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Signalling Pathways in the Unfolded Protein Response: Development from Yeast to Mammals

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The accumulation of unfolded proteins in the endoplasmic reticulum (ER) under ER stress conditions activates a series of homoeostatic responses collectively termed the unfolded protein response (UPR). The UPR is unique in which the molecular mechanisms it uses to transmit signals from the ER lumen to the nucleus are completely different to those used for signalling from the plasma membrane. An ER stress signal is sensed and transmitted across the membrane by a transmembrane protein(s) in the ER. Interestingly, the number of such functional sensors/transducers, ubiquitously expressed, has increased with evolution, for example, one in *Saccharomyces cerevisiae*, two in *Caenorhabditis elegans* and *Drosophila melanogaster*, and three in mammals. Accordingly, mammalian cells are able to cope with ER stress in a more sophisticated manner. Here, I summarize the mechanisms and activation consequences of UPR signalling pathways in yeast, worm, fly and mammalian cells. I also discuss how they have evolved to counteract ER stress effectively.

Key words: endoplasmic reticulum, phase shift, protein degradation, protein folding, quality control.

Abbreviations: bZIP, basic leucine zipper; ER, endoplasmic reticulum; ERAD, ER-associated degradation; eIF2α, eukaryotic translation initiation factor 2; UPR, unfolded protein response.

Proteins must be correctly folded and assembled to fulfil their functions as assigned by genetic code. All living cells have developed systems to counteract unfolding or misfolding of proteins. A typical example of such a homoeostatic response is found in the endoplasmic reticulum (ER), into which newly synthesized secretory and transmembrane proteins are translocated. The ER contains a number of molecular chaperones and folding enzymes (collectively termed ER chaperones hereafter) and provides an optimal environment for the productive folding of these proteins. Proteins remaining unfolded or misfolded even after the assistance of ER chaperones are retrotranslocated back to the cytosol, where they are ubiquitinated and degraded by the proteasome through a process termed ER-associated degradation (ERAD). These two mechanisms, productive folding and ERAD, ensure the quality of proteins that pass through the ER and allow only correctly folded molecules to move along the secretory pathway (*1*, *2*). However, the ER quality control system is compromised under a variety of conditions, collectively termed ER stress, resulting in the accumulation of unfolded proteins in the ER. Essentially, all eukaryotic cells cope with ER stress and maintain the homoeostasis of the ER by activating the unfolded protein response (UPR) (*3*, *4*).

A prototype of the UPR was first discovered in the 1970s, when analysis of virus-transformed mammalian cells identified GRP78 and GRP94 as proteins inducible by glucose starvation, with GRP standing for glucose-regulated protein (*5*). Substantial analysis of the UPR began in the late 1980s, when the accumulation of unfolded proteins in the ER was found to trigger the induction of GRP78 (found to be identical to the major ER chaperone BiP) and GRP94 (found to be an ER chaperone of the Hsp90 family) (*6*). This finding in turn implied that the UPR is a homoeostatic response that maintains the protein-folding environment in the ER by suppressing the proteotoxicity of accumulated unfolded proteins.

ER stress signals emanating from the lumen of the ER are sensed and transmitted across the membrane by a transmembrane protein(s) in the ER. The number of such functional UPR transducers, ubiquitously expressed, has increased with evolution, for example, one (Ire1) in *Saccharomyces cerevisiae*, two (ire-1 and pek-1) in *Caenorhabditis elegans* and *Drosophila melanogaster*, and three (IRE1, PERK and ATF6) in mammals. The UPR is unique in that the molecular mechanisms it uses to transmit signals from the ER lumen to the nucleus are completely different from those used for signalling from the plasma membrane. Thus, Ire1/Ire-1/IRE1 is an ER-membrane-bound

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(type I) endoribonuclease and pek-1/PERK is an ER-membrane-bound (type I) protein kinase, whereas ATF6 is an ER-membrane-bound (type II) transcription factor. Thanks to these three pathways, mammalian cells are able to cope with ER stress by bringing about a general attenuation of translation to decrease the burden on the folding machinery (via the PERK pathway; see below); inducing the transcription of ER chaperones to augment folding capacity; and inducing the transcription of ERAD components to enhance degradation capacity (via IRE1 and ATF6 pathways; see below). The combined effect of these responses aims to ensure the maintenance of the ER (*3*, *4*).

