Pulmonary endoplasmic reticulum stress:

Scars, Smoke and Suffocation

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Abbreviations used:

ER (Endoplasmic Reticulum); UPR (unfolded protein response); ISR (integrated stress response); CHOP (C/EBP homologous protein); GADD34 (Growth Arrest and DNA Damage protein 34); ERO1 (Endoplasmic Reticulum Oxidoreductase 1); IRE1 (Inositol requiring enzyme 1); RIDD (Regulated IRE1 Dependent RNA Decay); ATF4 (Activating Transcription Factor 4); XBP1 (X-Box Binding Protein 1); ATF6 (Activating Transcription Factor 6); ERAD (ER associated degradation); SFTPC (surfactant protein C); SFTPA (Surfactant protein A); ABCA3 (ATP Binding Cassette Subfamily A Member 3); HPS1/4 (Hermansky Pudlack syndrome 1 and 4); BLOC3 (biogenesis of lysosome-related organelles complex 3); IPF (idiopathic pulmonary fibrosis); UIP (usual interstitial pneumonia); IIP (idiopathic interstitial pneumonias); NSIP (non-specific interstitial pneumonia); CMV (Cytomegalovirus); EBV (Epstein–Barr virus); HHV (Herpesviridae); TERT (Telomerase Reverse Transcriptase); TERC (Telomerase RNA Component); PTEN (Phosphatase And Tensin Homolog); PINK1 (PTEN-induced putative kinase 1); TGFβ (Transforming Growth Factor Beta); FGF2 (Fibroblast growth factor 2); NFκB (Nuclear Factor Kappa B); AP-1 (Activator protein 1); IL8 (interleukin 8); JNK (Jun N-terminal kinase); EMT (epithelial-to-mesenchyme transition); 4PBA (4-phenyl butyric acid); COPD (chronic obstructive pulmonary disease); PERK (protein kinase R-like ER kinase); eIF2α (Eukaryotic

Translation Initiation Factor 2 Subunit Alpha); GRP94 (Glucose regulated protein 94kDa); MUC2 (Mucin 2); PDI (Protein disulphide isomerase); Nrf2 (Nuclear Factor, Erythroid 2); HIF (Hypoxia-Inducible Factor); COX (cytochrome-c oxidase); PDK (pyruvate dehydrogenase kinase); HSP70 (Heat shock protein 70kDa); VKOR (Vitamin K epoxide reductase); PRDX4 (Peroxiredoxin 4); GPx7/8 (Glutathione Peroxidases 7 and 8); ROS (reactive oxygen species); NPGPx (non-selenocysteine containing phospholipid hydroperoxide glutathione peroxidase); PAH (Pulmonary arterial hypertension); TUDCA (tauroursodeoxycholic acid); NO (nitric oxide); SERCA (Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase); RyR2 (Ryanodine Receptor 2); MTMP (mitochondrial permeability transition pore); MAM (mitochondrial associated ER-membranes); BiP (Binding immunoglobin protein); LC3 (Microtubule Associated Protein 1 Light Chain 3); ATG5 (Autophagy Related Gene 5); VEGFA (vascular Endothelial Growth Factor A)

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Abstract

Protein misfolding within the endoplasmic reticulum (ER stress) can be a cause or consequence of pulmonary disease. Mutation of proteins restricted to the alveolar type II pneumocyte can lead to inherited forms of pulmonary fibrosis, but even sporadic cases of pulmonary fibrosis appear to be strongly associated with activation of the unfolded protein response (UPR) and/or the integrated stress response (ISR). Inhalation of smoke can impair protein folding and may be an important cause of pulmonary ER stress. Similarly, tissue hypoxia can lead to impaired protein homeostasis (proteostasis). But the mechanisms linking smoke and hypoxia to ER stress are only partially understood. In this review, we will examine the role of ER stress in the pathogenesis of lung disease by focusing on fibrosis, smoke and hypoxia.

Introduction

The lung is a critical barrier between the external environment and the internal milieu. Transcriptional targets of CHOP allow the resumption of normal cellular processes during the dissipation of acute ER stress, supporting the resumption of protein synthesis and oxidative protein folding, via GADD34 and ERO1 upregulation respectively [1]. Failure to do so is implicated in a wider range of pulmonary diseases [2]. For many years, the cancer causing genotoxicity of inhaled pollutants has be studied in detail, but more recently it has become clear that additional effects on protein folding homeostasis (proteostasis) are also key to the development of lung disease [3, 4]. Failure of proteostasis specifically within the endoplasmic reticulum (so-called ER stress) has been shown to play an important role in the pathogenesis of many human diseases ranging from airway pathology to cancer [3-5].

The response to ER stress called the Unfolded Protein Response (UPR) and the consequences of phosphorylating eIF2α (the Integrated Stress Response, ISR) have been described in depth elsewhere [3, 4, 6]. We shall therefore not discuss the individual pathways in great detail here apart from the following brief overview (Figure 1). When proteins misfold inside the ER, a signal must be transmitted across the membrane to the cytosol and then the nucleus. Three individual classes of ER stress sensor achieve this, but the outputs are essentially: (i) to lower the rate of protein synthesis and enhance the folding capacity of the ER while (ii) promoting disposal of terminally misfolded proteins and, finally (iii) to trigger cell death if homeostasis cannot be restored. The reduction in translation is achieved by the combined actions of PERK and IRE1. PERK is a kinase that selectively phosphorylates eIF2α to lower the rate of translation initiation [7, 8]. By contrast, IRE1 is an endonuclease capable of degrading the mRNAs of some secretory proteins to prevent their synthesis in a process called Regulated IRE1 Dependent RNA Decay (RIDD) [9]. Following the initial reduction in protein synthesis, a complex transcriptional programme is induced by three parallel, yet interacting, pathways subordinate to three transcription factors -- one for each ER stress sensor (Figure 1). PERK causes the preferential translation of ATF4 [10]; IRE1α initiates the unconventional splicing of *XBP1* mRNA

into an active form [11-13]; and ATF6 is processed proteolytically into a soluble transcription factor at the Golgi apparatus [14, 15]. To enable the cell to adapt to its current load of ER client proteins these transcription factors induce genes encoding chaperones, enzymes required for new proteins to undergo post-translational modification, and components of the ER associated degradation (ERAD) machinery. The mechanisms by which cell death is activated are less clear, but appear to occur following prolonged activation of ER stress signalling [1, 16].

