The integrated stress response in pulmonary disease

Giulia Emanuelli[1,2,3], Nikou Nassehzadeh-Tabriz[1,3], Nick W. Morrell[2] and Stefan J. Marciniak[1,2]

Affiliations: [1]Cambridge Institute for Medical Research (CIMR), University of Cambridge, Cambridge, UK. [2]Division of Respiratory Medicine, Dept of Medicine, University of Cambridge, Cambridge, UK. [3]Equal first authors.

Correspondence: Stefan J. Marciniak, Cambridge Institute for Medical Research (CIMR), University of Cambridge, Cambridge CB2 0XY, UK. E-mail: sjm20@cam.ac.uk

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By integrating signals from multiple stressors, the integrated stress response plays key roles in the pathophysiology of lung disease. Understanding the mechanisms involved will identify novel means of therapy. https://bit.ly/2Bg2kj4

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ABSTRACT The respiratory tract and its resident immune cells face daily exposure to stress, both from without and from within. Inhaled pathogens, including severe acute respiratory syndrome coronavirus 2, and toxins from pollution trigger a cellular defence system that reduces protein synthesis to minimise viral replication or the accumulation of misfolded proteins. Simultaneously, a gene expression programme enhances antioxidant and protein folding machineries in the lung. Four kinases (PERK, PKR, GCN2 and HRI) sense a diverse range of stresses to trigger this "integrated stress response". Here we review recent advances identifying the integrated stress response as a critical pathway in the pathogenesis of pulmonary diseases, including pneumonias, thoracic malignancy, pulmonary fibrosis and pulmonary hypertension. Understanding the integrated stress response provides novel targets for the development of therapies.

The integrated stress response

The airway epithelium faces many stresses, both extrinsic, *e.g.* inhaled toxins, and intrinsic, *e.g.* protein misfolding [1, 2]. Stress-responsive pathways determine whether these stresses are withstood or if they cause disease. A variety of insults trigger the phosphorylation of eukaryotic initiation factor (eIF)2α [3]. Since eIF2α phosphorylation integrates signals from diverse sources, its downstream pathway has been named the "integrated stress response" (ISR) (figure 1). Four stress-sensing kinases trigger the ISR [4]: protein kinase R (PKR) responds to cytosolic double-stranded (ds)RNA [5]; PKR-like endoplasmic reticulum kinase (PERK) detects endoplasmic reticulum (ER) stress [6, 7]; heme-regulated inhibitor (HRI) responds to iron deficiency and oxidative stress [8]; while general control non-depressible (GCN)2) is activated during amino acid starvation [9, 10].

The eIF2 complex recruits methionyl-transfer (t)RNA to the ribosome at the beginning of protein synthesis. During cycles of translation initiation, eIF2 hydrolyses its bound GTP, which is replenished by the guanine nucleotide exchange factor eIF2β. However, when eIF2α is phosphorylated it binds avidly to eIF2β inhibiting further GTP exchange. As a result, protein synthesis is attenuated, although a subset of transcripts is translated more efficiently thanks to regulatory elements within their 5′-untranslated regions [11, 12]. The transcription factors ATF4 and CHOP are induced in this way by the ISR and upregulate hundreds of ISR target genes. One of these encodes PPP1R15A (also known as GADD34), which forms a

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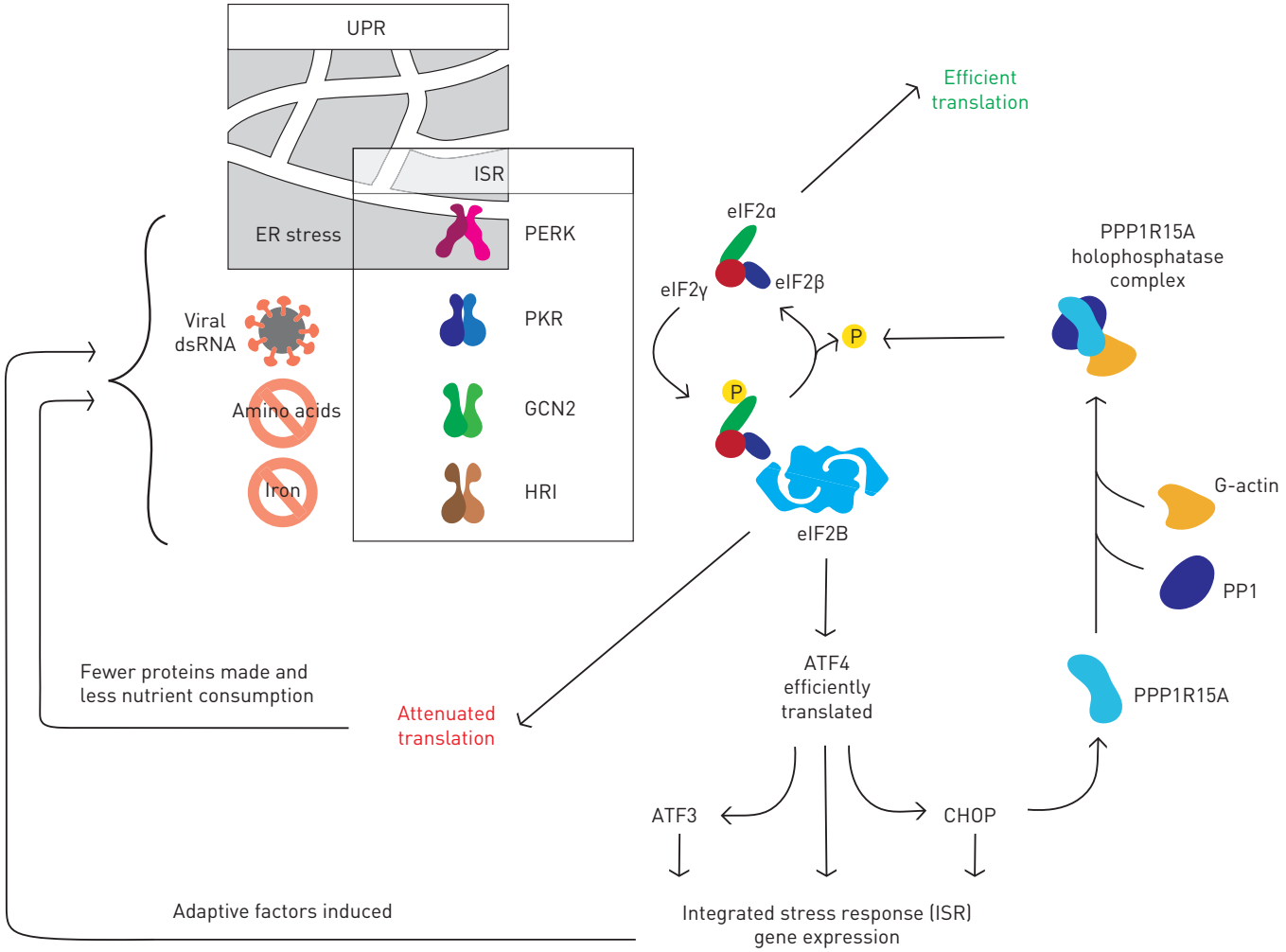

FIGURE 1 The integrated stress response (ISR) is triggered by stress-sensing kinases that phosphorylate eukaryotic initiation factor (eIF)2α, a component of the eIF2 translation initiation complex. Protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) responds to endoplasmic reticulum (ER) stress, and so the ISR overlaps with the unfolded protein response (UPR). PKR detects viral double-stranded (ds)RNA. General control non-depressible (GCN)2 is activated by amino acid deficiency. Heme-regulated inhibitor (HRI) responds to iron depletion. Phosphorylated eIF2α binds avidly to eIF2β to inhibit most translation, but some mRNAs including those encoding the transcription factors ATF4 and CHOP are translated more efficiently. The resulting gene expression restores homeostasis by enhancing oxidative protein folding in the ER; promoting amino-acyl transfer (t)RNA synthesis; and inducing antioxidant genes. PPP1R15A (also known as GADD34) is eventually induced and in complex with PP1 and G-actin dephosphorylates eIF2α to terminate the ISR.

complex with protein phosphatase (PP)1 and G-actin to generate an eIF2α-specific phosphatase that terminates the ISR [13]. This negative feedback mechanism allows the ISR to be activated transiently, but premature inactivation of the ISR can cause cell death [14, 15].

