The integrated stress response

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Abstract

In response to diverse stress stimuli, eukaryotic cells activate a common adaptive pathway, termed the integrated stress response (ISR), to restore cellular homeostasis. The core event in this pathway is the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) by one of four members of the eIF2α kinase family, which leads to a decrease in global protein synthesis and the induction of selected genes, including the transcription factor ATF4, that together promote cellular recovery. The gene expression program activated by the ISR optimizes the cellular response to stress and is dependent on the cellular context, as well as on the nature and intensity of the stress stimuli. Although the ISR is primarily a pro-survival, homeostatic program, exposure to severe stress can drive signaling toward cell death. Here, we review current understanding of the ISR signaling and how it regulates cell fate under diverse types of stress.

See the Glossary for abbreviations used in this article.

Introduction

The integrated stress response (ISR) is an elaborate signaling pathway present in eukaryotic cells, which is activated in response to a range of physiological changes and different pathological conditions [1–5]. Such stresses commonly include cell extrinsic factors such as hypoxia, amino acid deprivation, glucose deprivation, and viral infection [2,4–8]. However, cell intrinsic stresses such as endoplasmic reticulum (ER) stress, caused by the accumulation of unfolded proteins in the ER, can also activate the ISR [9]. Furthermore, in the context of cancer biology, the ISR can be triggered by oncogene activation [10,11]. The common point of convergence for all the stress stimuli that activate ISR is phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) on serine 51 [1]. In mammalian cells, this is catalyzed by a family of four

serine/threonine (S/T) eIF2α kinases that are activated by distinct stress stimuli [12]. eIF2α phosphorylation causes a reduction in global protein synthesis while allowing the translation of selected genes including activating transcription factor 4 (ATF4), aiding cell survival and recovery [13]. However, if the cellular stress is severe, either in intensity or in duration, it will overwhelm the capacity of the adaptive response to resolve it and additional components become activated to execute cell death. Dephosphorylation of eIF2α signals termination of the ISR and return to normal protein synthesis (Fig 1) [14–16]. It is likely that the duration and level of eIF2α phosphorylation, as well as ATF4 regulation and its interactions with other proteins, determine the ultimate ISR outcome resulting from different environmental and physiological stresses [16–18].

Here, we outline current knowledge of the molecular mechanisms of the ISR, highlighting the importance of eIF2α phosphorylation/dephosphorylation and how signaling events downstream of ATF4 ultimately regulate the cell responses to the particular stress insult. We also discuss the ISR pathway in the context of cell survival and cell death in mammalian cells, and describe pharmacological modifications of the pathway that may have therapeutic value.

Activation of the integrated stress response

The eIF2α kinases act as early responders to disturbances in cellular homeostasis. There are four members of the family: PKR-like ER kinase (PERK) [19], double-stranded RNA-dependent protein kinase (PKR), heme-regulated eIF2α kinase (HRI), and general control nonderepressible 2 (GCN2) (Fig 1) [5,12]. All four eIF2α kinases share extensive homology in their kinase catalytic domains, but possess distinct regulatory domains [9,20–24]. Each eIF2α kinase dimerizes and autophosphorylates for full activation [25]. However, each kinase responds to distinct environmental and physiological stresses (Fig 1), which reflects their unique regulatory mechanisms [12].

For example, PERK, found in metazoans, is located in the endoplasmic reticulum (ER) membrane where its luminal domain is normally bound by 78-kDa glucose-regulated protein (GRP78). ER stress (e.g. due to the accumulation of unfolded proteins in the ER, or perturbations in cellular energy, calcium homeostasis, or redox status) causes the activation of PERK. Two models for the activation of PERK by ER stress have been put forward [26,27]. The classical

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Glossary

β-TrCP	F-box protein β-transducin repeat-containing protein
5′UTR	5′ untranslated region
AARE	Amino acid response element
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein 1
APAF-1	Apoptotic peptidase-activating factor 1
ASNS	Asparagine synthetase
ATF/CREB	Activating transcription factor/cyclic AMP response element binding protein
ATF	Activating transcription factor
Atg	Autophagy-related protein
BBC3	p53 upregulated modulator of apoptosis
BCL-2	B-cell lymphoma 2
Becn1	Beclin-1
BIM (BCL2L11)	BCL-2 interacting mediator of cell death
BNIP3L	BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like
bZIP	Basic leucine zipper domain
C/EBPβ	CCAAT/enhancer-binding protein beta
CARE	C/EBP-ATF response element
CDS	Coding sequence
C_H1	Constant heavy chain domain 1
CHOP	C/EBP homologous protein
CK1	Casein kinase 1
CK2	Casein kinase 2
CLOCK	Circadian locomotor output cycles protein kaput
CMT	Charcot–Marie–Tooth disease
CReP	Constitutive repressor of eIF2α phosphorylation
DR5	Death receptor 5
eIF	Eukaryotic translation initiation factor
ER	Endoplasmic reticulum
ERO1α	Endoplasmic oxidoreductin-1-like protein
FOXO	Forkhead box O
Gabarap	Gamma-aminobutyric acid receptor-associated protein
Gabarapl2	Gamma-aminobutyric acid receptor-associated protein-like 2
GADD34	Growth arrest and DNA damage-inducible protein GADD34
GCN2	General control non-depressible protein 2
GRP78	78-kDa glucose-regulated protein
HIV	Human immunodeficiency virus
HRI	Heme-regulated eIF2α kinase
HSP70 and 90	Heat shock proteins 70 and 90
HSV	Herpes simplex virus
IAPs	Inhibitor of apoptosis proteins
IBTKα	α-isoform of inhibitor of Bruton's tyrosine kinase
IRE1	Inositol-requiring enzyme-1
IRES	Internal ribosome entry site
ISRIB	Integrated stress response inhibitor
ISR	Integrated stress response
Map1lc3b (LC3)	Microtubule-associated proteins 1 light chain 3 beta
MCL-1	Myeloid cell leukemia-1
MEFs	Mouse embryonic fibroblasts
mTORC1	mTOR complex 1
mTOR	Mammalian target of rapamycin
NAIP	Neuronal apoptosis inhibitory protein
NBR1	Neighbor of BRCA1 gene 1
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NRF2	Nuclear factor erythroid 2-related factor 2
NSRE-1	Nutrient-sensing response elements 1
NSRE-2	Nutrient-sensing response elements 2
NUPR1	Nuclear protein 1
OLA1	Obg-like ATPase 1
p300	Histone acetyltransferase p300
PDX1	Pancreatic and duodenal homeobox 1
PERK	PKR-like ER kinase
PHD1	Prolyl hydroxylase 1
PHD3	Prolyl hydroxylase 3
PI3K	Phosphatidylinositol-3 kinase
PIC	Pre-initiation complex
PKA	Protein kinase A
PKR	Double-stranded RNA-dependent protein kinase
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1
PP1c	Protein phosphatase 1 catalytic subunit
PP1	Protein phosphatase 1
PPP1R15A	Protein phosphatase 1 regulatory subunit 15A
PPP1R15B	Protein phosphatase 1 regulatory subunit 15B
REDD1 (Ddit4)	Regulated in development and DNA damage response 1
Rpl7	60S ribosomal protein L7
RSK2	Ribosomal protein S6 kinase alpha-2
SCF^{βTrCP}	SCF E3 ubiquitin ligase complex
Siah 1/2	E3 ubiquitin protein ligases 1 and 2
Sqstm1	Sequestosome 1
SyK	Spleen-associated tyrosine kinase
TFE3	Transcription factor E3
TFEB	Transcription factor EB
TNFRSF10B	TNF receptor superfamily member 10b
TRAIL	TNF-related apoptosis-inducing ligand
TRIB3, SKIP3	Tribbles homolog 3
TXNIP	Thioredoxin-interacting protein
uORF	Upstream open reading frame
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
WARS	Tryptophanyl-tRNA synthetase
XBP1	X-box binding protein 1
XIAP	X chromosome-linked inhibitor of apoptosis
ZIPK	Zipper-interacting protein kinase

model proposes that upon the accumulation of misfolded or unfolded proteins in the ER lumen, GRP78 becomes dissociated from PERK, leading to its autophosphorylation and activation [9,22,28,29]. However, recent research suggests an alternative mechanism whereby PERK may be activated directly by binding of unfolded or misfolded proteins to its luminal domain [26,30]. While there is somewhat limited direct evidence for the latter model of PERK activation, it is supported by evidence from another ER sensor of unfolded proteins, inositol-requiring enzyme-1 (IRE1), whose ER luminal domain exhibits strong sequence homology with that of PERK and which in yeast has been shown to be directly activated by

