

# The unfolded protein response (UPR) pathway in *Cryptococcus*

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**Abbreviations:** ATF6, activating transcription factor 6; bZIP, basic domain/leucine zipper; DTT, dithiothreitol; ER, endoplasmic reticulum; HOG, high osmolarity glycerol response; HU, hydroxyurea; IRE1, inositol-requiring protein 1; MMS, methyl methane sulfonate; PERK, protein kinase RNA-like ER kinase; PKA, protein kinase A; PKC, protein kinase C; RIDD, regulated Ire1-dependent decay; TM, tunicamycin; UPR, unfolded protein response; UTR, untranslated region; XBPI, X-box binding protein-1

Unique and evolutionarily conserved signaling pathways allow an organism to sense, respond to, and adapt to internal and external environmental cues at its biological niche. In eukaryotic cells, the unfolded protein response (UPR) pathway regulates endoplasmic reticulum (ER) homeostasis upon exposure to environmental changes causing ER stress. The UPR pathway of *Cryptococcus neoformans*, an opportunistic fungal pathogen, which causes life-threatening meningoencephalitis in immunocompromised individuals, consists of the evolutionarily conserved Ire1 kinase, a unique bZIP transcription factor, Hx11, and the ER-resident molecular chaperone Kar2/BiP. Although the *Cryptococcus* UPR pathway regulates ER stress, antifungal drug resistance, and virulence in an Ire1/Hx11-dependent manner, Ire1 has Hx11-independent roles in capsule biosynthesis and thermotolerance. In this review, we highlight the conserved and unique features of the *Cryptococcus* UPR pathway compared with other fungal UPR systems and its importance in the pathogenesis of cryptococcosis and discuss future challenges in this field.

## Introduction

The growing elderly population, the extensive use of immunosuppressive agents after organ transplantation or cancer therapy, and the AIDS pandemic has led to an increased number of immunocompromised individuals in past decades. As a result, systemic fungal infection has emerged as a major infectious disease. Life-threatening invasive fungal diseases are mainly caused by opportunistic fungal pathogens such as *Candida*, *Cryptococcus*, and *Aspergillus*.<sup>1</sup> With the onset of the AIDS pandemic, the basidiomycetous yeast *Cryptococcus* has emerged as one of the top ten

fatal invasive mycoses, because untreated cryptococcal infection causes lethal meningoencephalitis.

*Cryptococcus* includes two major pathogenic species, *C. neoformans* and *C. gattii*, which have been recently classified as separate species based on distinctive morphological and biochemical characteristics and infection patterns.<sup>2,3</sup> *C. neoformans* is the most commonly isolated clade worldwide and mainly infects immunocompromised populations. On the other hand, *C. gattii* was considered to be geographically restricted to tropical and subtropical regions of the world, but has become more recently isolated from infected immunocompetent individuals in non-tropical regions, such as the Pacific Northwest.<sup>4</sup> Nearly 1 000 000 cases of HIV/AIDS-related cryptococcal meningitis occur worldwide every year, causing more than 620 000 deaths.<sup>5</sup>

*Cryptococcus* is ubiquitous in environmental niches such as soil, trees, and bird guano. Infectious propagules, in the form of spores or dried yeast cells, are inhaled through the respiratory tract, leading to pulmonary infection. Subsequently, *Cryptococcus* disseminates from the lung into multiple organs through the bloodstream. This pathogen has a particular tropism to the central nervous system and traverses the blood brain barrier, resulting in meningoencephalitis.<sup>6</sup> During the progression of infection, *C. neoformans* deploys diverse virulence strategies to survive and proliferate in each of the host's biological niches. Two well characterized virulence factors are the antiphagocytic polysaccharide capsule and the antioxidant melanin.<sup>7</sup> Stimulated by several factors such as serum, iron limitation, and physiological CO<sub>2</sub> levels,<sup>8,9</sup> the capsule is composed of approximately 88% glucuronoxylomannan and 10% galactoxylomannan, and interferes with macrophage phagocytosis or confers direct immunosuppressive activity.<sup>10,11</sup> Melanin, which is a brown pigment made of polyphenol complexes, protects cells from environmental UV radiation and oxidative stress in the form of scavenging reactive oxygen species generated by the host defense system during infection. Melanin also enables *Cryptococcus* to escape from the lung to the central nervous system.<sup>12–14</sup>

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During infection, *Cryptococcus* experiences dramatic environmental transitions, such as thermal shock, oxidative stress, and high CO<sub>2</sub> levels in the host. Therefore, the ability to sense, respond to, and adapt to environmental changes is essential for its survival and proliferation in the host. *Cryptococcus* exhibits evolutionarily conserved and unique signaling pathways, including HOG (high osmolarity glycerol response), Ras, cAMP/PKA (protein kinase A), Ca<sup>2+</sup>/calcineurin, and PKC (protein kinase C) pathways, to overcome these external stresses.<sup>15-20</sup> In this review, we focus on the conserved and unique features of the *Cryptococcus* unfolded protein response (UPR), which has recently been shown to play an essential role in endoplasmic reticulum (ER) stress response, in comparison to those of the model yeast, *Saccharomyces cerevisiae*, and other fungal species. Furthermore, we discuss the potential of the UPR pathway as a novel antifungal therapeutic target and future challenges facing this field.