UPR MECHANISM IN YEAST

The use of the budding yeast *S. cerevisiae* as a model in the 1990s led to major progress in understanding the molecular mechanisms of the UPR. Specifically, this yeast was used for the molecular cloning of the UPR transducer Ire1, which is present in the ER (*7*, *8*), as well as that of the UPR-specific transcription factor Hac1 (*9*, *10*). This was followed by the discovery of the Ire1-mediated splicing of HAC1 mRNA, which connects the event in the ER to that in the nucleus (*9*, *11*). Because the HAC1 intron has the ability to block translation, Hac1 is not translated from HAC1 unspliced mRNA but from HAC1 mature mRNA (*11*, *12*). This unconventional (spliceosome independent) splicing not only allows the translation of HAC1 mRNA but also joins the DNA binding domain and transcriptional activation domain, which are separated by the HAC1 intron in HAC1 unspliced mRNA (*13*). Accordingly, yeast cells are able to induce the transcription of a number of ER chaperones with potency only under ER stress via a *cis*-acting element present in their promoter regions (*14*).

Importantly, microarray analysis, a new technology invented in the late 1990s, has increased the number of UPR target genes drastically to 381, of the total 6,607 yeast genes listed in the Saccharomyces genome database as of June 14, 2009. These 381 include not only ER chaperones but also numerous proteins working at various stages of secretion, specifically proteins involved in translocation, protein folding, protein degradation, glycosylation in the ER, lipid/inositol metabolism, ER-Golgi transport, Golgi-ER retrieval, glycosylation in the Golgi apparatus, vacuolar targeting, distal secretion and cell wall biogenesis. Based on these findings, it was proposed that activation of the yeast UPR induces specific remodelling of the secretory pathway to minimize either the amount or concentration, or both, of unfolded proteins in the ER (*15*). Although the IRE1 and HAC1 genes are not essential, IRE1- or HAC1-knockout yeast cells are sensitive to ER stress. Importantly, translation is not attenuated under ER stress in yeast cells owing to the absence of pek-1/PEK/PERK, while ER chaperones and ERAD components are transcriptionally induced simultaneously via the Ire1-Hac1 pathway owing to the absence of ATF6 in yeast cells. ER chaperones and ERAD components may deal with accumulated unfolded proteins in a competitive manner.

UPR MECHANISM IN METAZOANS

In metazoan cells, the UPR transducer Ire1 is well conserved as Ire-1 in worm (*C. elegans*) (*16*), IRE1 in fly (*D. melanogaster*) (*17*) and IRE1α/β in mammalian cells [IRE1α is expressed ubiquitously (*18*, *19*), whereas IRE1β is expressed only in the gut (*20*)]. Interestingly, however, the transcription factor down-stream of Ire1 is switched from Hac1 to xbp-1 or XBP1 in metazoan cells (*16*, *21*, *22*) (Fig. 1, right part). Although both Hac1 and xbp-1/XBP1 encode a basic leucine zipper (bZIP)-type transcription factor and their mRNA is subjected to Ire1-mediated unconventional splicing in response to ER stress, their primary amino acid sequences are not similar. The evolutionary pressure that caused this switching

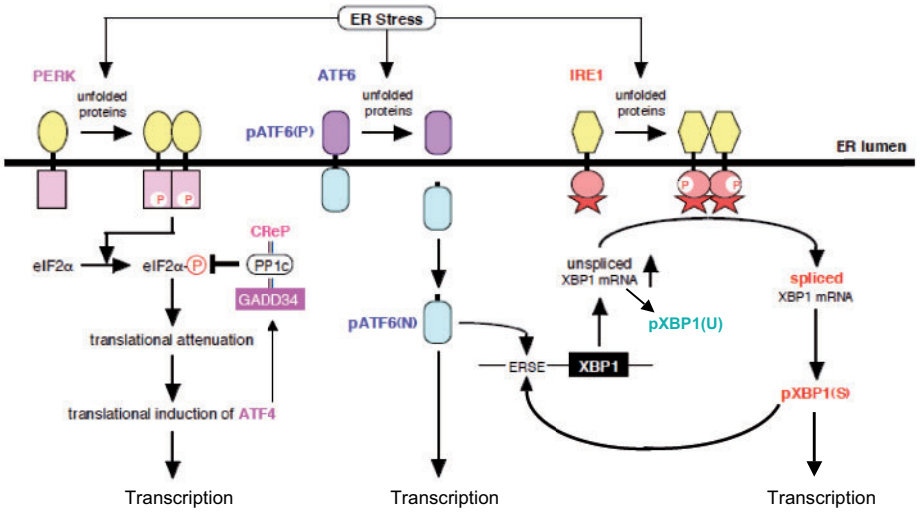

Fig. 1. **UPR mechanism in mammals.**

of transcription factors down-stream of Ire1 remains to be determined.