In this review, we shall examine the role of ER stress in the pathogenesis of lung disease by focusing on three main themes: the originals of pulmonary fibrosis, the effects of inhaled pollutants on the airway epithelium, and the wider consequences of impaired oxygenation that occurs during respiratory failure.

Pulmonary fibrosis

ER stress is associated with familial pulmonary fibrosis - Up to 30% of cases of pulmonary fibrosis have a genetic basis and, by studying these, ER stress has emerged as a potential pathogenic mechanism [17]. Type II pneumocytes of the alveolar epithelium secrete pulmonary surfactant to prevent surface tension from collapsing the airways. This surfactant is composed of abundant lipids combined with a small number of proteins, one of which is surfactant protein C (SFTPC). Autosomal dominant mutations of *SFTPC* can present as familial pulmonary fibrosis suggesting a toxic gain-of-function. Paucity of cases of biallelic *SFTPC* mutation raises the possibility of a gene-dosage effect with only a single case being reported of a child born alive who was homozygous for an *SFTPC* mutation [18]. Deletion of exon 4 (Δexon4) of *SFTPC* was the first such mutation to be reported [19], but others including L188Q quickly followed [20]. Both of these mutations and numerous others disrupt the C-terminal BRICHOS domain of the SFTPC pro-protein (Figure 2). This domain is believed to function as a self-chaperone to facilitate the folding of SFTPC [21]. Consequently, these mutations hinder normal folding of SFTPC and impair its exit from the ER. In heterologous expression models, SFTPC Δexon4 accumulates in the ER or in heavily ubiquitinated aggresomes and this is accompanied by activation of all three arms of the UPR [22-26]. Expression of SFTPC L188Q in mouse type II

pneumocytes also triggers the UPR *in vivo* but this is not sufficient to cause the development of pulmonary fibrosis [27]. However, SFTPC L188Q-expressing mice suffer exaggerated levels of fibrosis when challenged with other fibrogenic agents. This may be a generalizable phenomenon, since the misfolding of other proteins within the ER of type II pneumocytes is also associated with the development of pulmonary fibrosis. Surfactant protein A (SFTPA) is a hydrophilic surfactant protein with roles in innate immunity [28]. It too can be mutated in autosomal dominant pulmonary fibrosis and adenocarcinoma [29]. The pulmonary fibrosis-associated mutations of *SFTPA,* G231V and F198S, trigger the splicing of *XBP1* mRNA and induce the expression of BiP. They do not, however, appear to exert a dominant negative effect on the trafficking of wild-type SFTPA suggesting, once again, that their toxicity is through a toxic gain-of-function mechanism.

Pulmonary surfactant is assembled and stored by specialized structures within type II pneumocytes called lamellar bodies. Its lipid component is transported into these organelles by the ABCA3 transporter [30] and so loss of ABCA3 function causes an autosomal recessive deficiency of surfactant [31, 32]. However, a subset of *ABCA3* mutations is toxic owing to protein misfolding. For example, the R280C and L101P mutants of *ABCA3* are retained in the ER, induce expression of BiP, and sensitize cells to other ER stressing agents [33]. Mistrafficking of surfactant components has similar effects. Hermansky Pudlack syndrome (HPS) is a rare, recessive, form of oculocutaneous albinism caused by mutations in a variety of genes affecting lysosome maturation that in some patients can cause pulmonary fibrosis. It can be caused by mutations of *HPS1* and *HPS4,* which are components of the BLOC3 complex (biogenesis of lysosome-related organelles complex-3), a Rab32/38 guanine nucleotide exchange factor involved in the biogenesis of lysosome-related organelles [34]. The defective membrane trafficking resulting from these mutations leads to increased expression of CHOP and ATF4 in lung tissue, suggesting involvement of the UPR, or at least the ISR, in the pathogenesis of the disease [35].

ER stress is associated with sporadic idiopathic pulmonary fibrosis (IPF) - Eighty-five percent of biopsy-proven cases of familial disease display histological changes consistent with so-called usual interstitial pneumonia (UIP) [36]. These changes are typical of the more common sporadic disorder idiopathic pulmonary fibrosis (IPF), and this similarity prompted a search for evidence of ER stress in sporadic IPF too. Activation of the UPR has now been detected reproducibly in the lungs of individuals with sporadic IPF [25, 37, 38]. There is also evidence for UPR activation in the second most common form of sporadic idiopathic interstitial pneumonias (IIPs) called nonspecific interstitial pneumonia (NSIP) [39]. Not only can activation of the UPR be detected in explanted lungs from individuals with late stage pulmonary fibrosis, but also in relatively early diagnostic biopsies. Although the association between sporadic IPF and activation of the UPR is strong, it remains correlative owing to the absence of a clear mechanistic link. To address this, a number of lines of investigation are being pursued. Chronic viral infection of the lung epithelium has been implicated in the pathogenesis of IPF (reviewed in [40]). The DNA of herpes viruses (including CMV, EBV, HHV-7, HHV-8) can be detected in up to 97% of tissue samples from patients with IPF compared with 36% of controls [41]. This may be relevant because CMV infection has been shown to activate the UPR in cultured cells [42]. Of course, the presence of viral DNA in tissue does not equate with infection, but herpes antigens, which are more indicative of chronic infection, were detected in 65% of individuals with UIP but were absent in all controls tested [25]. These viral antigens co-localized with markers of an activated UPR in the airway epithelium. Nevertheless, the lack of viral antigens in many cases of IPF despite evidence of an active UPR suggests other ER stress-inducing agents may also be involved in the pathogenesis of pulmonary fibrosis. For example, inhaled smoke is a plausible candidate (see later) since smoking cigarettes is a known risk factor for IPF and components of smoke can cause ER stress [43-51]. Equally, airborne particulate matter can activate the UPR in cultured cells and in the murine lung [52, 53], while exposure to airborne particulate matter is associated with more rapid progression of IPF [54]. It is likely that other, as yet unrecognised factors, may be involved including expression of other misfolding proteins or disorders of cellular proteostasis.

ER stress is associated with secondary pulmonary fibrosis – In a subset of patients, the development of pulmonary fibrosis can be attributed to a specific trigger. And for some of these triggers, ER stress has emerged as a plausible mediator of toxicity. For example, some individuals develop lung fibrosis due to the inhalation of asbestos fibres and are said to have asbestosis. Their pulmonary histology closely resembles that of sporadic IPF and so a shared pathogenic mechanism seems plausible. When explanted type II pneumocytes are exposed to asbestos fibres, activation of IRE1 can be detected [55]. The UPR is also detected in macrophages exposed to asbestos *in vitro*, and in the bronchial fluid of mice exposed to asbestos and humans suffering from asbestosis [56]. The antiarrhythmic drug amiodarone, which causes pulmonary toxicity in about 5% of patients and can also cause pulmonary fibrosis in mice, has also been shown to induce ATF6 and CHOP in cultured mouse lung epithelial cell lines [57, 58]. Another drug, bleomycin, is well known to cause pulmonary inflammation and fibrosis (so much so that it is commonly used to model interstitial lung disease in mice). Bleomycin can also activate the UPR following even a single intra-tracheal administration [59, 60].