unfolded or misfolded proteins [26,30]. PERK activation in response to glucose deprivation has been reported in neuronal cells and in pancreatic β cells [31,32] where it was shown that it is the ATP depletion that leads to the activation of PERK through the inhibition of sarcoplasmic/ER Ca^{2+}-ATPase pump. It is also interesting to note that in cancer cells, oncogene activation can trigger the ISR through PERK. For example, amplified HRAS induces PERK/eIF2α/ATF4 and cellular senescence in a premalignant model of melanoma [10] and increased activity of proto-oncogene MYC elicits the activation of the PERK/eIF2α/ATF4 resulting in cellular transformation and tumorigenesis [11].
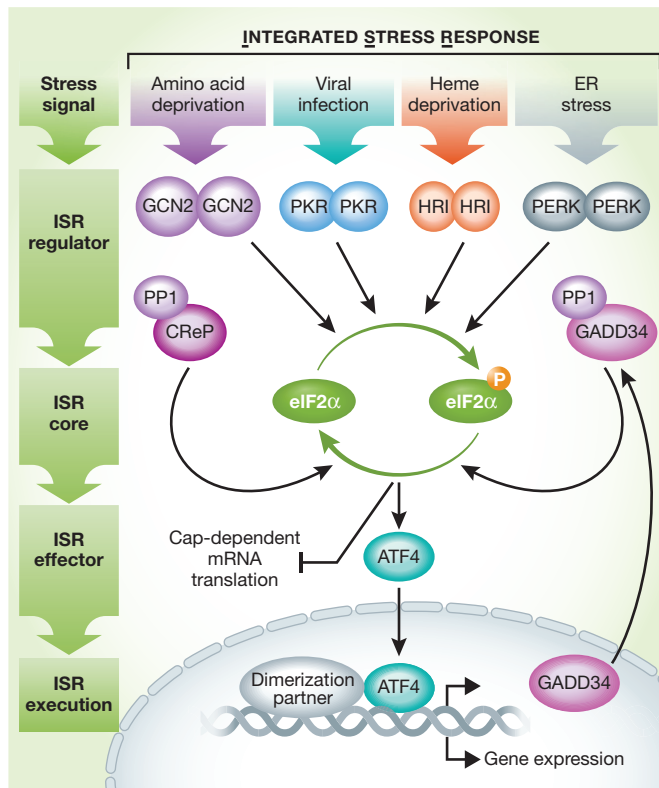
Figure 1. Integrated stress response signaling.
ER stress, viral infection, and other cellular stress signals activate PERK, PKR, HRI, and GCN2 kinases that converge on phosphorylation of eIF2α, the core of ISR. This leads to global attenuation of Cap-dependent translation while concomitantly initiates the preferential translation of ISR-specific mRNAs, such as *ATF4*. ATF4 is the main effector of the ISR. It forms homo- and heterodimers that bind to DNA targets to control the expression of genes involved in cellular adaptation. Termination of the ISR is regulated by the constitutively expressed CReP and stress-inducible phosphatase GADD34 that dephosphorylate eIF2α. For more details, see main text and Table 1. Arrows denote activation or induction, while blunt lines indicate inhibition.

GCN2 is highly conserved from yeast to humans [33]. Mechanistic insights into GCN2 regulation, largely derived from studies in yeast, indicate that GCN2 binds to deacylated transfer RNAs (tRNAs) via a histidyl-tRNA synthetase-related domain to become active in response to amino acid deprivation [34]. GCN2 activation has also been reported in response to a prolonged glucose deprivation in tumor cells, although this is probably an indirect effect resulting from the consumption of amino acids as an alternative energy source in the absence of glucose [7]. GCN2 can also be activated by UV light in mouse embryonic fibroblast (MEF) cells and human keratinocytes [35,36]. The mechanism of GCN2 activation by UV light is unclear and at least two models have been suggested, including UV-induced cross-linking between tRNA and GCN2, or the rapid consumption of arginine due to UV activation of nitric oxide synthetase [33,35,36].

The mammalian PKR is activated mainly by double-stranded RNA (dsRNA) during viral infection [37,38]. Dimerization of PKR via its C-terminal kinase domain results in autophosphorylation at T446 and the subsequent functional activation of the kinase [39–41]. Thus, the presence of dsRNA during viral infection results

in PKR activation and the subsequent inhibition of viral and host protein synthesis through eIF2α phosphorylation [37,42,43]. Interestingly, other stresses such as oxidative and ER stress [44,45], growth factor deprivation, cytokine or bacterial infection [46,47], ribotoxic stress [48], stress granules [49], and heparin [50] have also been shown to stimulate PKR, but in a dsRNA-independent manner. Interestingly, PKR can also be stimulated by caspase activity in the early stage of apoptosis, indicating a role for the inhibition of protein synthesis in this cell death program [51].

Unlike the other eIF2α kinases that have a broad tissue distribution, HRI is mainly expressed in erythroid cells where it is involved in erythrocyte differentiation during erythropoiesis [52]. HRI couples the translation of globin mRNAs with the availability of heme for the production of hemoglobin and thus protects erythroid cells against the accumulation of toxic globin aggregates in iron deficiency [52–55]. Similar to the other eIF2α kinases, HRI activation requires dimerization and autophosphorylation of its kinase domain [56,57]. Regulation of HRI kinase activity by heme is mediated by the two heme-binding domains that are located in the N-terminus and the kinase insertion domain [56,57]. Binding of heme to the N-terminus is stable and it co-purifies with HRI, whereas heme binding to the kinase insertion domain is reversible and inhibits HRI kinase activity [54,56]. Heme inhibits HRI kinase activity *in vitro* and *in vivo* by promoting disulfide bond formation between HRI monomers, keeping them in an inactive dimer conformation [52,58,59]. However, in the absence of heme, non-covalent interactions between HRI molecules occurs, resulting in an active HRI dimer [52,58,59]. HRI can also be activated by other stresses including arsenite-induced oxidative stress, heat shock, osmotic stress, 26S proteasome inhibition, and nitric oxide [52,60–63]. Interestingly, activation of HRI by these diverse stresses is independent of heme and occurs with the aid of heat shock proteins HSP90 and HSP70; however, the exact mechanism of HRI activation remains to be investigated [63].

The eIF2α kinases have overlapping functions and, as such, can act cooperatively to specifically tune cellular responses to a wide variety of stressors, hereafter referred as cellular stress, unless otherwise stated. For example, GCN2 contributes to ER stress-induced eIF2α phosphorylation in *Perk*$^{-/-}$ MEF cells [64]. Conversely, PERK can compensate for *Gcn2* loss in a genetically engineered mouse model of soft tissue sarcoma [65] and in a *Gcn2*-knockout 5XFAD mouse model of Alzheimer's disease [66], as well as for *Hri* loss in mouse hepatocytes [58]. Further support for signaling redundancy between PERK and GCN2 comes from experiments on HeLa cells stressed by protein overload where knockdown of either *PERK* or *GCN2* was compensated by a rapid upregulation of the other kinase [67]. Additionally, both PERK and GCN2 become activated during nucleofection [68] and together with PKR can regulate host response to viral infection [8,69,70]. All eIF2α kinases become activated in response to oxidative stress [2,55,71,72]. PERK and PKR kinases activate the ISR to effectively manage heat stress and to limit the aggregation and accumulation of the denatured proteins in the ER of human endothelial cells and MEFs, respectively [73,74]. Possibly, in such cases when eIF2α kinases act cooperatively, the cellular response is determined not only by ISR activation but also by the activation of other specific substrates of eIF2α kinases. Recently, it has been reported that in *in vitro* models of several types of tumors, upon ER stress, amino acid starvation, and oxidative

stress, the ISR can be also modulated by OLA1, a GTPase that inhibits *de novo* formation of the eIF2 ternary complex, providing a secondary mechanism for the inhibition of global mRNA translation while permitting ATF4 protein synthesis [75].

Termination of the ISR

Dephosphorylation of eIF2α is central to ISR signal termination to restore protein synthesis and normal cell functioning [15]. It is mediated by protein phosphatase 1 (PP1) complex that recruits a PP1 catalytic subunit (PP1c) and one of the two regulatory subunits. In mammals, phosphatase activity is regulated by either PPP1R15A (also known as growth arrest and DNA damage-inducible protein, GADD34), which is induced as part of the ISR, or by the constitutively expressed paralogue PPP1R15B (also known as constitutive repressor of eIF2α phosphorylation, CReP) that is responsible for targeting the enzyme to eIF2α (Fig 1) [15,76]. CReP normally operates in a complex with PP1c in unstressed cells to sustain translational homeostasis by maintaining low levels of eIF2α phosphorylation [76]. In contrast, GADD34 expression is induced downstream of phosphorylated eIF2α and ATF4 during the later stages of the ISR to significantly increase eIF2α dephosphorylation [77]. Thus, the GADD34–PP1 complex acts as an important negative feedback loop to restore protein synthesis once the ER stress has been resolved, and as such aids in cell survival [78,79]. However, eIF2α dephosphorylation may also be important to accommodate the translation of accumulated mRNAs of stress-responsive genes during the ER stress and oxidative stress [16]. It may also facilitate the execution of cell death when cellular homeostasis cannot be restored, by allowing the synthesis of death-inducing proteins and also other proteins that accumulate in the cell and aggravate the proteotoxicity and oxidative stress [16,80]. The importance of eIF2α dephosphorylation is highlighted by the fact that *Ppp1r15a* and *Ppp1r15b*-double-knockout mice exhibit early embryonic lethality, which can be rescued by eIF2α S51A mutation that prevents eIF2α phosphorylation [81].

Recently, in addition to PP1c and GADD34, G-actin was identified as a crucial and conserved component of the eIF2α phosphatase complex, providing an insight into the importance of cytoskeletal dynamics in regulating the ER stress-induced ISR [82,83]. It is possible that the eIF2α phosphatase complex contains other important regulatory factors that may differ among cell types or species. The mechanism of ISR termination may also involve CReP degradation as indicated by a recent study [84]. Interestingly, the role of GADD34 in a cell may not be limited to aiding cellular recovery after protein synthesis shut off, but it may also play an important, independent role in apoptosis induction [77,85,86].