In addition to ire-1/IRE1, worm, fly and mammalian cells also express pek-1, PEK and PEK/PERK, respectively, as a second UPR transducer (*16*, *17*, *23*) When activated upon ER stress, pek-1/PEK/PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), which leads to the general attenuation of translation. Thus, metazoan cells are able to decrease the burden on the ER when the protein-folding environment is compromised, in marked contrast to yeast cells, which continue protein synthesis even under ER stress conditions. Paradoxically, PERK-mediated translational attenuation induces translation of mRNA, encoding the bZIP-type transcription factor ATF4, which activates transcription of genes involved in amino acid metabolism and resistance to oxidative stress (*24*, *25*). Simultaneously, transcription of GADD34 is induced, resulting in recruitment of protein phosphatase 1c, which dephosphorylates eIF2α (*26*). Thus, translation levels attenuated by activated PERK return to normal automatically with time (*27*) (Fig. 1, left part).

Further, worm and fly cells express atf-6 (*16*, *17*), whereas mammalian cells express ATF6α and ATF6β [both expressed ubiquitously (*28*, *29*)] as third UPR transducers. Owing to the marked difference in functionality of atf-6/ATF6α in the UPR, the UPR mechanism in mammals and that in *C. elegans* and *D. melanogaster* are described in different sections.

UPR MECHANISM IN MAMMALS

Extensive genome-wide searches using microarray or chromatin immunoprecipitation analyses by several laboratories have consistently shown that mammalian XBP1 is involved in the induction not only of genes working for protein folding and degradation in the ER but also of many genes working for the secretory pathway and others, similar to the case of Hac1 in *S. cerevisiae*. These findings indicate that XBP1 is a multifunctional transcription factor (*30–33*). Interestingly, however, the IRE1-XBP1 pathway is dispensable to the induction of major ER chaperones such as GRP78/BiP and GRP94 in mammalian cells, but is required for induction of a subset of ER chaperones and most ERAD components (*30*, *34–36*). This indicates diversity in the transcriptional induction system in mammals.

Mammalian ATF6α/β, the third UPR transducers, are constitutively expressed as type II transmembrane proteins in the ER, designated pATF6α/β(P). These are activated by ER stress-induced proteolysis (*28*, *29*), which occurs in the Golgi apparatus via the action of Site-1 and -2 proteases (*37*). Thus, pATF6α/β(P) relocates from the ER to the Golgi apparatus upon ER stress via COP II vesicles (*38*). This cleavage results in the release of their N-terminal fragments, designated pATF6α/β(N), which enter the nucleus and activate transcription (*39*) (Fig. 1, centre part). Analysis of mouse embryonic fibroblasts deficient in ATF6α or ATF6β revealed that ATF6α and not ATF6β is required for transcriptional induction of major ER chaperones as well as of ERAD components (*40*, *41*). Microarray analysis identified only 30 genes among the 14,729 mouse genes as ATF6α-target genes. Notably, among these 30, seven were ER chaperones, five were ERAD components, and six were ER proteins, whereas the remaining 12 targets were miscellaneous and could not be further categorized. This classification in turn led to the proposal that ATF6 is a transcription factor specialized in the regulation of ER quality control proteins (*42*). Detailed analysis showed that ATF6α is solely responsible for the transcriptional induction of major ER chaperones and that ATF6α heterodimerizes with XBP1 for the induction of major ERAD components (*41*). Thus, ATF6 has gained the ability to induce the canonical UPR target genes in higher eukaryotes.