### General Features of UPR Pathways in Yeast and Higher Eukaryotes

The ER is a dynamic organelle with essential roles in protein synthesis, folding, modification, secretion, lipid synthesis, calcium storage and signaling. The accumulation of toxic unfolded or misfolded proteins in the ER triggers “ER stress”, which can also be induced by altered calcium homeostasis and glycosylation, oxidative stress, nutrient starvation, pathogen infection, and activation of inflammation.<sup>21</sup> Mounting evidence links ER stress to human diseases as diverse as diabetes, viral infection, Alzheimer disease, cancer, and inflammation.<sup>22,23</sup> To mitigate ER stress, eukaryotic cells activate conserved UPR signaling pathways, which regulate the expression of numerous genes encoding ER chaperones and folding enzymes as well as proteins involved in diverse cellular processes.<sup>21,24</sup>

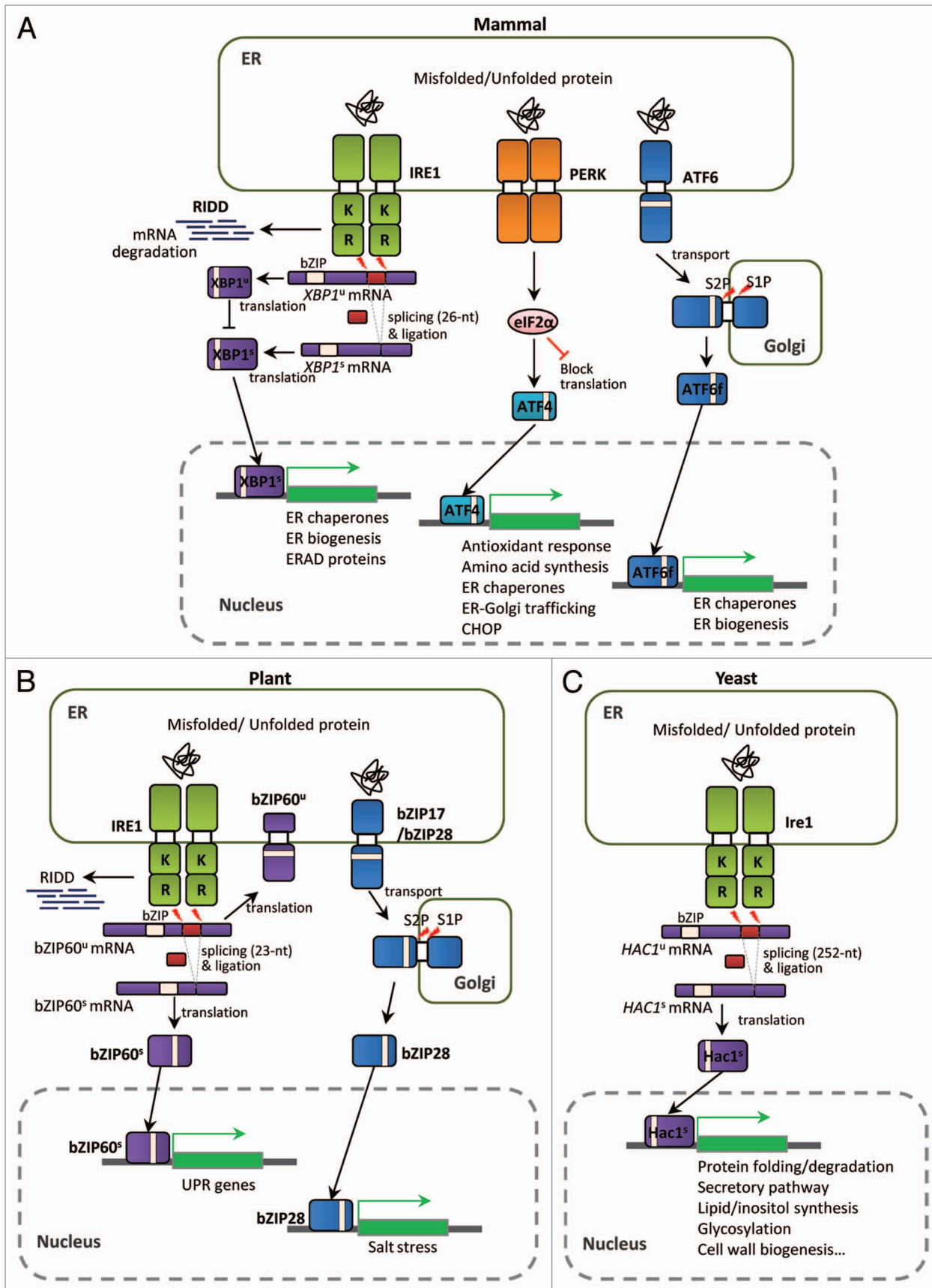
In mammalian cells, UPR-inducing signals are transduced via three UPR sensors localized at the ER membrane: inositol-requiring protein 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Fig. 1A).<sup>21</sup> Among these, the IRE1-mediated UPR pathway is the most evolutionarily conserved in eukaryotes. In humans, this pathway consists of IRE1, a type I transmembrane Ser/Thr kinase with

an endoribonuclease (RNase) domain, and its downstream transcription factor, XBP1 (X-box binding protein-1). During ER stress, the *XBP1* mRNA undergoes unconventional, spliceosome-independent splicing owing to the RNase activity of Ire1. The activated XBP1 transcription factor is subsequently translated from the spliced *XBP1* mRNA.<sup>21</sup> The unspliced *XBP1* mRNA is also translated, but generates a negative regulator of the UPR pathway.<sup>25,26</sup>

In plants, two branches of the UPR pathway were discovered to play essential roles in ER stress response (Fig. 1B). Similar to mammalian ATF6, the ER membrane-associated basic domain/leucine zipper (bZIP) transcription factors bZIP17 and bZIP28 undergo proteolytic activation to regulate ER stress response gene expression.<sup>27</sup> Recently, an IRE1-mediated *bZIP60* mRNA splicing event was identified in plants. Unspliced *bZIP60* mRNA encodes a membrane-associated protein with a bZIP domain and a single transmembrane domain. ER stress stimulates IRE1 to mediate an unconventional splicing event in the *bZIP60* mRNA, removing a 23 nt intron to produce a bZIP factor lacking the transmembrane domain but possessing a putative nuclear targeting signal. The resulting bZIP60 protein translocates to the nucleus to activate UPR target genes.<sup>28</sup>