Components of the ISR pathway

eIF2α at the core of the ISR

eIF2α, eIF2β, and eIF2γ together form the eIF2 complex and eIF2α is the main regulatory subunit of this complex since it contains both the phosphorylation and RNA binding sites. Under normal conditions, eIF2 plays a key role in the initiation of mRNA translation and recognition of the AUG start codon [87,88]. It forms a

ternary complex with GTP and Met-tRNA_i that binds the 40S ribosome subunit, and together with two small initiation factors, eIF1 and eIF1A, it forms the 43S pre-initiation complex (PIC) [89,90]. The 43S PIC is recruited to the 5′methylguanine Cap of mRNA in a process that is facilitated by the eIF4F complex among others. The eIF4F complex consists of the Cap-binding protein eIF4E, eIF4G that constitutes as a scaffold protein, and the RNA helicase eIF4A [91]. The interaction between eIF4G and eIF3 further stabilizes the PIC, leading to its migration to the AUG start codon [89,90,92]. Upon binding of the Met-tRNA_i anticodon and the AUG start codon, eIF1 dissociates from the complex and GTP on eIF2 is hydrolyzed with the aid of eIF5. This leads to the dissociation of the eIF2–GDP complex from the 40S ribosomal complex and its recycling for another round of initiation of mRNA translation. Exchange of GDP for GTP is catalyzed by a guanine nucleotide exchange factor eIF2B, and this converts eIF2 back to its active form [87,88].

In response to ISR activation, phosphorylated eIF2α blocks the eIF2B-mediated exchange of GDP for GTP, thereby preventing the formation of the 43S PIC. This results in the global attenuation of 5′Cap-dependent protein synthesis and concomitant translation of selected mRNAs that contain a short upstream open reading frame (uORF) in their 5′ untranslated region (5′UTR). Examples of these preferentially translated mRNAs include *ATF4, ATF5, CHOP,* and *GADD34* (Fig 2) [92–94]. These mRNAs do not require Cap recognition by the eIF4F complex, and their translation relies on a re-initiation mechanism or the direct recruitment of ribosomes to internal ribosome entry sites (IRES) [6,95].

Phosphorylation of eIF2α occurs at S51. Homozygous mutation at this site effectively prevents eIF2α phosphorylation permitting normal mRNA translation even under ER stress or glucose deprivation [96]. Early work showed that mice with homozygous mutation of the eIF2α phosphorylation site die shortly after birth due to prolonged hypoglycemia, demonstrating the importance of translation initiation in glucose metabolism [96,97]. These data suggest that eIF2α phosphorylation is crucial for sustaining proper physiological function of the liver and pancreas after birth, as eIF2α phosphorylation is required for the induction of gluconeogenic enzymes and insulin [96]. Also, it is noteworthy that eIF2α S51A/S51A MEF cells are hypersensitive to ER stress and require supplementation by nonessential amino acids and a reductive agent, which indicates that eIF2α phosphorylation protects against metabolic and oxidative stress [96]. Moreover, cells with homozygous eIF2α S51A mutation are unable to induce autophagy upon viral infection, starvation, and ER stress [98,99]. Thus, the phosphorylation of eIF2α at S51 appears to be crucial for stress-induced autophagy (this will be discussed in a later section).

ATF4 is the best characterized effector of the ISR

ATF4 is a basic leucine zipper (bZIP) transcription factor that belongs to the activating transcription factor/cyclic AMP response element binding protein (ATF/CREB family) [100–102]. ATF4 has several dimerization partners that influence its regulation of gene transcription and can guide cellular outcome. In fact, ATF4 is a key deciding factor in cellular fate in response to ISR activation. It is regulated at the transcriptional, translational, and post-translational level, and moreover, its ability to interact with other transcription factors provides a further level of regulation. A consequence of this
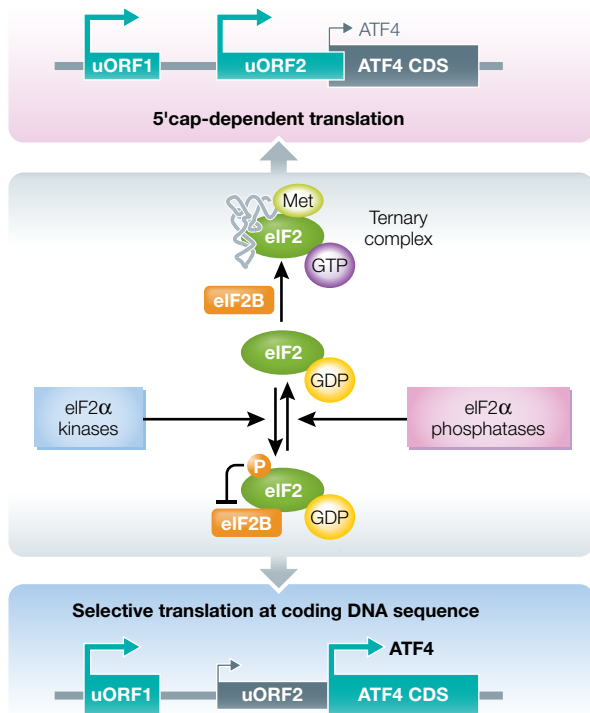
Figure 2. Model for eIF2-mediated translational control of ATF4.
During the ISR, phosphorylation of eIF2α subunit of eIF2 complex inhibits eIF2B-mediated exchange of eIF2-GDP to eIF2-GTP, thus reducing the formation of ternary complex consisting of eIF2-GTP-methionyl-initiator tRNA. Under normal conditions, due to abundance of the ternary complex, ribosomes initiate scanning at upstream open reading frame uORF1 of ATF4 transcript and quickly re-initiate at uORF2. uORF2 overlaps out-of-frame with ATF4 coding DNA sequence (CDS) preventing ATF4 translation. During cell stress, limiting ternary complex availability leads to longer ribosomal scanning along the ATF4 transcript enabling re-initiation of translation at the ATF4 CDS.

intricate regulation is that despite the common mediator, the ISR produces distinct tailored responses to different cellular stresses, with the activation of different ATF4 target genes also being highly dependent on stress intensity and the cellular context [2,101,103].

ATF4 functions as a transcription factor ATF4 has an important role in regulating both normal metabolic and redox processes as well as acting as a master transcription factor during the ISR. ATF4 has a vital role in many tissues and plays a central function in the regulation of obesity, glucose homeostasis, energy expenditure, and neural plasticity. *Atf4*-knockout mice reveal critical roles for ATF4 in embryonic lens formation [104,105], fetal liver hematopoiesis [106], and bone development [107]. *Atf4*-knockout mice are also smaller and leaner [106], suggesting a role for ATF4 in lipid metabolism. They have decreased fat mass caused by a reduction in the volume rather than the number of cells, suggesting that the deletion of ATF4 provides an increased fat mobilization and suppressed the fatty acid synthesis [108]. Knockout of *Atf4* in mice has also revealed a physiological role in thermoregulation as these mice exhibit higher core body temperatures in response to cold stress [109]. ATF4 has also been implicated as a vital mediator of muscle

weakness and atrophy in aging muscles as the reduction in its expression improves muscle quality, strength, and mass in mice [110]. Although the role of ATF4 in models of learning and memory formation is somewhat controversial, with both positive and negative effects suggested [111–114], targeted knockdown of *Atf4* in the mouse hippocampus indicated that it is necessary for memory formation and neural plasticity [115].

During stressful conditions, elevated translation of *ATF4* mRNA facilitates transcriptional upregulation of stress-responsive genes. ATF4 regulates the transcription of its target genes through binding to C/EBP-ATF response element (CARE) sequences that can mediate the transcriptional activation in response to various stimuli [116–118]. During amino acid starvation, these sequences function as amino acid response element (AARE) and ATF4 is the only transcription factor known to bind all known AARE sequences [103,119,120]. ATF4 can form homodimers and heterodimers with several other bZIP transcription factors, including its downstream target CHOP [100,120]. Recent studies have connected ATF4 and CHOP with autophagy induction in mammalian cells [6,103,121]. ATF4 and/or CHOP can bind to the specific promoter *cis* element in their target genes, leading to the induction of the AARE-dependent transcription of genes involved in autophagy activation upon starvation [103]. Moreover, the formation of an ATF4–CHOP heterodimer increases its binding affinity for AARE and this is reported as an early event in transcriptional induction of autophagy genes [103]. Upon ER stress and amino acid depletion in mouse cells, ATF4 alone, or together with CHOP, preferentially binds to proximal promoter regions of target genes, including *Atf3, Ppp1r15a, Trib3, Wars*, and *Rpl7* [122]. Although it is well established that ATF4 alone regulates the expression of genes involved in amino acid transport and biosynthesis, Kaufman and colleagues showed that in response to ER stress, ATF4 and CHOP interact to regulate common genes involved in cellular amino acid metabolic processes, mRNA translation, and the unfolded protein response (UPR) [122].

Although ATF4 mainly acts as a transcriptional activator of the cohort of genes involved in cellular stress adaptation, it can also act as a transcriptional repressor [100]. Some examples of the repressor activity of ATF4 include repression of CRE-dependent transcription of genes such as enkephalin and chromogranin B [101,123]. The repressive effect of ATF4 on transcription can be linked to the inhibition of induction and maintenance of long-term memory by ATF4 [124]. ATF4 is implicated in amino acid metabolism through the induction of asparagine synthetase (ASNS) during both the amino acid and UPR signaling where it binds promoters that contain nutrient-sensing response elements 1 and 2 (NSRE-1 and NSRE-2) [120]. Together, these two elements function as an enhancer and mediate the activation of the *ASNS* gene in response to ATF4, regardless of whether the initial stimulus was amino acid limitation or ER stress [120,125]. ATF4 was identified as a major regulator of *VEGF* expression induced by oxidized phospholipids, a common lipid component of atheroma, thus implicating ATF4 as a pathogenic factor in vascular damage [126]. Recently, several studies have shown ATF4 to be a link between stresses such as glucose starvation or ER stress and mammalian target of rapamycin (mTOR) inhibition through upregulation of cytoprotective Sestrin2 [127–129].

