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The role of endoplasmic reticulum stress on reducing recombinant protein production in mammalian cells

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ABSTRACT

Therapeutic recombinant protein production relies on industrial scale culture of mammalian cells to produce active proteins in quantities sufficient for clinical use. The combination of stresses from industrial cell culture environment and recombinant protein production can overwhelm the protein synthesis machinery in the endoplasmic reticulum (ER). This leads to a buildup of improperly folded proteins which induces ER stress. Cells respond to ER stress by activating the Unfolded Protein Response (UPR). To restore proteostasis, ER sensor proteins reduce global protein synthesis and increase chaperone protein synthesis, and if that is insufficient the proteins are degraded. If proteostasis is still not restored, apoptosis is initiated. Increasing evidence suggests crosstalk between ER proteostasis and DNA damage repair (DDR) pathways. External factors (e.g., metabolites) from the cellular environment as well as internal factors (e.g., transgene copy number) can impact genome stability. Failure to maintain genome integrity reduces cell viability and in turn protein production. This review focuses on the association between ER stress and processes that affect protein production and secretion. The processes mediated by ER stress, including inhibition of global protein translation, chaperone protein production, degradation of misfolded proteins, DNA repair, and protein secretion, impact recombinant protein production. Recombinant protein production can be reduced by ER stress through increased autophagy and protein degradation, reduced protein secretion, and reduced DDR response.

1. Introduction

Biotherapeutics are one of the fastest growing segments of the pharmaceutical industry. In 2023, the global biotherapeutics market was valued at $478.20 billion and is expected to grow to $709.91 billion by 2028 [1]. Since the market release of Humulin (recombinant insulin) in 1982, biotherapeutic molecules have been developed to treat numerous conditions, including cancer, heart disease, multiple sclerosis, anemia, and rheumatoid arthritis [2]. The production of biotherapeutics is generally more complex and expensive than small molecule drugs,

Abbreviations: ATF4, Activating Transcription Factor 4; ATF6, Activating Transcription Factor 6; AEJ, Alternative End Joining; BER, Base Excision Repair; Bid, BH3 interacting-domain death agonist; CHOP, CCAAT/enhancer-binding protein homologous protein; CHO, Chinese Hamster Ovary; ChIP, Chromatin Immunoprecipitation; CNHEJ, Classical NHEJ; CD, Cytosolic Domain; DDR, DNA Damage Repair; DSB, Double Stranded Breaks; ER, Endoplasmic Reticulum; ERAD, ER Associated Degradation; EIF2α, Eukaryotic translation initiation Factor 2α; HSP70, BIP, Heat Shock Protein 70; HR, Homologous Recombination; IRE1α, Inositol-Requiring transmembrane kinase/Endoribonuclease 1α; LD, Luminal Domain; LAMP3, Lysosome Associated Membrane Protein 3; MMR, Mismatch-Mediated Repair; MtDNA, Mitochondrial DNA; MMP, Mitochondrial Membrane Potential; MEF, Mouse Embryonic Fibroblast; NHEJ, Non-Homologous End Joining; Nrf2, Nuclear factor erythroid-derived 2-like 2; NER, Nucleotide Excision Repair; γH2A.X, Phosphorylated Histone H2A.X; PERK, PRK-like Endoplasmic Reticulum Kinase; ROS, Reactive Oxygen Species; RIDD, Regulated IRE1α-Dependent Decay; SSA, Single Strand Annealing; SSB, Single Stranded Breaks; S1P, Site 1 Protease; S2P, Site 2 Protease; XBP1s, Spliced X-Box Binding Protein-1; SREBF1, Sterol regulatory element binding factor 1; TGN, Trans Golgi Network; TMD, Transmembrane Domain; UPR, Unfolded Protein Response; XBP1, X-Box Binding Protein-1.

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often involving recombinant DNA, engineered cell lines, specialized media components, and complex purification approaches that yield products sensitive to environmental conditions and transportation stresses. It is essential, therefore, to continue to investigate improvements to biotherapeutic production processes that increase yield per unit feed, thereby reducing the production costs of the biotherapeutics.

Biotherapeutics can be divided into distinct classes: blood-derived products, vaccines, engineered cell therapies, nucleic acids, and recombinant proteins, which is the focus of this review. Recombinant proteins, ranging from insulin to monoclonal antibodies, were the first biotherapeutics to be approved and continue to dominate the biotherapeutics landscape (e.g., there are more than 7000 current clinical trials for monoclonal antibodies) [3]. Mammalian expression systems are ideal for production of recombinant protein therapeutics since they provide proper protein folding, disulfide bond formation, glycosylation, and other post translational modifications [4], with Chinese hamster ovary (CHO) cells being the most used host cells due to their similar post-translational modification machinery to human cells.

Recombinant protein production yield is a function of the number and specific productivity of the host cells. There are many factors that affect yield in industrial cultures, e.g., the excessive burden on cells' protein expression machinery, local nutrient deprivation, mixing stresses, and high passage numbers. Current industrial attempts to overcome these problems rely on sequential selection of the highest yielding cell lines expressing the therapeutic protein - a process that could be improved or sped up by understanding the mechanisms that affect productivity of the cell lines [5]. The handling of proteins, their modification and folding occurs in the endoplasmic reticulum (ER) which are tightly regulated and determines cell function and survival. Prior reviews focused on ER stress, and approaches to increase recombinant protein yields, including chaperone protein engineering and cell selection [6–11]. In contrast, this review examined research from cell biology, bioprocessing, and DNA damage response from cancer studies to highlight mechanisms that could be monitored to increase recombinant protein production. The impacts of ER stress on protein synthesis are clear and well-established. However, increasing evidence indicates that ER stress interplays with genome integrity and damage repair mechanisms of host cells, which affects cell viability and thereby protein production. Recombinant protein production generally requires the product to be secreted into the culture medium to simplify purification. ER stress can alter this secretory behavior. Maintaining cell health and specific productivity are essential for achieving long-term productivity of recombinant proteins through economically feasible processes. Mounting evidence links ER stress to these essential processes, namely, regulation of the trafficking of secretory vesicles and genome stability.

