI to become competent and assume an active form, Hsc70 negatively regulates HRI and inhibits HRI activation that occurs in response to GSSG and other oxidants, heat shock, denatured proteins, and presumably in the presence of newly synthesized proteins that are still bound to polyribosomes like in cycloheximide treated heme-deficient or hemin supplemented reticulocyte lysates [135, 283]. These observations suggest that HRI works like a cytosolic sensor of unfolded proteins and its activation appears to be somewhat similar to the activation of PERK, an ER stress sensor for unfolded proteins, that is discovered later. Consistent with these results, activation of HRI in response to Shigella infection results in the expression of HSPB8, a heat shock protein expression. This chaperone liberates from HRI and also it is expressed particularly via the HRI-eIF2α-P-ATF4 axis [150]. Recent studies further established that HRI promotes inflammatory responses during bacterial infection, by evoking the ISR pathway and is also implicated in regulating signalosome formation involving amyloid-like fibrils, clearance of cytosolic protein aggregates resulting due to inhibition of ubiquitin-proteasome system [146]. HRI presence or activation in non-erythroid cells or tumor cells is not known previously. The current information, however, suggests the importance of HRI activation occurs through proteasome inhibition in mouse embryonic fibroblasts and response to diffusible gases in NT2 neuroepithelial and C2C12 myoblast cells [137, 138]. HRI activation in the CA1 region of the dorsal hippocampus is shown to be necessary for retention of object recognition memory in rats [139], and a recent study proposed a novel role for neuronal HRI that senses and responds to compromised function of the proteasome and to restore proteostasis [140]. Also, small chemical molecules are found to activate HRI in tumor cells and reduce cell proliferation [141, 284]. Mitochondria that generate cellular energy are involved in the biosynthesis of heme, and iron-sulfur clusters in addition to producing nucleotides and playing a role in amino acid metabolism [285]. Indeed, activation of the ISR pathway that occurs as a consequence of protein misfolding within mitochondria (UPR^{mt}) has been shown to increase the expression of mitochondrial chaperones such as Hsp60, Hsp10, mtDnaJ, and ClpP in a CHOP-dependent manner [146]. The two recent studies, one carried out with CCCP-treated HAP1 cells and the other carried out with oligomycin-treated HEK293T cells [279, 280] have implicated activation of HRI kinase during mitochondrial dysfunction and suggested that a short form of DELE1 (DAP3-binding cell death enhancer 1) produced by OMA1 (overlapping activity with m-AAA protease), identified as a metalloprotease, located in the inner mitochondrial membrane, moves to the cytosol and interacts with HRI kinase and activates it. However, OMA1 is activated when the mitochondrial membrane potential is lowered or on depolarization and it degrades OPA1, a GTPase that is located in the inner mitochondrial membrane. Although our studies have not identified any link, based on our observations here, we suspect the activation of HRI kinase may be due to a relay in mitochondrial oxidative stress to cytosol that may be disrupting the HRI Hsp90 interaction. This notion is consistent with one of our results that the addition of geldanamycin derivative, 17-AAG, an inhibitor of Hsp90, inhibits CCCP-induced eIF2α phosphorylation (**Fig. 4A**) thus suggesting, the requirement of Hsp90 for HRI activation as reported earlier [149]. Also, as mentioned above, Hsc70 is found to regulate negatively HRI. Hence, it is likely mitochondrial oxidative stress produced by CCCP may be causing a limitation on molecular chaperones due to the accumulation of unfolded proteins either in mitochondria, in the cytosol, or in both and the activation of the ISR pathway appears to be important to express the necessary heat shock proteins.