ATF4 acts in combination with other proteins Due to the presence of a leucine zipper domain, ATF4 can interact with other proteins

forming homodimers and/or heterodimers. When ATF4 is not bound to its DNA target, it exists as a monomer [130]. Transcriptional selectivity of ATF4 is modulated by the formation of heterodimers with other bZIP or AP-1 members. These interactions of ATF4, with other transcription factors or interacting partners, influence the outcome of ISR signaling; for example, interactions with ATF3 enhance cellular efforts to re-establish homeostasis, while interactions with CHOP promote cell death upon ER stress [131,132]. Consequently, by association with other proteins and transcription factors, ATF4 can modulate the transcriptome of the cell in response to diverse stress stimuli. Some interactors, such as PHD1 [133], PHD3 [134] during hypoxia, and TRIB3 [135] upon ER stress and amino acid starvation, can effectively decrease ATF4 transcriptional activity. Interestingly, TRIB3 is a downstream target of ATF4, and thus, its activation provides a negative feedback loop to control ATF4 activity during ER stress and amino acid deprivation [131,135]. The interacting partners that cooperate with ATF4 are summarized in Table 1. It seems that the interaction between ATF4 and a precise set of bZIP family members and co-activators regulates specific gene transcription, deciding on the signaling outcome. This interaction network must be therefore highly coordinated and time dependent to induce and modulate gene transcription tailored to the cellular demand.

Translational regulation of ATF4 The primary mechanism by which the levels of ATF4 protein are adjusted in response to different stresses, such as ER stress, hypoxia, or oxidative stress, is through translational regulation [17,136,137]. The mechanism is highly conserved, with yeast regulating levels of Gcn4p (a functional homologue of ATF4) using a very similar mechanism [138,139]. Since yeast do not have PERK, global inhibition of translation is related to the activation of Gcn2p (the yeast homologue of GCN2) and eIF2α phosphorylation upon amino acid depletion [139]. This results in preferential synthesis of Gcn4p, a transcription factor that controls the expression of genes involved in amino acid synthesis and transport [138].

Translational control of yeast *GCN4* requires four uORFs [140]. When the ternary complex is abundant, ribosomes initiate scanning at uORF1 and re-initiate at inhibitory downstream uORFs that precludes the translation of *GCN4* coding sequence (CDS). Under amino acid depletion, more time is required for the ribosome to re-initiate translation, due to low levels of ternary complex, so that ribosomes scan through uORF4 and re-initiate at the *GCN4* CDS [141]. In mammals, mouse *Atf4* has features similar to the *GCN4* [13]. The mouse gene contains two uORFs (uORF1 and uORF2) that are located 5′ to the *Atf4* CDS (Fig 2), while human *ATF4* mRNA contains three upstream open reading frames (uORF1, uORF2, and uORF3) [142]. Under normal cellular conditions, the translation of mouse *Atf4* mRNA is initiated at uORF1, which encodes a peptide just three amino acids in length, and re-initiates at uORF2. This precludes translation of *Atf4* mRNA because, unlike *GCN4*, the uORF2 (uORF3 in human) sequence overlaps with the *Atf4* CDS in an out-of-frame manner [100,142–144]. In stressed cells, ribosomes scan through uORF2 and re-initiate at the *Atf4* CDS [5,143].

Thus, upon eIF2α phosphorylation, re-initiation of translation at the *Atf4* CDS results in approximately fivefold higher ATF4 expression (Fig 2) [13,87,143]. However, Walter's group recently observed high levels of peptide synthesis from both *ATF4* uORFs during ER stress and oxidative stress conditions [145]. These authors suggest

that this deviation from the current re-initiation model may indicate that either only a few ribosomes skip uORF2 or that a combination of re-initiation and scanning past uORF2 ("leaky scanning") occurs. Interestingly, apart from the ribosome re-initiation mechanism, in HeLa and THP-1 cells the translation of an alternatively spliced variant of *ATF4* mRNA is reported to be also controlled by an IRES sequence in 5′UTR [95].

Transcriptional regulation of ATF4 Under non-stressed conditions, the levels of *ATF4* mRNA transcripts available are low [87]. *ATF4* gene transcription is induced in response to different stresses and is mediated by different transcription factors [17]. For example, nuclear factor-like 2 (NRF2) induces *ATF4* in response to oxidative stress [126], CLOCK regulates *ATF4* expression in response to the chemotherapeutic drugs cisplatin and etoposide [146], TFEB and TFE3 induce *ATF4* transcription under ER stress and starvation conditions [147], and a pancreas-specific transcription factor (PDX1) mediates transcriptional induction of *Atf4* during ER stress in mouse islet β cells [148]. Interestingly, in a positive feedback loop, ATF4 downstream gene targets, such as NUPR1, can also elevate *ATF4* mRNA levels [149].

In contrast, hypoxia does not alter *ATF4* mRNA levels, although it can increase ATF4 levels by translational and post-translational mechanisms [136]. Furthermore, there is evidence of transcriptional repression of ATF4 by some cellular stressors. For example, C/EBPβ, a member of the C/EBP family, has been identified as a transcriptional repressor of *Atf4* during UV irradiation, revealing a novel role for C/EBPβ in regulating the ISR [150]. Thus, despite an increase in eIF2α phosphorylation upon UV irradiation, ATF4 induction is not observed because of diminished levels of available *ATF4* transcript [150,151]. Transcriptional repression of *ATF4* is also observed in nonalcoholic fatty liver and nonalcoholic steatohepatitis [152].

ATF4 post-translational modifications and protein stability ATF4 is an unstable protein with a half-life of < 1 h under normal conditions [102,153,154]. Several post-translational modifications regulate ATF4 stability, including phosphorylation and ubiquitination, which are the two most significant in this regard (Table 2). ATF4 degradation is mediated by the ubiquitin-proteasome pathway [153]. Proteasomal inhibitors such as bortezomib and MG132 cause an increase in ATF4 protein levels as a dual consequence of inhibited proteasomal degradation (which stabilizes the protein), and ISR activation (which is triggered by the depletion of intracellular amino acids as a result of proteasome inhibition) [153,155–157]. Phosphorylation of ATF4 at S219 (within its degradation motif sequence DSGICMS) is essential for its interaction with the F-box protein β-transducin repeat-containing protein (β-TrCP), the receptor component of SCF E3 ubiquitin ligase complex (SCF^βTrCP) that targets ATF4 for proteasomal degradation (Fig 3) [153]. Also, phosphorylation of ATF4 at S224 within the same motif, and proline-directed phosphorylation at multiple proline residues surrounding β-TrCP have been implicated in regulating ATF4 interaction with β-TrCP, indicating the tight control of ATF4 degradation [153,158].

Phosphorylation of ATF4 at S245 by ribosomal protein S6 kinase α-2 (RSK2) or S254 by protein kinase A (PKA) can enhance its transcriptional activity during osteoblast and osteoclast differentiation [107,159]. In addition, there are several other kinases that have been shown to interact with ATF4, for example, zipper-interacting

Table 1. ATF4 interacting partners.

Interacting partner	Name	Function	References
β-TrCP	F-box protein β-transducin repeat-containing protein	Interaction of β-TrCP with ATF4 controls ATF4 stability and inhibits its transcriptional activity	[153]
CENP-F	Centromere protein F	Interacts with ATF4 and negatively regulates ATF4 transcriptional activation in interphase	[256]
CEP290	Centrosomal protein of 290 kDa	Interacts with ATF4 and induces its transcriptional activity	[257]
CHOP	C/EBP homologous protein	Interaction between ATF4 and CHOP results in negative regulation of ATF4-dependent transcriptional activation of the ASNS gene ATF4 and CHOP interact to induce target genes involved in protein synthesis and the UPR	[122,258]
PHD3	Prolyl hydroxylase 3	Regulates ATF4 protein stability	[134]
FAM175B (Abro1)	BRISC complex subunit Abro1	Interaction with ATF4 is crucial for the cytoprotective function of Abro1 in response to oxidative stress	[259]
FIAT	Factor inhibiting ATF4-mediated transcription	Modifies early osteoblast activity by interacting with ATF4	[260]
FOS	Proto-oncogene c-Fos	Forms heterodimers with ATF4	[261]
Fra1	Fos-related antigen 1	Forms heterodimers with ATF4	[261]
GABA(B)	Gamma-aminobutyric acid type B	Interacts with ATF4 in mouse brain during the postnatal development	[262]
GPE-1	GPE1-binding protein	Forms heterodimers with ATF4 that regulate G-CSF gene expression	[263]
HIF1A	Hypoxia inducible factor 1-alpha	Interaction with ATF4 initiates bone angiogenesis in hypoxic osteoblasts	[264]
Jun	Proto-oncogene c-Jun	Forms heterodimers with ATF4	[261]
NIPK	Neuronal cell death-inducible putative kinase (TRIB3)	Interacts with ATF4 and inhibits ATF4 transcriptional activity	[265]
Nrf-2	Nuclear factor erythroid 2-related factor 2	Interaction between ATF4 and Nrf2 regulates the expression of heme oxygenase-1 gene	[266]
p300	Histone acetyltransferase p300	Interaction with p300 stabilizes ATF4 and increases its transcriptional activity	[164]
RPB3	RNA polymerase II subunit 3	Interaction with RPB3 enhances ATF4 transcriptional potential in muscle differentiation	[267]
RSK2 (RPS6KA3)	Ribosomal protein S6 kinase alpha-2	ATF4 and RSK2 interact to regulate osteoblast differentiation and function	[107]
RUNX2	Runt-related transcription factor 2	Interaction with ATF4 stimulates osteoblast differentiation and bone formation	[268]
SATB2	DNA-binding protein SATB2	Regulates skeletal development and osteoblast differentiation through the direct interaction with ATF4 and enhances its activity	[269]
SKIP3	Orthologue of the *Drosophila* TRIB3 and rat NIPK	Interaction between SKIP3 and ATF4 is associated with ATF4 proteolysis	[161]
Tax of HTLV-1	Trans-activating transcriptional regulatory protein of HTLV-1	ATF4 interacts with Tax and triggers Tax transactivation of HTLV	[270]
TRIB3	Tribbles homolog 3	Interacts with ATF4 and negatively regulates the ATF4-dependent transcription.	[135,197]
Zhangfei	HCF-binding transcription factor Zhangfei	Forms heterodimers with ATF4 and enhances its transcriptional activity	[271]
ZIP	Zipper-interacting protein kinase	Forms heterodimers with ATF4, which inhibits the apoptotic activities mediated by ZIP	[160]

protein kinase (ZIPK) [160], casein kinases 1 and 2 (CK1 and CK2) [158], and SKIP3 [161], but their full role in regulating ATF4 activity in response to physiological stimuli and hypoxia, respectively, remains to be addressed.