Towards a mechanistic link between ER stress and pulmonary fibrosis – Current models suggest that pulmonary fibrosis develops when a susceptible pulmonary epithelium confronts an environmental trigger. In such models, the factors rendering the epithelium vulnerable include age and genetics, while those acting as trigger might include viral infections and inhaled toxins. Studies suggest that cells already burdened with ER protein misfolding are less able to cope with the second proteotoxic insult. For example, it has been shown that expression of proteins liable to misfold, such as the Z variant of α_1-antitrypsin, can sensitize cells to drugs that impair protein folding, resulting in exaggeration of the UPR [61]. This may be relevant to the mice expressing L188Q *SFTPC* that do not spontaneously develop pulmonary fibrosis, but respond in an exaggerated manner to bleomycin with higher levels of apoptosis and fibrosis [27]. Similar synergy has been observed when wild type mice are treated with bleomycin in combination with the ER stress-inducing toxin, tunicamycin. Likewise, the level of apoptosis and the degree of ER

stress caused by viral infection are both enhanced in cells already expressing the misfolding Δexon4 mutant of *SFTPC* [62].

The incidence of IPF increases with age [63] and around 15% of cases of familial pulmonary fibrosis are related to mutations of the telomerase genes *TERT* and *TERC* that cause accelerated telomere shortening and premature senescence [64]. Interestingly, the ability of cells to withstand protein misfolding and ER stress also falls with age (reviewed in [65]) and older mice mount an exaggerated ER stress response following infection with herpes virus, which is associated with more pulmonary fibrosis [66]. The mechanism for this association might relate to a reduced capacity to turn over defective organelles, since ageing and ER stress both reduce the levels of PINK1 (PTEN-induced putative kinase 1) within lung epithelium [67]. This results in impaired mitochondrial quality control with increased release of pro-fibrotic mediators such as TGFβ and FGF2 and elevated levels of apoptosis.

ER stress may also induce inflammatory signaling through activation of NFκB and AP-1. Expression of Δexon4 *SFTPC* induces the NFκB pathway [62, 68] and cells over-expressing misfolding mutants of *SFTPC* release IL8 owing to JNK/AP-1 activation [69]. IL8 is a potent chemo-attractant for neutrophils and is present at elevated levels in lung tissue, bronchoalveolar lavage fluid, sputum and serum from individuals with IPF patients compared with controls [70-72]. But increased inflammation is not a major feature in the L188Q *SFTPC* expressing mouse [27]. Indeed, there was no excess of pulmonary inflammation in transgenic animals expressing low-levels of mutant protein compared to controls even following treatment with bleomycin, suggesting that ER stress may not promote fibrosis through effects on inflammation. This chimes with theories in which IPF is not principally an inflammatory disorder, although microinjury and acute exacerbations of IPF do seem to correlate spatially and temporally with episodes of inflammatory cell activity (reviewed in [73]). Instead, current research is focusing on epithelial injury followed by aberrant repair as the initiating events in IPF. Prolonged exposure of primary alveolar epithelial cells to TGFβ induces an epithelial-to-mesenchyme transition (EMT) into collagen-producing myofibroblasts [74]. Such EMT has been observed in the lungs of

patients with IPF, in murine models of TGFβ-mediated lung injury, and in bleomycin-induced pulmonary fibrosis [75, 76]. Intriguingly, ER stress has been shown to trigger EMT in a variety of tissues including the lung [77, 78], while TGFβ appears to promote a more myofibroblast-like phenotype in pulmonary fibroblasts in an UPR-dependent manner [79]. Cultured lung epithelium forced to express either the L188Q or Δexon4 mutants of *SFTPC* or treated with the drug tunicamycin, which induces ER stress directly, underwent EMT in a src kinase-dependent manner [75, 76]. Moreover, inhibition of src or treatment with the chemical chaperone 4-phenyl butyric acid (4PBA) both ameliorated EMT suggesting a causal link between ER stress and this phenotypic change [60]. The precise mechanisms remain to be elucidated, but the IRE1-XBP1 pathway has been implicated in driving EMT during pulmonary fibrosis through increased expression of the transcription factor Snail [80]. Other cell types in the lung may also be susceptible to ER stress-induced phenotypic changes. For example, bleomycin-induced activation of the UPR in alveolar macrophages has been shown to induce a pro-fibrotic M2 phenotype leading to increased secretion of TGFβ [81].

Apoptotic death of epithelial cells is a recognized feature of pulmonary fibrosis (reviewed in [82]). Transgenic mice have been used to identify a cytotoxic effect of misfolding *SFTPC* mutants during lung development [23]. Exaggerated apoptosis also occurs in the lungs of mice expressing L188Q *SFTPC* when challenged with bleomycin and in cells expressing L188Q or Δexon4 *SFTPC* [20, 24, 25, 27]. This toxicity has been attributed to increased activation of caspase 4 and activation of JNK [26, 83]. Similarly, ER stress is thought to mediate cell death in models of pulmonary fibrosis associated mutation of *ABCA3* [33]. Although correlative, it is interesting that markers of the unfolded protein response co-localize with apoptosis in fibrotic lungs from individuals with sporadic disease [37, 38].

Smoke

In addition to the potential link between smoke, ER stress and pulmonary fibrosis, proteomic analysis has identified elevated levels of ER chaperones in the lungs of patients with chronic obstructive pulmonary disease (COPD) [84]. This sparked interest in a potential role for smoke-

induced ER stress as a mediator of COPD. Despite apparent discrepancies in some of the effects of cigarette smoke in different disease models, smoke-induced ER stress appears to be initiated by perturbations in redox that lead, in many instances, to the triggering of cell death pathways [44-51, 85, 86].