2. ER stress and the UPR

Many conditions that involve a dysregulation of proteostasis lead to the induction of ER stress. Stresses from the environment, e.g., chemicals, pathogens, genetic manipulation, oxidative stress, and cytokines, induce ER stress and alter cellular protein demands. Increasing protein production in the cells can overwhelm the quality control machinery in the ER, leading to a buildup of unfolded or misfolded proteins which induces ER stress [12]. To cope with ER stress the unfolded protein response (UPR) is activated to restore proteostasis. The UPR is initiated

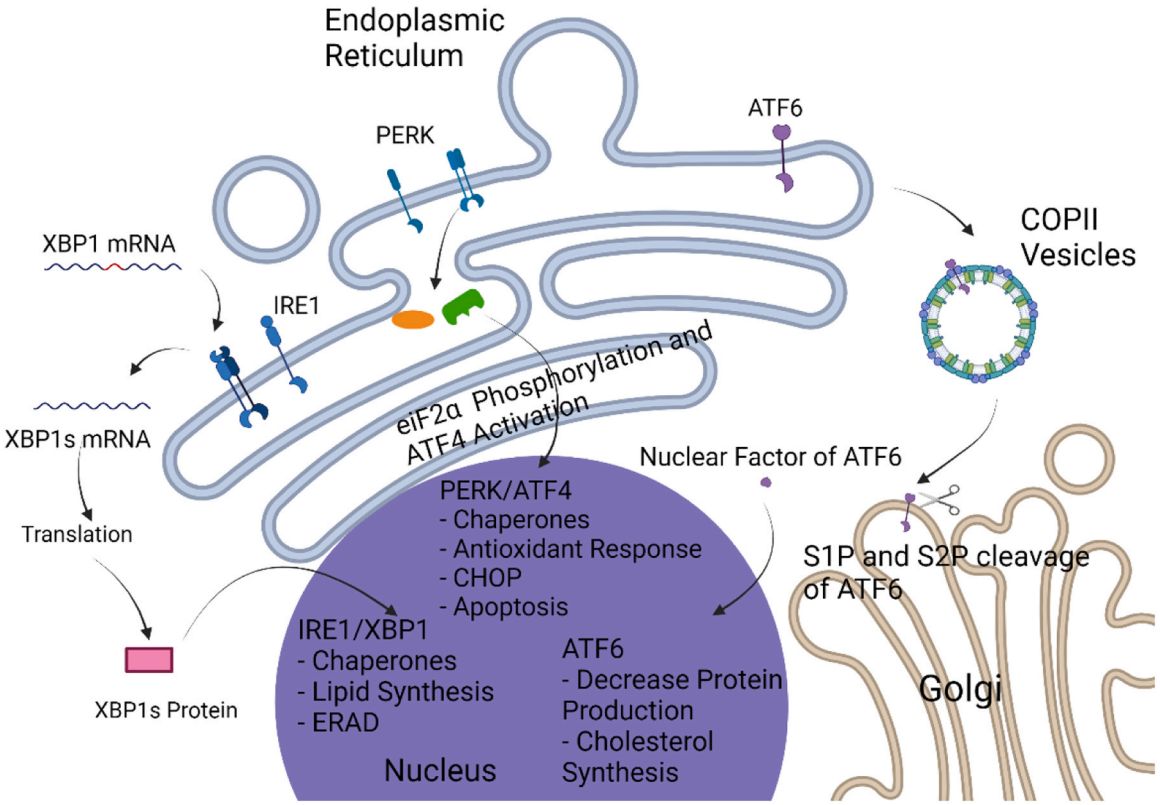

Fig. 1. : Summary of the Unfolded Protein Response (UPR). The UPR consists of three protein sensors of ER stress: IRE1α, PERK, and ATF6. In response to ER stress from protein overproduction or stress cues in bioreactors, IRE1α dimerizes and oligomerizes creating an active site for the splicing of XBP1 mRNA which is then translated into the XBP1s transcription factor. PERK dimerizes to phosphorylate and activate transcription factors eIF2α and ATF4. Activated ATF6 is trafficked to the Golgi where site-1-protease (S1P) and site-2-protease (S2P) cleaves the ER portion and cytosolic portion of the protein to create the nuclear factor of ATF6. Together these branches decrease global protein expression, increase chaperone expression, increase proteolysis, and - if ER stress persists – activate apoptosis. ER stress signaling therefore can alter the number of cells producing recombinant protein and the amount of protein each cell produces.
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by 3 sensor proteins: Inositol-Requiring transmembrane kinase/endoribonuclease 1α (IRE1α), PRK-like endoplasmic reticulum kinase (PERK), and Activating Transcription Factor 6 (ATF6). UPR activation (1) decreases global protein expression, (2) *increases* chaperone protein expression, (3) increases protein degradation and secretion, and, if these measures do not restore proteostasis, (4) signals apoptosis [11] (Fig. 1).

IRE1α, a ubiquitously expressed serine-threonine kinase, is a type I transmembrane protein of the ER with an N-terminal luminal domain (LD) that acts as an ER stress sensor, a C-terminal cytosolic domain (CD) containing both Ser/Thr kinase and endoribonuclease (RNase) activities, and a transmembrane domain (TMD) that senses membrane lipid saturation [13,14]. During ER stress, misfolded proteins bind BH3 interacting-domain death agonist (Bid), releasing IRE1α and allowing the LD of IRE1α to dimerize/oligomerize and initiate signaling [15].

Dimerization/oligomerization facilitates trans-autophosphorylation and the activation of the RNase domain [16]. This active form of IRE1α catalyzes the unconventional processing of the mRNA encoding X-Box binding protein-1 (XBP1) by splicing a 26-nucleotide intron from the XBP1 mRNA to generate the coding sequence for an active transcription factor, spliced XBP1 (XBP1s) [17]. XBP1s upregulates genes involved in enhancing ER protein-folding capacity and degrading unfolded or misfolded proteins [18]. In addition to catalyzing the splicing of XBP1, IRE1α induces regulated IRE1-dependent decay (RIDD), which results in the degradation of RNAs including mRNAs, microRNAs, and ribosomal RNAs [19,20] with XBP1-like endomotifs (consensus sequence CNG|CAGN). However, RIDD can also degrade mRNAs without such motifs through a more promiscuous, endomotif-independent processing that requires phospho-oligomers [21]. Through RIDD, IRE1α promotes the degradation of mRNAs encoding ER-targeted proteins to reduce the protein load in the ER [22,23].