In *S. cerevisiae*, a conserved Ire1 sensor and an XBP1 ortholog, Hac1, were discovered and characterized as key components in the yeast UPR pathway (Fig. 1C).<sup>29</sup> Ire1 senses ER stress in the ER lumen and undergoes autophosphorylation and dimerization for its own activation. Subsequently, activated Ire1 removes an unconventional intron from the *HAC1* mRNA, resulting in an active bZIP transcription factor.<sup>30</sup> Unlike the human *XBP1* mRNA, the unspliced *HAC1* mRNA is not translated because of long-range base pairing between the *HAC1* 5'-untranslated region (5'-UTR) and its intron. The Ire1-dependent unconventional splicing of the *HAC1* mRNA is critical for activation of a plethora of UPR target genes.<sup>31</sup> Recent studies on the UPR pathways of another ascomycetous yeast, *Yarrowia lipolytica*, as well as pathogenic fungi such as *Candida albicans*, *Aspergillus fumigatus*, and *Alternaria brassicicola*, revealed that the *HAC1* orthologs of these organisms are also subject to unconventional splicing upon ER stress.<sup>32-35</sup> Their UPR targets include genes with functions in ER stress, protein secretion, morphological differentiation, and

**Figure 1 (See opposite page).** Unfolded protein response (UPR) pathways in eukaryotes. **(A)** The mammalian UPR pathway consists of three ER-transmembrane sensor proteins, IRE1, PERK, and ATF6. Activation of IRE1 cleaves the 26 nt intron of *XBP1*<sup>u</sup> mRNA, and the activated XBP1<sup>s</sup> bZIP transcription factor upregulates many essential UPR target genes. On the other hand, the translated XBP1<sup>u</sup> protein appears to sequester XBP1<sup>s</sup> protein in the cytosol. IRE1 also controls selective mRNA decay (RIDD). Activation of PERK blocks general protein synthesis and increases the specific translation of *ATF4* mRNA via phosphorylation of eIF2 $\alpha$ . The ATF4 bZIP transcription factor induces expression of UPR target genes. ATF6 is a type II ER transmembrane protein with a bZIP domain. Upon ER stress, ATF6 is translocated to the Golgi and processed proteolytically by site-1 protease (S1P) and site-2 protease (S2P); the ATF6 fragment with the bZIP domain (ATF6f) is then released and translocates to the nucleus to activate UPR genes. **(B)** The UPR pathway in *Arabidopsis thaliana* consists of two branches: one involving endoribonuclease IRE1 and the other involving the proteolytic processing of membrane-associated bZIP transcription factors (bZIP17/28). Upon ER stress, IRE1 removes the 23 nt intron of *bZIP60* mRNA, resulting in a bZIP protein lacking a transmembrane domain (bZIP60<sup>s</sup>) via frameshift translation. The bZIP60<sup>s</sup> transcription factor translocates to the nucleus to activate UPR target genes. Similar to mammalian ATF6, the membrane-associated bZIP transcription factors (bZIP17/28) are processed at the Golgi by S1P and S2P, releasing the truncated versions of bZIP17/28 into the nucleus to activate UPR target genes. Regulated IRE1-dependent decay of specific mRNAs in *Arabidopsis* has also been observed recently. **(C)** The yeast *Saccharomyces* UPR pathway is composed of the Ire1 kinase and the Hac1 bZIP transcription factor. Accumulation of unfolded or misfolded proteins in the ER lumen causes Ire1 to dimerize and *trans*-autophosphorylate through its kinase domain. The activated Ire1 kinase removes the unconventional intron (252 nt) of the *HAC1* mRNA and a tRNA ligase, Rlg1, joins the two exons without the help of conventional spliceosomes. Spliced *HAC1* mRNA is translated to produce an active Hac1 protein, which translocates to the nucleus to upregulate expression of UPR target genes encoding ER-resident chaperones and other proteins. “K” and “R” in Ire1 indicate the kinase and ribonuclease domains, respectively.



**Figure 1.** For figure legend, see page 342.

fungal virulence.<sup>33-35</sup> However, these orthologs have a shorter unconventional intron (19, 20, or 29 nt) than that of *S. cerevisiae* (252 nt).

The basic features of the Ire1-dependent UPR signaling pathway appear to be well conserved in most eukaryotes. However, recent reports are revealing unexpected variations, such as the lack of Hac1 homolog in the fission yeast *Schizosaccharomyces pombe*<sup>36,37</sup> and the lack of *HAC1* homolog splicing in *Candida glabrata*.<sup>38</sup> Moreover, some protozoans do not possess Ire1 or Hac1/Xbp1 homologs.<sup>39,40</sup> Therefore, it seems that the Ire1-dependent UPR pathway has undergone extensive divergence during evolution, particularly in terms of the regulatory mechanism of its downstream bZIP transcription factors.

### Core Components of the *Cryptococcus* UPR Pathway

*Cryptococcus* has an evolutionarily conserved Ire1 kinase/endonuclease as its sole UPR pathway sensor in the ER and is not likely to contain other UPR sensors such as PERK and ATF6.<sup>41</sup> The *Cryptococcus* Ire1 kinase is highly homologous to the *S. cerevisiae* Ire1 and to IRE1 $\alpha$  and IRE1 $\beta$  in humans. Notably, however, *Cryptococcus* has a unique bZIP transcription factor encoded by *HXL1*, which is structurally and phylogenetically distant from yeast Hac1/human XBP1. Like other Hac1/XBP1 orthologs, Hxl1 contains a bZIP domain at the N-terminus (Fig. 1). However, Hxl1 expression does not rescue the ER stress-sensitive phenotypes of the *S. cerevisiae* *hac1* $\Delta$  mutant, the way that Hac1 orthologs do in other ascomycetes, such as *Trichoderma reesei* and *C. albicans*.<sup>34,42</sup>

Upon ER stress, the endonuclease activity of Ire1 removes an unconventional intron, thereby converting unspliced *HXL1* mRNA to spliced *HXL1* mRNA.<sup>41</sup> Although *HXL1*<sup>u</sup> and *HXL1*<sup>s</sup> mRNAs encode the same bZIP domain at the N-terminus (60 to 125 aa), the *HXL1*<sup>s</sup> mRNA produces the active Hxl1 protein with an extended C-terminal domain (406 aa of *Hxl1*<sup>u</sup> to 426 aa of *Hxl1*<sup>s</sup>) via frameshift translation of its C-terminal exon region. The Ire1-mediated *HXL1* mRNA splicing event is well conserved in different serotypes of *Cryptococcus* strains, including serotype A *C. neoformans* var *grubii* (the H99 strain), serotype D *C. neoformans* var *neoformans* (JEC21 and B-3501A strains), and serotype B *C. gattii* (R265 and WM276 strains).<sup>41</sup>

