The Unfolded Protein Response Is Induced by the Cell Wall Integrity Mitogen-activated Protein Kinase Signaling Cascade and Is Required for Cell Wall Integrity in *Saccharomyces cerevisiae*

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The yeast cell wall is an extracellular structure that is dependent on secretory and membrane proteins for its construction. We investigated the role of protein quality control mechanisms in cell wall integrity and found that the unfolded protein response (UPR) and, to a lesser extent, endoplasmic reticulum (ER)-associated degradation (ERAD) pathways are required for proper cell wall construction. Null mutation of *IRE1*, double mutation of ERAD components (*hrd1Δ* and *ubc7Δ*) and *ire1Δ*, or expression of misfolded proteins show phenotypes similar to mutation of cell wall proteins, including hypersensitivity to cell wall-targeted molecules, alterations to cell wall protein layer, decreased cell wall thickness by electron microscopy, and increased cellular aggregation. Consistent with its important role in cell wall integrity, UPR is activated by signaling through the cell wall integrity mitogen-activated protein (MAP) kinase pathway during cell wall stress and unstressed vegetative growth. Both cell wall stress and basal UPR activity is mediated by Swi6p, a regulator of cell cycle and cell wall stress gene transcription, in a manner that is independent of its known coregulatory molecules. We propose that the cellular responses to ER and cell wall stress are coordinated to buffer the cell against these two related cellular stresses.

INTRODUCTION

The yeast cell wall is an essential structure that protects the cell from lysis during budding, mating, and periods of environmental stress (Klis *et al.*, 2002). The yeast cell wall is a complex lattice of protein and carbohydrate that is constructed from, and by, proteins delivered by the secretory pathway (Lesage and Bussey, 2006). Accordingly, genome-wide screens for genes involved in yeast cell wall biosynthesis have consistently identified protein and vesicular trafficking genes (Firon *et al.*, 2004). In addition, cellular secretion has been specifically shown to be required for the incorporation of mannoprotein (Conde *et al.*, 2003), 1,3- β -glucan (Abe *et al.*, 2003), 1,6- β -glucan (Brown *et al.*, 1993), and chitin (Santos and Synder, 1997) into the yeast cell wall.

Protein quality control, in contrast, is one important aspect of cellular secretion that has not been extensively studied in terms of its role in yeast cell wall integrity. Misfolded or misprocessed proteins are toxic to the cell; consequently, mechanisms to identify, refold, and/or remove such proteins are crucial for cellular viability (Sayeed and Ng, 2005). Two general mechanisms of protein quality control are operative in yeast as well as in higher eukaryotes. The first mechanism is endoplasmic reticulum (ER)-associated degradation (ERAD; Meusser *et al.*, 2005). Through ERAD, proteins resistant to chaperone-mediated refolding are identified, retro-translocated from the ER, tagged with ubiquitin, and, ultimately, degraded by the 26S proteasome. ERAD is constitutively active and, during unstressed vegetative growth, seems sufficient to process the load of misfolded proteins in yeast (Spear and Ng, 2001).

When the cell encounters conditions that increase unfolded proteins, a second mechanism called the unfolded protein response (UPR) is activated to compensate for elevated levels of ER stress (Ron and Walter, 2007). UPR is an ER-to-nucleus signaling pathway that is initiated by ER stress and induces the transcription of a large number of genes. In yeast, UPR is triggered when unfolded proteins are detected by the transmembrane sensor Ire1p. Ire1p contains protein kinase and endoribonuclease activities that are essential to its role in UPR (Cox *et al.*, 1993; Mori *et al.*, 1993). Ire1p oligomerizes in the presence of unfolded proteins and undergoes autophosphorylation, which in turn activates its RNase activity (Shamu and Walter, 1996). Ire1p RNase activity is specific for the mRNA of the transcription factor Hac1p, its only known substrate. In yeast, *HAC1^u* mRNA ("u" for uninduced) is constitutively transcribed but is not translated due to the presence of an inhibitory intron. Activated Ire1p removes the intron from *HAC1^u* and tRNA ligase rejoins the two exons to generate *HAC1ⁱ* ("i" for induced). *HAC1ⁱ* is then efficiently translated and the resulting Hac1p transcription factor translocates to the nucleus where it initiates the transcriptional program of UPR (Travers *et al.*, 2000).