Because the 252-nt HAC1 intron has the ability to block translation, as mentioned above, yeast HAC1 unspliced mRNA is not translated under normal conditions (*11*, *12*). In contrast, the XBP1 intron, consisting of 23 and 26 nt in worm and mammalian cells, respectively, is too short to block translation. Thus, XBP1 unspliced mRNA is constitutively translated to produce pXBP1(U): U stands for unspliced, and the translational product of XBP1 spliced mRNA is referred to as pXBP1(S) (*21*, *22*) (Fig. 1, right part). pXBP1(U) and pXBP1(S) share an N-terminal 166 aa containing the bZIP domain. Since the pXBP1(S)-specific C-terminal region (210 aa) functions as a transcriptional activation domain, ER stress-induced splicing of XBP1 mRNA joins the DNA-binding domain and activation domain to produce a highly active transcription factor, pXBP1(S), as in yeast (*21*). Interestingly, the pXBP1(U)-specific C-terminal region (95 aa) contains two characteristic domains, which are absent from pXBP1(S) (*43*), a nuclear exclusion signal that allows pXBP1(U) to shuttle between the nucleus and cytoplasm, and a degradation domain that targets not only pXBP1(S) but also pATF6α/β(N) in proteasome-mediated degradation via bZIP-domain-mediated direct interaction between pXBP1(U) and pXBP1(S) as well as pATF6α/β(N) (*43*, *44*). This result is consistent with the *in vitro* demonstration of dimer formation between the bZIP domains of XBP1 and ATF6 (*45*). Thanks to this newly gained mechanism, mammalian cells are able to shut off transcription of pXBP1(S)- and pATF6α/β(N)-target genes rapidly when ER stress is resolved. This rapid shut-off is important because over-expression of ER chaperones in the absence of ER stress by active transcription factor, namely Hac1 in yeast and pATF6α(N) in mammals, is known to be toxic to cells and to decrease the rate of cell growth. Levels of ER chaperones must accordingly be kept under fine control (*13*, *46*). It should be noted that pXBP1(U) does not interact with ATF4, a transcription factor down-stream of PERK, and therefore has no effect on turnover rate of ATF4 (*44*, *45*). This is probably because ATF4 is activated not only by ER stress but also by other stimuli such as amino acid starvation and viral infection; the lack of interaction between pXBP1(U) and ATF4 allows the cell to avoid undesired degradation of ATF4 induced in response to non-ER stress by pXBP1(U), which is constitutively synthesized. This process exemplifies the multiple layers of tight regulation found in the UPR.

PHENOTYPES OF KNOCKOUT MICE DEFICIENT IN UPR TRANSDUCERS

Knockout mice deficient in UPR transducers have been produced and characterized. IRE1α-knockout mice exhibit embryonic lethality (*19*, *34*), whereas IRE1β-knockout mice are normal provided they are not subjected to insult of the gut (*20*). Mice deficient in XBP1, a transcription factor downstream of IRE1, also show embryonic lethality via a primary defect in liver development (*47*). Specific deletion of XBP1 in immune cells blocks terminal differentiation of B cells into antibody-secreting plasma cells (*48*). PERK-knockout mice develop diabetes mellitus and exocrine pancreatic dysfunction after birth (*49*). These results clearly indicate the importance of the UPR in the function of professional secretory organs such as plasma cells, liver and pancreas. Although ATF6α- and ATF6β-knockout mice develop normally, ATF6α/β double knockout causes embryonic lethality (*41*). As no double knockout embryos were observed as early as at embryonic day 8.5, complete absence of ATF6 function seems to cause blockage of mouse embryonic development at a very early stage, as in the case of BiP knockout (*50*). The molecular basis of the tissue-specific defects observed in knockout mice deficient in ubiquitously expressed UPR transducers (IRE1α, PERK and ATF6α/β) remains to be determined.