As with IRE1α, ER stress promotes dimerization of the LD of PERK. PERK belongs to the eukaryotic translation initiation factor 2α (eIF2α) kinase subfamily, containing a Ser/Thr kinase domain in the cytosol. Upon dimerization of the LD, the cytosolic kinase domain undergoes activation by trans-autophosphorylation [24]. Activated PERK phosphorylates eIF2α at Ser 51, and phosphorylated eIF2α impedes global translation initiation, decreasing the protein expression load in the ER. However, activated eIF2α also increases the translation of activating transcription factor 4 (ATF4) [25]. Under prolonged ER stress, ATF4 activates CCAAT/enhancer-binding protein homologous protein (CHOP), which contributes to the upregulation of apoptotic pathways [26,27].

ER stress reduces protein expression by phosphorylating eIF2α, which prevents eIF2ß guanine nucleotide exchange factor from converting eIF2α back into the active form. This prevents recognition of mRNAs and further translation. Chaperone proteins (e.g., HSP70), essential proteins (e.g., insulin receptor), and viral RNAs (e.g., picornaviral RNAs) avoid decreased expression by using an internal ribosome entry site (IRES) that is recognized by ribosomes in an eIF2α-independent manner [28]. IRESs are currently used in antibody production to ensure both chains of the antibody are expressed once the mRNA is recognized at the ribosome [29]. Further use of IRESs to avoid decreased stress-induced translation is worth considering.

While IRE1α and PERK are type I transmembrane proteins with single α-helical TMDs and cytosolic kinase domains, ATF6α is a type II transmembrane transcription factor containing several α-helical TMDs and a DNA-binding domain with a basic leucine zipper motif [30]. Upon ER stress, ATF6 localizes to the Golgi apparatus and is further cleaved by site 1 and 2 proteases (S1P, S2P) allowing translocation of ATF6 to the nucleus to form the active transcription factor pATF6a [31]. An important role of ATF6 is to upregulate molecular chaperones and folding enzymes to increase the protein folding capacity of the ER. Additionally, if ER stress persists, ATF6 and PERK can work synergistically to induce CHOP and apoptosis [32].

3. ER stress induced protein degradation

3.1. ER Associated Degradation (ERAD)

ER stress activates ER associated degradation (ERAD) as an additional mechanism for restoring proteostasis and protein quality control in the cell. The accumulation of misfolded proteins activates the UPR and ERAD clears the misfolded proteins through the cytosolic ubiquitin-proteasomal degradation pathway [33]. Proteins for degradation are identified, exported from the ER, ubiquitinated, and transported to the proteasomes for destruction.

ERAD depends on chaperone protein function. Chaperone proteins (Table 1) were first identified from cellular responses to heat stress. Therefore, many of the chaperone proteins are named heat shock protein (HSP) followed by their approximate size in kDa (an alphabet-based system is replacing these historical names [34]). Given the importance of chaperone proteins to protein synthesis and production, the field of "chaperone engineering" was developed to optimize the expression levels of key chaperone proteins to maximize recombinant protein production. Previous attempts at chaperone engineering of mammalian cell factories achieved varying levels of effectiveness. HSPA5 overexpression in CHO cells has been shown to reduce production of von Willebrand factor (a blood glycoprotein that promotes platelet adhesion) but did not change production of macrophage colony-stimulating factor [35], indicating substrate-specific effects. siRNA silencing of HSPA5 improved secretion of recombinant tissue plasminogen activator in CHO cells [36]. Thus, chaperone proteins appear to impact production rates in a cell/client-specific manner [37]. Investigations into chaperone engineering at industrial scales remains largely unstudied because similar results are accomplished by cell line evolution and selection to create high expressing cell lines that tend to have high levels of stabilizing/refolding chaperones and low levels of degrading or apoptotic chaperones [38,39]. An individual chaperone protein can interact with several different substrates and an individual substrate can interact with many chaperone proteins complicating which chaperone protein to overexpress to increase the expression of a given recombinant protein. Recent developments in computer modeling and protein identification may mitigate these challenges of chaperone engineering. The ability to predict which chaperone proteins will interact with a given client may allow for genetic alterations to create a better cell line for biomanufacturing.

3.2. ER induced autophagy and degradation

In addition to ubiquitin-proteasome degradation, autophagy traffics material to lysosomes for degradation (Fig. 2). Autophagy-lysosomal degradation can be divided into three categories: microautophagy, chaperone-mediated autophagy, and macroautophagy [64]. Microautophagy is the basal level breakdown of bulk materials, such as organelles and proteins by the lysosome through direct encapsulation [64]. Chaperone-mediated autophagy (CMA) occurs when chaperone proteins like Hsc70/HSPA8 recognize the CMA-targeting motif of misfolded proteins and translocate the protein to the lysosome for degradation [65]. CMA targets the KFERQ or similar motif and through careful sequence selection can be avoidable for recombinant proteins [66] (Dice, Autophagy, 2007). Macroautophagy, commonly known as autophagy, starts with the formation of a phagophore from ER-associated components that matures into a new membrane bound vesicle called an autophagosome [64]. The autophagosome fuses with lysosomes for degradation of the autophagosome's contents [64]. In contrast with proteasomal degradation, autophagy can degrade protein aggregates, organelles, and insoluble material [64]. For proteins that can be degraded by either proteasomes or autophagy, the half-life of the protein dictates the degradation pathway, with longer half-life proteins being more prone to degradation by autophagy [67].

Chemical and physiological inducers of ER stress can promote

Table 1
Chaperone Proteins Potential Impact on Secretion of Recombinant Therapeutic.