ER Stress is associated with exposure to smoke and pollutants in vitro – *In vitro* exposure of cells to cigarette smoke and other airborne pollutants can trigger the UPR [44-51]. When primary bronchial epithelial cells from non-smoking individuals were exposed for a brief period to whole cigarette smoke, transient PERK-dependent phosphorylation of eIF2α was observed followed by induction of ATF4 and GADD34 [44]. Similar results have been obtained for a variety of lung cancer and extra-pulmonary cell lines, with induction of UPR targets including BiP, XBP1 and GRP94 suggesting that smoke and aqueous smoke extracts are *bona fide* inducers of ER stress [44-48]. However, not all studies using primary airway epithelial cells have consistently reported ER stress to be caused by cigarette smoke [49, 50]. It has been suggested that the airway epithelium of patients with COPD may be more prone to smoke-induced ER stress compared with non-smoking controls, which could account for some of these differences [49]. The mechanisms underlying this differential susceptibility is unclear, but the cells of patients with COPD are reported to show elevated basal levels of UPR markers, perhaps indicating that they have a pre-existing defect in ER homeostasis. Moreover, volatile exhaust fumes from diesel engines, which can trigger the UPR in a dose-dependent manner [50], appear to activate the UPR in primary airway cells from COPD patients but not from non-smoking controls, supporting a notion that some individuals are innately more susceptible to airway ER stress [51]. Alternatively, this may indicate that the epithelium of COPD lungs is more susceptible to ER stress for other reasons, for example an abnormal microenvironment.

Despite good evidence for activation of PERK and ATF6 by cigarette smoke *in vitro*, the involvement of the IRE1α-XBP1 axis is less straightforward. Little or no splicing of XBP1 mRNA was observed when fibroblasts or lung-derived cancer cell lines were treated with aqueous smoke extract or volatile smoke respectively [44, 45]. Indeed, it has been suggested that

cigarette smoke can inhibit splicing of XBP1, ameliorating IRE1α-XBP1 signalling even when cells are treated with strong inducers of the ER stress such as tunicamycin [44, 53]. It is noteworthy that, in contrast to many other tissues, the lung and gut express two IRE1 paralogues at appreciable levels [87]. Unlike the ubiquitously expressed IRE1α isoform, IRE1ß has been suggested to possess only low levels of XBP1-directed RNAse activity and so plays only a minor role in the IRE1-XBP1 pathway; instead, it seems to be more important in RIDD [88, 89]. Indeed, in lung cells treated with diesel fume particulates, despite a low level of XBP1 splicing, increased levels of RIDD have been observed [53]. This refinement of the UPR in the lung and gut appears related to the secretion of mucin, because *Ire1b*$^{-/-}$ deficient mice aberrantly accumulate mucin within the ER of goblet cells owing to increased stability of *MUC2* mRNA [90]. IRE1ß may therefore function to regulate mucin production by degrading excess *MUC2* mRNA. This raises the intriguing possibility that IRE1ß-directed therapies might plausibly be developed to regulate mucus production in smokers with COPD.

ER Stress is following exposure to smoke and pollutants in vivo – The lung tissue of individuals with COPD has been shown to express elevated levels of UPR and ISR markers [84, 91]. Similarly, the lungs of rats exposed to cigarette smoke for two to four months have increased levels of phosphorylated eIF2α and express the ISR target CHOP [92]. However, studies in mice have been less consistent. When mice were exposed to the smoke of a single cigarette, activation of all three arms of the UPR could be detected within twelve hours [85], and mice chronically exposed to fine particulate matter induced both the ISR and UPR in their lungs [53]. However, in other studies, protein lysates of the lungs of mice exposed chronically to cigarette smoke failed to show evidence of a UPR [49, 93, 94]. This might indicate a species difference in the response to cigarette smoke, since transcriptional profiling also revealed few genes that are commonly modulated between smoke-exposed mice and humans with COPD [95]. Alternatively, it may represent a problem of detection sensitivity because immunohistochemistry has revealed localized increases in BiP and CHOP protein selectively in the bronchial epithelium of mice chronically exposed to smoke [93], while mRNA levels of BiP and CHOP were increased following prolonged smoke exposure when measured by northern

blot [47]. It is worth noting that the susceptibility of mice to developing emphysema or cancer following exposure to cigarette smoke is highly strain dependent [96-99], but it is as yet unclear if the UPR shows similar strain effects in mice.

The role of redox in smoke-induced ER stress – At least some of the impairment of ER homeostasis caused by cigarette smoke is mediated through effects on cellular redox status. Cigarette smoke contains many potent oxidizing agents and so the lungs of humans and mice exposed to smoke have elevated levels of oxidized macromolecules [46, 100]. Similar biochemical changes are observed when human airway epithelial cells are treated with smoke condensate *in vitro* [101, 102]. Importantly, co-administration of an anti-oxidant, such as N-acetylcysteine, along with smoke attenuates the induction of both the ISR and UPR, while reducing the level of cell death [45, 47, 48, 85, 86].

An important function of the ER is the formation of disulphide bonds in many of its client proteins during oxidative protein folding [103]. This requires the ER to maintain a finely balanced redox environment that permits the formation of disulphide bonds, while also allowing aberrant disulphides to be reduced. This may explain the sensitivity of the ER to the noxious effects of oxidants in smoke. Protein disulphide isomerase (PDI) is a redox sensitive chaperone that undergoes cycles of oxidation and reduction through interactions with the enzyme ERO1 [103]. This generates disulphide bonds within PDI that are passed on to its client proteins during oxidative protein folding. Exposure to a variety of constituents of smoke, including acrolein, hydroxyquinones or peroxynitrites, leads to nitrosylation of PDI on cysteine and tyrosine residues impairing this enzymatic activity [46]. The resulting protein misfolding within the ER leads to ER stress. Treatment of cells or mice with acrolein rapidly induces GADD34, which appears to mediate some of acrolein's toxicity [104]. Just as GADD34 deficient mice are protected from the toxicity of ER stress [1], *Gadd34*$^{-/-}$ mice exposed chronically to intra-nasal acrolein are more resistant to cell death and have better preserved pulmonary architecture [104]. Inhibition of GADD34 might therefore be expected to protect the lung from smoke-induced ER stress, but although putative GADD34 inhibitors have been reported, their effectiveness remains

somewhat controversial [105, 106]. Nevertheless, salubrinal, a drug that is said to inhibit the dephosphorylation of eIF2α, also protects cells from smoke [107].

Chronic exposure to cigarette smoke leads to the induction of anti-oxidant pathways and detoxification enzymes both in humans and rodents [84, 108]. Although many of these genes are induced by the oxidative stress-response transcription factor Nrf2 [109, 110], the ISR also participates by inducing anti-oxidant genes via ATF4 [4, 111]. It has been suggested that Nrf2 and ATF4 may even heterodimerize to regulate expression of some of their target genes [112, 113] and, more recently, Nrf2 has been shown to regulate some genes through driving increased expression of ATF4 [114]. Interestingly, a signature suggesting regulation by ATF4 was found amongst genes induced in the airway of smokers with COPD compared to smokers without COPD [115]. Subsequently, overexpression of ATF4 was shown to recapitulate this profile in cultured airway epithelial cells.