Infection

The ISR can be activated by viral infection. During replication of RNA viruses and complex DNA viruses, dsRNA can be detected by PKR [16]. In addition, enveloped viruses require high levels of membrane protein synthesis, which can trigger PERK [17]. Phosphorylation of eIF2α then antagonises viral protein synthesis while triggering ISR gene expression [1, 17]. Innate antiviral pathways induce interferon (IFN)-dependent genes including PKR, thus enhancing ISR activation [16]. Simultaneously, phosphorylation of eIF2α causes formation of stress granules [18], membraneless organelles formed by a liquid–liquid phase separation of ribonuclear proteins, which sequester viral factors, block viral gene expression and recruit viral sensors such as PKR [19]. The importance of the ISR in defending against viral infection is illustrated by the wide variety of evasion mechanisms that viruses have evolved to overcome its effects, including decoy dsRNAs, PKR degradation, viral RNA sequestration, blockade of PKR dimerisation, pseudosubstrates that compete for PKR's activity and even expression of a viral eIF2α phosphatase (table 1) [5, 20–44]. In addition, some viruses inhibit IFN signalling to prevent induction of PKR [46].

Influenza

Influenza A causes half a million deaths annually [47]. Two decades ago, it was discovered that influenza circumvents PKR by means of a nonstructural protein called NS1 [26] (table 1). NS1-deleted strains of influenza are enfeebled, but their virulence is restored in PKR-deficient cells [45]. NS1 was initially thought to enable influenza to evade PKR through activation of the co-chaperone $p58^{IPK}$ [48]. Indeed, $p58^{IPK}$ was named because of its putative role as a 58-kDa inhibitor of PKR [49]. However, subsequent work revealed that $p58^{IPK}$ resides exclusively in the ER lumen [50], while PKR is cytosolic, making a direct interaction impossible. By contrast, through its putative RNA-binding domain, cytosolic NS1 can bind to PKR, probably mediating its inhibition directly [45]. Supporting this, mutations of this NS1 domain restore PKR activation and stress granule formation, impairing influenza replication [51]. In addition, NS1 can inhibit PKR indirectly by promoting the expression of vtRNA [52], a form of long noncoding RNA found in ribonucleoprotein particles called vaults (vt) [53]. For example, vtRNA2-1 directly inhibits PKR activity [54]. Influenza A infection or isolated expression of NS1 enhances the production of vtRNAs in human and mouse cell lines [52]. By contrast, NS1-deleted viruses cause the production of little vtRNA, and depletion of host vtRNAs leads to PKR activation, which reduces influenza replication [52].

PKR activation may not always be beneficial. Different strains of influenza vary in their ability to activate or evade the ISR [55, 56]. Some highly pathogenic avian influenza viruses (*e.g.* H5N1, H7N7 and H7N9) induce severe pulmonary inflammation owing to excessive production of IFN-β, interleukin (IL)-6 and IL-8 [55]. This response appears to be mediated by PKR triggering a cascade that leads to phosphorylation of TRIM28, a regulator of many immunomodulatory genes. This raises the possibility that PKR-directed therapies might modify the immune response to highly pathogenic strains of influenza. The complex roles of PKR during inflammation are discussed in more detail later.

Coronaviruses

Coronaviruses primarily cause respiratory and enteric disease. Many human coronaviruses including hCoV-229E, OC43, NL63 and HKU-1 cause only mild respiratory symptoms [57], but the zoogenic

TABLE 1 Viral-mediated inhibition of protein kinase R (PKR) activity

	Virus(es)	Viral protein	Mode of PKR inhibition	References
ssRNA				
Orthomyxoviridae	Influenza A Influenza B	NS1	Direct interaction/dsRNA-mediated interaction	[26, 45]
Coronaviridae	Infectious bronchitis virus	NSp2	Inhibition of phosphorylation	[21]
	hCoV-229a	NSp15	dsRNA sequestration	[22]
	MERS-CoV	NS4a	dsRNA sequestration	[23, 27]
Bunyaviridae	Rift Valley fever virus	NSs	Proteasome-mediated degradation	[28]
Filoviridae	Ebolavirus	VP35	Unknown/possible dsRNA sequestration	[29]
Retroviridae	HIV	Tat	Pseudosubstrate/direct interaction	[9]
		TAR RNA	Decoy dsRNA	[10]
Flaviviridae	Hepatitis C virus	NS5a	Blocking of dimerisation/direct interaction	[32]
		E2	Pseudosubstrate/direct interaction	[33]
Reoviridae	Reoviruses	σ3/σ4	dsRNA sequestration	[34]
dsRNA				
Herpesviridae	Human cytomegalovirus	pTRS1/pIRS1	Interaction and relocalisation/dsRNA sequestration	[35]
dsDNA				
Herpesviridae	Herpes simplex virus	γ134.5	Dephosphorylation of eIF2α substrate	[24, 36, 37]
		US11	dsRNA sequestration/direct interaction	
		US3/UL13	Inhibition of activation	
	Kaposi's sarcoma herpes virus	vIRF2	Direct interaction and inhibition of phosphorylation	[38]
		vIRF3 (LANA2)	Inhibition of PKR-induced apoptosis	[39]
	Epstein–Barr virus	EBER RNAs	Decoy dsRNA	[40]
		SM	dsRNA sequestration/direct interaction	[41]
Poxiviridae	Vaccinia virus	E3L (p25/p20)	dsRNA sequestration/direct interaction	[42, 43]
		K3L	Pseudosubstrate/direct interaction	[44]
Adenoviridae	Adenovirus	VAI RNA	Decoy dsRNA/pseudoactivator	[40]

Summary of viral-encoded proteins that mediate evasion of PKR-mediated innate immune response. Viruses are classified by family and type of genome. MERS-CoV: Middle East respiratory syndrome-coronavirus; ss: single-stranded; ds: double-stranded; NSp: nonstructural protein; VP: viral protein; TAR: transactivation responsive; vIRF3: viral IRF3-like protein; EBER: Epstein–Barr virus encoded RNAs; VAI: adenovirus-associated RNA-I; eIF: eukaryotic initiation factor.

coronaviruses severe acute respiratory syndrome (SARS)-CoV-1, SARS-CoV-2 and Middle East respiratory syndrome (MERS)-CoV cause severe damage to the respiratory epithelium, which can be fatal [58]. The coronavirus genome encodes a number of structural proteins including spike, envelope, membrane, nucleoprotein and hemagglutinin-esterase [59]. The expression of virus-specific accessory proteins modulates activation of the ISR during infection [6], and being enveloped, coronaviruses impose significant stress on the ER, triggering PERK [21, 60–62]. Of note, although PERK is activated by expression of SARS-CoV-1 spike protein and accessory protein 3a [61, 62], rather than promoting an antiviral ISR, it has been suggested that the accompanying unfolded protein response (UPR) favours viral protein synthesis through upregulation of ER chaperones.