We also analyzed here the CCCP-induced ISR pathway in the presence of NAC, a known antioxidant, and ISRIB, a recently discovered ISR inhibitor that promotes a decameric eIF2B complex from its sub-complexes and enhances the GDP/GTP exchange activity that recycles eIF2.GDP to eIF2.GTP [54]. Using these compounds, we studied here CCCP-induced activation of AKT, AMPK, autophagy, and inhibition in eIF4E-BP1 phosphorylation to determine any cross-talk between ISR and other substrates belonging to different signaling pathways. Although both NAC and ISRIB attenuate CCCP-induced ATF4 and CHOP of the ISR pathway (**Fig. 6**), the mechanism of action of these two agents appears to be different. NAC, a known antioxidant, may be clearing CCCP-induced ROS that is generated by mitochondria. In contrast, ISRIB inhibits CCCP-induced ATF4 and CHOP, transcriptional factors which are implicated in cell survival and death by enhancing the activity of eIF2B and general protein synthesis. Consistent with this idea that CCCP induces ROS, NAC neutralizes all the CCCP-induced signaling pathways here. ISRIB, on the contrary, promotes eIF2α phosphorylation albeit at a slower rate (**Fig. 6F** vs **4A**), restores eIF4E-BP1 phosphorylation partially (**Fig. 7C** vs **7A**), mitigates CCCP-induced suppression in protein synthesis somewhat less efficiently than NAC (**Fig. 7F,** lane 6 vs 4), and inhibits the activation of AKT (**Fig. 9C**), but not, AMPK (**Fig. 8D**). The slower rate of eIF2α phosphorylation observed in ISRIB and CCCP-treated cells (**Fig. 6F**) may be due to increased GDP/GTP exchange activity of eIF2B by ISRIB as it promotes the formation of an active eIF2B complex [54]. This interpretation is consistent with a study that was conducted a long time ago where it was shown that free eIF2 was phosphorylated at least 10 times more efficiently by HRI than the eIF2 in eIF2.eIF2B complex (referred then as RF or Reversing factor) and the eIF2 in the complex was phosphorylated upon addition of GDP almost on par with free eIF2 suggesting the rate of eIF2.GDP recycling to eIF2.GTP by eIF2B determines eIF2α phosphorylation [74].

Our findings suggest a cross-talk between eIF2α phosphorylation and mTORC1-mediated eIF4E-BP1 and S6K phosphorylation as suggested recently [184]. This notion is supported by the following observations: CCCP-induced inhibition in phosphorylation of eIF4E-BP1 and protein synthesis are mitigated efficiently by NAC, whereas ISRIB restores them partially (**Fig. 7C** vs **7A & Fig. 7F**). Thus, we find an inverse correlation between eIF2α phosphorylation and eIF4E-BP1 phosphorylation. Analysis of CCCP-induced activation of AMPK, which is inhibited by NAC but not by ISRIB (**Fig. 6D** vs **A**) here in our studies suggests that CCCP-induced oxidative stress primarily regulates AMPK activity as has been suggested [276]. HepG2 cells are tuned to survive under glycolysis and low ATP levels. Smaller fluctuations in ATP may not increase AMPK unless the anabolic pathways are up-regulated, and ATP levels do not commensurate with anabolism. Indeed, both NAC and ISRIB treatments mitigated CCCP-induced inhibition in protein synthesis suggesting that there may be a decline in ATP levels to meet the anabolic demands that may be contributing to the activation of AMPK, in addition to oxidative stress. Our findings suggest that oxidative stress or ROS generation rather than enhanced metabolic demands is the cause for AMPK activation. Consistent with this idea, CCCP-induced AMPK activation is mitigated by NAC but not by ISRIB. Analysis of autophagy marker, LC3II, in CCCP-treated cells suggests that CCCP promotes autophagy, a kind of cell survival. While both AMPK and ATF4 are implicated in stimulating autophagy, our observations suggest that CCCP-induced autophagy is regulated by ATF4 but not by AMPK activation. This is because CCCP-induced autophagy is inhibited by both ISRIB (**Fig. 8D**) and NAC (**Fig. 8A**), which are also found to attenuate the ISR pathway. In contrast, AMPK activation is inhibited by NAC only but not by ISRIB. These findings are consistent with the idea that ATF4 is shown to stimulate genes involved in autophagy [177] and that autophagy is independent of AMPK activation [278].