Protein Family	Protein	Alternative names	Description	Potential Role in Protein Production
HSPA [40] (HSP70)				
	HSPA1	Hsp72	Binds, stabilizes, and folds newly synthesized proteins [41].	Resists apoptosis under stress. Secreted as a cytokine by cancer cells, may carry proteins with it [42].
	HSPA5 [43]	BiP, Grp78, Mif−2, HSP70	Under normal conditions, binds, stabilizes, and folds newly synthesized proteins. Under stress conditions, regulates protein production and ER calcium levels.	Under stress conditions HSPA5 ubiquitinates misfolded proteins for degradation
	HSPA8 [44]	Hsc70, Hsc71, Hsp71, Hsp73	Assists in protein folding with HSPBs.	Decreased expression under ER stress may prevent clathrin coated vesicles from disassembling
HSPB [45] (less than 40 kDa)				
	HSPB1	HSP27, HSP28	Forms large complexes that become phosphorylated under stress releasing dimers and tetramers to stabilize misfolded proteins.	Works with HSPAs to refold or tag proteins for degradation
HSPC [46] (HSP90)				
	HSPC1,2,4,5	HSP90AA1, HSPN, LAP2, HSP86, HSPCA, HSP89, HSP90, HSP90A, HSP90N, HSPCAL1, HSPCAL4, FLJ31884	Contains ATPase activity and folding domains of DNAJ and HSPA; proteins that repair DNA damage are HSPC1 clients	Activation of HSPCs promote cell survival through apoptosis inhibition
	HSPC3	HSP90AB1, HSPC2, HSPCB, D6S182, HSP90B, FLJ26984, HSP90-Beta	HSPC3 interacts with a non-overlapping set of client proteins compared with HSPC1	Activation of HSPCs promote cell survival through apoptosis inhibition
HSPH [45]				
	HSPH1–4		Acts as a nucleotide exchange factors for HSPAs and prevents protein aggregation.	Improves HSPA turnover and prevents aggregation.
DNAJ				
	DNAJA1-C30 [45]		Assists HSPA in supporting protein folding with ATPase domain	Under normal conditions, improves protein folding; when overexpressed, increases degradation
	DNAJB1	Hsp40, Hdj1, jDj−1, HSPF1, Sis1, RSPH16B	Increases cell proliferation and reduces p53-linked apoptosis [47]	Increases cell number
	DNAJB4	human liver DnaJ-like protein, HLJ−1, Hlj1, DjB4, Dnajw	Increases ERAD [48]	Increases degradation
	DNAJB9	ER-resident protein ERdj4, Mdg1, mDj7	Recruits ERAD proteins, Derlin−1, and BiP [49]	Increases degradation
	DNAJB12	Dj10,mDj10	Degradation of DNAJB12 allows BOK accumulation and apoptosis [50]	Increases cell number
	DNAJB14	ER-resident EGNR9427, PRO34683, FLJ14281	Promotes ERAD [51]	Increases degradation
	DNAJC3	ER-resident protein ERdj6, p58, Prkri, protein kinase inhibitor p58 ($p58^{IPK}$)	Promotes ERAD [52]	Increases degradation
	DNAJC5	cysteine string protein (Csp) alpha	Promotes cell proliferation, neurotransmitter release, and misfolding-associated protein secretion [53]	May increase secretion of proteins with high cysteine content
	DNAJC8	splicing protein spf31, Hspc315, Hspc331	Regulates glycosylation [54]	May increase protein production in glucose-starved cells.
	DNAJC9	DnaJ protein SB73, HdjC9	Regulates cell cycle through H3 and H4 binding [55]	Inhibits cell proliferation
	DNAJC10	ER-resident protein ERdj5, macrothioredoxin (MTHr), JPD1	Regulates disulfide-bonds and traffics misfolded proteins to proteasomes [56]	May improve secretion of high sulfide content proteins. Likely to reduce secretion in stress states
	DNAJC13	DNA J-domain containing protein Rme−8 (RME−8), mKIAA0678, Gm1124	Increases autophagy [57]	Increases degradation
	DNAJC14	Hdj3, hDj−3, LYST-interacting protein 6 (LIP6), dopamine receptor-interacting protein of 78 kDa (DRIP78, Drip−78)	Under ER stress, promotes unconventional secretion [58]	May increase secretion
	DNAJC16	ERdj8, mKIAA0962	Controls size of autophagosomes [59]	Increases autophagy.
	DNAJC21	Dnaja5, GS3, Jjj1	Assists in folding of nuclear ribosomes and 60 S ribosome maturation [60].	Improves ribosome activity
	DNAJC23	ER-resident protein ERdj2, Sec63L, Dnajc23	Accompanies small proteins to secretory vesicles [61].	May increase protein production of small proteins
	DNAJC26 [62]	Cyclin-G-associated kinase (GAK), auxilin−2	Recruits clathrin for endocytosis and assists in clathrin disassembly.	Increases membrane turnover, enhancing production
CCT				
	CCT1–6		Assists in cytoskeleton formation [63].	Protein secretion depends on proper cytoskeleton formation

autophagy, and are context-specific, depending on the intensity and duration of the stress and the cell type. Autophagy is considered pro-survival [68]. The n-glycosylation inhibitor tunicamycin has been shown to upregulate autophagy in LNCaP cells [69], while the ER-calcium transport inhibitor thapsigargin does not alter autophagosome creation but does prevent autophagosome degradation in MEFs [70]. Glucose starvation can either increase or decrease autophagy [71]. The fatty-acid palmitate upregulates autophagy [72]. Activation of the JNK, AMPK1, MAPK8, ATK1, mTOR, or BECN1 pathways is associated with increased autophagy [73]. Degradation by autophagy is counter-balanced with proteasome-mediated degradation, with ER stressors that downregulate autophagy being associated with upregulation of