The target genes regulated by the UPR pathway play critical roles in counteracting ER stress. Transcriptome profiling analysis of the *S. cerevisiae* UPR pathway defined the “UPR regulon”, which includes genes involved in the protein secretion and modification pathway as well as ER-resident chaperones.<sup>24</sup> As a member of the UPR regulon, an ER-resident molecular chaperone Kar2/BiP ortholog was identified and functionally characterized in *C. neoformans*.<sup>41,43</sup> The expression of *C. neoformans* *KAR2* is tightly regulated in an Ire1- and Hxl1-dependent manner upon ER stress. Kar2 is essential for viability of *C. neoformans* as it is in *S. cerevisiae* and *C. albicans*. Constitutive expression of *KAR2* by the strong histone H3 promoter partially restores a subset of Ire1- and Hxl1-dependent phenotypes, including the ER stress response, thermotolerance, and cell wall integrity.<sup>43</sup> In summary,

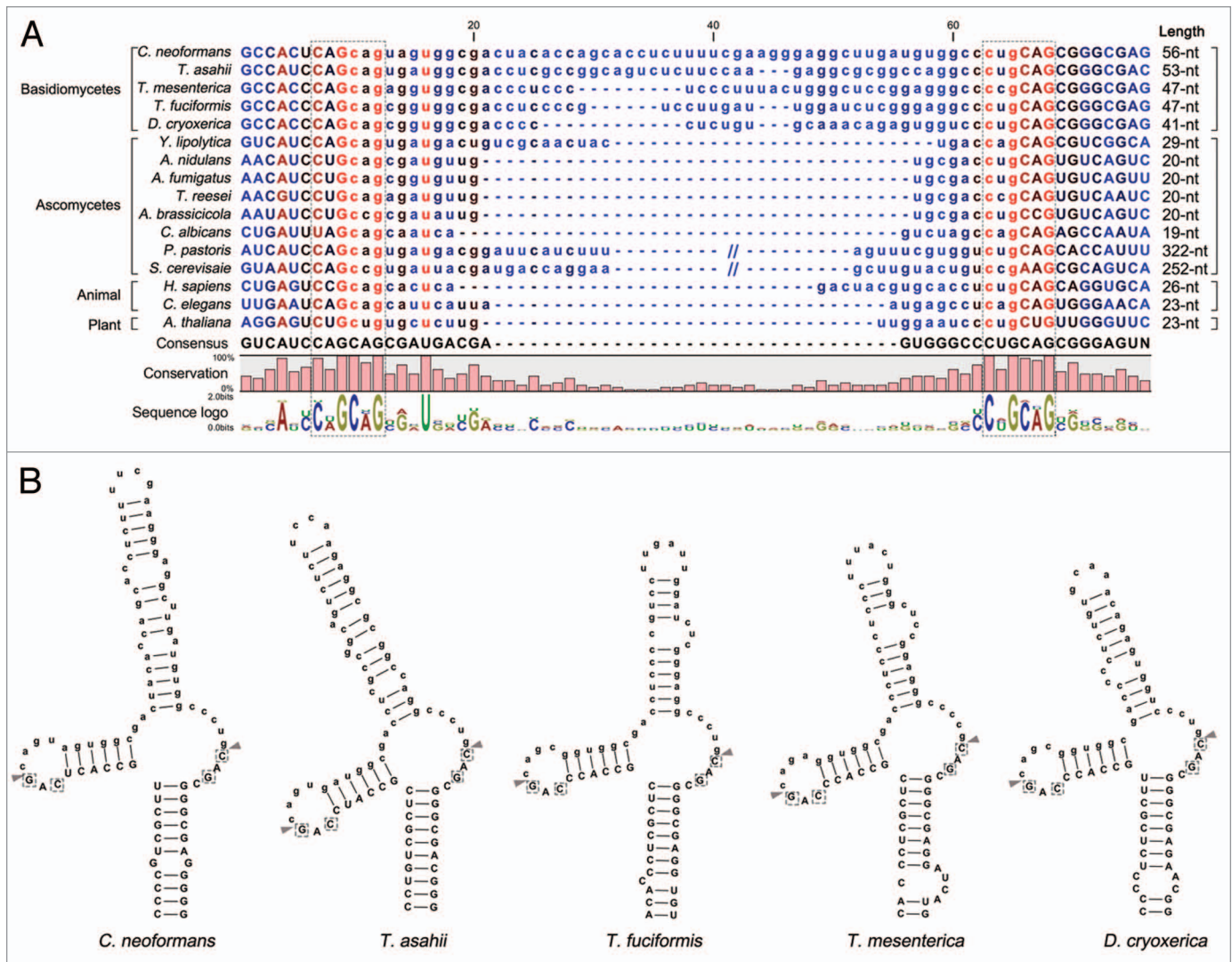
*Cryptococcus* contains both evolutionarily conserved and unique UPR components.

### Structural Characteristics of Non-Canonical Introns of *HXL1* Orthologs in the Basidiomycota

A recent study by Hooks and Griffiths reported the conserved structure of the non-canonical, spliceosome-independent introns of *HAC1/XBP1* homologs in 128 of the 156 eukaryotic species that were examined.<sup>36</sup> Interestingly, they could not identify *HAC1/XBP1* homologs in several fungal phyla including basidiomycota. Using the bZIP sequence of Hxl1 as query, however, we identified Hxl1 homologs with well conserved non-canonical intron structure in some basidiomycetous fungi, including *Trichosporon asahii* (A1Q2\_03745, e-value 5e-12), *Tremella mesenterica* (fgeneshtM\_kg.7\_#\_94\_#\_318\_2\_CCOI\_CCON, 1.2E-15), *Tremella fuciformis* (Tr21-F09, 3E-08), and *Dioszegia cryoxerica* (fgenesht1\_kg.80\_#\_88\_#\_Locus1962v1rpk301.65, 7.2E-17) (Fig. 2A). As in the short intron-containing *HAC1/XBP1* orthologs in filamentous fungi and animals, the putative splicing sites “CAG|CAG” and “C(U/C)G|CAG” are observed at both of the putative intron borders in basidiomycetous *Hxl1* homolog mRNAs (Fig. 2A), and the spliced proteins are expected to be generated by +1 translational frameshifting after removing the unconventional intron. Furthermore, as previously reported in other fungi and animals,<sup>32,42,44</sup> the secondary mRNA structures of the unconventional introns of basidiomycetous *HXL1* homologs are predicted to form stem-loop structures (Fig. 2B), which are probably recognized by Ire1 orthologs.<sup>44</sup>