Although early reports suggested that PERK is able to phosphorylate Nrf2 directly leading to its activation [116, 117], subsequent studies have demonstrated that the effects of PERK on target gene expression are entirely dependent on phosphorylation of eIF2a [118]. The relevance of Nrf2 to UPR signalling therefore remains uncertain.

A role of ER stress in smoke-induced cell death – Cigarette smoke is highly noxious and smoke-induced cell death appears to play an important role in the pathogenesis of COPD [119]. Several studies have suggested that ER stress may mediate some of this toxicity. Although the mechanisms by which ER stress triggers apoptosis are complex and only partly understood (reviewed in [120]), the recovery of protein synthesis during late stages of the UPR is thought to contribute to ER stress-induced cell death [1]. This recovery of protein synthesis is mediated by GADD34, which is induced via a number of pathways including ATF4 and its target CHOP [1, 121]. Perhaps for this reason, CHOP deficient cells and animals are resistant to ER stress-induced cell death [1, 122]. Unsurprisingly, because the PERK-ATF4 axis is triggered by cigarette smoke, the induction of CHOP is also observed [47, 48]. Expression of dominant-negative constructs of PERK or CHOP have been reported to abolish cigarette smoke-induced

apoptosis, suggesting that the ISR directly links smoke to cell death [47, 48]. This may involve the PERK-ATF4-CHOP-GADD34 axis because loss of GADD34 also renders mice more resistant to the pulmonary toxicity of acrolein [104]. However, the inhibition of PERK in order to protection airway epithelia from cigarette smoke is brought into question by the observation that knockdown of PERK using RNA interference also exaggerates smoke-induced apoptosis in normal human bronchial epithelial cells [107]. This likely reflects both the importance of PERK-mediated attenuation of translation during the response to ER stress and the beneficial effects of the ISR during oxidative stress. However, ameliorating ER stress by overexpression of the chaperone BiP or the transcription factor XBP1 can enhance cell viability following exposure to smoke [47, 123]. Therefore chemical chaperones might offer similar protection, but this needs to be tested formally.

Hypoxia

Hypoxia is a common feature of many pulmonary diseases and has relevance to many others, notably cancer [124]. A master regulator of the cellular response to hypoxia is Hypoxia-Inducible Factor (HIF)-1, a basic helix-loop-helix transcription factor consisting of α and β subunits [125]. Under normoxic conditions, HIF-1α is hydroxylated in an oxygen-dependent manner, promoting its rapid ubiquitination and degradation by the proteasome (reviewed in [126]). During hypoxia, reduced levels of hydroxylation lead to persistence of HIF-1α, heterodimerization with HIF-1β, and the induction of genes involved in the hypoxia response. Molecular oxygen is also necessary for efficient protein folding within the ER and so, in addition to activation of HIF-1-dependent pathways, deficiency of oxygen also serves as a potent trigger for the UPR.

The molecular basis for ER stress during hypoxia - Although a topic of intense investigation, the precise mechanisms by which hypoxia induces ER stress remain incompletely understood. A variety of processes involved in ER function are either directly or indirectly dependent upon molecular oxygen. The generation of ATP, which is necessary for many processes within the ER, consumes oxygen at the mitochondria and during prolonged hypoxia the ratio of ATP:AMP

falls in cultured cells [127, 128]. *De novo* synthesis of secretory proteins at the ER involves energy expenditure during gene transcription, mRNA translation, chaperone assisted protein folding, protein degradation, intracellular trafficking, and secretion [129-134]. These processes can therefore be affected when ATP availability falls owing to reduced mitochondrial respiration. The decreased generation of ATP observed in hypoxic conditions can be attributed both to the requirement of cytochrome-c oxidase (COX) for oxygen as an electron accepter and the inhibition of mitochondrial respiration by pyruvate dehydrogenase kinase (PDK) 1, a transcriptional target of HIF-1 [135, 136].

The most energy expensive component of secretory protein synthesis is mRNA translation itself [137]. Limiting GTP has been linked to increased rates of translation errors in reconstituted bacterial systems, which could conceivably give rise to missense mutations [138, 139]. Yet it would seem unlikely that the ATP depletion seen in prolonged hypoxia would promote sufficient levels of translational errors to induce ER stress through protein misfolding. Indeed, hypoxia rapidly reduces the rate of global translation prior to detectable drops in ATP being occurring [127]. The entry into the ER of newly synthesised proteins is mediated by the action of ER resident chaperones, which also prevent protein aggregation. BiP is the most abundant chaperone within the ER and, as a member of the HSP70 family of proteins, hydrolyses ATP during cycles of interactions with its substrates [140]. However, the energetic cost of chaperone driven protein folding is relatively small and therefore unlikely to be affected by the levels of ATP depletion seen during hypoxia. In luciferase refolding experiments, a similar HSP70 chaperone required only five molecules of ATP to unfold a misfolded protein substrate, which represents an energy cost several orders of magnitude lower than that of mRNA translation [141]. Therefore, at levels of ATP depletion necessary to impair protein folding, mRNA translation would already have dropped dramatically and so no new polypeptide chains would be made.

Proteins entering the ER often undergo post-translation modifications that guide protein folding toward the native state. Disulphide bond formation is one such modification, the disruption of which leads to protein misfolding and ER stress [142]. This process is facilitated by protein

disulphide isomerase (PDI), which oxidises a pair of cysteines in a folding protein to generate a disulphide bond. The reduced PDI molecule then shuttles electrons to ERO1, which in turn is reoxidised using molecular oxygen [143]. This requirement of ERO1 for molecular oxygen suggests that hypoxia might drive ER stress by limiting the formation of disulphide bonds. Intriguingly, ERO1 is a prominent target of HIF-1α up-regulation during hypoxia [144]. A number of additional ER-disulphide forming pathways have been identified more recently. Vitamin K epoxide reductase (VKOR), Peroxiredoxin 4 (PRDX4), and glutathione peroxidases 7 and 8 (GPx7/8) have all been shown to form oxidizing equivalents in the ER, and cells lacking ERO1 display only a slight delay in disulphide formation [145-149]. Whilst VKOR uses oxygen as a terminal electron acceptor, PRDX4 and GPx7/8 use hydrogen peroxide instead, although the source of ER hydrogen peroxide remains obscure. While mitochondrial respiration can generate hydrogen peroxide, recent findings suggest that the ER membrane is relatively impermeable to this, implying the existence of an as yet unknown ER-localised hydrogen peroxide generating system [150]. Interestingly, the co-translational formation of disulphide bonds can occur in the absence of oxygen, whereas post-translational formation of disulphide bonds and their isomerisation is strongly inhibited in anoxic conditions [151]. This suggests that a subset of ER client proteins, specifically those requiring the co-translational formation of disulphide bonds, may fail to fold in the absence of oxygen, providing a plausible mechanism by which hypoxia might induce ER stress. It is possible that limiting oxygen may have additional effects on the post-translational maturation of proteins. For example, hypoxia has been reported to inhibit N-linked glycosylation of some proteins [152], although this is not a universal finding and so requires further investigation [151].