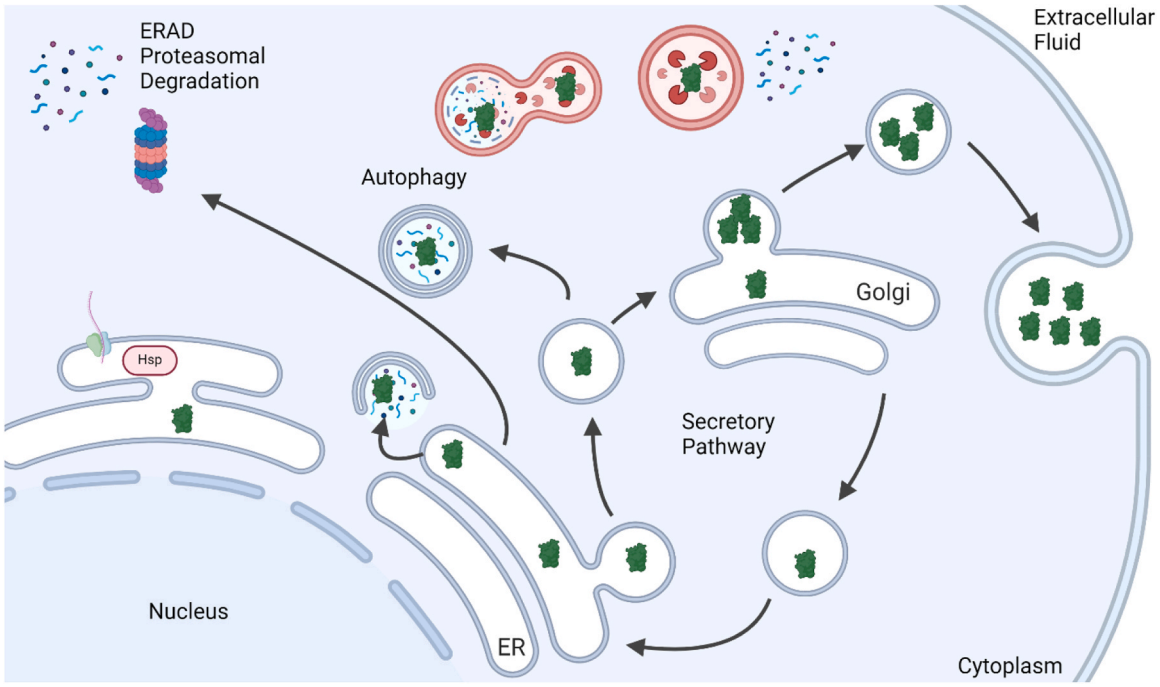

Fig. 2. : Recombinant proteins exit the secretory pathway for degradation. Proteins can be ubiquitinated, transported across the ER membrane, and trafficked to proteasomes for degradation (ERAD). Chaperone-mediate autophagy can remove proteins from the secretion pathway be transporting them across the ER membrane and into autophagosomes. Finally, proteins can be rerouted from the canonical secretion pathway by being encapsulated in vesicles leaving the ER or Golgi which include ATG proteins that merge with autophagosomes rather than progressing toward the cell membrane for release.
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proteasomal degradation [66].

Autophagy-lysosomal degradation is used for eliminating cellular machinery associated with protein production. The phospholipids that make autophagosomes originate in the ER. Degradation of ribosomes by autophagy reduces protein production and secretion [74]. Build-up of non-soluble protein aggregates induces ER shedding of phagophores for targeted degradation of bulk ER contents [75]. That said, protein secretion of inflammasome associated proteins such as IL-1ß can increase under some stress conditions (starvation) [76]; however, it is unlikely therapeutic recombinant proteins use this atypical, autophagy-mediated secretion pathway because of the lower overall secretory flux. Nonetheless, the autophagy inhibitor 3-methyladenine has been used to improve therapeutic protein secretion in CHO cells [77,78].

4. The role of the ER in protein secretion

Secretion of recombinant proteins begins in the rough ER (Fig. 3), where soluble proteins are translated and inserted into the ER lumen. In the ER, proteins are post-translationally modified, including disulfide bond formation and glycosylation. Proteins are subsequently transported in COPII-coated vesicles from the ER to the Golgi apparatus, which is made up of a series of cis and trans compartments (cisternae). The trans Golgi network (TGN) is responsible for protein secretion, where clathrin-coated vesicles bud off the TGN and traffic to the plasma membrane. Simultaneously, retrograde transport from the Golgi to the ER is occurring in COPI-coated vesicles. The TGN also transports vesicles to the early endosome, where the fate of the vesicle contents is determined by the proteins present on the vesicle membranes (e.g., Rab4, Rab5, Rab7, or Rab11) [79]. Transport of membrane bound vesicles requires cytoskeletal rearrangement which under stress is mediated by HSPBs. While there are additional mechanisms available to the cell for protein secretion, including: lysosome secretion, transport through protein transporters, multivesicular body secretion, and membrane blebbing, the canonical secretion pathway, through the TGN, is the most common mechanism for protein secretion [80].

4.1. Links between the UPR and secretion machinery

The UPR also directly regulates the secretion machinery. IRE1α regulates protein secretion through XBP1s. XBP1 overexpression increases the capacity of the ER and secretory machinery and thereby the secreted proteins [81]. Upregulating XBP1s increased IL-6 and IgM secretion in CHO-K1 cells expressing the human placental secreted alkaline phosphatase (SEAP) [82]. Additionally, IRE1α may impact protein secretion through RIDD, as the mRNA of many secreted proteins, e.g., secretory μ chains of IgM heavy chains, are RIDD targets [83]. Activated PERK also impacts secretion by interacting with actin-binding protein Filamin A. Cells without PERK do not form ER-plasma membrane contact sites and have dysregulated F-Actin/G-actin localization [84]. Proper F-actin mechanics, which depends on its localization, are required for protein secretion [85]. Impacts of ATF6 on secretion are poorly studied. ATF6 overexpression reduced the secretion of some amyloid disease-causing proteins through increased quality control and degradation [86]. All three branches of the UPR modulate lipid synthesis and processes in the ER, altering lipogenesis, lipolysis, triglyceride synthesis, fatty acid content, cholesterol synthesis, and phospholipid content [87]. Over expression of sterol regulatory element binding factor 1 (SREBF1) which activates *de novo* lipogenesis and fatty acid synthesis has been used to engineer the lipid composition of CHO cells to increase productivity at the expense of cell survival [88].

In summary, protein secretion is known to be a limiting factor in recombinant protein therapeutic production. Current studies have difficulty resolving the effects of increased degradation and decreased secretion as both processes are regulated by both the UPR and endomembrane systems. In general, ER stress is to be avoided, since it traffics secretory proteins to degradation pathways, i.e., autophagy and proteasomal, and decreases the overall production of therapeutic proteins.

