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Targeting the Integrated Stress Response in Ophthalmology

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Abstract

The Integrated Stress Response (ISR) is a powerful and conserved signaling pathway that allows for cells to respond to a diverse array of both intracellular and extracellular stressors. The pathway is classically responsible for coordination of the cellular response to amino acid starvation, ultraviolet light, heme dysregulation, viral infection, and unfolded protein. Under normal circumstances it is considered pro-survival and a necessary mechanism through which protein translation is controlled. However, in cases of severe or prolonged stress the pathway can promote apoptosis, and loss of normal cellular phenotype. The activation of this pathway culminates in the global inhibition of cap-dependent protein translation and the canonical expression of the activating transcription factor 4 (ATF4). The eye is uniquely exposed to these stressors due to its environmental exposure and relative isolation from the circulatory system which are necessary for its function. In this review we will summarize the growing body of evidence of the role of the ISR in the context of ophthalmology, with special interest on the cornea and anterior segment. We will discuss how this pathway is critical for the proper function of the tissue, its role in development, as well as how targeting of the pathway could alleviate key aspects of diverse ophthalmic diseases.

Keywords

ATF4; ISR; ISRIB

Introduction:

The Integrated Stress Response (ISR) is a critical pathway that is required for cellular response to external and internal cellular stressors[1]. Principally, it provides a mechanism by which a cell can alter its protein production dynamics and shut down global cap-dependent protein synthesis.[2] This functions as a brake on protein synthesis allowing the cell time to

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adapt to the new environment by slowing the rate of protein synthesis and therefore decreasing demand for energy[3] and amino acids.[4] Simultaneously, the cell can increase the production of proteins involved with cell survival,[5] amino acid synthesis,[6] and autophagy[7] through the key transcription factor of the ISR, activating transcription factor-4 (ATF4). The activation of the ISR is canonically carried out in response to signaling from four kinases that are principally responsive to; amino acid and glucose deprivation (general control non-depressible protein 2, GCN2), [4, 5] heme deficiency (heme-regulated inhibitor, HRI),[8] viral infection (double-stranded RNA-dependent protein kinase, PKR),[9] hypoxia[10] and the accumulation of unfolded proteins in the endoplasmic reticulum (PKR like endoplasmic reticulum kinase, PERK).[11] Activation of the ISR results in a reduction of global protein synthesis and an induction of specific genes that aid in cellular recovery.[1, 2] A short-lived ISR is a pro-survival response aimed at relieving stresses and regaining protein homeostasis, while a sustained ISR can result in cell death.[12] Therefore, it is commonly believed that the ISR is a double-edged sword whose actions have been correlated with a diverse field of human diseases including neurodegeneration,[13] endocrine disorders,[14] cancer,[15, 16] autoimmune disorders,[17] and infections.[18]

Unlike other tissues, the eye is uniquely exposed to all the potential stressors that can activate the ISR being both exposed to the environment, and being relatively isolated form the circulatory system[19, 20]. The response to these stressors in the eye is also limited, neovascularization in the eye has been associated with deleterious diseases and loss of vision.[21, 22] Viral infection of the eye is also pernicious with the eye being a privileged site that does not, and indeed cannot, utilize the full extent of the immune system to clear infection.[23, 24] The activation and consequences of an active ISR have often been observed, or eluded to, in diverse ocular morbidities affecting all tissues of the eye. Reports of ISR involvement have been published in, macular degeneration,[21] neurodegeneration,[25] diabetic retinopathy,[26] cataracts,[27] dry eye disease,[28] and keratoconus.[29] In animal models, the ablation of ATF4 and other components of the pathway leads to profound ocular phenotypes in development which leads us to suggest that ISR and ATF4 are key modulators of ophthalmic diseases.[30–32] Critically, the ISR is considered a druggable target, with several compounds that target the pathway under investigation for diverse pathologies.[33–35] In this review, we update the pathophysiologic roles of the ISR in ocular diseases, with special interests in disorders of the anterior segment. We further explore the potential for small molecules in modulating key components of the ISR to modulate their activity, and finally discuss the available animal models of ISR disruption, their ocular phenotypes, and their usefulness in studying the role of the ISR in the eye.

Integrated Stress Response (ISR)

The ISR is the central node by which the cell coordinates the dynamics of protein production in response to environmental and intracellular stressors.[1, 2] The ability to decrease the rate of protein production and alter phenotype in response to acute and chronic stress situations is critical for survival of the cell, and the whole organism.[36] In humans, the ISR sensors comprise four eIF2α kinases:[37] GCN2,[38] HRI,[8] PKR,[39, 40] and PERK.[11, 41] These kinases respond to diverse stresses: GCN2 to amino acid and glucose deprivation,[42] HRI to iron deficiency,[43] PKR to viral infection,[9] and PERK to protein misfolding in the ER, the so-

called ER stress.[44] Upon activation of its respective stimulus each kinase dimerizes, auto-phosphorylates, then phosphorylates Ser51 of eIF2α as demonstrated [37]in Figure 1.

The core mediator of the ISR response is the eIF2α subunit, being a key element in the eukaryotic initiation factor 2 complex (eIF2, consisting of α, β, and γ subunits),[1, 37] this is a central subunit required for the initiation of mRNA translation. Under normal conditions, the eIF2 complex binds a GTP, and a met-tRNA molecule and presents these to the 40S ribosomal subunit.[45] This assembly is a key rate limiting step in the initiation of mRNA translation,[46, 47] therefore the function of the ISR is to regulate the availability of eIF2α by decreasing the pool of the subunit available to initiate ribosome assembly[1, 2] (Figure 2).