The unconventional introns of basidiomycetous *HXL1* homologs (41 to 56 nt) are longer than those of mammalian *XBP1* and most ascomycetous *HAC1* orthologs (19 to 29 nt), but much shorter than the *S. cerevisiae* *HAC1* intron (252 nt).<sup>32-34,42,44,45</sup> In contrast to the *S. cerevisiae* *HAC1* intron, the shorter introns of other fungal *HAC1* orthologs and mammalian *XBP1* do not contain sequences complementary to their 5'-UTR regions and thus translational attenuation for negative regulation of UPR activation may not occur.<sup>31,45</sup> This suggests that there must be other regulatory mechanisms for repressing the unspliced version of *HAC1* mRNA (transcriptionally, translationally, or posttranslationally) under normal, unstressed conditions. In mammalian cells, the unspliced *XBP1* mRNA is translated under unstressed conditions, but the resulting XBP1 protein is degraded rapidly by the proteasome, thus it is generally undetectable.<sup>25</sup> Interestingly, the *Cryptococcus* *HXL1* intron similarly lacks a sequence complementary to any of its 5'-UTR regions, presenting the possibility that unspliced *HXL1* mRNA could be translated, but that the Hxl1 protein may be subject to rapid degradation. Another possibility is translational upregulation of Hxl1 as reported in *C. albicans*, *T. reesei*, *A. nidulans*, and *A. niger*, which express Hac1 mRNAs with truncated 5'-UTRs in response to stress to be translated more efficiently.<sup>34,42,46</sup> It will be quite an intriguing endeavor to define the regulatory mechanism of Hxl1 activation and the unique features that distinguish it from other yeast and fungal Hac1 homologs.





**Figure 2.** Conservation of the putative unconventional splicing sites of *HXL1* homologs in basidiomycetes. **(A)** The unconventional intron sequences of *HAC1/XBP1/HXL1* homologous mRNAs are aligned. Flanking exon sequences are denoted by uppercase letters and intron sequences by lowercase letters. Length indicates nucleotide length of the non-conventional intron that can be removed by Ire1. The conserved sequences of 5'- and 3'- splicing junctions are represented by sequence logo and dotted box. **(B)** Predicted secondary mRNA structures of *HXL1* homologs in some basidiomycetous fungi. Putative splicing sites in 5' and 3' intron borders are located in the loop regions of stem-loop structures. The putative Ire1-mediated splicing sites and introns are indicated with arrowheads and written in lower case, respectively. Conserved sequences in splicing junctions are indicated by dotted boxes. Alignment and RNA secondary structure prediction were performed with the CLC main benchwork 6.8.4 (CLC bio). Nucleotide sequences were retrieved from the NCBI database and fungal genomics resource at JGI<sup>61</sup>: *Cryptococcus neoformans* (*HXL1*, CNAG\_06134), *Trichosporon asahii* (A1Q2\_03745), *Tremella mesenterica* (TREMEDRAFT\_57223), *Tremella fuciformis* (bZIP1, GU723640.1), *Dioszegia cryoxerica* (fgenes1\_kg.80\_#\_88\_#\_Locus1962v1rpk301.65), *Yarrowia lipolytica* (*HAC1*, XM\_500811.1), *Aspergillus nidulans* (*hacA*, AJ413273), *Aspergillus fumigatus* (*hacA*, XM\_743634), *Trichoderma reesei* (*hac1*, AJ413272), *Alternaria brassicicola* (*HacA*, AB01954.1), *Candida albicans* (*HAC1*, EF655649), *Pichia pastoris* (*HAC1*, FN392319), *Saccharomyces cerevisiae* (*HAC1*, NC\_001138.5), *Homo sapiens* (*XBP1*, NM\_005080), *Caenorhabditis elegans* (*xbp-1*, AF443190), *Arabidopsis thaliana* (bZIP60, AY045964).

## Pleiotropic Roles of the UPR Pathway in *Cryptococcus*

The primary function of the UPR in *S. cerevisiae* is to relieve ER stress via expression of molecular chaperone genes.<sup>24</sup> The Ire1/Hxl1-dependent UPR pathway also promotes resistance to ER stress in the serotype A *C. neoformans* (the H99 strain) background.<sup>41</sup> Both *ire1Δ* and *hxl1Δ* mutants are highly susceptible to ER stress agents (e.g., tunicamycin [TM; an N-glycosylation inhibitor] and dithiothreitol [DTT; a reducing agent]). Hxl1

appears to be the only bona fide ER stress response transcription factor downstream of Ire1, since the expression of spliced *HXL1* mRNA completely restores wild-type resistance of the *ire1Δ* mutant to ER and cell wall stresses.<sup>41</sup> Similarly, we observed that deletion of *IRE1* or *HXL1* resulted in increased susceptibility to ER and cell wall stresses in the serotype D *C. neoformans* and *C. gattii* R265 (serotype B/C) strain backgrounds (unpublished data by Y.S.B.), indicating that the major roles of the UPR pathway are evolutionarily conserved among pathogenic *Cryptococcus* species.

In addition to its conserved role in the response to ER stress, the *C. neoformans* UPR pathway is also involved in resistance to genotoxic stresses, which activate the unconventional splicing of *HXML1* mRNA and Ire1/Hxl1-dependent *KAR2* induction.<sup>43</sup> Supporting this, both *ire1Δ* and *hxl1Δ* mutants show increased susceptibility to DNA damaging agents, including hydroxyurea (HU; a ribonucleotide reductase inhibitor) and methyl methane sulfonate (MMS; a DNA alkylating agent that induces DNA base mispairing and blocks replication). DNA damages caused by genotoxic stress are likely to result in production of mutated, abnormal proteins, which may be misfolded or unfolded to trigger ER stress.