UPR MECHANISM IN WORM AND FLY

The ire-1 null mutant is viable but sensitive to ER stress in *C. elegans* (*16*), as in *S. cerevisiae*. Microarray analysis showed that 202 of the 4,050 genes examined are ER stress inducible, 170 of which relied on the ire-1-xbp-1 pathway. As in *S. cereviasiae*, the Ire-1 pathway is responsible for induction of most canonical UPR target genes, including ER chaperones and ERAD components in *C. elegans* (*51*). This is also the case for *D. melanogaster* (*17*). These findings indicate that the ATF6 pathway is not functional in *C. elegans* or *D. melanogaster*, even though their genomes each contain a single atf-6 gene. Indeed, RNAi analysis in *C. elegans* showed that few genes depend on ATF6 for their induction in response to ER stress; and ER chaperones and ERAD components are fully induced in the atf-6 knockdown worm. Moreover, atf-6 (RNAi) worm or atf-6 null mutant

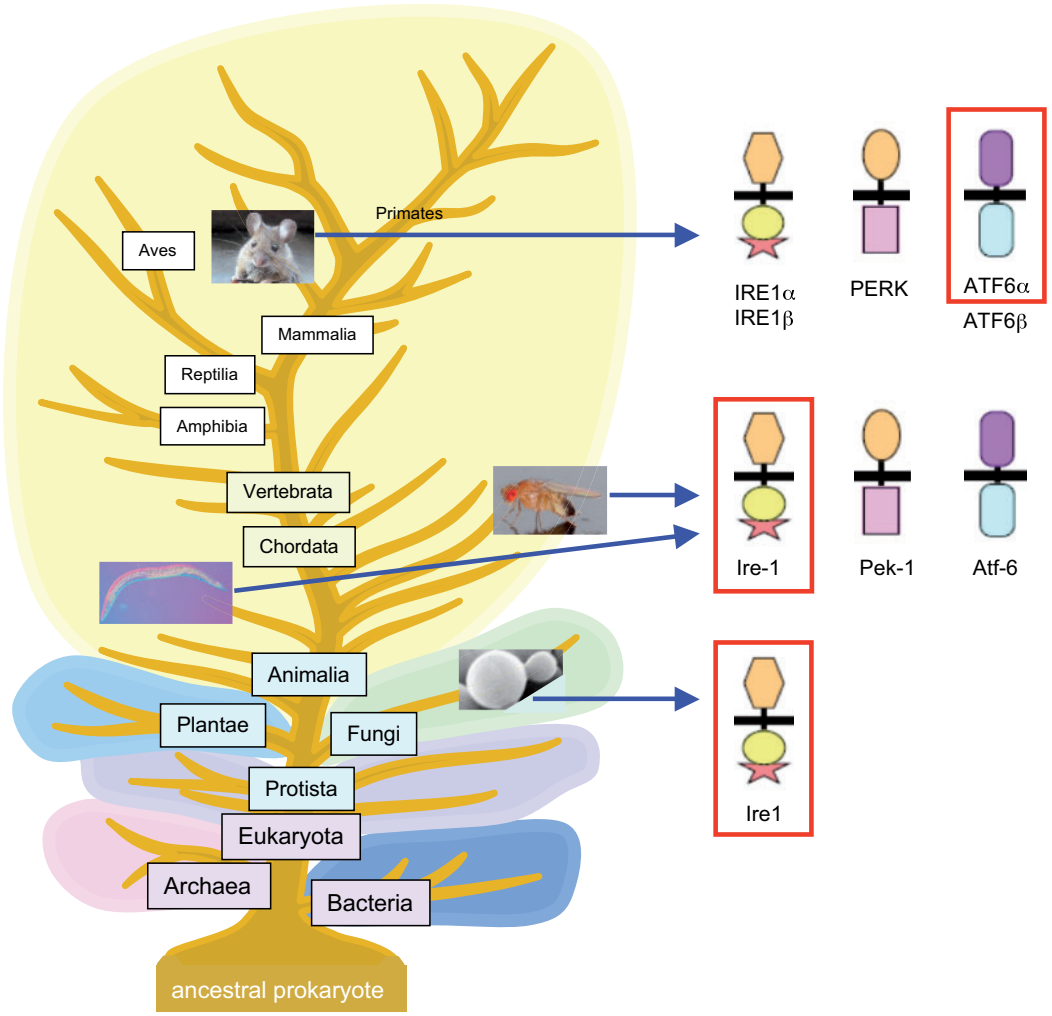

Fig. 2. **Evolution of the UPR.** The UPR transducers in red boxes each indicate a major regulator of ER quality control proteins.

worm exhibited no obvious phenotype compared with the wild-type worm (*51*). These findings imply that the major regulator of ER quality control proteins switched from IRE1 to ATF6 during evolution from fly to mammals (Fig. 2). It would be interesting to know why and when this switch occurred.