Under cellular stress, any of the above-mentioned kinases can phosphorylate Ser51 of eIF2α (peIF2α). This event causes the subunit to increase its affinity for the guanine nucleotide exchange factor, eIF2B.[46] Once bound peIF2α inhibits the ability of the eIF2B to exchange GDP for GTP resulting in a decrease in the available pool of free GTP-eIF2 and decreasing the rate of translational initiation[33]. The amount of eIF2B in the cell is significantly less than eIF2α so only a small increase in peIF2α can sequestrate all the available eIF2B and effectively shut down cap dependent mRNA translation.[48, 49] This reduction of global protein synthesis via the 5' cap-dependent mRNA translation serves a number of cytoprotective roles.[37] In conditions of amino acid shortage[50] or iron deficiency,[51] it reduces the rate at which these nutrients are consumed, during viral infection it slows viral replication by impeding viral protein synthesis[52, 53]. While in ER stress, its activation decreases the rate of proteins entering the ER, thereby relieving the overburdened organelle.[54]

However, certain mRNAs that encode for proteins participating in cell responses to cellular stress are not affected or are indeed enhanced in the presence of eIF2α phosphorylation. Examples of these mRNAs include the ATF4,[55] C/EBP homologous protein (CHOP),[56] and growth arrest and DNA-damage 34 (GADD34).[57] This subset of mRNAs do not require 5' cap recognition during translation, instead utilizing a re-initiation mechanism based on the direct recruitment of ribosomes to internal ribosome entry sites.[55–57] As Figure 1 demonstrates, ATF4 being the best characterized effector of the ISR, is a transcription factor which has several dimerization partners that collaborate in the regulation of gene transcription and direct cellular outcomes[36] in amino acid transportation,[58] oxidative stress,[50] glucose metabolism,[59] autophagy,[60] angiogenesis[61] and protein homeostasis.[62]

Timely termination of the ISR allows protein synthesis to recover once the ISR stimuli is relieved. As Figure 1 shows, at low stressed conditions, the constitutive repressor of eIF2α - protein phosphatase 1 (CReP-PP1) complex[63] sustains a low level of peIF2α by removing phosphate group and restoring translational homeostasis. However, during chronic stress, ATF4 mediates activation of CHOP, which cooperates with ATF4 to induce growth arrest and GADD34 expression. GADD34 then couples with PP1 to terminate the ISR by dephosphorylation of peIF2α.[64] This feedback mechanism allows for protein synthesis to continue and contributes to increased ER stress and induction of cell apoptosis.[65] Therefore, the downstream execution of ATF4 can be cell apoptosis,[66] cell-cycle arrest,[67] and senescence.[68]

In brief, acute activation of the ISR leads to temporary shutdown of the universal protein synthesis, whilst simultaneously activating pro-survival genes through ATF4 activation. However, in conditions such as chronic stress, prolonged activation of the ISR may activate CHOP or other pro-apoptotic genes and lead to cell death.[2]

Pathophysiological association of ISR and Ocular Diseases

As Figure 1 summarizes, ISR activation by different stresses converge in the phosphorylation of eIF2α and the increase of ATF4. Therefore, alongside with the expression of ISR regulators (kinases), peIF2α and ATF4 are the common indicators of ISR activity in ocular disorders[2]. In addition, CHOP expression frequently suggests the pro-apoptotic state or cell death as the result of ISR activation in disease models.[65]

(1) Cornea and Conjunctiva—Fuchs endothelial corneal dystrophy, a disorder of the corneal endothelium that is characterized by loss of endothelial cells and abnormalities of Descemet's membrane, may result in progressive corneal edema.[69, 70] Enlargement of rough ER of endothelial cells has been demonstrated in Fuchs dystrophy specimens. Meanwhile, significantly higher peIF2α and CHOP expression were quantified in Fuchs dystrophy corneal endothelium as compare to the non-Fuchs dystrophy controls.[71] Okumura et al also reported elevated PERK activation and CHOP expression in cultivated human Fuchs endothelial cells as compared to its normal controls.[72] Both studies have shown that accumulation of unfolded proteins may induce corneal endothelial cell apoptosis.

Keratoconus, a multifactorial disease that is characterized by progressive thinning and weakening of the cornea, could lead to severe visual impairment in young adults.[73] Mass spectrometric analysis of keratoconic corneal proteins has suggested increased ER stress, oxidative stress, and apoptosis in the keratoconic stromal proteome.[74] We have reported elevated peIF2α and ATF4 in cultivated stromal keratocytes and corneal buttons from keratoconus patients as compared to those from normal controls.[29] Our findings indicate abnormal ISR activity may participate in keratoconus pathogenesis.

Another corneal disease known to be related to abnormal ISR is granular corneal dystrophy type 2 (GCD2), which is caused by the mutation in the transforming growth factor β-induced *(TGFBI)* gene. Diseased GCD2 corneal fibroblasts have been shown to accumulate mutant *TGFBI*-encoded protein (TGFBIp).[75] Choi et al has also demonstrated a higher level of PERK activities in GCD2 corneal fibroblasts, and GCD2 fibroblasts were more susceptible to ER-stress induced cell death than were the normal controls.[76]

Herpes simplex virus type 1 (HSV-1) is an alphaherpesvirus that is recognized as the most common cause of corneal blindness in developed countries.[77] Ocular involvement can present as primary infection or recurrence from latent disease.[78] HSV-1 is known to be able to disarm the ISR of host cells in their early stage of infection.[79] Us11, a HSV-1 protein, can bind dsRNA and block PKR activation.[80] HSV-1 also expresses an ortholog of GADD34 called γ34.5, that results in eIF2α dephosphorylation to ensure protein synthesis and offset the activation of PKR and PERK during viral infection.[81] By maintaining a pool of unphosphorylated eIF2α, HSV-1 avoids antiviral cellular translational arrest, meanwhile, it prevents potentially hurtful downstream ISR transcription from ATF4 and CHOP, and the

induction of autophagy.[24] The ability of countering antiviral response of infected cells promotes HSV-1 neuroinvasion and periocular replication following corneal infection.[82]

ER stress was also known to involve in the pathogenesis of chronic inflammatory and autoimmune diseases, including dry eye. Pflugfelder's group has demonstrated interferon-γ (IFN-γ) could induce unfolded protein response and the expression of ATF4, and thus resulted decreased mucin synthesis and reduced goblet cell proliferation in the cultivated conjunctival goblet cell harvested from C57BL mice. Interestingly, the conjunctival goblet cells cultivated from IFN-γ knockout or IFN-γ receptor knockout mice had increased proliferation as compared to that from the wild type. In other words, inflammatory related ER stress participates in the mucin-deficiency dry eye via modulating the mucin production and goblet cell proliferation.