Oxidative stress can also enhance PI3K-dependent AKT phosphorylation [264]. Consistent with this idea, it is observed here that NAC mitigates efficiently CCCP-induced AKT activation (**Fig. 9A**). CCCP-induced AKT activation is also inhibited in cells transfected with HRI siRNA (**Fig. 9E**) or treated with ISRIB (**Fig. 9C**), where ATF4 expression is inhibited, suggesting that the ISR pathway may be involved in regulating AKT activity. Interestingly, AKT phosphorylation or activation is also reduced in the initial phase of CCCP treatment (**Fig. 5B**). Considering the fact that NAC reduces AKT activation that occurs in the later phases of CCCP treatment, it is likely that CCCP-induced diminution in AKT phosphorylation in the absence of NAC may be due to an increase in the antioxidant activity of CCCP-treated cells to maintain a redox balance. Also, ISRIB that does not have any antioxidant activity like NAC can inhibit CCCP-induced AKT activation. Taking together these findings, we propose that CCCP may be inducing activation of the pentose phosphate pathway (PPP) that generates NADPH which is further enhanced by ISRIB. Further, ISRIB-induced changes in protein expression may also be regulating the cellular antioxidant activity to overcome CCCP-induced ROS. These possibilities, however, have to be investigated further [263, 264]. Consistent with these suggestions, very early studies have suggested that purified eIF2B is bound by NADPH, and oxidation of NADPH or the addition of NAD reduces eIF2B activity [286]. However, it is not known whether ISRIB that activates eIF2B by promoting a decameric complex from its subcomplexes can promote the binding of any reduced pyridine dinucleotides to the subunits of eIF2B. CCCP treatment for 36 hrs causes cell death that is not inhibited by z-VAD-fmk suggesting that it induces necrotic or non caspase-mediated cell death that is protected both by NAC and ISRIB (**Fig. 10F** & **H**).

Further studies have to be carried out to determine the fate of the surviving cells and whether they can proliferate or go into a senescence mode that leads to death. Overall, these findings open up further avenues to understand the mechanism of ISR activation by HRI when mitochondria are stressed, its role if any, in the regulation of cytosolic and mitochondrial chaperones or on mitochondrial UPR, and the cross-talk between ISR and other signaling pathways and organelles during mitochondrial dysfunction, and in maintaining mitochondrial homeostasis. Also, it may be interesting to analyze the importance of PPP activation in AKT activation, as suggested above in CCCP-treated cells and whether ISRIB stimulates this pathway. Also, the significance of AKT inhibition by ISRIB in cell survival requires to be determined.

6. Summary

ISR is an evolutionarily conserved intracellular signaling complex pathway in eukaryotic cells, that is activated in response to a plethora of internal or external stresses to restore cellular homeostasis. ISR has two arms: translational attenuation of general mRNAs via phosphorylation of eIF2α and transcriptional induction of a cadre of genes (which contain uORFs) such as ATF4 and CHOP, which govern the expression of genes participating in redox metabolism, chaperones, autophagy, and cell death, most likely apoptosis. In this research dissertation, we show that CCCP-induced mitochondrial malfunction triggers an ISR, in which eIF2α phosphorylation enhances the expression of genes such as ATF4 and CHOP. Activation of HRI kinase, a cytosolic eIF2α kinase, is essential for CCCP-induced eIF2α phosphorylation, but not GCN2, PERK, or PKR, which is in line with other very recent studies [279, 280]. CCCP also stimulates phosphorylation of AKT, a cell proliferation and survival regulator, and AMPK, a cellular energy sensor, and suppresses phosphorylation of mTORC1 substrates, S6K, and eIF4E-BP1. In addition, CCCP inhibits translation in HepG2 cells, as demonstrated by the incorporation of [S^{35}] methionine into proteins, and stimulates autophagy, leading to non caspase-mediated cell death. NAC, an anti-ROS, neutralizes all of these events, indicating that CCCP-induced oxidative stress or ROS is solely responsible for and/or the root cause of these occurrences. ISRIB, an inhibitor of ISR signaling, lowers CCCP-induced ATF4 & CHOP expression, AKT phosphorylation, and autophagy. ISRIB efficiently reduces CCCP-stimulated autophagy although it fails to inhibit AMPK activation, revealing that the ISR signaling regulates CCCP-induced autophagy rather than AMPK activation. ISRIB, unlike NAC, does not inhibit CCCP-induced eIF2α phosphorylation. However, it slows down CCCP-induced eIF2α phosphorylation initially. This