It was reported that during infection with SARS-CoV-1, both PKR and PERK are phosphorylated [63]. In a kinome-wide short interfering (si)RNA screen of 293T cells expressing the SARS-CoV receptor angiotensin-converting enzyme-2, both PKR and PERK were necessary for cells to restrict SARS-CoV-1 replication. However, others have found that, although activated, PKR was unable to reduce viral titres, suggesting that SARS-CoV-1 can evade PKR's antiviral activity [64]. It is unclear whether the net effect of PKR is to limit infection or to exacerbate deleterious inflammation *in vivo*. Recent reports describe how the β-coronavirus endoribonuclease nonstructural protein (nsp)15 interferes with host RNA sensing [22, 65, 66] (table 1). Mutations of nsp15 in mouse hepatitis virus and HCoV-229E enhance the phosphorylation of PKR and eIF2α in infected macrophages [22, 65]. This increases the induction of type I IFN and reduces viral titres. Consistent with this, deletion of nsp15 renders the virus unable to replicate in cells except those lacking PKR. Conversely, MERS-CoV inhibits the formation of stress granules in a manner dependent upon another viral protein ns4a [23] (table 1). Ns4a contains a dsRNA-binding motif and can antagonise the activation of PKR, perhaps by preventing dsRNA sensing [23, 27].

Infectious bronchitis virus (IBV), a γ-coronavirus causing respiratory disease in birds, suppresses the ISR to enable viral protein synthesis [21]. *In vitro*, IBV infection of ISR-deficient eIF2α^{AA} cells (in which a serine-51 to alanine mutation blocks phosphorylation) results in elevated viral replication confirming the importance of eIF2α phosphorylation for antiviral activity. During IBV infection, activation of PKR is suppressed by viral protein nsp2 (table 1). However, nsp2 is only a partial inhibitor of PKR and so, although it prevents a marked reduction of protein synthesis, there is sufficient residual ISR signalling to permit PPP1R15A expression, which then supports protein synthesis and therefore IBV replication. Similarly, depletion of CHOP, which induces PPP1R15A, suppresses viral replication [60]. Disappointingly, the putative PPP1R15A inhibitor, salubrinal, failed to block IBV replication. However, we have shown that salubrinal has little effect on PPP1R15A and most likely antagonises the UPR through other mechanisms [67] (table 2).

Other respiratory viruses

Infection with respiratory syncytial virus (RSV) triggers formation of stress granules [100]. These are maintained throughout the infection *via* PKR-mediated eIF2α phosphorylation, and contain viral RNA. Similarly, human enterovirus (EV)-D68, which causes severe epidemic respiratory disease, triggers stress granules to form, although these are only transient and wane within 14 h [101]. This disaggregation of stress granules correlates with increased virus production and, since PKR remains active, appears to be mediated by cleavage of the stress granule protein G3BP1 by virally encoded factors.

Porcine reproductive and respiratory syndrome virus (PRRSV, also known as beta-arterivirus suid 1) infects pigs and inflicts a great economic strain on the farming industry, but frequent antigenic variations have hampered the development of a vaccine [102–105]. PRRSV primarily infects alveolar macrophages where it inhibits PKR to enable its own replication [102]. However, early pharmacological activation of PKR with polyinosinic-polycytidylic acid can block viral replication (table 2). Interestingly, UPR signalling by PERK and IRE1α appears to be more important than PKR in promoting stress granule formation during PRRSV infection [104, 105], but PERK may also play a role in severe pneumonias caused by co-infection with PRRSV and bacteria, since the resulting eIF2α phosphorylation inhibits IFN and tumour necrosis factor (TNF) production. Curiously, ISR activation during PRRSV infection might increase viral titres [103]. ATF4 is held in the cytosol by the viral proteins nsp2 and nsp3, preventing its migration to the nucleus. While impairing normal ISR signalling, this mislocalisation of ATF4 to sites of viral replication also benefits the virus through a poorly understood protein–RNA interaction.

Bacterial infection

We identified a role for the ISR during infections with *Pseudomonas aeruginosa* [106]. This opportunistic bacterium, which can cause morbidity in patients with bronchiectasis, secretes several virulence factors, among which pyocyanin and AprA can trigger the UPR and ISR [106]. Exposure of human bronchial epithelial cells to such factors caused a rapid induction of PPP1R15A, which protected against

TABLE 2 Integrated stress response (ISR)-modifying drugs

	Drug	Putative mode of action	Cautions	References
ISR inhibitors				
PERK	GSK2656157	Targets ATP binding site of PERK	Inhibits RIPK1	[68, 69]
	GSK2606414	Targets ATP binding site of PERK	Inhibits RIPK1 and PKR Weakly activates GCN2	[69, 70]
	4-PBA	Reduces ER stress by unclear mechanism	Affects all arms of the UPR	[71]
	TUDCA	Reduces ER stress by unclear mechanism	Affects all arms of the UPR	[71]
HRI	Aminopyrazolindane		Not commercially available	[72]
PKR	C16	Targets ATP binding site of PKR		[73]
	C22	Targets ATP binding site of PKR		[73]
	2-Aminopurine	Targets ATP binding site of PKR		[74]
GCN2	6D	Targets ATP binding site of GCN2	Not commercially available	[75]
	6E (aka GCN2iA)	Targets ATP binding site of GCN2	Not commercially available	[75, 76]
eIF2β	ISRIB	Stablises eIF2β dimers	Cell lines can acquire ISRIB resistance mutations	[77–79]
	Dibenzoylmethane	Cells insensitive to p-eIF2α	Mechanism of action unclear	[80]
	Trazodone	Cells insensitive to p-eIF2α	Mechanism of action unclear	[80]
ISR activators				
PERK	CCT020312	Enhances PERK activation	Mechanism of action unclear	[81]
	Tunicamycin	Induces ER stress: inhibits N-glycosylation	Activates all arms of the UPR	[82]
	Bortezomib	Induces ER stress: inhibits the proteasome	Pleotropic effects of proteasome inhibition	[83]
	Montelukast	Enhances PERK signalling Mechanism unclear	Leukotriene receptor antagonist	[84]
HRI	BTdCPU			[85]
	cHAUs			[86]
PKR	Interferon	Increases expression of PKR	Pleotropic effects of interferon signalling	[87]
	poly I:C	RNA mimetic	Requires transfection to enter cell	[88]
	BEPP	Mechanism of action unclear		[89]
GCN2	Histidinol	Inhibits histidinyl-tRNA synthetase		[90]
	Tryptophanol	Inhibits tryptophan-tRNA synthetase		[91]
	Halofuginone	Inhibits prolyl-tRNA synthetase		[92]
	L-asparaginase	Depletes extracellular asparagine		[93]
PPP1R15A	Salubrinal	Putative PPP1R15 inhibitor	Concerns that effects may be PPP1R15 independent	[67, 94]
	Guanabenz	Putative PPP1R15 inhibitor	Concerns that effects may be PPP1R15 independent	[67, 95, 96]
	Sephrin1	Putative PPP1R15 inhibitor	Concerns that effects may be PPP1R15 independent	[67, 97, 98]
PPP1R15A and B	Jasplakinolide	Depletes G-actin required for PPP1R15 function	Pleotropic effects of actin stabilisation	[99]