(2) Lens—Lens epithelial cells coordinate the development of the entire ocular lens. During cellular maturation, most lens fiber cells lose their nuclei and mitochondria.[83] However, the anterior epithelial cells remain mitotically active as a stem cell niche producing secondary fiber cells.[84] The ISR is highly associated with lens development and degeneration as the absence of secondary lens fibers and severe microphthalmia[30] have been shown in ATF4 deficient mice. In addition, interruption of lens autophagy could lead to the loss of lens differentiation and the failure of lens resistance to stresses, resulting in cataract formation.[85, 86] Cataract, which has long been the leading cause of blindness globally,[87] can be induced by senility and physical and chemical stresses.[88] A large variety of ER stressors were found to induce the production of reactive oxygen species in cultivated lens epithelium with or without the induction of cell apoptosis.[89] Increased level of ATF4 and CHOP were documented in human aged lenses.[89] The activation of ER stress pathways were also reported in high-myopia related cataract[90] and congenital cataract.[27] Accordingly, ATF4 related pathways is key for the evolution of normal lenses. The ER stress and its consequences may be one of the initiation factors of many types of cataracts.[27, 91]

(3) Retina and Glaucoma—Accumulation of unfolded protein activates the PERK pathway and its related pro-apoptotic circuits, which are implicated in the pathogenesis of many diseases involving different layers of the retina and optic nerve.[92] Diabetic retinopathy (DR), a leading cause of visual loss in the working-age population, has long been recognized as a microvascular disease.[93] The pathologies in DR include pericyte loss and vascular endothelial cell apoptosis in responses to hyperglycemia.[94] Zhang et al have proved intermittent high glucose can induce ER stress in human retinal pericytes, and is related to the inflammatory responses in DR mouse models.[26, 95] Elevated ATF4 was further demonstrated in the aqueous humor and vitreous of proliferative diabetic retinopathy patients by Wang et al. In contrast to DR, age-related macular degeneration (AMD) is the top cause of blindness among the aged group in the developed world.[96] AMD is characterized by dysfunction of retinal pigment epithelium (RPE) cells and their over expression of vascular endothelial growth factor (VEGF).[21, 97] Roybal et al reported oxidative stress activates the secretion of VEGF in human RPE cells by an ATF4 dependent mechanism. The ATF4 complex binds to the *VEGF* gene and transactivates its expression.[98] Therefore, activation of the ISR under oxidative stress may be one of the contributing factors

to neovascularization in retinal diseases.[99] Alongside DR and AMD, another key contributor to global visual impairment is glaucoma, a group of optic neuropathies characterized by progressive loss of retinal ganglion cells.[100] ER stress was documented to play a major role in the pathogenesis of myocilin mutation associated glaucoma and glucocorticoid-induced ocular hypertension in mouse models.[25, 101] Trabecular meshwork tissue lysate form glaucoma patients also demonstrated increased ER stress markers including ATF4 and CHOP as compared to those age-matched normal controls.[102]

In summary, the ISR aids in cellular adaptation and defense in the eye. Inactivation of the ISR by viral proteins allows viral replication and tissue destruction. Moreover, the eye is consistently under oxidative and metabolic stresses, chronic stimulation of the ISR may lead to cell death, and ISR stimulation is observed in both anterior and posterior segment ocular disorders.

Modulators of ISR and their possible therapeutic applications

Many toxins or drugs induce extensive ISR,[103] including arsenic,[104] tunicamycin,[105] thapsigargin,[106] and mitomycin C.[107] Environmental arsenic contamination in drinking water has been a global health issue that was associated with many cancers.[108] Arsenic generates oxidative stress and mediates ER and mitochondrial cross-talks that results in apoptosis via ATF4 regulated pathways.[104] Arsenic induced-VEGF production in retinal pigmented epithelium via eIF2α-ATF4 branch has suggested non-hypoxic stresses also contributed to VEGF expression.[98] Tunicamycin, an antibiotic mixture produced by *Streptomyces lysosuperificus*, has antimicrobial activity against bacteria, fungi, and viruses. Tunicamycin not only impairs protein glycosylation, but also depletes the calcium in ER which further aggravates unfolded proteins stress.[105] Tunicamycin has not been used as human medicine[103] due to its toxicity, but has been broadly applied as an ER stressor in studying various pathological and physiological processes of diabetes and asthma.[109, 110] Thapsigargin is a highly potent drug isolated from the plant *Thapsia garganica L* (Linnaeus), [106] its cytotoxicity is derived from its ability to inhibits calcium transport leading to calcium depletion in ER. Therefore, in addition to ER stress-related cell death, thapsigargin induces concomitant increase in free cytosolic calcium is also a potent pro apoptotic signal in cells. [106] Mitomycin C, is a reactive oxygen species (ROS)-generating anticancer drug. Mitomycin C can induce human fibroblast apoptosis via PERK pathway,[107] and its anti-fibrotic effect has been applied in many ophthalmic surgeries such as pterygium excision[111] and glaucoma filtering surgery.[112]

Other than these general ISR inducers, there are several approaches for therapeutic activation or inhibition of the ISR by modulation of specific targets: (1) counteracting the effect of peIF2α; (2) activation or inhibition of the eIF2α kinases; (3) inhibition of the eIF2α phosphatase; (4) post-translational modification of ATF4; (5) altering the ATF4 downstream pathways. Here we elaborate the most studied small molecules targeting ISR pathways, especially the highly potent ISR inhibitor, ISRIB. We also summarize the molecules that have been applied in the ophthalmic field (Figure 3). Detailed review of small molecules modulating the ISR network are listed.[2, 33–35, 113]