may be due to an increase in eIF2B activity by ISRIB as has been suggested earlier and is consistent here with partial recovery in translation in CCCP-treated cells. Our observations that ISRIB partly restores the phosphorylation of eIF4E-BP1 that is suppressed by CCCP, suggest that phosphorylation of eIF2α regulates eIF4-EP1 phosphorylation [184]. Our findings are consistent with idea that CCCP-induced oxidative stress leads to eIF2α phosphorylation and ATF4 expression which is known to stimulate genes in autophagy play a pro-survival role together with AKT activation and regulates mTOR mediated eIF4E-BP1 phosphorylation.

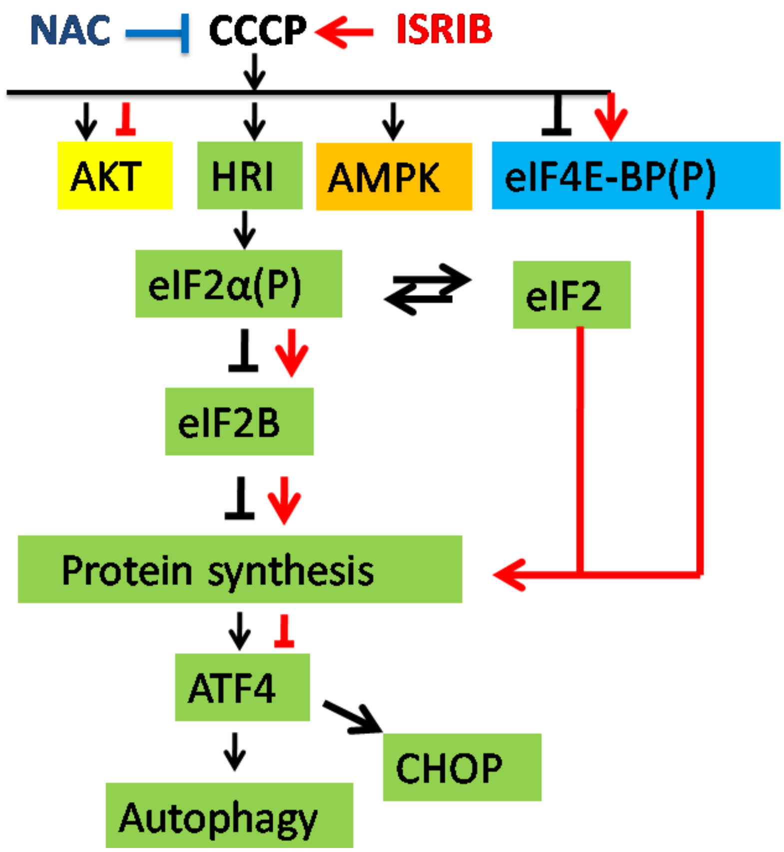

Fig. 11. Graphical Abstract. NAC efficiently inhibits CCCP-induced activation of HRI, ISR (green boxes) AKT & AMPK, and suppresses eIF4E-BP1 phosphorylation, translation & autophagy suggesting that CCCP-induced oxidative stress mediates all these occurrences. In contrast, ISRIB (Red lines) which is known to activate eIF2B, inhibits ISR and lacks presumably an anti-oxidant activity, promotes CCCP-induced eIF2α phosphorylation but slowly, reverses partly CCCP-induced suppression in eIF4E-BP1 phosphorylation, reduces AKT activation and autophagy but does not affect AMPK activation. This may be due to ISRIB enhanced guanine nucleotide exchange activity of eIF2B that may be reducing eIF2α phosphorylation and is inversely correlated to eIF4E-BP1 phosphorylation and translation. Its ability to reduce CCCP-induced AKT activation may be due to the activation of PPP which is consistent with a decline in AKT activation in the initial phase of CCCP treatment. CCCP-induced ISR, rather than AMPK presumably is involved in autophagy.