PERK: protein kinase R (PKR)-like endoplasmic reticulum kinase; HRI: heme-regulated inhibitor; GCN: general control nondepressible; eIF: eukaryotic initiation factor; 4-PBA: 4-phenylbutyric acid; ER: endoplasmic reticulum; UPR: unfolded protein response; TUDCA: tauroursodeoxycholic acid; C16: CAS 608512-97-6 [6,8-dihydro-8-(1H-imidazol-5-ylmethylene)-7H-pyrrolo[2,3-g]benzothiazol-7-one]; C22: CAS 852547-30-9 (5-chloro-3-[(3,5-dichloro-4-hydroxyphenyl)methylidene]-2,3-dihydro-1H-indol-2-one); ISRIB: integrated stress response inhibitor [trans-2-(4-chlorophenoxy)-N-(4-(2-(4-chlorophenoxy)acetylamino)cyclohexyl)acetamide]; CCT020312: [6-bromo-3-[5-(4-bromo-phenyl)-1-(3-diethylamino-propionyl)-4,5-dihydro-1H-pyrazol-3-yl]-4-phenyl-1H-quinolin-2-one]; cHAUs: [1-([1,4-trans)-4-arylox-ycyclohexyl)-3-arylureas]; polyI:C: polyinosinic-polycytidylic acid; BEPP: [1H-benzimidazole-1-ethanol, 2,3-dihydro-2-imino-α-(phenoxymethyl)-3-(phenylmethyl)-monohydrochloride].

Pseudomonas-induced cytotoxicity. Surprisingly, the upstream eIF2α kinase involved proved to be the iron sensor, HRI. Iron availability is necessary for bacterial growth and its availability is limited by the host as a means to protect against bacterial infection [107]. It appears that in the airway, further depletion of iron from the microenvironment by *P. aeruginosa* is sensed by HRI to trigger a cytoprotective PPP1R15A response. Other studies have confirmed the phosphorylation of eIF2α during the exposure of human lung epithelial cells to pyocyanin [108, 109]. It has also been suggested that this *P. aeruginosa*-mediated activation of the ISR triggers a cytoprotective autophagic response [108].

Interventions against infection

The innate antiviral role of the ISR makes it an attractive target for the development of antiviral therapies. In a repurposing-screen of drugs approved for human use, an anti-asthma medication, montelukast, was found to inhibit influenza A replication (table 2) [84]. It appeared to exert a mild antiviral effect by enhancing PERK signalling. Accordingly, high concentrations of guanabenz, a putative inhibitor of eIF2α phosphatases, impaired viral protein synthesis [84] and a derivative of guanabenz called sephin1 blocked replication of RSV and EV-D68, but not influenza A in human cells [110]. However, the results could not be replicated *in vivo* owing to the toxicity of the high concentrations of sephin1 required, and there remains doubt as to whether the effects of guanabenz or sephin1 can be attributed to inhibition of PPP1R15A (table 2) [67, 97].

A challenge to the development of vaccines that impart cell-mediated immunity in the lung is the short lifespan of airway-resident memory T (T_{RM})-cells [111]. Unlike the long-lived T_{RM} cells of other tissues, airway T_{RM} cells are almost completely lost through apoptosis by 180 days following exposure to influenza or Sendai viruses [111]. For durable immunity, it is therefore important to understand what accounts for this short survival. Transcriptomic analysis revealed GCN2-dependent activation of the ISR as a unique signature in airway-resident T_{RM} [111]. This appears to be a function of the nutrient-poor microenvironment of the airway but is reversible, since airway T_{RM} cells cultured *ex vivo* in amino acid sufficient conditions are rescued. In theory, antagonism of GCN2 might prolong airway T_{RM} cells, but the consequences of this for immunopathology and inflammation are unclear. The abnormal pulmonary vascular phenotypes of GCN2-deficient individuals, may serve as a warning in this regard (discussed later).

Inflammation

The airway epithelium has a role in regulating the immune microenvironment and the ISR seems to be important in this. Toll-like receptors (TLRs) enable bronchial epithelial cells to sense pathogen associated molecular patterns (PAMPS) such lipopolysaccharide (LPS), a component of bacterial cell walls (figure 2). TLR activation induces PKR *via* IFNs as part of the inflammatory response, but other ISR factors including ATF3 and PPP1R15A are upregulated through less well understood mechanisms [112].

Early work showed that intense PERK activation can activate NFκB *in vitro* by inhibiting the synthesis of IκB, a short-lived negative-regulator of NFκB [113]. However, it is unclear if sufficiently high levels of phosphorylated eIF2α are achieved *in vivo* to induce this mechanism in response to ER stress. Nevertheless, PERK activation seems to enhance inflammatory signalling *in vivo* in response to PAMPs. For example, in bronchial epithelial cells treated with LPS, PERK activation increases proinflammatory IL-6 and IL-8 production *via* increased p38 and ERK signalling [114]. Furthermore, CHOP can dimerise with other members of the C/EBP transcription factor family to regulate the transcription of cytokines including IL-6 [115, 116]. Translational regulation of IκBα might nonetheless be important in activated macrophages and dendritic cells, where induction of indoleamine 2,3-dioxygenase, an enzyme that metabolises tryptophan, can trigger GCN2 and the ISR *via* amino acid sensing [117]. This enhances the secretion of IL-6 in response to LPS and is mediated, at least in part, by inhibition of IκB synthesis (figure 2).

LPS administration increases PKR phosphorylation in mouse lungs [118, 119]. In murine alveolar macrophages, PKR is critical in TLR-stimulated cytokine secretion and appears to contribute to lung damage in the context of sepsis [120]. A variety of PAMPS transactivate NOD-like receptor (NLR) inflammasomes [121]. During bacterial infection, complement activation generates a C5a fragment, which is chemotactic for neutrophils and enhances their capacity to phagocytose and kill bacteria [122]. One function of C5a appears to be the stimulation of PKR, which activates the NLRP3 inflammasome through direct interaction, promoting the release of HMGB1 (high mobility group box 1) (figure 2) [123, 124]. Conversely, HMGB1, which is a DNA binding protein with inflammatory cytokine activity, can itself activate PKR during macrophage M1 polarisation in acute lung injury, suggesting the possibility of a positive feedback loop [125]. Inhibition of PKR with C16 opposes both NLRP3 activation and HMGB1 release from LPS-challenged macrophages (table 2). Consequently, administration of C16 to mice prior to LPS challenge, inhibits PKR activation and so reduces the concentration of pro-inflammatory cytokines in lung lavage, limiting pulmonary damage [118, 119].

Barotrauma caused by mechanical ventilation can also induce pulmonary inflammation and acute lung injury [126]. In cell models of alveolar epithelial stretch, or if the lung is hyperinflated mechanically *in vivo*, PERK-dependent ISR signalling is triggered, possibly *via* stretch-induced release of calcium from the ER. This may mediate some of the toxicity of barotrauma, since pharmacological inhibition of PERK limits alveolar permeability and production of IL-8 during acute inflammation induced by cyclic strain [126, 127].