Most notably, the UPR pathway governs the thermotolerance and virulence of *Cryptococcus*.<sup>41</sup> The ability to survive and proliferate at physiological body temperature is an essential virulence factor for most pathogens. Both Ire1 and Hxl1 are required for growth of *Cryptococcus* at temperatures above 30 °C, and deletion of either gene abolishes its ability to grow at 37 °C. This is likely to be the reason *Cryptococcus* UPR mutants are avirulent and rapidly cleared during the initial infection stage in the host lung.<sup>41</sup> Unlike the case in ER and cell wall stress responses, however, Ire1 and Hxl1 appear to have both redundant and unique roles in thermotolerance based on several observations. First, *ire1Δ* and *hxl1Δ* mutants exhibit different levels of temperature sensitivity. In fact, the *hxl1Δ* mutant is more thermosensitive than the *ire1Δ* mutant. Second, the expression of spliced *HXML1* mRNA only partly restores thermotolerance in the *ire1Δ* mutant. Third, the *ire1Δ* mutant harboring the spliced version of *HXML1* mRNA is still attenuated in virulence, whereas *ire1Δ* and *hxl1Δ* mutants are completely avirulent in a murine model of systemic cryptococcosis. Therefore, Ire1 modulates the thermotolerance and the virulence of *C. neoformans* in an Hxl1-dependent as well as -independent manner (Fig. 3).

### Hxl1-Independent Ire1 Functions in the *Cryptococcus* UPR Pathway

Hac1 is the only known Ire1 substrate in *S. cerevisiae* and no Hac1-independent functions of the UPR pathway have been identified in this yeast model. Although the functions of the UPR pathway are widely conserved in response to ER stress and cell wall stress, Ire1 and its downstream factors, Hac1 in fungi and XBP1 in humans, appear to have distinct roles in response to different environmental cues. Comprehensive gene expression analysis of the UPR pathways in *Caenorhabditis elegans* and *A. fumigatus* strongly support the divergent regulation of Ire1 and Hac1 orthologs.<sup>47,48</sup> Moreover, in metazoans, the endoribonuclease activity of Ire1 was directly involved in controlling the expression of a subset of genes encoding ER proteins by degrading mRNA to reduce the unfolded protein load in the ER lumen.<sup>49,50</sup> It was thus proposed that Ire1 selectively degrades groups of ER-bound mRNAs to relieve the burden of unfolded proteins in the ER lumen via the regulated Ire1-dependent decay (RIDD) pathway.<sup>49,50</sup> Furthermore, IRE1 $\alpha$  is part of a signaling platform called the UPRosome where several cofactors and adaptor proteins are assembled and function to

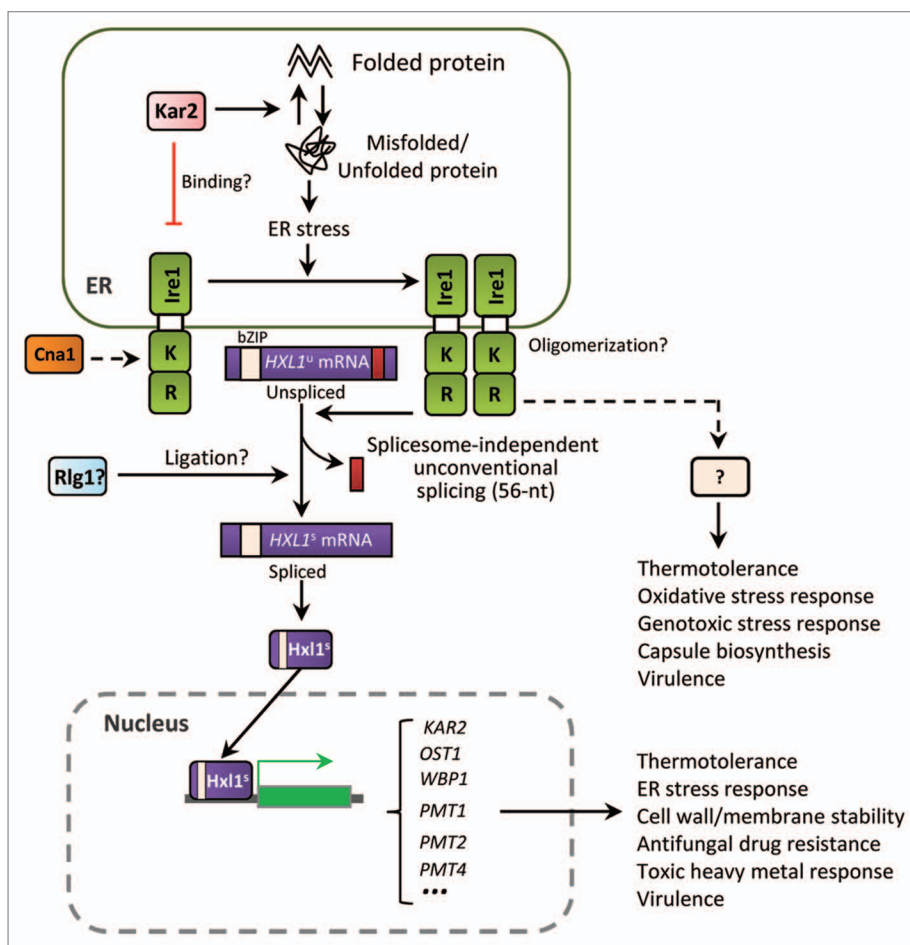
modulate the kinetics and amplitude of downstream signaling in mammals.<sup>51</sup>