It is well known that hypoxia promotes the production of reactive oxygen species (ROS), which could conceivably contribute to activation of the UPR [153, 154]. Whilst the ER is undoubtedly sensitive to changes in redox, the impact of ROS on ER proteostasis is unclear. For example, although hydrogen peroxide is present within the lumen of the ER, its reaction with proteins is energetically unfavourable and so it is unlikely to contribute directly to protein misfolding [155]. However, cellular hydrogen peroxide can also give rise to lipid peroxides, which have been

shown to cause ER stress in cultured cells [156]. This lipid peroxidation is accompanied by depletion of reduced glutathione, the most abundant reductive buffering component in the ER, and the generation ROS [156]. The extent to which other hypoxia-induced ROS, such as superoxide, impact of ER proteostasis is unclear. It has been proposed that ROS might alter ER proteastasis indirectly via an ER resident protein named non-selenocysteine containing phospholipid hydroperoxide glutathione peroxidase (NPGPx) [157]. When exposed to ROS, NPGPx forms an internal disulphide bond that is subsequently transferred to the ER chaperone BiP, leading to its inactivation. As the master regulator of ER proteostasis, BiP, via NPGPx, may provide another link between hypoxia induced ROS and ER stress.

Pulmonary hypertension - Pulmonary arterial hypertension (PAH) describes an increase in blood pressure within pulmonary arteries, characterised by vasoconstriction of the pulmonary vasculature and excessive proliferation of smooth muscle cells, ultimately leading to right heart failure. A wide range of drivers of PAH are known, a number of which are associated with tissue hypoxia, for example COPD, ILD, and habitation at high altitude [158]. Recently, links have been identified between hypoxia induced ER stress and progression of PAH, with evidence pointing to effects in a number of tissues including vascular smooth muscle [159], the endothelium [160], and hypertrophy in the right ventricle [161]. Notably, expression of the protein Nogo-B, a modulator of ER structure, was seen to be up-regulated downstream of ATF6 in response to hypoxia in smooth muscle cells of pulmonary arteries [162]. This was associated with disruption of ER-mitochondrial contacts, reducing phospholipid and calcium transfer between the organelles and suppressing mitochondrially driven apoptosis. Strikingly, *Nogo-B* knockout mice were completely resistant to hypoxia induced PAH. Moreover, treatment with the chemical chaperones 4PBA and tauroursodeoxycholic acid (TUDCA) reversed hypoxia-induced PAH in mice, with reduced ATF6 signalling and mitochondrial calcium depletion [159]. Indeed, activation of all three arms of the UPR has been observed in the lungs of mice during chronic hypoxia induced PAH [161]. The subsequent inflammatory response was attenuated in a 4PBA dependent manner. It is worth noting that the mechanism(s) by which these chemical chaperones suppress UPR activation remains under discussion. 4PBA has been suggested to

bind hydrophobic regions of unfolded and partially folded proteins, thereby inhibiting aggregation [163]. TUDCA is also thought to inhibit mitochondrial dysfunction and apoptosis [164-166]. However, the precise mechanisms still need to be understood if more effective therapies based on them are to be developed.

Ischaemia - Restriction of blood flow to tissues and organs causing nutrient deprivation and hypoxia is described as ischaemia. Although distinct from the hypoxia of pulmonary failure, the models used to study extra-pulmonary ischaemia may shed light on the pathologies of both conditions and so will be considered here. It is important to stress, however, that we use these observations only to glean clues as to the role of impaired oxygenation on proteostasis in peripheral tissue, and they should not be considered models of impaired perfusion of lung parenchyma. PERK is activated in the mouse brain by ischaemia followed by reperfusion, leading to phosphorylation of the translation initiation factor eIF2α [167]. Mice lacking the transcription factor CHOP, which is up-regulated by eIF2α phosphorylation, were protected against ischemia induced neuronal cell death [168]. Whilst transcriptional targets of CHOP are cytoprotective in the face of acute ER stress, CHOP drives the resumption of protein synthesis and oxidative protein folding, via GADD34 and ERO1 upregulation respectively [1]. As such, the PERK arm of the UPR is cytotoxic in the face of chronic ER stress and likely contributes to neuronal apoptosis during ischemia. In addition to hypoxia-induced activation of the UPR, reperfusion is associated with the generation of nitric oxide (NO) that is proposed to further exacerbate ER stress and CHOP induction [169]. NO is thought to perturb ER calcium homeostasis, in part by the inhibition of SERCA, which maintains calcium concentrations in the ER, and by promoting glutathionylation of the ryanodine receptor RyR2 leading to calcium efflux [170, 171]. The high concentration of calcium in the ER is though to promote the activity of ER resident chaperones and folding factors, many of which bind calcium [172]. Recently, depletion of ER calcium was shown to inactivate the ER chaperone BiP by inducing its homo-oligomerisation and thereby preventing interaction with unfolded protein substrates [173]. This mode of chaperone inactivation likely contributes to the sensitivity of ER protein folding to luminal calcium disturbances, although its prevalence during hypoxia remains to be established.

In addition, ER stress induced calcium release promotes cell death by opening of the mitochondrial permeability transition pore (MPTP), leading to inner membrane depolarization and induction of apoptosis [174]. This process is promoted by the accumulation of calcium channels at direct contact sites between the ER and mitochondrial known as mitochondrial associated ER-membranes (MAM) [175].