(1) Counteract the effect of peIF2α: eIF2B activator—As Figure 2 illustrated, eIF2B catalyzes the GDP-GTP exchange on eIF2. While the eIF2α is phosphorylated, it transforms from a substrate into potent inhibitor of eIF2B and reduces its catalyzing ability.[1, 46] A small molecule ISR inhibitor, ISRIB, discovered by Walter's group, binds in the pocket formed at the junction of eIF2B β- and δ-subunits, driving the subunits to fully assemble the eIF2B decamer complex with GDP exchange ability.[114–116] ISRIB, therefore, rescues protein translation by stabilizing and increasing eIF2B complex abundance and counteracting low levels of peIF2α in treated cells.[116, 117] However, when the concentration of peIF2 exceeds a certain threshold-that is, when the ISR is strongly activated, more eIF2B are trapped by peIF2, and ISRIB can no longer restore the drained pool of eIF2B complex. Thus, ISRIB does not abolish the ISR's cytoprotective effects in cells in which the ISR is strongly activated.[1] ISRIB has good potency (nM) and brain penetrance, and optimization of ISRIB has led to the discovery of analogs with picomolar activity.[117, 118] In vitro, ISRIB treatment partially restores the cellular protein translation rates in cultivated neuronal model of amyotrophic lateral sclerosis.[119] In vivo, ISRIB partially recovers the cellular protein translation rates in prion-infected mice.[120] ISRIB also reverses some aspects of cognitive deficit in traumatic brain injury mice[121] and Down syndrome mice.[122] In the ophthalmic field, our group was the first to report that activation of the ISR can recapitulate the keratoconus phenotype in normal corneal keratocytes in vitro. Conversely, by blocking the ISR with ISRIB, the production of matrix metalloprotease (MMP)-9 is reduced and the synthesis of collagen in keratoconic fibroblasts increases in vitro, relieving many of the hallmarks of keratoconus.[123] Therefore, targeting of the ISR through small molecules may be a promising therapeutic path for ophthalmic disease.

(2) Target the eIF2α kinases

Activators of eIF2α kinases: Pharmacological activation of ISR signaling can be achieved by activating eIF2α kinases. Halofuginone,[124] histidinol,[116] asparaginase,[125] and arginine deiminase[126] are known to be GCN2 activators; BTdCPU activates HRI;[127] BEPP and poly (I:C) are PKR activators;[128, 129] and CCT020312[130, 131] is a selective PERK activator. These potential drugs were mostly studied for cancer treatment, and the application in ophthalmology fields is just emerging.[33, 35] In vivo, oral administrated or intraperitoneal injected halofuginone has shown potent inhibitory effects on angiogenesis progression in a mouse corneal neovascularization model.[132] Arginine deiminase catalyzes the deimination of proteins, a nonreversible post-translational conversion of protein-bound arginines to protein-bound citrullines. Elevated levels of protein deimination are reported in human neurological diseases,[133] the retina tissue of AMD patients,[134] and the optic nerve of open-angle glaucoma eyes.[135] These findings suggest GCN2 may be one of the treatment targets of AMD and glaucoma. Poly (I:C), polyinosinic:poycytidylic acid, has similar structure to the double-stranded RNA which leads to PKR activation.[129] In vivo, topical and systemic administrated Poly (I:C) has been known for decades to induce tear interferon against HSV in rabbit and human eyes.[136, 137]

Inhibitors of eIF2α kinases: Pharmacological inhibition of the ISR can be achieved by inhibition of eIF2α kinases. Three structurally similar compounds indirubin-3'-monoxamie, SP600125 and a SyK inhibitor inactivate GCN2;[138] amino-pyrazolindine inhibits HRI;[139]

C16 and 2-aminopurine are frequently used for inhibition of PKR;[140, 141] and GSK260614 and its analogue GSK2656157 inactivate PERK.[142, 143] However, the application of eIF2α kinase inhibitors in ophthalmic researches is scarce. In vitro, GCN2 inhibitors, Indirubin-3'-monoxamie, SP600125, and SyK inhibitor decrease the phosphorylation of eIF2α in mouse embryonic fibroblast cells after UV irradiation.[138] Yet, these compounds are poorly specific for GCN2, and additional studies on structure-activity relationship are essential to increase their specificity and potency in treating eye diseases. In vitro, Jiang et al has reported GSK2606414, the highly selective PERK inhibitor, suppressed RPE cell proliferation in a dose-dependent manner. Meanwhile, GSK2606414 treatment reduced the level of peIF2α, CHOP, and VEGF mRNA expression in RPE cells under ER stress.[144]

(3) target eIF2α phosphatase—Dephosphorylation of eIF2α is the key step of ISR termination and restoration of protein synthesis for normal cellular functions. As shown in Figure 1 and 3, in mammals, two phosphatases are responsible for the dephosphorylation of peIF2α, the CReP[63] and GADD34.[145] As the name suggested, CReP normally operates in unstressed cells for maintaining low level of peIF2α. While GADD34 expression is induced by ATF4 and CHOP, and acts as a negative feedback loop to resume protein synthesis once the stress is relieved.[64] Salubrinal and its analogue SAL003, are inhibitors of both GADD34 and CReP.[123, 146] Guanabenz and its derivative, Sephin 1 are known to inhibit GADD34.[146] Nelfinavir, a HIV protease inhibitor downregulates CReP and increases peIF2α.[147] Among these peIF2α phosphatase inhibitors, salubrinal has been shown to result in high levels of peIF2α. It has also been demonstrated that salubrinal blocked the replication of HSV in cell culture models.[148, 149] In vitro, Salubrinal treatment protects trabecular meshwork cells against ER stress[150] and RPE cells against toxins.[151, 152] However, we found SAL003 stimulates the ISR and leads to apoptosis of normal fibroblasts cultivated from donor corneas.[123]

(4) Post-translational modification of ATF4—There are no reported molecules that can directly bind and inhibit ATF4. Transcription factors are largely poor drug targets due to the inefficiency of small molecules to halt protein-DNA and protein-protein binding interfaces.[153] However, ATF4 function is dependent on extensive post-translational modifications, particularly phosphorylation, that modulates its transcriptional activity and degradation. Ribosomal S6 kinase (RSK2) and protein kinase A (PKA) are two kinases that phosphorylate ATF4 and enhance its transcriptional activity.[154, 155] Reversible RSK2 inhibitors SL0101 and BI-D1870, and the irreversible inhibitor fmk have been developed.[156–158] PKA inhibitors H-89 and KT 5720 are also available yet with low selectivity.[159] Recently, another PKA antagonist Rp-8-Br-cAMPS with better selectivity has been developed.[160] However, RSK2 and PKA both work on multiple protein responses for different cellular functions,[161, 162] and the use of these compounds as ATF4 inhibitors will need careful validation to support that the observed effects can be contributed to ATF4 inhibition. As yet, the application of ATF4 inhibitor in treating eye disease has not been reported.