Notably, *Cryptococcus* Ire1 also appears to have Hxl1-independent functions. One such function is the regulation of antiphagocytic capsule biosynthesis, as the *ire1Δ* mutant is defective in capsule biosynthesis. This defect cannot be restored by either *KAR2* overexpression or integration of the spliced *HXML1* mRNA.<sup>41,43</sup> Furthermore, phenotypic analyses revealed that Ire1 generally plays more pleiotropic roles than Hxl1 in response to diverse environmental cues. For example, the *ire1Δ* mutant, but not the *hxl1Δ* mutant, shows increased sensitivity to diamide,<sup>41</sup> flucytosine, and *tert*-butyl hydroperoxide (unpublished data by YS Bahn). In response to heavy metal stress (e.g., CdSO<sub>4</sub>), the *hxl1Δ* mutant shows increased resistance, while the *ire1Δ* mutant shows increased susceptibility (unpublished data by YS Bahn). In response to ER stress and thermal shock, representative UPR target genes, such as *KAR2*, *SEC61* (which regulates translocation of misfolded proteins out of the ER), and *DER1* (involved in ER-associated degradation), were shown to be upregulated in an Ire1/Hxl1-dependent manner, whereas expression of *PMT1* and *PMT4* (protein O-mannosyltransferase) genes appeared to be dependent on Hxl1 only.<sup>41</sup> These observations strongly suggest that Ire1 has bifurcated signaling branches (Fig. 3), one of which includes Hxl1 to execute conserved roles of the UPR pathway and the other, which excludes Hxl1. On the other hand, Hxl1 might also have an upstream signaling controller(s), other than Ire1.

### Crosstalk between the UPR Pathway and Other Signaling Pathways in *Cryptococcus*

As reported in UPR-defective mutants of other yeasts and fungi,<sup>33,34,52,53</sup> the *ire1Δ* and *hxl1Δ* mutants exhibit hypersensitivity to cell wall destabilizing agents, such as Calcofluor white (CFW) and Congo red (CR). Although TM treatment mainly results in defective N-glycosylation, it may also affect cell wall integrity. Indeed, the addition of an osmotic stabilizer (e.g., 1 M sorbitol) restores resistance to TM in the UPR mutants of *C. neoformans*. Furthermore, TM treatment triggers phosphorylation of the Mpk1 MAPK, a component of the cell wall integrity pathway in *C. neoformans*.<sup>41</sup> Notably, perturbation of the UPR pathway significantly increases Mpk1 phosphorylation levels (under both basal and stress conditions), suggesting that there is direct or indirect crosstalk between the UPR pathway and the Mpk1 MAPK pathway.<sup>41</sup>

Crosstalk between the UPR and calcineurin pathways is also likely in *Cryptococcus*. Perturbation of the calcineurin signaling pathway, which is involved in Ca<sup>2+</sup> homeostasis, cell wall integrity, thermotolerance, and virulence in *C. neoformans*, affects *HXML1* splicing and *KAR2* induction under certain conditions (e.g., high temperature) in *C. neoformans*.<sup>41</sup> It was recently reported that deletion of *CNBI*, the regulatory B subunit of the calcineurin phosphatase, decreases tolerance to ER stress in *Candida glabrata*, which is in agreement with the fact that *C. neoformans* and *C. gattii* *cna1Δ* mutants lacking the catalytic subunit of calcineurin show growth defects in response to ER stress.<sup>38,41,54</sup> Furthermore, in *C. glabrata*, *KAR2* expression is



**Figure 3.** The ER stress response and UPR pathways in *C. neoformans*. The *Cryptococcus* UPR pathway consists of the Ire1 kinase, a bZIP transcription factor Hxl1, and their target genes. Upon ER stress, the spliceosome-independent unconventional splicing event in *HXL1* mRNA occurs. Activated Hxl1 translocates to the nucleus and induces the expression of UPR target genes such as *KAR2*, which encodes an ER-resident molecular chaperone. The UPR pathway plays Ire1/Hxl1-dependent roles in ER stress response, antifungal drug resistance, and virulence. However, Ire1 also appears to have Hxl1-independent functions. Crosstalk between the UPR and calcineurin pathways via Cna1 is also indicated in *Cryptococcus*. Black arrows represent positive regulation or activation whereas red barred lines indicate negative regulation or repression. Dotted arrows indicate potential or unclear regulation.

regulated by the calcineurin pathway, but not by the UPR pathway. This species has lost the canonical Hac1-like transcription factor downstream of Ire1. Therefore, it is highly likely that cellular responses to ER stress involve crosstalk between Ire1 and calcineurin in *C. glabrata*.<sup>38</sup>

The UPR pathway may also engage in crosstalk with the mRNA degradation machinery in *C. neoformans*. Recently, Havel et al.<sup>55</sup> demonstrated that ER stress-responsive transcripts are regulated at the post-transcriptional level during adaptation of *C. neoformans* to host physiological temperature. During ER stress response and host temperature adaptation, the decay rates of ER stress-responsive transcripts, including *KAR2*, *OST2* (a subunit of the ER oligosaccharyltransferase complex), and *ALG7* (a lipid-linked *N*-oligosaccharyltransferase), are lower in cells null for the mRNA deadenylase-encoding *CCR4* gene.<sup>55</sup> Furthermore, *RBP4*, which encodes an RNA polymerase II subunit, was shown to be involved in destabilizing the *KAR2* transcript during temperature upshift.<sup>56</sup> Therefore, the mRNA degradation machinery regulated by Ccr4 and Rbp4 appears to provide an additional

level of control to the UPR pathway, contributing to the cellular response to ER stress. In summary, the Ire1/Hxl1-dependent UPR pathway serves as a hub in *C. neoformans*, interacting with other stress-related signaling pathways directly or indirectly to execute more efficient responses to various environmental cues.