In the bronchoalveolar lavage fluid of asthmatic patients the levels of CHOP and the ER chaperone BiP are higher than healthy controls [128]. Ovalbumin (OVA) and LPS administration produces an asthma-like

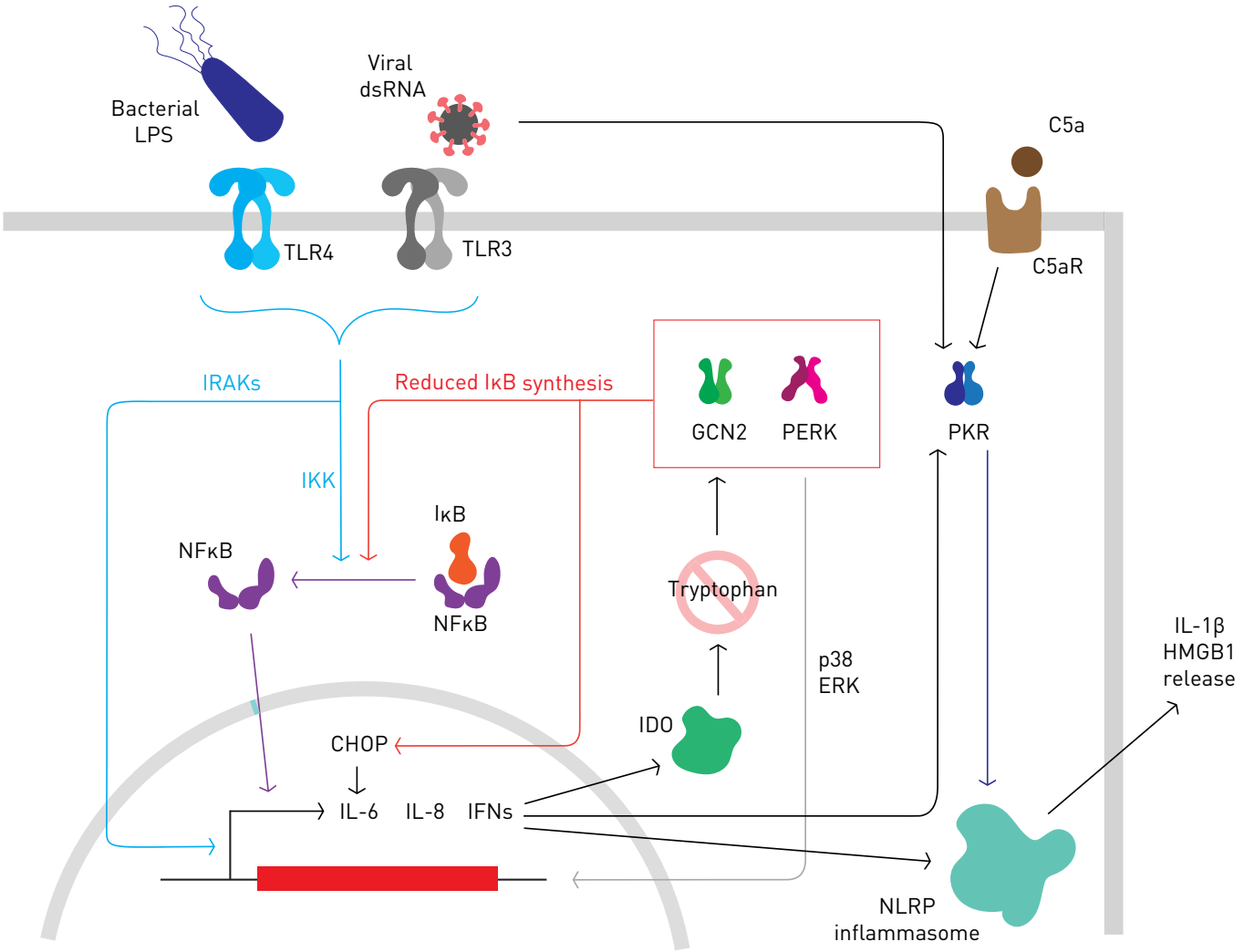

FIGURE 2 The integrated stress response and inflammation. Pathogen-associated molecular patterns (PAMPs) recognised by Toll-like receptors (TLRs) can trigger the innate immune response. Phosphorylation of IκB by IKK promotes its destruction and releases nuclear factor (NF)-κB to transactive pro-inflammatory genes including interleukin (IL)-6, IL-8 and interferons (IFNs). Induction of indoleamine 2,3-dioxygenase (IDO) depletes cells of tryptophan (at least activated macrophages and dendritic cells) to activate general control nondepressible (GCN)2. Activation of GCN2 or protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) can trigger NFκB signalling by reducing the synthesis of IκB, while CHOP increases IL-6 expression directly. Some evidence suggests PERK-mediated p38/ERK signalling might also contribute to inflammatory gene expression. PKR is induced by IFN and further activated if viral double-stranded (ds)RNA is present in the cytoplasm. Inflammatory mediators including C5a fragment can activate PKR, *e.g. via* C5a receptor (C5aR) signalling. PKR contributes to inflammation by stimulating the NLRP3 inflammasome to generate pro-inflammatory mediators including IL-1β and high mobility group box (HMGB)1.

state in murine models in which lungs show evidence of ER stress with induction of the same proteins as seen in patients. OVA/LPS-sensitised mice, treated with 4-PBA, which ameliorates ER stress *via* poorly understood mechanisms, show less IκB degradation therefore reduced NFκB signalling (table 2). This also results in attenuated inflammatory signals such as IL-10 expression and reduces infiltration of neutrophils and dendritic cells into the lung [128, 129].

In other situations, the ISR and pro-inflammatory signalling pathways appear to be antagonistic, suggesting some of these effects might be tissue specific. For example, in the gut, eIF2α phosphorylation caused by GCN2 during acute amino acid starvation suppresses intestinal inflammation [130]. Conversely, TLR4 activation appears able to stimulate eIF2β to antagonise the inhibition of translation caused by eIF2α phosphorylation, thus suppressing the translation of ATF4 and CHOP [131, 132].

Smoke and exhaust fumes

Inhaled pollutants can trigger the UPR and ISR [133–138]. The resulting cytotoxicity is thought to impair epithelial integrity and lead to COPD [139]. The particulate matter found in diesel exhaust induces ER stress in mouse lung tissue [140] and increases the expression of CHOP and PPP1R15A in human airway

epithelium [137]. This response appears to be accentuated in patients with COPD [138] and is even more marked if cells are co-exposed to bacterial components of nontypeable *Haemophilus influenzae* [141].