In addition to phosphorylation during ER stress, ATF4 stability and activity can be negatively regulated by prolyl hydroxylase domain protein 3 (PHD3) leading to hydroxylation at proline residues 60 and 235 [162]. This process is regulated by Siah 1/2

Table 2. ATF4 post-translational modifications and their role.

Modification	Residue	Function	References
Acetylation	K311	Inhibition of ATF4 ubiquitination	[164]
Hydroxylation	P60 P235	Possible decrease in ATF4 transcriptional activity	[162]
Phosphorylation	S219 S224	bTrCP-dependent degradation	[153,272]
	S245 S251 S254	Upregulation of ATF4 transcriptional activity	[107,159]
	S215	ATF4 stabilization	[273]
Ubiquitination	S219 D218	ATF4 degradation	[153]

ubiquitin ligases that target PHD1 and PHD3 for degradation and thus increase ATF4 stability [162]. These findings demonstrate an important link between phosphorylation and hydroxylation, as P235 is localized close to the β-TrCP recognition DSGICMS motif [162]. Interaction of ATF4 with PHD1, but not with PHD3, is a stabilizing mechanism for ATF4. However, both interactions lead to a decrease in ATF4 transcriptional activity [133,134,163]. Additionally, ATF4 stability can be enhanced by histone acetyltransferase p300, independently of its catalytic activity, resulting in the inhibition of ATF4 ubiquitination [164].

Cellular outcome of the ISR

Although multiple stresses converge on eIF2α phosphorylation to activate the ISR, the cellular outcome is not always the same. The effect of ISR activation depends not only on the nature of the stress, its duration and severity, but also on the extent of eIF2α phosphorylation and translation of *ATF4* mRNA and other bZIP transcription factors [17,165]. It is commonly accepted that a short-lived ISR is an adaptive, pro-survival response aiming at resolving stress and restoring homeostasis, while a prolonged ISR can signal toward cell death induction [154]. Therefore, this dual effect of eIF2α

Figure 3. Schematic ATF4 protein structure.
ATF4 protein consists of 351 amino acids organized into several domains and motifs. It contains basic domain for DNA binding and leucine zipper domain involved in protein–protein interactions. ATF4 has an oxygen-dependent degradation domain, which is recognized by PHD3. ATF4 has β-TrCP recognition motif, which is a degradation motif. Depending on the phosphorylation events mainly at S219, ATF4 is recognized by β-TrCP and targeted for proteasomal degradation.

phosphorylation raises an important question concerning how the switch between pro-survival and pro-death signaling by ISR is regulated, as well as whether a threshold of cell stress signals exists that favors the activation of cell death proteins.

Cellular recovery signaling by the ISR

The ISR, together with other cellular adaptation pathways, functions as an important part of the cellular defense strategy in response to stress. It does this mainly through altering global protein synthesis and through the regulation of genes that promote pro-survival signaling such as through the activation of autophagy, or that counteract pathways that lead to cell death such as apoptosis or proteotoxicity (impairment of cell function due to the effects of misfolded proteins). Notably, there is also a cross-talk between the ISR and other pro-survival pathways such as the UPR, phosphatidylinositol-3 kinase (PI3K) signaling, autophagy, and the ubiquitin-proteasome system. These are discussed below.

One of the major effects of the ISR is on protein synthesis. The initial repression of global mRNA translation plays a very important role in promoting cell survival in the face of different stresses that activate the ISR. The accumulation of unfolded proteins in the ER induces a condition of ER stress, which is relieved by the reduced level of incoming proteins when global protein synthesis is inhibited [1]. PKR activation of the ISR during viral infection helps to reduce the translation of viral mRNAs, thus protecting the cells [8,42]. Under conditions of amino acid depletion, activation of the ISR by GCN2 reduces the need for amino acids for protein synthesis, thus alleviating this stress [34]. Activation of the ISR by HRI under conditions of low heme lessens the need for heme by attenuating the translation of globin mRNAs, thus reducing the stress and promoting survival [52]. It is important, however, to note that timely termination of the ISR also plays a key role in promoting long-term cell survival, by re-starting synthesis of essential proteins. This is achieved through dephosphorylation of eIF2α by the phosphatase GADD34, which is induced by ATF4 and its downstream targets CHOP and ATF3 [15,78,79,166].

Through the activation of macroautophagy, commonly known as autophagy, the ISR can regulate cell survival and cell death pathways. Autophagy promotes the bulk removal and degradation of unfolded proteins or aggregates as well as damaged organelles while also serving as a means to replenish depleted amino acids for building proteins and to provide energy to a starved cell. Although the precise mechanisms by which phosphorylated eIF2α leads to autophagy are still poorly understood, it is interesting to note that distinct intrinsic and extrinsic stresses that lead to the phosphorylation of eIF2α have also been shown to induce autophagy. For example, ER stress leads to an increased phosphorylation of eIF2α and the subsequent upregulation of certain autophagy receptors such as *SQSTM1, NBR1*, and *BNIP3L* in a PERK-dependent manner [167]. Pharmacological inhibition of PERK suppressed transcriptional upregulation of these autophagy receptors in mammalian cells [167]. In addition, PERK-mediated phosphorylation of eIF2α mediates increased ATG12 and LC3 conversion upon the expression of polyQ72 aggregates in C2C5 cells [98]. In fact, the PERK arm of the UPR can regulate all stages of autophagy including induction, vesicle nucleation, phagophore elongation, and maturation [168]. Consistent with this, another study showed that ER stress caused by bluetongue virus induces autophagy through a PERK-eIF2α pathway
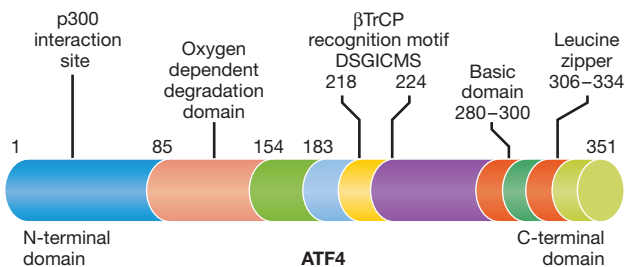
in infected cells [169]. In that study, both pharmacological inhibition of PERK activity and genetic silencing of eIF2α were shown to significantly reduce the level of the autophagy marker LC3 [169]. Furthermore, the induction of autophagy during hypoxia is also dependent on PERK-mediated eIF2α phosphorylation, as the disruption of PERK signaling or exposure of non-phosphorylatable eIF2α S51A mutant to hypoxia decreases the levels of *MAP1LC3B* and *ATG5* transcripts [121]. It has also been shown that GCN2-mediated phosphorylation of eIF2α is essential for the induction of autophagy upon amino acid starvation in tumor cells [7]. While *GCN2*-knockout cells displayed the reduced level of LC3, the eIF2α S51A-mutant cells could not induce LC3 processing [7]. Similarly, S51 of eIF2α has also been demonstrated to be important for amino acid starvation-induced autophagy in yeast and MEFs [99]. Together, these findings place eIF2α phosphorylation as the central link between different stresses and induction of autophagy.

Downstream of eIF2α phosphorylation, ATF4 has been shown to be required for autophagy induction. However, it has been suggested that mechanisms directed from eIF2α phosphorylation other than selective translation of *ATF4* mRNA might also be involved in the activation of autophagy [170]. During hypoxia, ER stress, or amino acid deprivation, there is ATF4-dependent transcriptional upregulation of essential autophagy genes involved in autophagosome biogenesis and function, for example, *MAP1LC3B* and *ATG5* [6,103,167]. ATF4 can also lead to an increased expression of regulated in development and DNA damage response 1 (REDD1; also known as Ddit4), which suppresses mTOR complex 1 (mTORC1) activity leading to autophagy induction upon ER stress and starvation [171,172]. ATF4 activation due to amino acid deprivation stimulates the upregulation of several genes involved in autophagy including *Atg3, Atg5, Atg7, Atg10, Atg12, Atg16, Becn1, Gabarap, Gabarapl2, Map1lc3b,* and *Sqstm1* [103]. Through the upregulation of key autophagy genes, the ISR contributes to increased autophagic flux that helps the cell to deal with the stress caused by hypoxia and nutrient deprivation through the enhanced recycling of cytoplasmic components and maintenance of cellular biosynthetic capacity and ATP levels, for example, by supplying amino acids for *de novo* protein synthesis and providing substrates for the tricarboxylic acid cycle, such as amino acids and free fatty acids [7,173]. It is worth noting that different autophagy genes can have different levels of dependence on ATF4 and CHOP signaling and that the activation of such genes is determined by the ratio of ATF4 and CHOP proteins that are bound to specific promoter *cis* elements, thus allowing subtle tuning of gene expression that can be tailored specifically to meet cellular needs [103]. Interestingly, a cytoprotective function of eIF2α phosphorylation against conditions that mimic viral infection or induce ER stress has been reported to occur through the activation of pathways that promote cell survival, such as PI3K and its downstream target Akt/mTOR [174]. In this regard, it is also of interest to consider the effect of proteasome inhibition on survival signaling by the ISR. Proteasome inhibition, for example, using bortezomib, leads to the activation of the ISR through GCN2 as a result of depletion of amino acids for protein synthesis [157]. In mammalian cells, the depletion of amino acids due to proteasome inhibition also activates autophagy through mTOR in an attempt to restore amino acid levels [157]. Supplementation with critical amino acids that are depleted by proteasome inhibition attenuates eIF2α phosphorylation and suppresses autophagy [157]. Thus, amino acid depletion constitutes

a link between autophagy induction and ISR activation in an attempt to promote the survival of cells.