(5) Target pathways downstream of ATF4—As Figure 1 and 3 demonstrate, ATF4 transcriptionally regulates genes involved in different cell functioning.[33] Disruption of the

pathways downstream of ATF4 is also a valid approach to interfere the effects of ISR. Insufficient vascular supply results in hypoxia, nutrient deprivation, lower ATP production and protein misfolding. These factors lead to ATF4 expression in ischemic tissue,[163] and drive the expression of VEGF[22] and pro-angiogenesis cytokine, interleukin (IL)-8.[164] Targeting VEGF or pro-angiogenesis interleukins has long been the promising treatment for ocular vascular neogenesis disorders.[165] Another potential therapeutic target is autophagy, the catabolic process responsible for lysosome-dependent degradation and recycling.[166] The expression of ATF4 and CHOP upregulate autophagy genes,[167] and dysregulated autophagy disturbs cellular homeostasis and has been linked to many ocular disorders, including dry eye, corneal dystrophy, keratoconus, cataract, glaucoma, and AMD.[85, 168] The other key mechanism for maintaining cellular homeostasis is the ER associated protein degradation (ERAD), which through the accumulation of unfolded proteins activates the PERK-ATF4 pathway.[169] Bortezomib is a specific inhibitor of the proteasome that impedes ERAD[170] which has shown neuro-protective effects in retinal ischemia-reperfusion injury in rat[171] and anti-inflammatory effects in experimental autoimmune uveitis (EAU) mice after intraperitoneal injection.[171, 172]

Animal models in ISR research

Investigations of the ISR is an emerging field based on its established role in assorted human pathologies[2] and because the pathway is considered a “druggable target”. Nonetheless, most mechanistic studies have been implemented in cell culture systems exposed to the stressors. In Table 1, we summarize relevant studies in animal models that have revealed novel and specialized functions of the ISR in distinct organisms *in vivo*.[173] The most profound of which is the ATF4 knockout mouse which presented with lens anomaly and microphthalmia.[30]

(1) *ATF4* knock out mice—ATF4 is a member of the cAMP-responsive element-binding protein (CREB) family and has multiple roles in cell differentiation and organ morphogenesis.[30, 174, 175] ATF4 heterozygous mice are viable, yet only 30% of ATF4 knockout ($CREB^{-/-}$) mice survive to maturity.[176] ATF4 deficiency leads to mutations in eye-lens development and hair-growth, accompanied by pancreatic hypoplasia skeletal defects, and growth retardation.[30, 59, 174, 176, 177] Tanaka et al generated $ATF4^{-/-}$ mice and noted severe microphthalmia and lens degeneration due to cell apoptosis without the formation of lens secondary fiber cells. Rescue of the ATF4 mutant phenotype by lens-specific gene expression not only recovered its lens secondary fibers but also induced hyperplasia of these fibers.[30] Hettmann et al have also demonstrated microphthalmia due to absence of lens on $ATF4^{-/-}$ mice.[176] They noted the defect appeared to be specific for the lens, as no gross anomalies in the retina or cornea could be detected. Interestingly, the $ATF4^{-/-}$ and $p53^{-/-}$ double-homozygous mutant mice resulted in a marked suppression of microphthalmia, bringing together the DNA damage response, and the ISR in development. The lens developed in the double mutant model; however, it contained a lower number of fibers as compare to that of the wild type. Therefore, the authors proposed that there may be both p53-dependent and p53-independent effect of ATF4 deficiency participating in the differentiation and survival of lens fiber cells.[176] Overall, these animal studies support the functions of ATF4 in modulating cell cycle, apoptosis, and metabolism in diverse tissues.

(2) EIF2α kinase gene knockout mice—In the 1990s, the physiologic functions of eIF2α kinases were elaborated. Kinase gene knockout mice were bred for development and phenotype observation[51] as listed in Table 1. Interestingly, *Gcn2*$^{-/-}$ mice have no observable phenotype mutation in a non-stressed status but present with developmental delay while being fed with leucine deficit diet.[178] *HRI*$^{-/-}$ mice are viable, fertile, and without gross abnormalities. Mild macrocytosis and hyperchromia were observed in the *HRI*$^{-/-}$ mice in the absence of stress that indicates the physiological adaptation of red blood cells to iron deficiency.[51] *Pkr*$^{-/-}$ mice have normal development and similar response to vaccine and influenza infection, which indicate the interruption of PKR is not sufficient to eliminate eIF2α phosphorylation and other eIF2α kinase family members may compensate for loss of PKR functions.[179] *Perk*$^{-/-}$ mice are viable, and the phenotypes involve strong changes of pancreatic β-cell secretory function and its survival. Therefore, *Perk* knockout animals have impaired glucose metabolism and develop early diabetes and growth retardation.[180, 181] *Perk*$^{-/-}$ mice also exhibit severe osteopenia because PERK signaling is also important for the differentiation of osteoblasts.[181–183] PERK signaling has divergent roles in neural tissues and cognitive function. Conditional disruption of PERK in the adult mouse forebrain was associated with altered behaviors.[184]

(3) EIF2α phosphatase gene knockout mice—Two somatic genes, *Ppp1r15a* and *Ppp1r15b*, encode the proteins GADD34[64] and CReP,[185] that recruit PP1 to form complex phosphatase to dephosphorylate peIF2α. The *Ppp1r15a*$^{-/-}$ mice have a normal phenotype; however, the ER stress response in their embryonic fibroblasts are different as compare to that of the wild type.[186] In contrast, the *Ppp1r15b*$^{-/-}$ newborns exhibit severe growth retardation, impaired erythropoiesis, and none survives the first day of postnatal life.[186]

(4) *eIF2α* gene knock in mice—In all eukaryotic cells, eIF2α is part of a major assemblage in the initiation of protein translation. The phosphorylation at serine 51 interrupts normal function of eIF2α and causes global reduction of protein synthesis except ATF4 and CHOP.[187] A knock in mouse model that has been developed by substitution serine 51 for alanine, creating a non-phosphorylatable eIF2α. Homozygous eIF2α^{S51A} mutant mice die within 24 hours of birth due to impaired gluconeogenesis and hypoglycemia.[188] Heterogenous eIF2α^{S51A} mutant mice develop normally into adulthood. Nevertheless, theses mice become obese and glucose intolerant when fed a high-fat diet.[189] Conditional destruction of eIF2α in pancreatic β-cell led to severe diabetic mice.[190]

(5) *CHOP* knock out mice—CHOP is a well-characterized downstream target of the eIF2α-ATF4 signaling branch. Deletion of *Chop* in mice does not alter animal survival and development. The literature using CHOP-deficient animals for disease models are broad, and here we feature a few studies. CHOP knockout mice have reduced cell apoptosis in lung and kidney induced by ER stressors.[191] In the nervous system, exceptionally, CHOP knockout mice showed increased apoptosis in the hippocampal cells and decreased performance in memory-related behaviors.[192] However, CHOP deficiency provides significant neuroprotection in the context of brain ischemia.[193] Taken together, these selected studies suggest, at least in neural models, CHOP may play a protective role or enhance apoptosis depending on the disease context.