Interestingly, 324 of 4,050 genes required at least one of the UPR transducers (ire-1, xbp-1, perk-1 and atf-6) for their constitutive expression, 84 of which depended on atf-6. Constitutive expression of these 84 genes helps worms to alleviate ER stress, particularly in the absence of transcriptional induction of ER chaperones and ERAD components, as ire-1 and atf-6 double mutation causes synthetic lethality. It appears that worm cells express Site-2 protease but not Site-1 protease; these two proteases are known to cleave mammalian ATF6α/β in response to ER stress, as mentioned above. It remains to be determined whether worm or fly atf-6 is activated by ER stress-induced proteolysis as in mammalian cells, and if so, whether its cleavage is constitutive to carry out the constitutive expression of the 84 genes.

The pek-1 null mutant worm is indistinguishable from the wild-type under normal growth conditions but sensitive to ER stress (*16*). Because double mutation of ire-1 and pek-1 causes embryonic lethality, pek-1-mediated translational attenuation is considered essential for worm development when transcription of ER chaperones and ERAD components is not induced in response to ER stress due to the absence of the ire-1-xbp-1 pathway. Nonetheless, it has yet to be demonstrated that translation is indeed attenuated after ER stress in *C. elegans*. In addition to translational control, microarray analysis showed that 47 of 202 ER stress-inducible worm genes depend on pek-1 for transcriptional induction. Although not yet demonstrated, pek-1-mediated translational attenuation is considered to cause translational induction of the transcription factor ATF4 in *C. elegans*, as in mammals. On the other hand, only 9 of 324 worm genes required pek-1 to maintain constitutive expression. As all nine genes also required the ire-1-xbp-1 pathway, however, the significance of the pek-1-mediated constitutively enhanced expression of these nine genes remains elusive.

Because RNAi of both atf-6 and pek-1 resulted in no particular phenotype, the ire-1-xbp-1 pathway is accordingly the principal pathway operating in worms to counteract ER stress. Nonetheless, through their development of pek-1-mediated translational attenuation and/or transcriptional induction of certain gene expression, or atf-6-mediated constitutive enhancement of certain gene expression, worm cells have developed the ability to survive and develop normally even in the absence of the ire-1-xbp-1 pathway.

Surprisingly, in *D. melanogaster*, it was shown that IRE1 activated upon ER stress cleaves not only XBP1 mRNA but also a specific set of mRNAs that encode secretory and transmembrane proteins (*17*). As this IRE1-mediated mRNA decay occurs during the cotranslational translocation process, this mechanism is postulated to allow the clearance of proteins jammed at the translocation channel and folding machinery, and therefore allow the efficient delivery of induced ER chaperones and ERAD components into the lumen after recovery from PERK-mediated translational attenuation. Very recently, two groups independently showed that this mechanism was conserved in mammals (*52*, *53*). Interestingly, however, one group proposed that sustained activation of this mechanism leads to apoptosis (*52*). The question of whether IRE1-mediated mRNA decay is beneficial or detrimental to the cell requires further investigation.

TIME-DEPENDENT PHASE SHIFT IN MAMMALS

The acquirement of three (IRE1, PERK and ATF6) functional signalling pathways allows mammalian cells (but not worm and fly cells) to perform multiple phase shifts to cope with ER stress effectively and determine the fate