The UPR that is activated by ER stress is mainly an adaptive response and encompasses the activation of the ISR. In this pathway, PERK, which phosphorylates eIF2α to activate the ISR, functions in concert with the other arms of the UPR, IRE1, and ATF6, to ameliorate proteotoxicity by enhancing the transcription of genes that promote protein folding and degradation of misfolded or aggregated polypeptides [175,176]. The interplay between the different arms of the UPR controls the cellular outcome [177]. The survival benefit provided by the ISR during ER stress reveals ATF4 as a focal point linking PERK-mediated translational control with IRE1- and ATF6-mediated gene expression [1]. It has been proposed that the relative duration of PERK and IRE1 signaling dictates the cellular outcome, with the sustained PERK activation promoting apoptosis and the prolonged IRE1 activation leading to cell survival [178,179].

The ISR also promotes survival signaling by negative regulation of cell death pathways, particularly apoptosis. For example, in response to ER stress, PERK-mediated activation of the ISR leads to an upregulation of cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2) in both tumor-derived cell lines and non-tumor cells [180–182]. ATF4 has also been shown to mediate bortezomib-induced upregulation of anti-apoptotic MCL-1 leading to resistance to the drug [183]. Both MCL-1 and cIAPs can inhibit apoptosis at different points in the pathway that are upstream and downstream of the release of cytochrome *c* from the mitochondria (a critical step in the intrinsic apoptosis pathway). PERK can also mediate a pro-survival role by the induction of microRNA 211 (miR-211), which attenuates a stress-dependent expression of CHOP and which was suggested by the authors as a mechanism that allows cells to re-establish homeostasis before the initiation of apoptosis [184]. However, despite having a beneficial role in restoring homeostasis, these mechanisms could also contribute to tumor development. Increased expression of miR-211 was detected in two mouse models of mammary carcinoma as well as in human B-cell lymphoma and was found to be PERK dependent [184].

Several other genes activated during ISR appear to contribute to pro-survival signaling, although their importance and function in determining cellular outcome remains to be fully addressed. An example is IBTKα, the α isoform of inhibitor of Bruton's tyrosine kinase, an important substrate adaptor for protein ubiquitination, which has emerged recently as an important pro-survival factor activated during ER stress [185]. Translational repression by eIF2α has also been implicated in nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway induction to protect against ER stress [186]. ATF4 also activates the transcription factor NUPR1, which regulates the expression of metabolic stress-responsive genes, especially those involved in DNA repair and cell cycle regulation, and therefore may be considered as a pro-survival factor [149,187].

Cell death signaling by the ISR

Within the ISR are embedded pathways that can lead to the induction of cell death if the adaptive response does not restore homeostasis. These are mainly regulated through the transcriptional activity of ATF4 and some of its downstream targets, particularly CHOP and ATF3. Post-transcriptional regulation of gene expression through the upregulation of microRNAs also plays a role in cell death signaling by the ISR. These will be discussed below.

One of the best studied mechanisms of ISR-induced cell death is through ATF4-mediated activation of CHOP. CHOP is a transcription factor that promotes cell death signaling through multiple mechanisms. In several cellular models, CHOP has been shown to induce cell death by upregulation of the BH3-only pro-apoptotic BCL-2 family members *BCL2L11* and *BBC3*, thus promoting ER stress-induced apoptosis [188,189]. CHOP can also contribute to cell death by enhancing the expression of one of the death receptors, DR5, that plays a role in the induction of apoptosis under ER stress [190]. In addition, CHOP induces the expression of the oxidase ERO1α that destabilizes the homeostasis of the oxidizing environment of ER [78]. Additionally, CHOP can further regulate gene expression by binding to other ATF/CREB family members, such as ATF4 or ATF3, thus altering their DNA binding specificity [103,191]. For example, CHOP–ATF4 heterodimers can upregulate *ATF5* expression, amplifying cell death signaling by regulating the expression of several pro-apoptotic genes such as *PMAIP1, APAF-1,* and *TXNIP* [192]. CHOP and ATF3 can interact to increase the expression of *TNFRSF10B* [191,193]. CHOP is also essential for the induction of *PPP1R15A*, which is important for both adaptation and cell death signaling [78]. It is important to note that although there is a well-established role for CHOP in cell death signaling, CHOP expression alone is not sufficient to induce cell death [194]. In fact, CHOP-deficient cells are only partially resistant to ER stress-induced cell death, indicating the role of other factors in mediating the cell death [195].

ATF4 itself can promote the cell death. It has been shown to be a direct transcriptional activator of the pro-apoptotic BCL-2 family member *PMAIP1*, which was found to mediate ER stress-induced cell death in neuroectodermal tumor cells [196]. ATF4 can also promote the cell death through the formation of heterodimers with CHOP (as described in the previous paragraph) or with ATF3, both of which are transcriptional targets of ATF4. ATF4–ATF3 heterodimers can increase the transcription of *PMAIP1* and thus promote apoptosis through the intrinsic pathway [132]. ATF4 and CHOP together can also induce *TRIB3*, a pseudokinase, which under normal conditions regulates NF-κB-induced gene expression, while during the cellular response to ER stress, it negatively regulates ATF4, thus limiting the transcription of other ATF4-dependent stress-responsive genes [135,197]. TRIB3 was identified as an ATF4 binding partner in a yeast two-hybrid assay and was shown to promote ATF4 degradation [161]. Moreover, TRIB3 can interact with CHOP and downregulate its own induction through a negative feedback loop whereby it represses ATF4-CHOP transactivation [131]. Notably, *TRIB3*-silenced HEK293 and HeLa cells display an increased resistance to ER stress-induced apoptosis [131]. Additionally, TRIB3 has also been shown to inhibit Akt activation in the liver cells, suggesting an alternative mechanism by which TRIB3 could promote the cell death [198]. In this regard, it is interesting to note that the loss of TRIB3 was shown to promote Akt-driven tumorigenesis via forkhead box O (FOXO) inactivation [199].

In addition, the ISR can modulate the cell death through the regulation of miR expression. PERK-mediated increases in miR30c-2 negatively regulate the expression of XBP1 (part of the pro-survival arm of the UPR) in mammalian cells [200]. Both ATF4 and NRF2 (which are directly phosphorylated by PERK) repress the miR-106b-25 cluster, which functions as an oncogene and whose overexpression has been documented in lung cancer, B-cell lymphoma, and gastric cancer [201,202]. ATF4- or NRF2-induced reduction in the miR-106b-25 cluster expression is accompanied by an increased expression of the pro-apoptotic BCL-2 family member BIM and the induction of apoptosis in mammalian cells [201].

Additionally, the ISR can also promote the cell death through translational and post-translational regulation of the caspase inhibitor X chromosome-linked inhibitor of apoptosis (XIAP) [203]. PERK has been shown to downregulate *XIAP* translation through the phosphorylation of eIF2α, while ATF4 promotes XIAP protein degradation through the ubiquitin-proteasome system [203]. This reduction in XIAP levels removes the brake on caspases and permits their activation with ensuing apoptosis [204,205]. It is worth noting that other IAPs such as cIAP1, cIAP2, Livin, Survivin, and NAIP can also be downregulated by the ISR in the cases of chronic PERK signaling [203].

During the prolonged ER stress, ATF4 induces an amino acid transporter network, which leads to mTORC1 activation, the inhibition of autophagy and the phosphorylation of multiple factors including eukaryotic elongation factor 2 (eEF2), and as such, positively regulates translation [165]. While this series of events could serve to restore cellular homeostasis, it can also contribute to cell death execution during chronic ER stress, as constant synthesis of nascent peptides without the production of mature proteins causes proteotoxic stress.

Although apoptosis is the main cell death pathway regulated by ISR, it can also trigger other forms of cell death. For example, the ISR can initiate ATF4-dependent necrosis, especially in response to glucose deprivation, as recently reported [206]. Intriguingly, ATF4-dependent apoptosis can be induced by 2-deoxyglucose in the same cellular model [206]. The ISR can also regulate autophagic cell death in human alveolar epithelial A549 cells in response to lipopolysaccharide treatment [207].

In the context of understanding the role of the ISR in cellular survival/death decisions, it is instructive to consider the utility of mathematical models. To gain a deeper understanding as to how UPR signaling produces different cellular outcomes, Erguler and colleagues developed a mathematical model, based on up-to-date literature, that integrates the three arms of the UPR and the associated genetic and post-translational interactions in a coherent mechanistic model [208]. This model reveals that the UPR has three distinct activity states depending on the level and duration of stress and on the availability of GRP78. The low, intermediate, and high activity states of the UPR are associated with three cellular outcomes of stress adaptation, tolerance, and initiation of apoptosis, respectively. This model revealed for the first time evidence of UPR-induced oscillations in translation attenuation and apoptotic signals, which provides valuable new insights into how the UPR switches between survival and death. Interestingly, the Erguler's mathematical model can also be considered in association with another model for mitochondrial BAX/BAK/BH3 apoptosis [209,210]. Importantly, the predictions of the Erguler model are supported by several experimental observations. For example, PERK signaling in that model is constantly elevated, indicating that the duration of PERK signaling is important for the life and death decision of a cell. However, the majority of the parameter values used in the model remain to be measured experimentally. The construction of a similar mathematical model to gain insight into the ISR is likely to prove helpful to understanding its role in cell death and survival decision making. Given the large amount of experimental data currently available on

the ISR, it is likely that a robust model could be developed that would yield valuable new observations that may be useful in the development of therapeutic approaches for relevant diseases.