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Table 1. Strains used in this study

Strain	Genotype	Source
W303 1A	<i>MAT a ade2-1, can1-100, his3-11, leu2-3, 112, trp1-1, ura3-1</i>	R. Fuller (Krysan <i>et al.</i> , 2005)
DKY104	W303 1A <i>MAT a ire1::TRP1</i>	D. Ng (Spear and Ng, 2003)
DKY103	W303 1A <i>MAT a leu2-3, 112::LEU2-GAL-CPY_{HA}*</i>	D. Ng (Spear and Ng, 2003)
DKY101	W303 1A <i>MAT a ire1::TRP1, leu2-3, 112::LEU2-GAL-CPY_{HA}*</i>	D. Ng (Spear and Ng, 2003)
BY4741	<i>MAT a his3Δ leu2Δ ura3Δ met15Δ</i>	Research Genetics (Huntsville, AL)
BY4742	<i>MAT α his3Δ leu2Δ ura3Δ lys2Δ</i>	Research Genetics
DKY1000	BY4741 <i>ire1Δ::KAN</i>	Research Genetics
DKY147	BY4742 <i>ire1Δ::NAT</i>	This study
DKY169	BY4741 <i>ssd1Δ::NAT</i>	This study
DKY171	BY4741 <i>ssd1Δ::NAT ire1Δ::KAN</i>	This study
DKY1001	BY4741 <i>ubc7Δ::KAN</i>	Research Genetics
DKY1002	BY4741 <i>hrd1Δ::KAN</i>	Research Genetics
DKY1003	BY4741 <i>doa10Δ::KAN</i>	Research Genetics
DKY153	BY4741 <i>ubc7Δ::KAN ire1Δ::NAT</i>	This study
DKY154	BY4741 <i>hrd1Δ::KAN ire1Δ::NAT</i>	This study
DKY185	BY4741 <i>doa10Δ::NAT</i>	This study
DKY191	BY4741 <i>doa10Δ::NAT ire1Δ::KAN</i>	This study
DKY125	BY4741 <i>gas1Δ::NAT</i>	This study
DKY126	BY4741 <i>gas1Δ::NAT ire1Δ::KAN</i>	This study
DKY1004	BY4741 <i>mpk1Δ::KAN</i>	Research Genetics
DKY155	BY4741 <i>mpk1Δ::KAN ire1Δ::NAT</i>	This study
DKY1005	BY4741 <i>mid2Δ::KAN</i>	Research Genetics
DKY1006	BY4741 <i>wsc1Δ::KAN</i>	Research Genetics
DKY1007	BY4741 <i>rlm1Δ::KAN</i>	Research Genetics
DKY1008	BY4741 <i>swi4Δ::KAN</i>	Research Genetics
DKY1009	BY4741 <i>swi6Δ::KAN</i>	Research Genetics
DKY1010	BY4741 <i>mbp1Δ::KAN</i>	Research Genetics
DKY1011	BY4741 <i>stb1Δ::KAN</i>	Research Genetics

Initially, the role of UPR was believed to be limited to protein quality control, but it has become clear that UPR plays a much broader role in cellular physiology (Sayed and Ng, 2005). For example, UPR has been linked to cytokinesis (Bicknell *et al.*, 2007), autophagy (Bernales *et al.*, 2006), haploid tolerance (Lee *et al.*, 2003), pseudohyphal growth (Schroder *et al.*, 2000), and lipid biosynthesis and membrane homeostasis (Cox *et al.*, 1997). Consistent with its broad role in cellular physiology, the transcriptional program of UPR includes genes involved in a wide range of cellular processes, including protein folding, ERAD, protein trafficking, lipid biosynthesis, and cell wall architecture (Travers *et al.*, 2000).