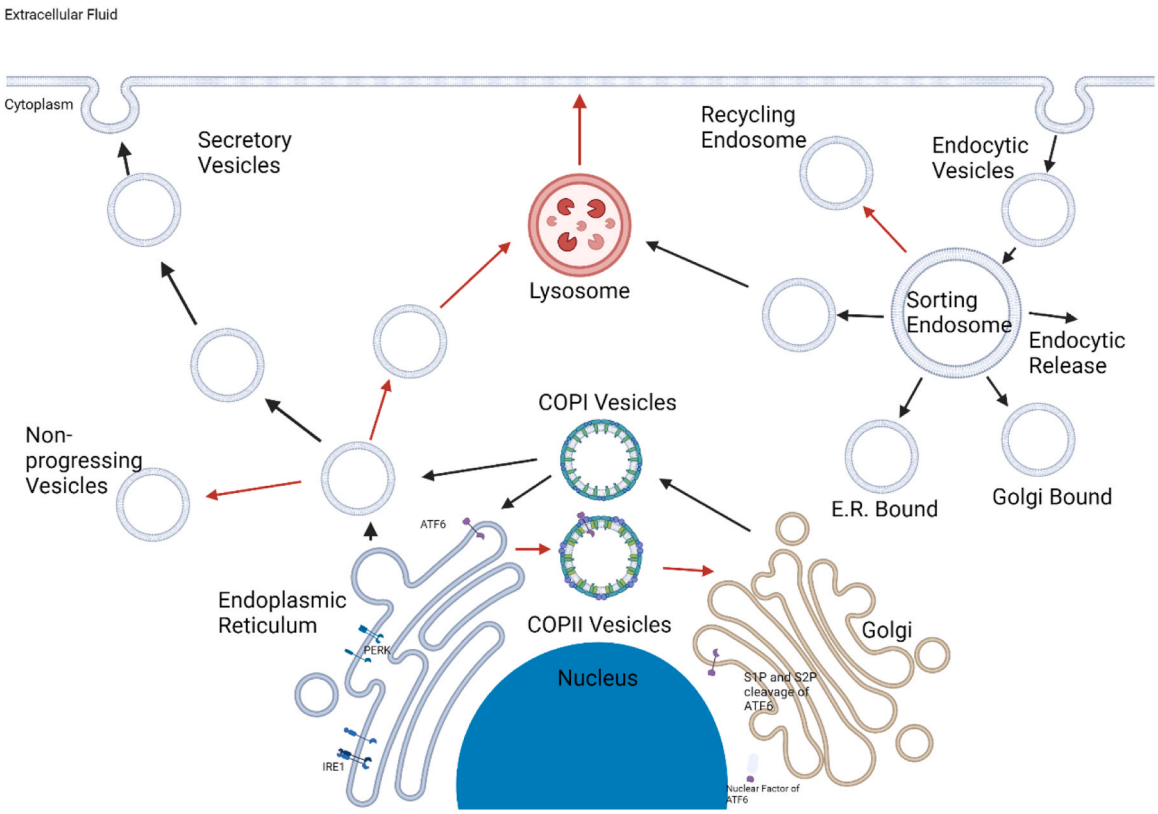

Fig. 3. : ER stress interrupts endomembrane homeostasis and secretion. The ER is intrinsically linked to the cell membrane, Golgi, lysosome, and other vesicles through control of shared membrane components. Beginning at the cell membrane, endosomes are trafficked to the early/sorting endosome. From the sorting endosomes, endosomal vesicles can be recycled or trafficked to the ER, Golgi, or lysosome. COPII coated vesicles transport material from the ER to the Golgi and COPI coated vesicles transport from the Golgi to the ER. Additional vesicles can leave the ER and Golgi for secretion or degradation in lysosomes. Lysosomal membrane components can be transported to the cell membrane to re-enter the endomembrane system. ER stress alters endosome recycling, lysosome recycling, ER to Golgi transport, and lysosome formation processes (red arrows).
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However, overexpressing XBP1 to increase secretory machinery size demonstrates a potential use of UPR-related genes to enhance protein production. Further studies are needed to determine the regulatory changes induced by XBP1 on the secretory pathway and chaperone-mediated protein synthesis that could lead to increased protein secretion for improved production of recombinant therapeutic proteins.

5. Genome instability in production hosts

Maximizing protein product yield requires high specific productivity in long-term culture. Maintaining CHO cell productivity in long-term culture has proven to be a major challenge [89–95]. One reason for declining productivity is reduction in transgene copy number due to genome instability [96,97]. Genome instability arises from DNA mutations and chromosomal rearrangements, resulting in subpopulations of CHO cells with lower copy number and reduced expression of the therapeutic protein [95]. Due to the increased metabolic demand to express the transgene, subpopulations with lower productivity outgrow higher productivity populations, resulting in reduced titers [98]. Approaches that enhance genome stability are important for cellular productivity and viability, which are critical for maintaining the yield of recombinant protein production in long-term culture.

Genome instability, defined as the increase in frequency of genomic alterations during cell division, was first reported in CHO cells in the 1970s [99] and has been extensively studied in relation to cancer [100–102]. Genome instability is linked to the inability of cells to properly perform DNA damage repair induced by double-strand breaks (DSBs) [103–105]. Accumulation of DNA DSBs compromises genome stability through chromosomal aberrations. Karyotype variation, which is a type of genome instability, has been identified in cultured CHO cells [96,106]. Furthermore, recent studies showed that variations in karyotype were related to artificial culture conditions (e.g. elevated oxygen levels, rapid cell division), which may increase DSBs [106–108]. Different lineages of CHO lines were associated with deficiencies in DNA repair [109,110]. A recent study showed that methotrexate, used for gene amplification in CHO cells, induced DSBs and subsequently led to extensive chromosomal rearrangement [111]. Furthermore, the ability of CHO cells to repair DSBs was shown to decrease with time (increased passage numbers), which could contribute to increased chromosomal instability and losses of productivity [112]. To enhance chromosomal stability and prevent loss in productivity, it is important to understand the mechanisms of DNA repair and their support of chromosomal/genome stability.

Genome instability has been associated with mutation of tumor suppressor gene p53 [103,113]. p53 is a transcription factor that plays an important role in cell cycle arrest, DNA repair, and apoptosis [113–115]. Interestingly, CHO-K1 cells possess a missense mutation at codon 211 [116]. This missense mutation does not affect CHO-K1 cells' ability to repair DNA damage induced by UV irradiation. However, a target of p53, p21, is not activated in CHO-K1 cells, which affects the cell's ability to induce G1 arrest [117,118]. Inability to induce G1 arrest could explain the increase in genome instability/chromosomal rearrangement as unrepaired DNA damage is propagated to the daughter cells. The p53 protein, known to be mutated in CHO-K1 cells [118,119], could explain, in part, the intrinsic genome instability of CHO cells. Thus, the intrinsic genome instability of CHO cells may be a result of

mutated p53 or reduced capacity to repair DNA DSBs.

5.1. DNA damage repair and mitochondria membrane potential pathways

A recent study found that CHO cell lines expressing difficult-to-express bispecific molecules generated from mitochondrial membrane potential (MMP)-enriched host outperformed the parental host [120]. High MMP influences production, while the loss of MMP leads to mitochondria dysfunction. Increasing evidence suggest that the ER and mitochondria crosstalk during ER stress [121]. The mitochondrial DNA (mtDNA) is more affected by DNA damaging agents than nuclear DNA, and unrepaired mtDNA can accumulate and lead to mitochondrial dysfunction [122]. Thus, DNA repair in the mammalian cells is important for proper mitochondrial function. To maintain genome integrity and mitochondrial function requires that DNA damage be repaired. DNA damage and repair pathways can be divided into subtypes depending on the type of DNA lesion. Table 2 compares the different DNA repair pathways.