Lungs from patients with COPD have elevated levels of ISR markers [142]. Rats and mice chronically exposed to cigarette smoke also express increased levels of ISR markers, although there appears to be species-dependent differences with few genes modulated in a concordant manner between mice exposed to smoke and the lungs of humans with COPD [140, 143, 144]. It has long been known that primary bronchial epithelial cells exposed to cigarette smoke display a transient PERK-dependent phosphorylation of eIF2α followed by induction of ATF4 and PPP1R15A [134]. Indeed, PERK-dependent eIF2α phosphorylation is a consistent feature of cells exposed to cigarette smoke or smoke extract and at least some of the cytotoxicity is mediated by ER stress-induced cell death [135, 145]. For example, the aldehyde acrolein found in smoke induces ER stress-mediated cell death in A549 cells through multiple pathways including perturbed ER calcium homeostasis [146]. Acrolein is highly oxidising, as are numerous components of smoke, and this impairs the function of many macromolecules in cultured airway epithelial cells [147, 148] and in the lungs of humans and mice [149, 150]. Oxidative stress appears to be a key trigger for ISR activation by smoke, since treatment with the antioxidant N-acetylcysteine attenuates ISR induction [133, 135, 136, 151]. Cultured cells or mice treated with acrolein induce PPP1R15A, which appears to be a toxic phenomenon since *Ppp1r15a*$^{-/-}$ mice are more resistant to cell death and have better preserved lung architectures [152]. This is reminiscent of the increased tolerance of *Ppp1r15a*$^{-/-}$ mice to ER stress [14]. Interestingly, salubrinal, which increases eIF2α phosphorylation through poorly understood mechanisms also protects cells from smoke (table 2) [153].

As cigarette smoke is a potent oxidative stress, induction of the ISR is likely to be a protective response because ATF4 induces many antioxidant genes [1, 9]. It has been suggested that NRF2, another regulator of the antioxidant response, and ATF4 heterodimerise to regulate the expression of target genes [154, 155]. NRF2 can also induce ATF4 to amplify this response [156, 157]. It should be noted that although early reports suggested that PERK could activate NRF2 directly [158, 159], it was subsequently shown that PERK-dependent antioxidant gene expression is entirely determined by phosphorylation of eIF2α [160]. Interestingly, differences in the vigour of these antioxidant responses might contribute to individual differences in the susceptibility to smoke, since an ATF4 target gene signature distinguish the airway of smokers with COPD compared to smokers without COPD [161].

Pulmonary hypertension

Pulmonary arterial hypertension (PAH) is a rare, fatal condition primarily affecting young adults. It involves vascular remodelling causing increased pulmonary vascular resistance and right-sided heart failure [162]. In 70% of familial cases and 20% of sporadic cases, heterozygous germ line mutations are identified in the gene encoding the bone morphogenetic protein type 2 receptor (*BMPR2*). However, these mutations are variably penetrant, suggesting that additional modifying factors exist. Two subtypes of PAH, pulmonary veno-occlusive disease (PVOD) and pulmonary capillary haemangiomatosis (PCH) were recently shown to be caused by biallelic mutations of *EIF2AK4*, which encodes GCN2 (figure 3) [163–165]. The PVOD/PCH subtypes have an even worse prognosis than classical PAH and currently have no effective treatments apart from lung transplantation [166]. In families affected by PVOD, mutant alleles of *EIF2AK4* segregate with the disease in a recessive manner, while in the general population such null alleles are vanishingly rare [163]. Remarkably, when histologically proven nonfamilial cases of PVOD were examined, 25% were also found to have pathogenic mutations of *EIF2AK4* [163]. Subsequently, biallelic mutations of *EIF2AK4* have been observed in some patients with classical PAH, suggesting that GCN2 may play a broader role in pulmonary vascular disease [167–171]. Individuals with PAH and biallelic mutations of *EIF2AK4* tend to be younger (aged <50 years) with lower transfer coefficients of the lung for carbon monoxide (<50%) and normal spirometry, leading some to suggest that *EIF2AK4* gene testing may be useful in the diagnosis and management of PAH [170, 172].

GCN2 is normally detectable in pulmonary vessel smooth muscle, interstitial tissue and macrophages, but is absent from individuals homozygous for loss-of-function alleles of *EIF2AK4* [163]. Interestingly, despite the loss of this kinase, lung tissue from patients with PVOD showed increased immunostaining for the downstream ISR targets CHOP and heme oxygenase (HO)-1, which are said to be absent from normal lung and the lungs of patients with classical PAH [173]. CHOP was increased in endothelial cells while HO-1 was found in the capillaries of haemangiomatosis foci. The mechanism by which loss of GCN2 enhances CHOP expression requires elucidation.

There is a suggestion of genotype–phenotype correlation in PVOD [174]. Individuals with PVOD due to mutations of *EIF2AK4* appear to develop more severe intimal fibrosis and less severe medial hypertrophy of the pulmonary arteries. They show greater smooth muscle hyperplasia of interlobular septal veins compared with individuals without the mutation and have more foci of PCH, rather than the more diffuse haemangiomatous changes seen in mutation-negative cases.