Conclusion and Perspectives

The ISR widely regulates cell survival and death via peIF2-ATF4 signaling and its downstream execution. The ISR contributes to many ocular diseases such as keratoconus, corneal dystrophy, HSV keratitis, cataract, DR, AMD, and glaucoma. The use of small molecules to counteract the peIF2α effects or target the eIF2α kinases/phosphatase is promising for treating ISR-related ocular disorders. However, drug selectivity and specificity need to be cautiously inspected. *In vivo* models such as gene knockout or knock-in mice are invaluable for studying ISR consequences in embryogenesis, development, and phenotypes. Together with ATF4 knockout mice that present with retarded lens development, future expansion of ocular ISR related disease models are mandatory for exploring potential therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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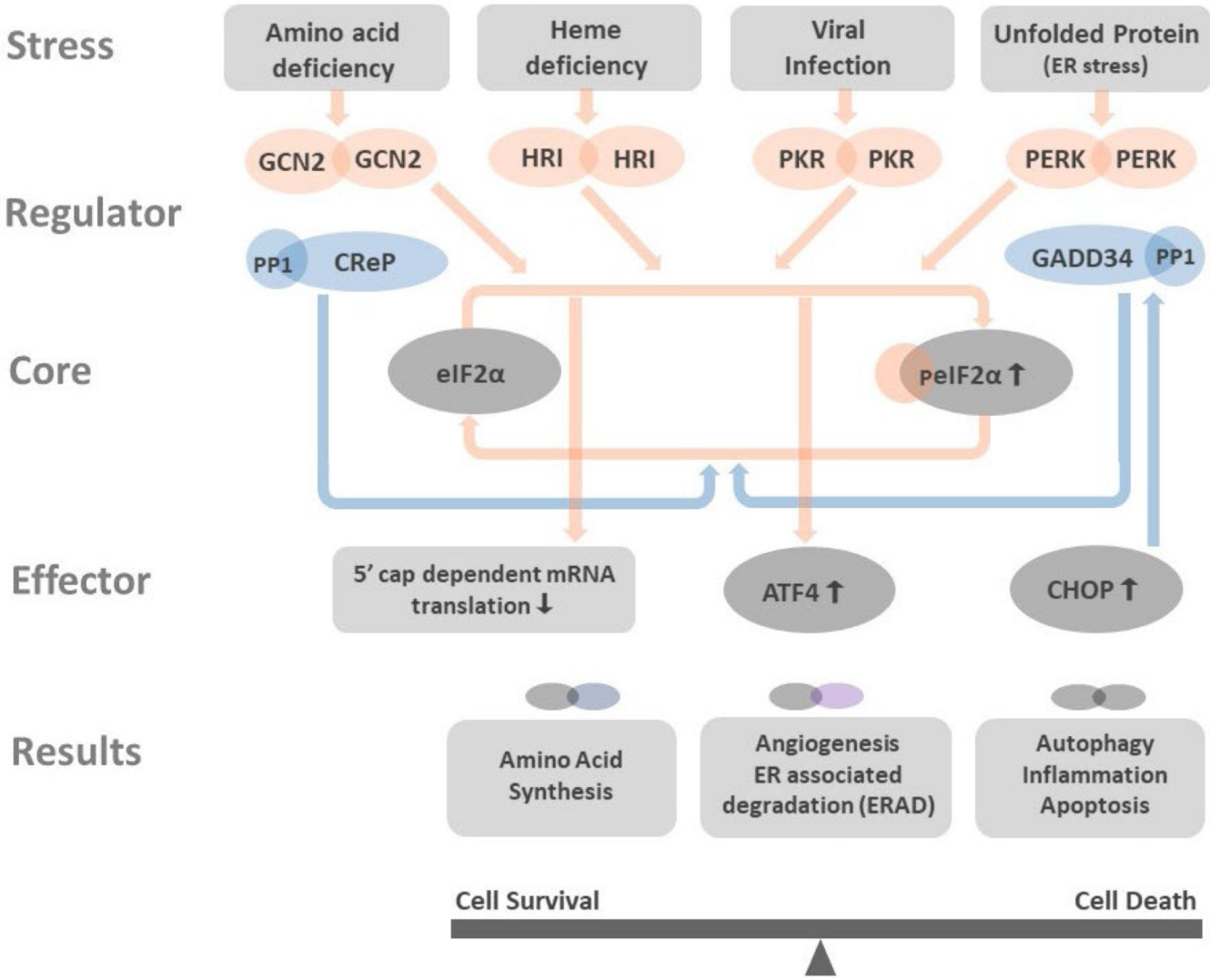

Figure 1: Integrated stress response affects cell functioning

Amino acid deficiency, heme deficiency, viral infection, and unfolded protein stress in the endoplasmic reticulum (ER stress) activate GCN2, HRI, PKR, and PERK. All these kinases phosphorylate eIF2α, the core member of ISR. Phosphorylated eIF2α (peIF2α) impedes the 5'cap-dependent mRNA translation (Figure 2 in details) and results in global reduction of protein synthesis. However, few genes as ATF4 and CHOP have alternative translation machinery and are less influenced by eIF2 dysfunction. The preferentially translated ATF4 is the key effector in ISR. ATF4 couples with another ATF4 or its interacting partners to form homo- and heterodimers that bind to DNA targets and control the expression of genes that participated in amino acid synthesis, angiogenesis, ERAD, autophagy, inflammation, and apoptosis. Depends on the disease context and the duration of ISR, the downstream processes of ISR can aid in cell survival or bring cell death. CHOP is one key factor that can be promoted by ATF4. CHOP not only activates autophagy, inflammation, and apoptosis, but also induces the expression of GADD34, a phosphatase coupled with PP1 to dephosphorylate peIF2α. The dephosphorylation of peIF2α terminates ISR and resumes

protein synthesis. At non-stressed cell, the CreP-PP1 complex phosphatase constantly operates for maintaining low level of peIF2α and protein homeostasis.