5.1.1. SSB repair

For single-strand breaks (SSBs), the repair pathways are mismatch-mediated repair (MMR), nucleotide excision repair (NER), and base excision repair (BER) [113,123,124]. SSBs are considered to be the most common type of DNA lesion in mammalian cells [125,126]. Accumulation of unrepaired SSBs can contribute to DNA replication stress, transcriptional stalling, and, subsequently, genomic instability [127,128]. Excision repair is a major DNA damage response pathway for SSBs. Its function includes recognition and removal of damaged bases/nucleotides from the DNA [129]. The two main repair pathways under excision repair are NER and BER. NER recognizes and removes localized, bulky DNA adducts that occur when DNA is covalently modified by chemicals, radiation, or mutagens (e.g. UV light or aromatic hydrocarbons) [130–133]. BER is used for smaller areas of DNA damage or apurinic/apyrimidinic sites induced by DNA alkylation (e.g., alkylating agent temozolomide), deamination (removal of amino group via DNA glycosylases), or oxidation (via reactive oxygen species (ROS)) [134–136]. DNA mismatch repair corrects base-base mismatches as well as insertion/deletion errors that occur during DNA replication and recombination [137–139]. Defects in MMR have been linked to increased mutation rate as defects in G2/M cell cycle arrest [123,140]. Both are key regulators of genome stability.

Table 2
Comparison among different DNA repair pathways.

Type of DNA Damage	Repair Pathway	Repair factors	Targets/Mechanism
Single-strand break	NER	XPC, RAD23, CETN2, TFIIH	DNA modification by covalent alteration, bulky DNA adducts [130–133]
	BER	DNA glycosylases, APE1, FEN1	DNA modification by small base damage or apurinic/apyrimidinic sites [134–136]
	MMR	MSH2, MSH3, MSH6, MLH1, PSM2	DNA modification by base-base mismatches, insertions/deletions [137–139]
Double-strand break	NHEJ	DNA-PKcs, XRCC4, LIG4	Available in all stages of cell cycle, less accurate compared to HR. Break ends ligatable (no long-range resection) [143]
	aEJ	Pol Q, RPA	RAD51 defective, long-range DNA resection at break site, required microhomology [155]
	HR	MRN, BRCA1, RPA, RAD51	Only available in G2/S phase of cell cycle, less error-prone, requires homology template [144–149]
	SSA	RAD52, XPF, ERCC1	RAD51 defective, long-range DNA resection at break site, required sequence repeats flanking break site [152–154]

5.1.2. DSB repair

For DSB repair, non-homologous end-joining (NHEJ) and homologous recombination (HR) are activated [124,141,142]. Accumulation of DSBs can lead to chromosomal aberrations, genome instability, and apoptosis [105,125]. The two major DSB repair mechanisms are classical NHEJ (cNHEJ) and HR. cNHEJ is a rapid repair pathway in which the two-ends of the DNA break can be quickly ligated without strict sequence requirements [143]. HR, unlike NHEJ, is a multistep process that requires sequence homology near the break site as well as a reference template (e.g. sister chromatid) to complete the repair [144–148]. This difference makes HR a higher fidelity DNA repair pathway than NHEJ. However, due to the requirement of a repair template, HR is available for DSB repair only during the S and G2 phase of the cell cycle [149]. It has been suggested that RNA-based repair templates (via RNA: DNA hybrids/R-loops) are used in HR repair near transcriptionally active regions of the genome [150,151]. In addition to NHEJ and HR, other DSB repair pathways such as single-strand annealing (SSA) and alternative end joining (aEJ) are also available depending on specific requirements such as sequence homology near the damaged site or the expression of specific repair factors [141,152–155].

5.2. Connection between genome integrity and ER stress/UPR

Emerging evidence suggests crosstalk between ER stress signaling and genome integrity [102,156–161]. XBP1-ChIP (chromatin immunoprecipitation) sequencing in mouse models identified XBP1 could regulate many DNA damage repair (DDR) repair genes [162]. DNA damage from the treatment of DNA damaging agent etoposide and γ-irradiation triggered the activation of c-Abl tyrosine kinase (part of the DSB repair pathways), which phosphorylates IRE1α [161,163]. Interestingly, Dufey and coworkers found the phosphorylation of IRE1α by c-Abl in mouse embryonic fibroblasts (MEFs) selectively activate RIDD to regulate downstream DNA damage signaling and response [161].

Methotrexate, previously used extensively in biomanufacturing further supports connections between ER stress and genome integrity. Given its role also in chemotolerance, these studies could provide insights on DNA repair pathways [164,165] and improvements to therapeutic protein production. Phosphorylation of IRE1α is elevated in chemotolerant cells [166]. Inhibiting IRE1α with various small molecular inhibitors (e.g., MKC8866, a salicylaldehyde analog, 4μ8 C, STF-083010) reduced chemotolerance [167–170] suggesting that activation of IRE1α signaling may enhance cell viability in the presence of DNA damage-causing agents like methotrexate. Thus, factors used in biomanufacturing that induce DNA damage could be mitigated through modulating IRE1α signaling.

Studies suggest potential roles of PERK in DDR. Knockdown of PERK increased ROS and oxidative DNA damage in cells [171]. Phosphorylation of PERK by nuclear factor (erythroid-derived 2)-like 2 (nrf2) transcription factor increased production of glutathione, an antioxidant [172–178]. Furthermore, knockdown of the PERK-ATF4-lysosome-associated membrane protein 3 (LAMP3)-arm was found to increase the radiosensitivity in human breast cancer [179]. Specifically, knockdown of LAMP3 limited DNA damage recognition by reducing the level of histone H2A.X phosphorylation (γH2A.X), and possibly diminishing the overall DNA damage repair capacity [179]. H2A.X is a variant of histone H2A. Unlike H2A, it is non-randomly distributed in the genome but is localized near/at the site of DSBs [180]. Upon the generation of DSBs, H2A.X is phosphorylated at the serine-139 position (γH2A.X) and serves as a checkpoint for downstream repair pathways such as HR and NHEJ [181]. Similar to IRE1α, activation of PERK signaling promotes DDR pathways, which contributes to enhancing genome stability and thereby could increase the yield of protein production in long-term culture.