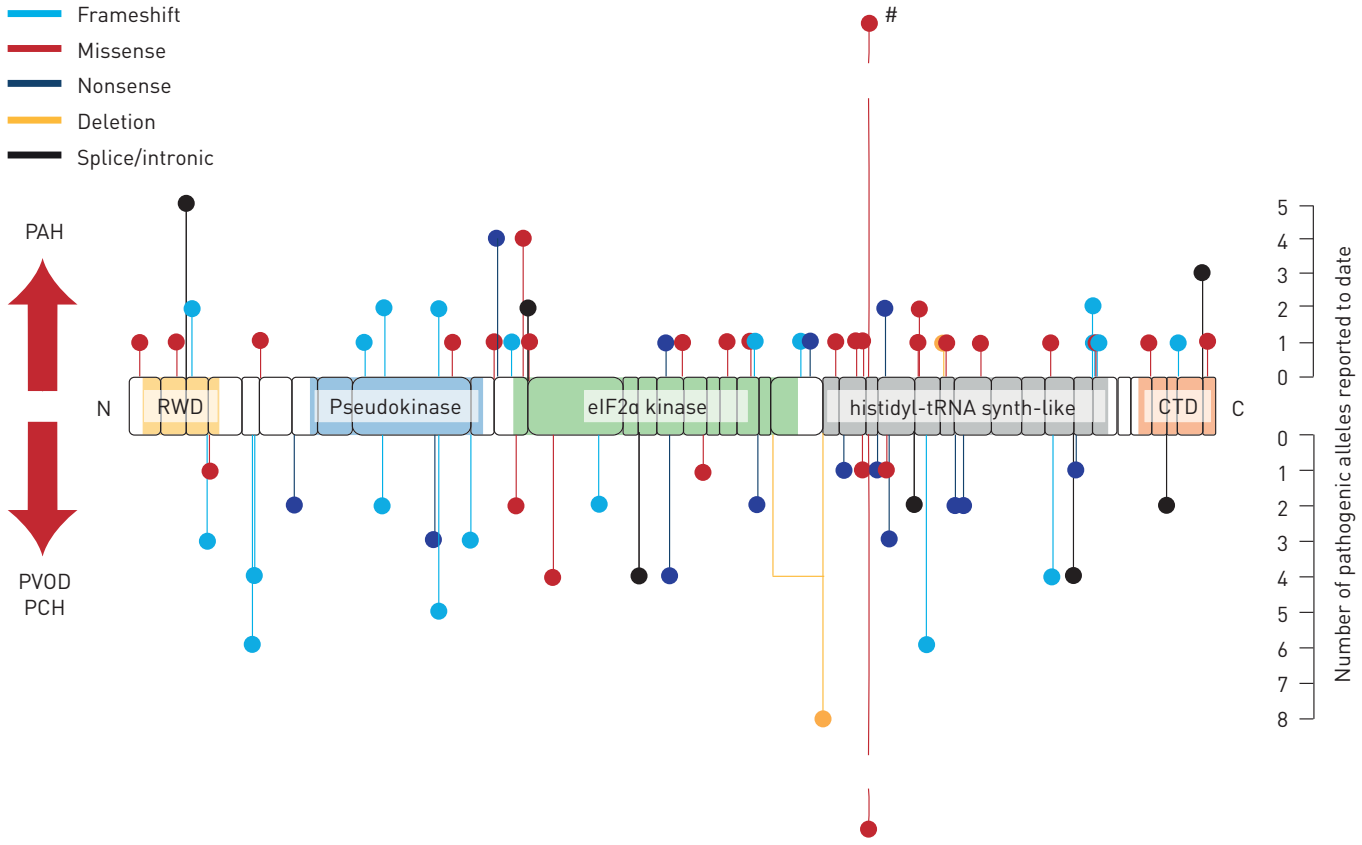

FIGURE 3 Variants of *EIF2AK4* associated with pulmonary vascular disease. Schematic representation of the general control nondepressible (GCN) 2 protein and its domains; boxes correspond to the 39 exons in *EIF2AK4*. Domains are highlighted: RWD (RING-finger proteins, WD repeat-containing proteins, yeast DEAD-like helicase), pseudokinase, eukaryotic initiation factor (eIF)2α kinase, histidyl-tRNA synthetase-like and carboxy-terminal domain (CTD). Predicted pathogenic variants are shown as lollipops: above the protein are likely pathogenic variants associated with pulmonary arterial hypertension (PAH), below are likely pathogenic variants associated with pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary haemangiomatosis (PCH). The lollipop length indicates the approximate number of such alleles reported in the literate allowing for incomplete reporting. Note, c.3344C>T (p.P1115L) in exon 23 in at the histidyl-tRNA synthetase-like domain has been reported in five families affected by PAH or PVOD (#). Potentially, 48 alleles have been described, but this may be confounded by overlaps between published reports.

Remarkably, while GCN2 protein is absent in those with biallelic *EIF2AK4* mutations, even patients with PVOD and wildtype *EIF2AK4* show reduced GCN2 expression. Moreover, patients with classical PAH can have reduced GCN2 protein expression suggesting that GCN2 might be relevant to many forms of pulmonary hypertension. Intriguingly, mitomycin C, which is a DNA alkylating agent that can induce PVOD in humans and rats [175], has been shown to reduce the expression of GCN2 in lung tissue [175]. This is associated with reduced phosphorylation of SMAD1/5/8, downstream mediators of BMP signalling, while depletion of GCN2 in pulmonary arterial endothelial cells also reduces the phosphorylation of SMAD1/5/9 in rats and human pulmonary arterial endothelial cells [173]. However, it has yet to be shown that restoration of GCN2 expression can rescue PVOD caused by mitomycin C.

The mechanistic links between loss of GCN2 and defects of the pulmonary vasculature are unclear, although we demonstrated recently that GCN2 expression can inhibit BMP signalling during development in *Drosophila*, in part by regulating the synthesis of components of the BMP signalling pathway [176]. Conversely, loss of GCN2 appears to impair the more complex BMP signalling found in mammals [173]. This was demonstrated both in *Eif2ak4*$^{-/-}$ rats and in pulmonary artery endothelial cells depleted of GCN2 by siRNA. Such an interaction between the ISR and BMP signalling might contribute to the incomplete penetrance of pathogenic *BMPR2* mutations. This is supported by observations in a family with autosomal dominant PAH caused by a *BMPR2* mutation, in which all living family members with diagnosed PAH also carried an *EIF2AK4* mutation [167].

The relationship between the ISR and cell survival may be relevant to its role in pulmonary hypertension. Dysregulated pulmonary artery endothelial cell proliferation and apoptosis are believed to be important factors in the development of PAH [177]. It has been proposed that the initial apoptosis of pulmonary endothelial cells is followed by hyperproliferation of apoptosis-resistant cells with increased expression of

anti-apoptotic factors including survivin [178]. Survivin is highly expressed in the pulmonary arteries of patients with PAH and in the monocrotaline-induced PAH rat model [179]. When a dominant negative mutant of survivin was expressed in monocrotaline-treated rats, established PAH was reversed resulting in prolonged survival [179]. In human pulmonary arterial endothelial cells, depletion of GCN2 by siRNA enhanced the proliferation and increased the expression of survivin, while inhibition of GCN2 with compound 2662034 also increased endothelial cell proliferation (table 2) [173]. This effect of GCN2 inhibition could be antagonised by exogenous BMP-9, which is known to reverse PAH in animal models [173, 180].

Inflammation is a recognised feature of PAH with increased circulating levels of IL-6, IL-8, IL-10 and IL-12p70 correlating with worse survival [181]. Inflammatory signalling appears to modify the penetrance of pathogenic alleles of *BMPR2* in humans and the TLR4 ligand LPS worsens PAH in *Bmpr2* mutant mice [182]. The ISR may play a role in the sensitisation of BMP-signalling-deficient animals to inflammation [183]. When pulmonary artery endothelial cells are treated with TNF, they produce the potent pro-inflammatory chemokine granulocyte–macrophage colony-stimulating factor (GM-CSF). Treatment with BMP2 suppresses GM-CSF production and so deficiency of BMPR2 results in enhanced TNF-mediated GM-CSF secretion. It appears that loss of BMPR2 activates $p38^{MAPK}$ to increase expression of PPP1R15A. The resultant dephosphorylation of eIF2α enhances translation of *GM-CSF* mRNA [183]. This suggests that ISR signalling normally limits inflammatory cytokine production in the pulmonary vasculature, and might explain why loss of an ISR kinase worsens PAH through enhanced translation of pro-inflammatory cytokines.

It appears that the ISR plays a role both to limit pulmonary endothelial cell proliferation and inflammatory cytokine production, while sharing a mutually regulating relationship with BMP signalling. It is therefore possible that ISR-directed therapies might have value in more common forms of PAH, not only in rarer genetic varieties.

Pulmonary fibrosis

The aetiology of pulmonary fibrosis is complex and is likely to involve many different triggers, including the ageing process. Prolonged inflammation or the expression of misfolded mutants of surfactant proteins can also lead to pulmonary fibrosis [2]. It has been noted that treating mice with tunicamycin, a toxin that causes ER stress, promotes lung fibrosis and causes mitochondrial dysfunction in primary type II alveolar epithelial cells (table 2) [82]. This is noteworthy because the type II alveolar epithelial cells from patients with idiopathic pulmonary fibrosis (IPF) also accumulate dysmorphic and dysfunctional mitochondria, as do other ageing animals. The integrity of a cell's mitochondria is maintained through the action of PINK1, the expression of which falls intriguingly with age and during ER stress. It is also downregulated in the lungs of patients with pulmonary fibrosis. PINK1-deficient mice are more susceptible to lung fibrosis induced by intratracheal instillation of bleomycin [82]. This drug drives epithelial to mesenchymal transition, fibroblast proliferation and subsequent extracellular matrix deposition in the lungs [184]. The repression of PINK1 seen during ER stress appears to be dependent upon ATF3, an ISR transcription factor, which binds directly to the PINK1 promoter [185]. Although circumstantial, it is interesting that the lungs of individuals with IPF show higher levels of ATF3 compared to age-matched controls [82]. In some stressful conditions, ATF3 is thought to contribute to CHOP expression [186]. CHOP, is also elevated in the lungs of patients with IPF and bleomycin-exposed mice, especially in areas affected by fibrosis that notably are also markedly hypoxic [187]. CHOP seems to play a crucial role in the development of these lesions since its deletion protects mice from fibrosis. Hypoxia can induce CHOP *via* ER stress and the UPR. Moreover, hypoxia (inspiratory oxygen fraction 14%) can worsen bleomycin-induced fibrosis, yet this is abrogated in $Chop^{-/-}$ mice. CHOP expression may have broader relevance in fibrosis, since CHOP is induced by silica, probably *via* ER stress, and inhibition of its induction by 4BPA ameliorates silicosis in rats [188, 189].