Ischaemic tissues tend to produce angiogenic factors that promote their reparative revascularisation, yet hypoxia-induced ER stress has been suggested to inhibit neovascularization though down-regulation of Netrin-1 mediated by the RIDD pathway [176, 177]. This might suggest that ER stress would have a negative effect of tissue responses to ischaemia, but in ischaemic post-conditioning intermittent interruption of blood supply during reperfusion has been found to offer some protection to tissues, notably the brain (reviewed in [178]). This is associated with reduced activation of the UPR, with decreased expression of CHOP, less apoptosis, and increased expression of the chaperone BiP [179, 180].

Malignancy - The majority of tumours outgrow their vascular supply and experience at least some degree of nutrient deprivation and hypoxia. As such, ER stress and the UPR are important in many aspects of hypoxia driven tumour biology [124]. The rapid reduction of mRNA translation during ER stress, facilitated by PERK phosphorylation of eIF2α [181], plays an important role in tumour cell survival during the onset of hypoxia. Disruption of this pathway, either by expression of a dominant negative PERK or the non-phosphorylatable S51A mutant of eIF2α, impaired cell survival in the face of prolonged hypoxia and gave rise to smaller tumours [182]. In addition, ATF4 and CHOP, downstream targets of PERK, are up-regulated in the hypoxic core of tumours [182]. PERK offers further protection during hypoxia via the induction of cytoprotective autophagy factors such as LC3 and ATG5, which are rapidly turned over during hypoxic exposure [183]. Accordingly, pharmacological inhibition of autophagy decreased viability of hypoxic tumour cells and sensitized human tumour xenografts to irradiation, providing promising avenues for potential therapeutic strategies [183]. Activation of IRE1 by ER stress is also important in promoting tumour cell survival in the face of hypoxia, via splicing of an mRNA

to encoding the active form of the transcription factor XBP1 [184]. Ablation of this pathway dramatically reduced tumour growth, sensitising cells to the hypoxic environment. Recently, a novel role for the IRE1-XBP1 axis during hypoxia was identified in triple negative breast cancer. Chen and colleagues showed that transcriptional targets of HIF-1α are also up-regulated by XBP1 in a cooperative manner, whereby both factors co-occupy hypoxia responsive target genes to promote tumourigenicity [185]. Another example of direct cross talk between the HIF-1 and ER stress pathways is evident in the amelioration of hypoxic conditions within tumours by promoting the pro-angiogenic factor VEGFA. VEGFA is a direct transcriptional target of both HIF-1 [186] and the UPR through the PERK-ATF4 [187, 188] or to a lesser extent IRE1-XBP1 axes [189]. Direct induction of the UPR appeared to synergise with hypoxia to produce higher levels of VEGFA expression than hypoxia alone. However, this cooperation occurred in an ATF4 independent manner, did not increase HIF-1α or HIF-1β expression or nuclear localization, but instead appeared to increase HIF-1 transcriptional activity [189]. These data propose distinct ATF4 driven and UPR-dependent HIF-1 driven regulation of VEGFA. The latter may provide an example of novel UPR pathways that might be targetable in future therapeutic strategies and will no doubt add to the great current interest in pharmacological manipulation of ER stress.

Concluding remarks

ER stress is therefore both a cause and consequence of pulmonary disease. The UPR signalling pathway is now understood to a remarkable level of precision and potent small molecules are currently available to enable its manipulation *in vivo*. The observation that some features of the UPR, notably the role of IRE1ß, seem to be particular to endoderm-derived structures such as the lung raises the possibility that tissue selective therapies may even be possible, if the precise function of these pathways can be unpicked. Despite exciting recent advances, much remains to be understood about the full extend of ER stress' involvement in lung disease, making this a thrilling time to be studying proteostasis in pulmonology.

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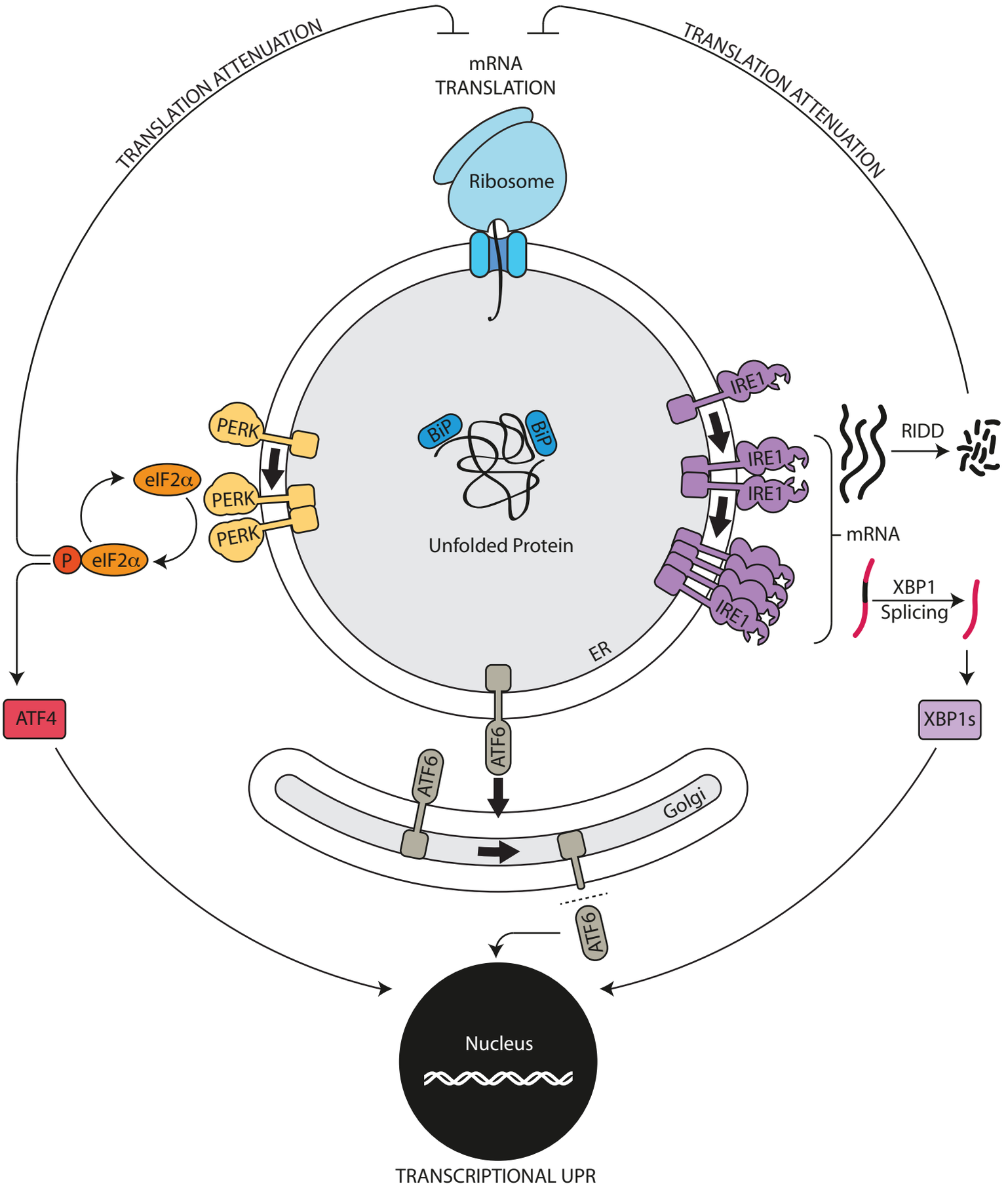
Figure Legends