### Potential for the UPR Pathway as a Novel Antifungal Therapeutic Target

The need for novel antifungal therapeutic targets and drugs has become urgent due to an increasing incidence of invasive fungal infections, toxic drug side effects, and the emergence of drug-resistant strains.<sup>57</sup> Recent studies suggest that the UPR pathway could potentially be exploited as a novel antifungal drug target, since fungal UPR pathways play a critical role in antifungal drug resistance.<sup>33,38,41,48</sup>

*A. fumigatus ireAΔ* and *hacAΔ* mutants exhibit increased susceptibility to azole drugs, such as itraconazole and voriconazole, which function through the inhibition of ergosterol



biosynthesis.<sup>48</sup> The expression levels of some ergosterol biosynthesis genes, including *ERG2*, *ERG11*, *ERG24*, and *ERG3*, decrease in both *ire1Δ* and *hac1Δ* mutants in *A. fumigatus* and subsequent ergosterol levels in both strains are reduced. In *Cryptococcus*, *ire1Δ* and *hxl1Δ* mutants also show significantly enhanced susceptibility to azole drugs, including fluconazole, ketoconazole, and itraconazole, although the mechanism appears to be different from that of *A. fumigatus* based on several observations. First, the expression levels of *ERG11* and *ERG3* in *ire1Δ* and *hxl1Δ* mutants are similar to those of wild type (WT) cells. Furthermore, *ire1Δ* and *hxl1Δ* mutants are also slightly more susceptible than WT to amphotericin B, which disrupts the ion homeostasis by binding ergosterol and forming channels in the plasma membrane. Amphotericin B therefore exerts antagonistic effects in combination with azole drugs, supporting the fact that perturbation of the UPR pathway does not affect ergosterol biosynthesis in *C. neoformans*. Second, azole treatment itself causes ER stress in *C. neoformans*. Treatment with fluconazole activates the UPR pathway via *HXL1* unconventional splicing and induces *KAR2* expression in WT cells.<sup>43</sup> Accordingly, the overexpression of *KAR2* partially suppresses azole sensitivity in *ire1Δ* and *hxl1Δ* mutants.

Among UPR pathway components, Hxl1 has several advantages as an attractive therapeutic target for the treatment of cryptococcosis. Hxl1 is conserved in different serotypes of both *C. neoformans* and *C. gattii*. The deletion of *HXL1* not only abolishes the virulence of *C. neoformans* var *grubii*, but also enhances azole susceptibility in *C. gattii* (R265 strain), *C. neoformans* var *neoformans* (JEC21 strain) (unpublished data by Y.S.B.), and *C. neoformans* var *grubii*.<sup>41</sup> This suggests that both mono-therapy (with an Hxl1 inhibitor) and combination therapy with a low dose of azole drugs could be very effective for the treatment of cryptococcosis. Particularly, combination therapy may reduce the hepatotoxicity caused by long-term exposure to high doses of azoles. Moreover, Hxl1 is structurally divergent from the host XBP1 transcription factor, suggesting that an Hxl1-specific inhibitor could be designed to avoid any adverse side effects to the host. Interestingly, recent works have revealed that Ire1 has an allosteric site in its dimer interface that may bind some drugs,<sup>58</sup> and small molecules that bind the kinase domain of Ire1 can enhance or reduce its activity.<sup>59</sup> Taken together, the core components of the *Cryptococcus* UPR pathway are potential therapeutic targets for the treatment of cryptococcosis.

## Conclusions and Future Perspectives

The functions of the UPR pathway governing the ER stress response and host adaptation are evolutionarily conserved in eukaryotes ranging from yeasts to mammals. In the pathogenic species of *Cryptococcus*, the UPR pathway has pleiotropic roles in regulating diverse environmental stress responses, the ER stress response, in vitro virulence factor production and in vivo virulence. The fact that UPR signaling components strongly promote antifungal drug resistance suggests that Hxl1, which is a transcription factor that is structurally divergent from the host XBP1, is an ideal target for antifungal drug development. Furthermore, as the first basidiomycetous fungus in which the UPR pathway

has been systematically characterized, *C. neoformans* will serve as an excellent model system to understand the conserved and unique features of the UPR in diverse fungal species.

There are several remaining issues to address in the *Cryptococcus* UPR pathway. Although the unspliced *HXL1* mRNA does not appear to be translated,<sup>41</sup> its translational repression mechanism remains an interesting subject to be defined. Considering a lack of any obvious long-range base pairing sequence between 5' UTR and the unconventional *HXL1* intron, the translational control mechanism of the unspliced *HXL1* mRNA should be different from that of the yeast *HAC1* mRNA. Moreover, it remains unclear to what extent the activation of *HXL1* is controlled by Ire1. We observed that some portion of *Cryptococcus HXL1* mRNA undergoes splicing even under unstressed conditions in the Ire1-dependent manner.<sup>41</sup> Nevertheless, almost a negligible amount of Hxl1 proteins seem to be produced under unstressed conditions. Considering that the Hxl1 protein has a PEST domain, which is involved in degradation,<sup>60</sup> it could be speculated that the Hxl1 protein remains unstable due to rapid degradation under unstressed conditions, as is the case in mammalian cells.<sup>25</sup> Systematic biochemical characterization of Hxl1 protein stability must be performed to address this issue.

Another issue is the identification of Ire1/Hxl1-, Ire1-, and Hxl1-specific regulons in *C. neoformans*. To this end, comparative transcriptome analysis of *ire1Δ* and *hxl1Δ* mutants under various stress conditions could provide useful information. Particularly, investigation of the RNA stability of Ire1-specific target genes would provide insight on the possible regulatory mechanism of Ire1 independent of Hxl1. Recently, studies in *S. pombe* and *C. glabrata* revealed that Ire1 degrades a subset of ER-localized mRNAs to relieve ER stress.<sup>37,38</sup> We also observed that upon ER stress or thermal shock, a subset of genes show increased mRNA levels in the *ire1Δ* mutant, but not in the *hxl1Δ* mutant, compared with the WT *C. neoformans*.<sup>41</sup> Thus, it could be speculated that Hxl1-independent roles of Ire1 may be partly mediated by the mRNA-decay machinery in *C. neoformans*.

Finally, it will be also important to elucidate the UPR pathway in *C. gattii*, which causes fatal disease even in immunocompetent individuals. The UPR mutants of *C. neoformans* var *grubii* are completely cleared at the initial stage of infection (within the lung) in an immunodeficient murine model of systemic cryptococcosis (A/Jcr mice).<sup>41</sup> Therefore, it will be interesting to examine whether the *C. gattii* UPR mutants could be similarly cleared in both immunocompetent and immunocompromised murine models. Such comparative analyses of *C. gattii* and *C. neoformans* UPR pathways should help us understand the differential pathogenic mechanisms of the two sibling species.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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