Thoracic malignancy

Having evolved from the ancestral general amino acid control pathway in yeast [190], the ISR in mammals plays a central role in regulating the availability of amino acids [9]. Cancers have a high demand for amino acids, both for new protein synthesis and to provide thiols for production of the antioxidant glutathione. As a tumour outgrows its vascular bed, both amino deprivation and ER stress trigger a cytoprotective ISR *via* GCN2 and PERK, respectively, elevating ATF4 and CHOP in its hypoxic core [191]. Consequently, the ability of cancer cells to phosphorylate eIF2α is critical for the growth of large solid tumours [191]. In hypoxic tumours, the ER generates increased levels of reactive oxygen species in part through CHOP-mediated induction of the ER oxidoreductase ERO1α [14, 192]. ERO1α expression enhances the capacity of a cell to generate disulphide bonds and so aids protein synthesis and tumour growth. Indeed, high ERO1α expression correlates with worse cancer prognosis and increased cancer cell growth [193,

194]. But excess generation of reactive oxygen species can be cytotoxic, and so one of the functions of the ISR is to defend against this.

In the lung, activated phospho-GCN2 is observed in carcinoma tissue, but less so normal tissues [195]. This is necessary for the expression of ATF4-target genes in lung cancer that promote the synthesis, import and mobilisation of amino acids. Increased ATF4-dependent production of glutathione may account for much of the resistance to cisplatin chemotherapy observed in multiple cell lines including those derived from lung cancers [196, 197]. PERK-mediated resistance to oxidative stress is also thought to be important in the resistance to radiotherapy [198, 199]. It has also been shown that drug-resistant KRAS-mutant lung cancers have elevated levels of ATF4 and are more susceptible than nonresistant lung cancer cells to inhibition of PERK [200]. PERK inhibition restores their sensitivity to the MEK inhibitor trametinib and raises to possibility of ISR-directed personalised therapies [200].

Analysis of The Cancer Genome Atlas reveals that advanced lung adenocarcinomas often exhibit an ATF4 target gene signature [201]. Correspondingly, in lung adenocarcinoma cell lines the drug ISRIB (integrated stress response inhibitor [trans-2-(4-chlorophenoxy)-N-(4-(2-(4-chlorophenoxy)acetylamino)cyclohexyl) acetamide]), which renders cells insensitive to eIF2α phosphorylation, blocked ATF4 expression and reduced both cancer cell proliferation and migration in amino acid deficient conditions (table 2) [201]. One of the mechanisms by which the ISR adapts cells to nutrient-poor environments is through enhanced import of amino acids. This too may produce specific vulnerabilities, since in nonsmall cell lung cancers the expression of the large neutral amino acid transporter LAT1 *in vivo* correlates with poor survival [202], while *in vitro* silencing of LAT1 in lung adenocarcinoma A549 cells can impair cancer cell growth [203]. The availability of amino acids can also be increased *via* activation of macro-autophagy, which liberates amino acids from long-lived proteins and damaged organelles. Accordingly, in hypoxic areas of tumour xenografts, increased expression of autophagy factors such as LC3 is observed, which is at least partially mediated by the GCN2/PERK–ATF4–CHOP axis [195, 199, 204].

Nutrient and oxygen deficiency both cause tissues to release signals that induce angiogenesis. It is well known that hypoxia leads to the stabilisation of the transcription factor HIF1α to promote angiogenesis [205]. However, the ISR also contributes to angiogenesis because ATF4 directly upregulates vascular endothelial growth factor (VEGF)A and downregulates inhibitors of angiogenesis [206–208]. Consequently, depletion of PERK prevents VEGFA secretion in response to glucose deprivation independent of HIF1α, while inhibition of PERK reduces xenograft vascularity and perfusion in mice [208]. The PERK inhibitor GSK2656157 reduces cancer growth *in vivo*, most likely *via* reducing angiogenesis and impairing amino-acid metabolism (table 2) [209].

Cancers are often associated with local immunosuppression [210]. Expression of the inflammatory ISR kinase PKR is reduced in many non-small cell lung cancers with lower expression correlating with poorer prognosis [211]. Conversely, pharmacological activation of PKR or its overexpression kills lung cancer cells [211, 212]. Recently, it was discovered that many lung cancer cell lines are dependent on ADAR1, an RNA deaminase [213]. Loss of ADAR1 leads to PKR activation and cell death, while loss of PKR rescues the lethality of *ADAR1* deletion. This suggests that PKR detects an RNA signal in lung cancer cells that is suppressed by ADAR1. It is unclear why lung cancer cells behave in this manner, but this raises the possibility of targeted therapies, for example ADAR1 inhibition, to trigger PKR-mediated lung cancer cell killing [213].

It is often stated that CHOP triggers cell death, but the reality is more nuanced. For example, expression of CHOP is an independent predictor of a poor prognosis in malignant pleural mesothelioma, suggesting CHOP expression is associated with increased tumour growth [214]. However, in chemoresistant mesothelioma, activation of the PERK–ATF4–CHOP pathway by bortezomib reverses the chemoresistance (table 2) [215]. Very few target genes of CHOP are prodeath factors, but rather are involved in oxidative protein folding, protein secretion and autophagy [14, 199, 216]. In fact, the recovery of protein synthesis and oxidative protein folding mediated by CHOP, may explain much of CHOP's relationship with ER stress induced cell death [14, 216]. The recovery of translation at later time points during the ISR is mediated by the CHOP target PPP1R15A, and consequently *Ppp1r15a*$^{-/-}$ mice are resistant to ER stress induced tissue damage, as they are protected from the toxicity of excessive protein synthesis [14]. In a murine model of medulloblastoma, loss of PPP1R15A increased phospo-eIF2α and promoted tumour growth, invasiveness and angiogenesis perhaps *via* the increased expression of VEGFA [217]. Although mutations of CHOP or PPP1R15A are uncommon in human cancers, their expression might be suppressed by other means. For example, in breast carcinoma, PERK-induced expression of microRNA mir211 promotes tumour cell survival in part by repressing CHOP expression [118]. Loss of PPP1R15A is also observed in the more aggressive sarcomatoid subtype of malignant pleural mesothelioma, and so low PPP1R15A expression correlates with a worse prognosis [218]. The mechanism by which PPP1R15A expression is suppressed in sarcomatoid mesothelioma remains unclear.

Conclusion

The ISR is central in responding to infection, oxidative stress and nutrient deprivation. Studies of human lung disease have revealed its importance in normal pulmonary physiology, perhaps relating to the uniquely oxidising and nutrient poor environment of the airway. The role of the ISR in regulating cell survival and inflammation make it an attractive target for the development of immunomodulators and anticancer therapies alike. More recent discoveries of the importance of GCN2 in maintaining pulmonary vascular health also raise the possibility that new treatments for pulmonary hypertension might be developed by targeting the ISR.

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