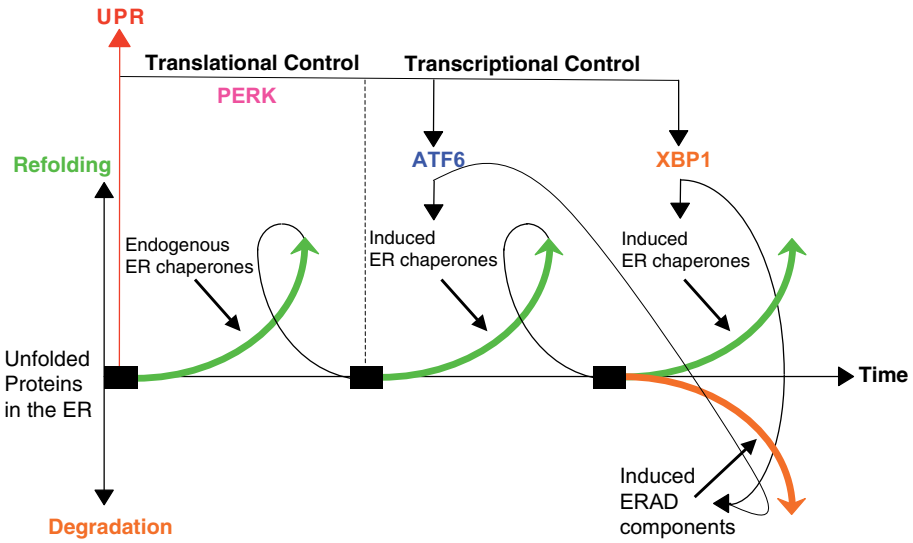

Fig. 3. **Time-dependent phase shift in mammalian UPR.**

of unfolded proteins accumulated in the ER (Fig. 3). When unfolded proteins are accumulated in the ER, mammalian cells undertake to first refold them using endogenous ER chaperones, which are abundantly expressed under normal conditions. PERK-mediated translational attenuation can decrease the burden on ER chaperones. If the unfolded proteins are refolded by the action of the endogenous ER chaperones, the UPR is finished. However, if unfolded proteins are not refolded completely, or continue to accumulate, the translational control phase is shifted to a transcriptional control phase, in which the active form of ATF6 is produced. Activated ATF6 induces ER chaperones, and if the unfolded proteins are refolded by the action of the induced ER chaperones, the UPR is finished. In this sense, the ATF6-mediated phase is unidirectional (refolding only). However, if unfolded proteins are still present in the ER, mammalian cells begin to synthesize active XBP1. Active XBP1 heterodimerizes with active ATF6 to induce ERAD components. Thus, as it induces not only ER chaperones but also ERAD components to degrade unfolded proteins, this phase is bi-directional (refolding plus degradation). This phase transition allows mammalian cells to maximally utilize proteins whose initial synthesis required the consumption of numerous ATP molecules. This also implies that the two signalling pathways (ATF6 and IRE1-XBP1) must be activated simultaneously for the execution of ERAD. The decision to induce ERAD components must be made carefully as ERAD consists of multiple ATP-dependent steps and thus also requires high energy. The involvement of three signalling pathways certainly gives versatility to mammalian cells, allowing them to execute phase transition, depending on the quality and/or quantity of unfolded proteins accumulated in the ER.

EVOLUTION OF THE UPR

Based on the published results, the following scenario to explain the evolution of the UPR is envisioned (Fig. 2). First, the IRE1 pathway evolved to counteract the accumulation of unfolded proteins in the ER. Because cells at this evolutional stage, such as *S. cerevisiae,* keep synthesizing proteins even under ER stress conditions, activated IRE1 induces transcription of not only ER chaperones and ERAD components but also numerous proteins working at various stages of secretion to minimize the amount, concentration, or both of unfolded proteins accumulated in the ER (*15*). Next, the PERK pathway emerged via gene shuffling between IRE1 and GCN2: GCN2 encodes a protein kinase, which phosphorylates eIF2α in response to amino acid starvation (*54*). Cells such as of *C. elegans* would now be able to attenuate translation and decrease the burden on the ER. This was perhaps advantageous in handling unfolded proteins in multicellular organisms, as these encounter not only environmental but also physiological ER stress during development or differentiation. The ATF6 pathway then evolved, which is specialized in regulating the transcription of ER quality control proteins. The difference between the IRE1 and ATF6 pathways in their mechanisms of activating downstream transcription factors allows cells such as those in mammals to perform a time-dependent phase shift to cope with ER stress in accordance with the quality, quantity or both of unfolded proteins accumulated in the ER (*35*, *41*). Finally, local quality control capability in the ER was strengthened by the development of various tissue-specific UPR transducers, such as IRE1β (*20*) and ATF6-like membrane-bound transcription factors, i.e. OASIS (*55*), CREBH (*56*), Luman/LZIP (*57*), Tisp40 (*58*) and BBF2H7 (*59*). It appears that ER stress is so threatening to the cell that ever more sophisticated tactics were created in accordance with the complexity of the biological systems in the cell or organism developed during evolution. A comprehensive understanding of the biological significance of the UPR awaits further extensive investigation.

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CONFLICT OF INTEREST

None declared.

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