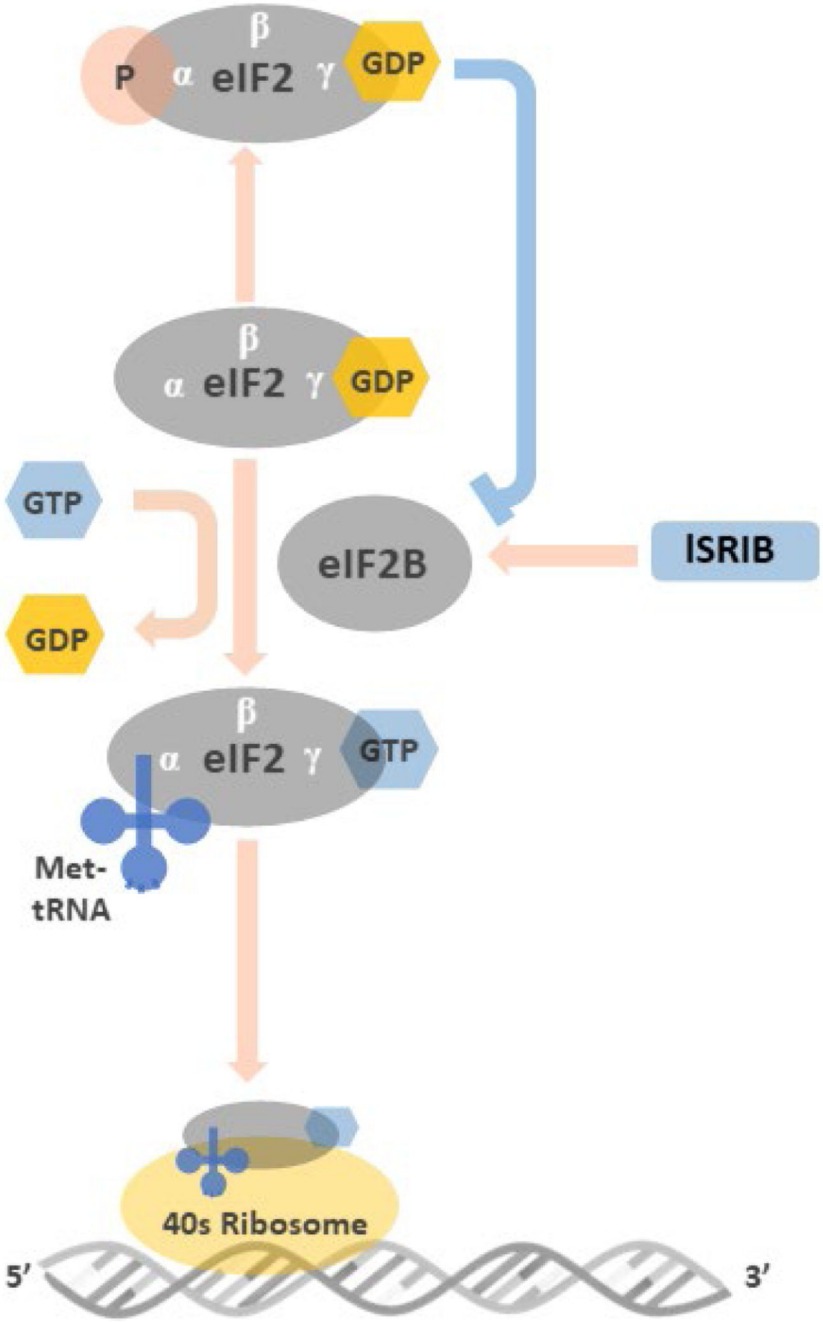

Figure 2: Illustration of the roles of eIF2 and peIF2 in the initiation of mRNA translation

Before binding to met-tRNA, the GDP bound to the γ subunit of eIF2 must be switched to GTP. This GDP-GTP exchange process is mediated by eIF2B. The met-tRNA-GTP-eIF2 ternary complex then delivers the met-tRNA to 40S ribosome, a key step for the initiation of mRNA translation. At the stressed condition, once the ISR is activated and the GDP-eIF2 is phosphorylated in its α subunit, the structural change makes GDP-peIF2 no longer a suitable substrate of eIF2B but transforms to an inhibitor of eIF2B. The inhibition of eIF2B by GDP-peIF2 further reduces the pool of functional GTP-eIF2 to bind met-tRNA, and lowers the

global protein synthesis via 5’cap-dependent mRNA translation. ISRIB rescues protein translation by stabilization and increasing eIF2B abundance that counteracts low level of GDP-peIF2α in treated cells.