Pharmacological modulation of ISR

Dysregulation of ISR signaling has important pathologic consequences linked to inflammation, diabetes, cancer, and neurodegenerative diseases [211–214]. Thus, pharmacological modulation of the ISR represents a promising therapeutic strategy. In recent years, encouraging progress has been made in this area through the development of several drugs that target ISR components to enhance or inhibit this signaling pathway. Interestingly, strategies aimed both to suppress and to enhance ISR signaling have been reported to inhibit tumor growth *in vivo*, which reflects ISR dual role in promoting cell survival and cell death [7,215].

Enhancers of ISR signaling

Pharmacological activation of ISR signaling can be achieved either by activating eIF2α kinases leading to eIF2α phosphorylation in the absence of upstream stress or by inhibiting eIF2α dephosphorylation using phosphatase inhibitors. Some of the compounds that have been shown to promote eIF2α phosphorylation include CCT020312 (a selective PERK activator that does not elicit a general UPR) [216]; BTdCPU and other related N,N'-diarylureas (HRI activators) [215]; histidinol, halofuginone, asparaginase, and arginine deiminase (GCN2 activators) [217–220]; and BEPP monohydrochloride (PKR activator) [221]. Drug-induced enhancement of ISR signaling through eIF2α kinases can be exploited in combined therapies against resistant cancers as it can lead to G1/S checkpoint activation through eIF2α-dependent depletion of cyclin D [216]. Halofuginone, for example, is a potent inhibitor of angiogenesis progression [222] and is being evaluated in several clinical trials.

Inhibitors of eIF2α dephosphorylation include drugs such as salubrinal [223] and guanabenz (2,6-dichlorobenzylidene aminoguanidine acetate) or its derivative Sephin1, which inactivate the binding of GADD34 to PP1c [224–226]. Salubrinal is an inhibitor of GADD34–PP1 and CReP–PP1 complexes that dephosphorylate eIF2α [223,225]. Although it is well established that salubrinal produces high levels of eIF2α phosphorylation and its action is independent of PERK, GCN2, PKR, or HRI, the mechanism by which salubrinal leads to eIF2α phosphorylation is still incompletely understood [225]. It has been suggested that salubrinal might inhibit GADD34–PP1 and CReP–PP1 complexes through the direct binding or that an additional indirect signaling may be involved [223]. Since protein phosphatases are involved in many pathological processes, salubrinal has been used to block the replication of herpes simplex virus (HSV) in both cultured cells and a mouse model of viral infection [223,227]. Salubrinal also produces beneficial effects in experimental models of Alzheimer's and Huntington's diseases [228,229].

In contrast, the mechanism of action of guanabenz and its derivative Sephin1 is better understood. Guanabenz acts by inhibiting the stress-induced phosphatase, GADD34, that causes the dephosphorylation of eIF2α [226]. However, it should be noted that guanabenz does not selectively inhibit GADD34 and that it has nanomolar affinity for the α2-adrenergic receptor and is used in the

treatment for hypertension [230]. Moreover, in mice, guanabenz exhibited an adverse effect by decreasing rotarod performance, which can be attributed to its adrenergic agonist activity as Sephin1 showed no such effect [224]. Similarly, in humans, treatment with guanabenz revealed the adrenergic side effects characterized by drowsiness and coma [230]. In contrast, Sephin1 (selective inhibitor of holophosphatase) selectively and safely inhibits stress-induced GADD34 *in vivo* [224]. Neither Sephin1 nor guanabenz inhibits the constitutive CReP, thus permitting low basal levels of eIF2α dephosphorylation; however, Sephin1 is devoid of α2-adrenergic activity and therefore has less adverse effects than guanabenz [224].

Some other drugs have also been shown to enhance the ISR. Nelfinavir is a HIV protease inhibitor that was recently shown to induce the ISR in several cell lines as well as in mice [231]. Nelfinavir treatment downregulates CReP levels and leads to a decrease in PP1 association with eIF2α. In this way, nelfinavir mediates an increase in eIF2α phosphorylation and thus ISR activation. Although the precise molecular target of nelfinavir is not yet known, it is apparent that nelfinavir itself does not activate any one of the four eIF2α kinases although it is dependent on basal activity of these enzymes. Regardless, nelfinavir seems to be a promising compound to restore homeostasis in pathologies characterized by elevated levels of eIF2α phosphorylation. Another drug, ONC201, is an anticancer drug that triggers cell death in various tumor types, which has recently been reported to trigger the ISR. Using the cells derived from various types of solid tumors, it was shown to activate ATF4 through PKR and HRI eIF2α kinases, leading to an increase in the abundance of TRAIL and its receptor DR5 [232]. Together, these data provide strong evidence that approaches aimed at enhancing or prolonging phosphorylation of eIF2α represent a promising strategy to treat a number of disorders associated with dysregulated ISR signaling.

Recent studies report the beneficial effects of enhancers of ISR in models of degenerative diseases, particularly where protein misfolding plays a role in disease pathogenesis. For example, guanabenz produces the beneficial effects in a rodent model of traumatic brain injury [233], in mouse models of multiple sclerosis [234], and in amyotrophic lateral sclerosis (ALS) [235]. Similarly, Sephin1, a guanabenz derivative, has been shown to prevent several molecular defects in mouse models of Charcot–Marie–Tooth syndrome (CMT) and ALS [224]. In diabetes, translational recovery during unresolved ER stress and thus increased protein synthesis promote apoptosis [236]. Pharmacological intervention with salubrinal applied during translational recovery upon chronic ER stress results in the attenuation of protein synthesis and protects against cell death [236]. Drugs such as salubrinal, guanabenz, and Sephin1 appear to act by slowing down protein synthesis, allowing increased time for protein folding within the ER, and thus protecting the cells from the deleterious effects of proteotoxicity.

The beneficial effects of enhanced ISR activity in some conditions are further supported by genetic manipulation of the ISR pathway in mouse model of diseases. Upregulation of PERK activity in oligodendrocytes attenuates disease severity in a mouse model of multiple sclerosis [237]. Enhanced eIF2 phosphorylation by GADD34 manipulation delays disease onset in a mouse model of ALS [238]. Furthermore, Wrabetz and colleagues showed that ablation of *Gadd34* in P0S63del transgenic mouse model of CMT restores motor function and attenuates demyelination [239].

Inhibition of ISR signaling

Inhibition of eIF2α kinases also represents a strategy to overcome resistant cancers, or to target neurodegenerative diseases and diabetes. Some examples of eIF2α kinase inhibitors include GSK2606414 and GSK2656157 that trap PERK in an inactive state and prevent its autophosphorylation [240,241]. PERK is involved in tumorigenesis and cancer cell proliferation, and both of the GSK inhibitors have been shown to exert antitumor activity against xenografts of human pancreatic cancer in mice [242,248]. Application of GSK2656157 significantly reduced the growth of human xenograft multiple myeloma in mice and also displayed antiangiogenic activity [248]. C16 is a small-molecule inhibitor that acts in an ATP-competitive manner to block autophosphorylation of PKR [242]. Aminopyrazolindane is an ATP-competitive HRI kinase inhibitor [243]. The ATP analogs indirubin-3′-monoxime, SP600125, and a SyK all inhibit GCN2; however, they are not GCN2 specific [244]. Another small-molecule inhibitor of the ISR is called integrated stress response inhibitor (ISRIB), which renders cells insensitive to eIF2α phosphorylation and thus inhibits the ISR downstream of eIF2α phosphorylation resulting in the attenuation of ATF4 synthesis (Fig 4) [245]. ISRIB restores the translational capacity and thus impairs the adaptation of cells to chronic ER stress. Additionally, ISRIB has been shown to prevent the formation of stress granules caused by eIF2α phosphorylation. ISRIB seems to be a promising tool for the treatment of neurodegenerative diseases that are associated with the formation of RNA–protein aggregates [245]. Inhibition of the translational block induced by ISR signaling can significantly enhance cognitive memory and, as such, represents a strategy to combat memory disorders [245].

Another approach to regulate ISR involves the modulation of ATF4 post-translational modifications. Phosphorylation of ATF4 at S251 can be blocked by SL0101, an RSK2 kinase inhibitor [246]. Inhibition of phosphorylation of ATF4 at S254 can be achieved by inhibiting PKA with H-89 [159]. Also, nuclear to cytoplasmic shuttling of ATF4 followed by its phosphorylation-dependent proteasomal degradation can be induced by the synthesized RPL41 peptide, which may have potential in cancer therapy [247].