Similarly, studies indicate that ATF6 also has a pro-survival/DNA repair role [182–184]. Knockdown of ATF6 increased irradiation-sensitivity and altered ROS regulation [184]. In addition, ATF6 knockdown decreased BRCA-1 expression and increased DNA damage and γH2A.X expression [183]. ATF6 also regulates catalase, an enzyme known to reduce hydrogen peroxide (a ROS) and oxidative DNA damage [185]. The role of γH2A.X is contradictory in the literature. One study suggest that an increase in γH2A.X is directly proportional to the amount of DNA damage, in contrast, another study indicated that γH2A.X is directly proportional to DNA damage repair since γH2A.X serves as a checkpoint for HR and NHEJ [181]. Table 3 lists connections between the UPR sensor proteins and DDR pathways. Interestingly, different synthetic pathways have been considered for producing etoposide, which is a popular chemotherapy drug [186]. However, since etoposide is very efficient at generating DNA damage, it might significantly affect the productivity of the cell of choice for biomanufacturing etoposide. Taken together, these studies suggest UPR sensor proteins contribute to DNA damage repair, a potential consideration for improving long-term cell viability and, in turn, protein production.

5.3. Factors contributing to DNA damage in CHO cells in industrial processes

Environmental and internal factors (e.g., metabolites and hormones) can affect UPR signaling and as discussed above, the viability and thereby the productivity of cells in a bioreactor (Table 4). Thus, understanding how these factors affect genome integrity would provide insight into improving cell viability and productivity. Accumulation of metabolic waste products, specifically ammonia and lactate, can lead to genome instability of CHO cells [190]. “Old” (passage 35) CHO cells have decreased productivity and reduced DNA damage repair (but not damage recognition) relative to “young” (passage 21) cells. Specifically, genes associated with HR (Rad51, XRCC2) were reduced [112].

Table 3
Connections between UPR and DNA repair pathways across cell types.

Cell lines	ER stress sensor/ pathway	Source of DNA damage/ cellular toxicity	Mechanism of DDR	Details
Hepatocellular carcinoma SKHep1, Hep3B, Huh7, HCCLM3	IRE1-XBP1	Tunicamycin, XRCC2 siRNA knockdown	XRCC2/HR	XBP1s reduces XRCC2 transcription/ mitochondrial DNA repair [187]
Human Multiple Myeloma SKO−007 (J3)	IRE1-XBP1	PARP and CHK1 inhibitors (AZD2461 and UCN−01)	HR/BRCA1	Inhibition of PARPs and CHK1 reduces XBP1s and increase DNA damage [188]
MEF, HEK	IRE1-RIDD	Etoposide	Cell cycle regulation/ chromatin remodeling	IRE1α-RIDD signaling induces cell cycle arrest and DNA repair [161]
Human lung carcinoma A549	Unspecified	Ionizing radiation (IR)	RAD51/HR	Tunicamycin used to induce ER stress which suppresses Rad51 protein via 26 S proteasome [189]
Human breast MDA-MB−468 and T47D	PERK	None added, observed elevated ROS	DSB repair, unspecified	PERK knockdown correlates with increased γH2A.X without any genotoxic agents [171]
Human breast MDA-MB−231 and MCF−7	PERK	Ionizing radiation (IR)	DSB repair, unspecified	Increases DNA damage repair signaling [179]

Table 4
ER stress induction by metabolites.

Metabolites/others	ER stress sensor	UPR activities	Cell line
Glucose	IRE1α	IRE1α phosphorylation, XBP1 splicing	Beta cells [196–198]
Palmitate, oleate	IRE1α, PERK, ATF6	IRE1α phosphorylation, PERK, eIF2a, XBP1 splicing	Beta cells [199]
Palmitate, oleate, Stearate	IRE1α, PERK	IRE1α phosphorylation, PERK, eIF2a, XBP1 splicing	Myeloid cells [200]
7-ketocholesterol*, 4-hydroxynonenal*	IRE1α, PERK, ATF6	IRE1α phosphorylation, PERK, eIF2a, XBP1 splicing	Endothelial cells [201]
Insulin	IRE1α	IRE1α phosphorylation, XBP1 splicing	Hepatocytes [202]
Glucagon/epinephrine	IRE1α	IRE1α phosphorylation, no XBP1 splicing	Hepatocytes [203]
Glucagon/(PA, OA, SA, LA at equimolar)	IRE1α	IRE1α phosphorylation, XBP1 splicing	Hepatocytes [204]
IL−4	IRE1α	IRE1α phosphorylation, XBP1 splicing	Macrophage [205]

Environmental factors, such as saturated and unsaturated fatty acids have different effects on CHO cells. Unsaturated fatty acids suppressed chemically-induced chromosomal aberrations unlike saturated fatty acids that shared the same number of carbons [191]. Monosaturated fatty acid oleate induced autophagy with minimal effects on apoptosis, while saturated fatty acids inhibited autophagy (which could improve therapeutic protein secretion [56,57]), generated ROS, and induced apoptosis [192]. Saturated fatty acid-induced apoptosis was further enhanced by a loss-of-function of p53 (important for genome integrity) [193,194]. Thus, fatty acids have multi-faceted effects, both beneficial and deleterious.

Ammonia stress induced by waste products causes mutations in the forms of indels and single nucleotide polymorphisms [190]. Many of these mutations were found in genes regulating DDR, which accelerated mutations and resulted in the propagation of genome instability and reduced productivity in culture [190]. Increased hydrodynamic stress (measured as average energy dissipation rate) decreased productivity and correlated with increased DNA damage in CHO cells [195].

In summary, many environmental and internal factors can lead to DNA damage and genome instability. Given that the UPR sensor proteins play a role on genome stability, more studies are needed to determine the contexts when the ER stress sensor proteins can be used to mitigate the effects of these environmental and internal factors that reduce cell viability and productivity.

6. Summary

Production of the recombinant proteins will continue to grow as an essential part of the development of biotherapeutics. Two important cellular processes to consider when optimizing recombinant protein production are cellular productivity and long-term viability. Both processes have been shown to be regulated by ER stress/UPR. Specifically, UPR can influence the balance between protein secretion and degradation (affects productivity) as well as DNA damage repair/genome stability (affects viability and in turn productivity). As such, it is important to consider how the bioprocess environment induces ER stress on host cells and how to manage that stress signaling to ensure maximal product

yields.

CRediT authorship contribution statement

Christina Chan: Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Kevin Chen:** Writing – original draft, Methodology, Conceptualization. **S. Patrick Walton:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **R. Chauncey Splichal:** Writing – original draft, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Corresponding author (CC) is an Associate Editor of BEJ. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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