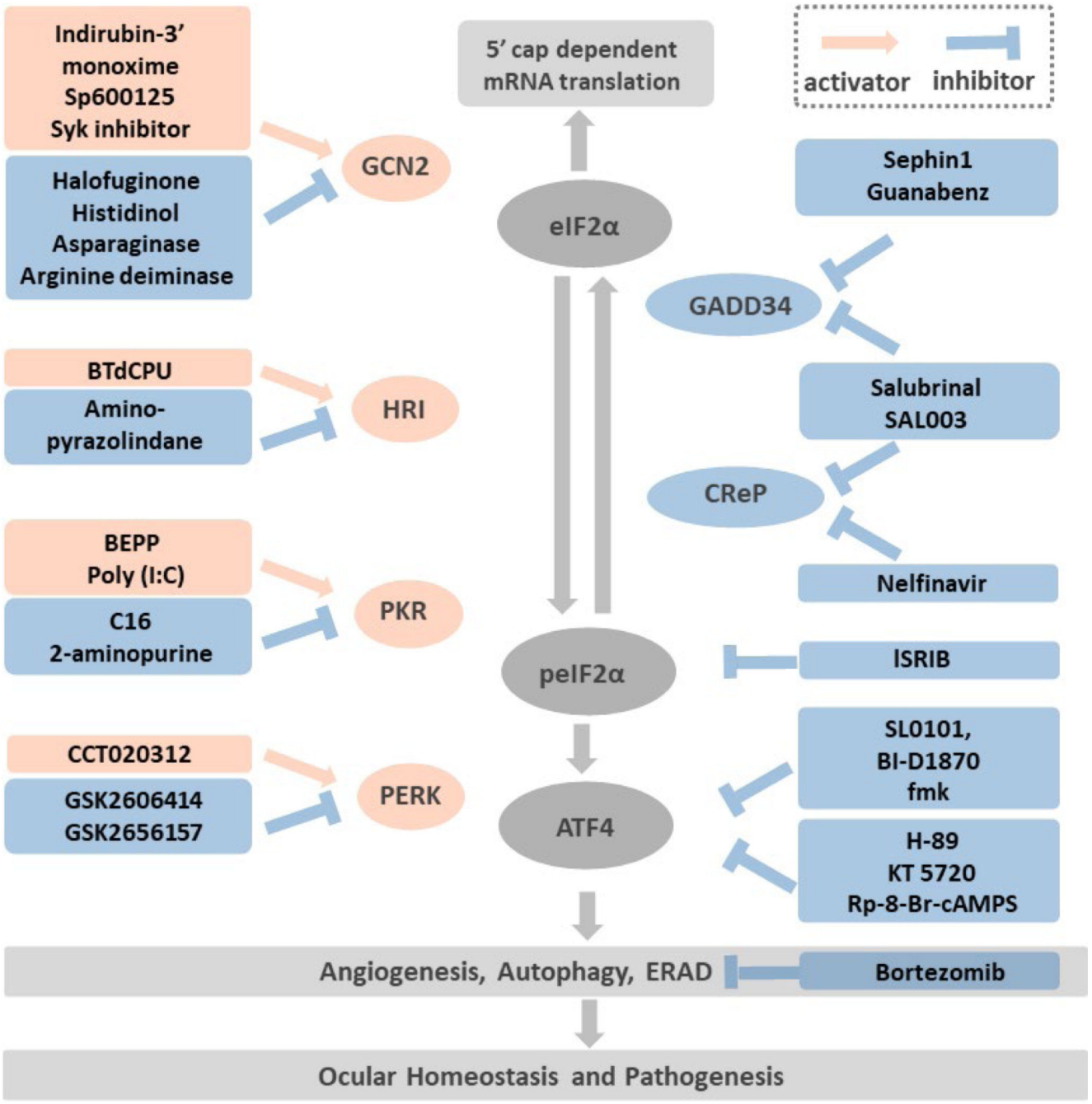

Figure 3: Targeting the druggable ISR pathways

Modulation of ISR can be achieved by: **(1)** Targeting the eIF2α kinases: the GCN2 activators include Indirubin-3' monoxime, Sp600125, Sky inhibitor; the GCN2 inhibitors consist of halofuginone, histidinol, asparaginase, arginine deiminase. The HRI activator BtdCPU and the inhibitor, amino-pyrazolindine. The PKR activators are BEPP and Poly (I:C). The PKR inhibitors are C16 and 2-aminopurine. The selective PERK activator is CCT020312 and the inhibitors are GSK2606414 and its more selective derivative GSK2656157. **(2)** Targeting the peIF2α phosphatase: Salubrinal and SAL003 inhibit both

the inducible phosphatase GADD34 and the constitutive expressed CReP. In addition, Sephin 1 and Guanabenz inactivate GADD34, while Nelfinavir inactivates CReP. **(3)** Antagonist to the peIF2α: as Figure 2 shows, peIF2α inhibits the eIF2B. ISRIB is hypothesized to stabilize eIF2B and offsets the peIF2α effects while the peIF2α concentration is low. **(4)** Inhibition of ATF4 post-translational phosphorylation: RSK2 and PKA are two kinases that phosphorylate ATF4 and increase its activity. The RSK2 inhibitors, SL0101, BI-D1870, fmk and the PKA inhibitors, H-89, KT 5720, Rp-8-Br-cAMPS are potential compounds that inhibit ATF4 phosphorylation. **(5)** Targeting pathways downstream of ATF4: among the cellular functions regulated by ATF4, angiogenesis, autophagy and ERAD played important roles in ocular homeostasis and pathogenesis such as dry eye, diabetic retinopathy, age-related macular degeneration. Bortezomib, a proteasome inhibitor impedes ERAD has been demonstrated with neuro-protective and anti-inflammatory effects *in vivo.*

Table 1:

Consequence of the genetic manipulation of ISR components in mice

Member	Model	Development	Phenotypes	Ref.
ATF4	CREB$^{-/-}$	Retarded	Defective lens development, and microphthalmia Anemia Dwarfisms and severe skeletal abnormalities	30, 59, 174, 176, 177
	CREB$^{+/-}$	Normal	No specific phenotype	
	CREB$^{-/-}$ and P53$^{-/-}$double KO	--	Normal eye morphology, lower number of lens fibers	
GCN2	GCN2$^{-/-}$	Normal	Developmental delay occurred if being fed with leucine deficit diet	178
HRI	HRI$^{-/-}$	Normal	Macrocytosis and hyperchromia	51
PKR	Pkr−/−	Normal	No specific phenotype	179
PERK	Perk$^{-/-}$	Retarded	Impaired insulin secretion and hyperglycemia; Growth retardation and abnormal bone development	180–183
	Perk$^{-/-}$	--	Acute diabetes	194
	Perk loxP/loxP & CamkIIα-Cre	--	Behavior change	184
GADD34	GADD34$^{-/-}$	Normal	No signs of disease or abnormal phenotypes at least the first 12 months of life	145
CReP	Ppp1r15b$^{-/-}$	Retarded	Half size, pale skin, impaired erythropoiesis, fail to survive in postnatal day 1	186
	Ppp1r15b$^{+/-}$	Normal	No specific phenotype	
EIF2α	Homozygous non-phosphorylatable KI (S51A)	Lethal	Severe hypoglycemia. Lethal within 24 hours of birth.	188
	Heterozygous non-phosphorylatable KI (S51A)	Normal	Glucose intolerance, increased weight gain if been fed with high fat diet, pancreatic β-cell failure.	189
	Conditional β-cell specific KO	--	Decreased insulin secretion, type 2 DM	190
CHOP	CHOP$^{-/-}$	Normal	Reduced performance in memory-related behaviors	192

KO: knock out; KI: knock in