Conclusions and future perspectives

Since it was first coined in 2002 by David Ron, Heather Harding, and coworkers [1,2], the term "integrated stress response" has garnered increasing attention as a useful way to organize our understanding of the effect of multiple stresses that converge on the phosphorylation of eIF2α through the activation of eIF2α kinases. There
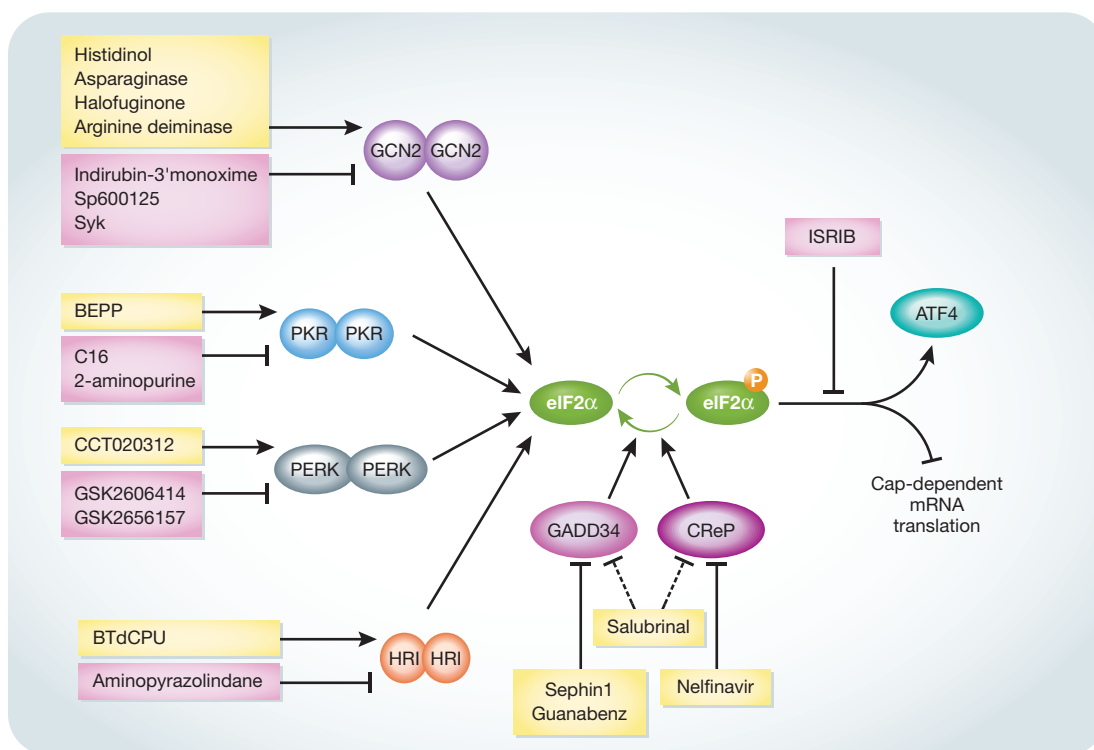
Figure 4. Pharmacological approaches for targeting the components of ISR.
Schematic representation of ISR signaling indicates the effect of eIF2α phosphorylation on translational control. eIF2α phosphorylation can be either stimulated through chemical activators of eIF2α kinases such as histidinol [220], asparaginase [217], halofuginone [253], arginine deiminase [219], BTdCPU [215], BEPP monohydrochloride [221], and CCT020312 [216], or prevented using indirubin-3′-monoxime, SP600125, and SyK to inhibit GSK2 [244], GSK2606414 and GSK2656157 to block PERK activation [240,254], C16 and 2-aminopurine to modulate PKR [242,255], and aminopyrazolindane to inhibit HRI [243]. Salubrinal [223] prolongs ISR through an unknown mechanism, while guanabenz [226] and Sephin1 [224] block GADD34, and nelfinavir [231] decreases CReP expression and affects CReP–PP1 complex binding to eIF2α. The consequences of eIF2α phosphorylation can be reversed using ISRIB [245]. See text for more details. Arrows point to enzyme activation, bar-headed lines point to enzyme inhibition, dashed lines indicate the lack of known target, yellow boxes indicate the activators of ISR signaling, and pink boxes indicate the negative regulators of ISR signaling.

are still gaps in our knowledge regarding many aspects of the ISR. For example, the role of cross-talk between the eIF2α kinases and its downstream targets in determining death or survival is unclear. It is not yet understood how cellular responses are tailored to address the different stresses that lead to eIF2α phosphorylation downstream of different eIF2α kinases. There is still much to be learned about the mechanisms that direct preferential mRNA translation during the ISR [194]. The fact that ATF4 upregulates less than 50% genes in MEF cells in response to ER stress rises the question about the existence of other effectors of ISR [2]. Also, yet to be clarified is how the regulation of specific target genes depends on the duration of eIF2α phosphorylation, type of stress, extent of stimuli, and cell type. Walter's group has recently shown that the cells exhibit noncanonical eIF2A-mediated synthesis of selected proteins during the ISR, thus highlighting divergent mechanisms employed by the ISR to regulate protein synthesis that we still do not fully understand [145]. Intriguingly, the authors suggested that the translation products of uORFs during the ISR could serve a variety of biological functions, for example, as antigens that shape an immune response.

The ISR is important in several diseases due to its role in cell death and survival, and thus, the possibility of targeting it to modulate pathological conditions is a strong rationale for understanding this in greater detail. It is evident that for protein folding diseases, such as certain neurodegenerative diseases, there are beneficial effects to slowing down translation, allowing more time for the ER to fold proteins properly. Targeting the ISR through the inhibition of GADD34, which catalyzes dephosphorylation of eIF2α, shows promise in models of several neurodegenerative diseases that are characterized by proteotoxicity [224]. In tumors, the activation of ISR has been implicated in several anticancer effects, including promoting cell survival. In fact, the cytoprotective ISR can be induced in response to anticancer therapies, thus promoting the development of chemoresistance, as it is the case in pancreatic ductal carcinoma cells and an orthotopic mouse model subjected to gemcitabine treatment [248]. Therefore, cancer treatment with chemotherapeutic drugs in combination with inhibitors to block the cytoprotective ISR may be beneficial. Also, there may be other drugs, already used in the clinic, that could be repurposed for the treatment of diseases where it is desirable to target the ISR.

For therapeutic manipulation of the ISR, it is important to recognize that the consequences of targeting ISR are not the same in all cell types and that there may be undesirable side effects. ATF4 has the potential to regulate more than 400 genes that are important in various, often opposing processes such as cell survival and cell death [115,122]. For example, research on ONC201-induced toxicity revealed that different stress response pathways are activated by the drug in solid tumors and hematological malignancies [232,249]. Clearly, when targeting the ISR, what needs to be considered is a global organism physiology and benefit for the whole body rather than for individual cells. For example, cells growing *in vitro* or as xenografts in mice are not subjected to physiological nonautonomous tumor microenvironment conditions. Knockdown of *GCN2* in human HT1080 sarcoma cells results in reduced tumor growth in xenograft mice model, while in a genetically engineered mouse model of soft tissue sarcoma, *Gcn2* knockdown has no effect on tumor progression due to a compensatory activation of PERK [65]. Thus, both PERK and GCN2 might need to be inhibited to

reduce the phosphorylation of eIF2α, and indeed, this has been shown to be the case in mouse fibroblasts [64].

Research conducted *in vitro* using the cultured cells isolated from tissues shows the ability of cells to autonomously respond to stress stimuli. However, recent advances on manipulating stress responses at an organismal level reveal multilayered intracellular cross-talk between and across the tissues to regulate organism homeostasis [250]. During aging in the nematode *Caenorhabditis elegans* (*C. elegans*), the accumulation of neurotoxic protein aggregates may influence protein homeostasis (proteostasis) in somatic tissues [251]. Furthermore, sensory neurons can downregulate noncanonical UPR activity in non-neuronal cells of *C. elegans* to suppress immune responses to pathogen infection [252]. This is an exciting, new area and understanding how higher organisms communicate local stress to the entire organism is yet to be clarified. Thus, understanding the consequences of targeting the ISR in different tissues, especially vulnerable cells such as neurons, cardiomyocytes, and pancreatic β cells, and different disease models, and the mechanisms engaged to regulate different responses across the whole organism are essential for the development of effective pharmacological agents. There are several physiological conditions that alter cell homeostasis, for example, high secretory demand on pancreatic β cells, hepatocytes, plasma cells, and barrier epithelial cells. Thus, pharmacological alterations of physiological responses might result in diabetes development, compromised immune response, hepatocarcinoma, and sepsis as unwanted side effects.

To gain an important insight into the physiological and pathological roles of the ISR, a better understanding of the molecular mechanisms linking the ISR to other cellular processes, such as autophagy, immunomodulation, metabolism, secretion, cell cycle regulation, and differentiation, would greatly facilitate approaches to modulate the ISR for therapeutic benefit. Considering autophagy as a key component of the ISR, there are still many unanswered questions concerning this in regard to signaling networks and links to biological processes that await further investigation [170]. Although a number of mechanisms underlying the interplay between the ISR and autophagy have been proposed, including upregulation of ATF4-regulated genes, a more comprehensive knowledge of how mTOR activity is regulated by ISR is required.

In conclusion, the ISR represents an important cell response that controls translation and mounts a cell protective response. It is poised at the balance of cell death and survival, and as such, it is an important target in the treatment for many disorders.

Sidebar A: In need of answers

i   What are the best therapeutic combinations that should be employed to usefully target the ISR in different disease conditions while minimizing unwanted side effects?
ii Does the ISR elicit extracellular signaling to regulate organismal homeostasis and could such extracellular signals be used as biomarkers of disease pathologies?
iii What are the molecular mechanisms that shift the balance between opposing cellular fates due to ISR activation?
iv Apart from ATF4, which other factors control the ISR?
v   How do post-translational modifications of ATF4 govern its interactions with binding partners to regulate gene expression?
vi How does the ISR regulate autophagy?

Conflict of interest

A.S. is a cofounder and director of Aquila Bioscience Ltd. A.G., A.S., K.M., M.L., and K.P.Z. are all cofounders of Cell Stress Discoveries Ltd.

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