Chapter-VI

7. Bibliography

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CCCP-induced mitochondrial dysfunction – characterization and analysis of integrated stress response to cellular signaling and homeostasis

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Keywords
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Mitochondrial dysfunction mediated by CCCP (carbonyl cyanide m-chlorophenyl hydrazone), an inhibitor of mitochondrial oxidative phosphorylation, evokes the integrated stress response (ISR), which is analyzed here by eIF2α phosphorylation and expression profiles of ATF4 and CHOP proteins. Our findings suggest that the CCCP-induced ISR pathway is mediated by activation of HRI kinase, but not by GCN2, PERK, or PKR. Also, CCCP activates AMPK, a cellular energy sensor, and AKT, a regulator implicated in cell survival, and suppresses phosphorylation of mTORC1 substrates eIF4E-BP1 and S6K. CCCP also downregulates translation and promotes autophagy, leading to noncaspase-mediated cell death in HepG2 cells. All these events are neutralized by NAC, an anti-ROS, suggesting that CCCP-induced mitochondrial dysfunction promotes oxidative stress. ISRIB, an inhibitor of the ISR pathway, mitigates CCCP-induced expression of ATF4 and CHOP, activation of AKT, and autophagy, similar to NAC. However, it fails to reverse CCCP-induced AMPK activation, suggesting that CCCP-induced autophagy is dependent on ISR and independent of AMPK activation. ISRIB restores partly, inhibition in eIF4E-BP1 phosphorylation, promotes eIF2α phosphorylation, albeit slowly, and mitigates suppression of translation accordingly, in CCCP-treated cells. These findings are consistent with the idea that CCCP-induced oxidative stress leading to eIF2α phosphorylation and ATF4 expression, which is known to stimulate genes involved in autophagy, play a pro-survival role together with AKT activation and regulate mTOR-mediated eIF4E-BP1 phosphorylation.

Introduction

As translation or protein synthesis is an energy-expensive and nutrient-consuming process, it is regulated at multiple levels, chiefly, by phosphorylation and dephosphorylation of protein factors involved in the initiation and elongation steps in protein biosynthesis, such as eIF2, eIF4E-BP1, eEF2 (eukaryotic elongation factor 2), and S6K, through the activation of various signaling pathways that include ISR, UPR, mTOR, MAPK (mitogen-activated protein kinase), AMPK, and protein kinase A/Ca^{2+}-dependent signaling [1-9].

Abbreviations
AKT, also known as protein kinase B; AMPK, AMP kinase; ATF4, activated transcription factor 4; CHOP, C/EBP homologous protein (GADD153); eIF2α, small or alpha subunit of heterotrimeric eukaryotic initiation factor 2; eIF4E-BP1, eukaryotic initiation factor 4E-binding protein 1; GADD-34, growth arrest and DNA damage-inducible protein; GCN2, general control nonderepressible eIF2α kinase; HRI, heme-regulated inhibitor or eIF2α kinase; ISRIB, inhibitor of ISR; mTORC1, mammalian target of rapamycin complex 1; NAC, *N*-acetyl cysteine; PKR, protein kinase RNA dependent; PEK/PERK, pancreatic or PKR-like endoplasmic reticulum resident eIF2α kinase; PPP, pentose phosphate pathway; UPR, unfolded protein response.

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Mitochondrial dysfunction: Characterization of the integrated stress response (ISR), cellular signaling, and homeostasis

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