Figure 1. The Unfolded Protein Response

Nascent proteins enter the ER and co-translationally associate with ER chaperones, e.g. BiP, and other components of the folding machinery. If they reached their correct fold, they are released from the ER to proceed along the secretory pathway via the Golgi. Accumulation of unfolded proteins in the ER triggers the unfolded protein response (UPR): IRE1 initiates unconventional splicing of mRNA encoding the transcription factor XBP1; ATF6 activation involves its trafficking to the Golgi apparatus where it is cleaved to release a transcription factor domain. Both XBP1 and cleaved ATF6 up-regulate components of the ER folding and degradation machineries. PERK phosphorylates eIF2α to inhibit translation of most proteins, while promoting the translation of ATF4. IRE1 can also reduce translation during stress, by degrading ER-localized mRNA transcripts in a process termed regulated IRE1-dependent decay (RIDD).

Figure 2. Structure of surfactant protein C.

(A) The crystal structure of the surfactant protein C (SFTPC) BRICHOS domain [DOI: 10.2210/pdb2yad/pdb] [190] and the NMR structure of the valine-rich α-helix of SFTPC in an apolar solvent [DOI: 10.2210/pdb1spf/pdb] [191] were used to generate a model of the SFTPC pro-protein. The linker region, predicted to be an unstructured polypeptide chain, is represented as a line. (B) Enlargement of the crystal structure of the BRICHOS domain of SFTPC. ER stress can be induced by mutations affecting (i) the conserved disulphide bond between cysteines 121 and 189 highlighted in blue; (ii) Leucine 188 highlighted in red; (iii) the region encoded by exon-4 highlighted in orange, which disrupts a disulphide bond between cysteines 120 and 148. Figures were generated using PyMOL v1.7.4.4, Schrödinger LLC.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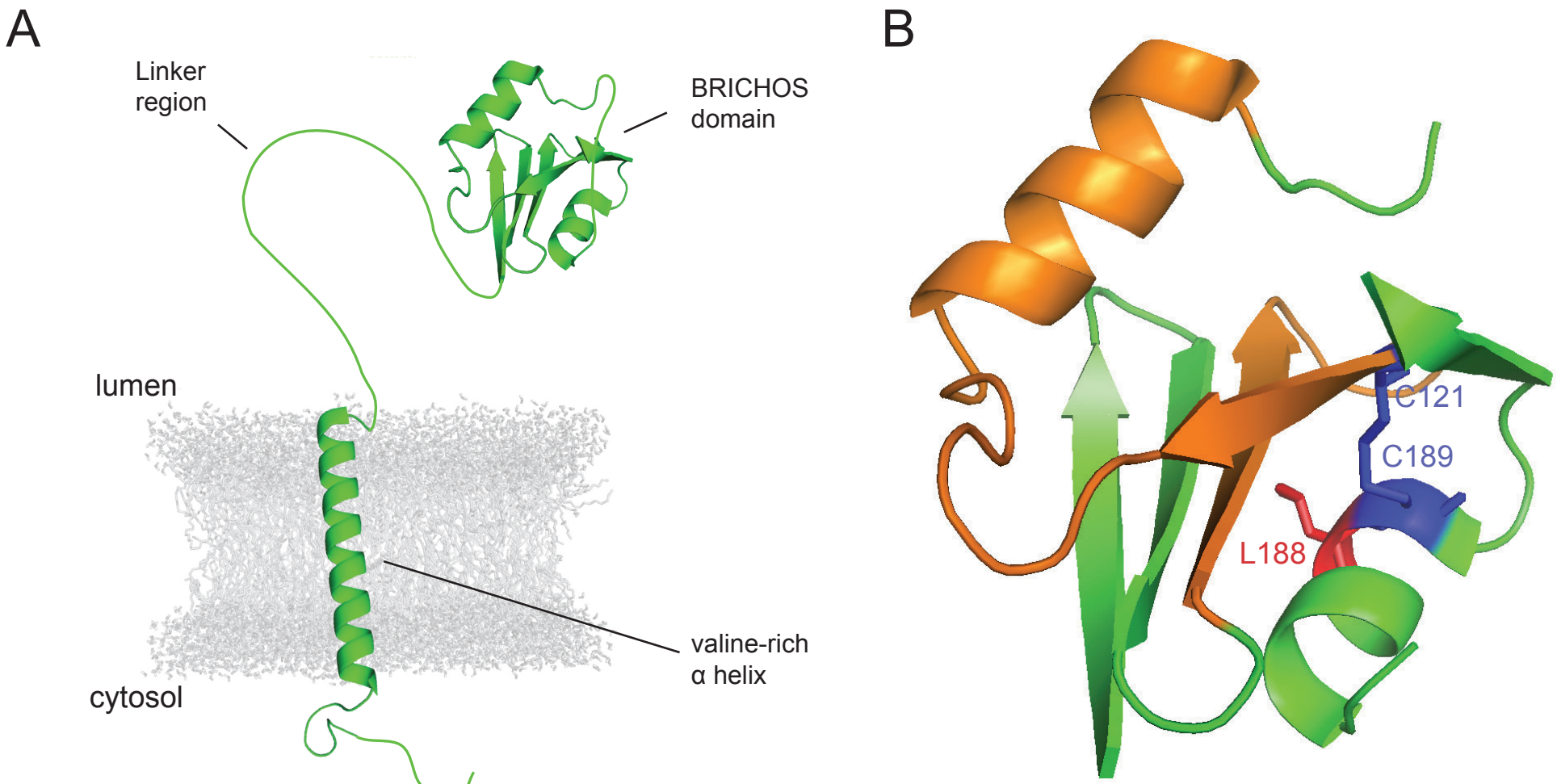
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