Although the role of UPR and, more generally, secretory protein quality control in yeast cell wall biosynthesis has not been extensively studied, a number of reports have provided evidence for a link between these two important processes in yeast. First, ER stress has been shown to trigger signaling through the cell wall integrity (CWI) mitogen-activated protein kinase (MAPK) signaling cascade (Bonilla and Cunningham, 2003), the most important mediator of the cellular response to cell wall stress (Levin, 2005). Second, null mutations in the components of the CWI pathway are hypersensitive to ER stress (Chen *et al.*, 2005). Third, Slt2/Mpk1p (hereafter noted as Mpk1), MAPK of the CWI pathway, stabilizes Hac1p during the concurrent application of heat stress and ER stress, leading to increased UPR activation during periods of dual stress (Pal *et al.*, 2007). Fourth, activation of Pkc1p has been reported to increase the degradation of misfolded carboxypeptidase Y (CPY*), suggesting cell wall stress may increase the capacity of the quality control systems during periods of stress (Nita-Lazar and Lennarz, 2005). Finally, cytosolic molecular chaperones such as Ydj1p, an Hsp40 chaperone (Wright *et al.*, 2007), and Sse1p, an Hsp110 chaperone (Shaner *et al.*, 2008), affect cell

wall integrity through interactions with CWI pathway components such as Mpk1p (Sse1p) and Pkc1p (Ydj1p).

Here, we show that *ire1Δ* mutants as well as strains expressing misfolded proteins have defects in cell wall integrity. In addition, double mutants that combine *ire1Δ* with mutations in components of the ERAD pathway show synthetic cell wall defects. We also demonstrate that cell wall stress activates UPR in a process dependent on both Ire1p and components of the CWI pathway. Finally, we provide evidence that both cell wall stress-induced and basal UPR activity is mediated by Swi6p in a process that is independent of its known transcriptional coactivators. Together, these results indicate that UPR and CWI signaling pathways form an interdependent regulatory circuit that allows yeast to respond to both intracellular ER stress and extracellular cell wall stress.

MATERIALS AND METHODS

Strains, Growth Conditions, and Materials

Yeast strains used in this study are listed in Table 1 and were derived from either S288C (BY4741) or W303 (CRY1) genetic backgrounds. Single yeast mutants in the BY4741 background were obtained from the yeast deletion collection (Invitrogen, Carlsbad, CA). Yeast strains were grown in yeast peptone dextrose (YPD) or synthetic drop out medium prepared according to standard recipes (Burke *et al.*, 2000). Expression of alleles from galactose-regulated promoters was induced by preculturing strains in 2% raffinose for 8 h before addition of 2% galactose. All incubations were performed at 30°C unless noted otherwise. DH5α *Escherichia coli* cells (Invitrogen) were used for routine plasmid manipulations. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. Oligonucleotides were synthesized by Integrated DNA Technologies (Corralville, IA).

Yeast Strain Construction

The *IRE1* open reading frame was deleted by standard polymerase chain reaction (PCR)-based one step gene-replacement with a *KANMX* cassette generated from the deletion collection *ire1Δ::KAN* strain (Johnston *et al.*, 2002).

Correct integration was determined by PCR with primers flanking the sequence used to generate the knockout cassette. All *ire1Δ* mutants displayed the expected hypersensitivity to deoxyglucose (Back *et al.*, 2005). The *KANMX* cassette was switched to the *NATMX* cassette according to a published procedure (Tong and Boone, 2006).

Plasmids

pJC104 contains four copies of the unfolded protein response element (4X-UPRE) fused to the *lacZ* gene in a *CEN/URA3* plasmid and was generously provided by P. Walter (Cox and Walter, 1996). pMB4 contains the *HAC1* promoter and open reading frame fused to a 6X hemagglutinin (HA) tag at the 3' end in a *CEN/LEU2* plasmid and was a gift of K. Cunningham (Johns Hopkins University, Baltimore, MD; Bonilla and Cunningham, 2003). pBD1265 contains *SWI6* under the control of its endogenous promoter in a 2μ plasmid with a *LEU2* marker and was a gift of L. Breeden (University of Washington, Seattle, WA).

Cell Wall Phenotypes Assays

Agar plates supplemented with Calcofluor white (CFW), Congo red, caffeine, and caspofungin were prepared according to recipes reported previously (Krysan *et al.*, 2005). Overnight cultures of each strain were adjusted to a cell density of 1 OD₆₀₀ unit. A three-step, 10-fold dilution series was prepared and spotted on to plates by using a metal frogger (VP Scientific, San Diego, CA). The plates were incubated at 30°C or 37°C for 2–5 d and photographed. Two independent isolates of each strain were tested in duplicate for each phenotype. Zymolyase sensitivity was assayed as described previously (de Nobel *et al.*, 2000).

Cell Aggregation Assay

Yeast were grown overnight to early stationary phase in YPD. The culture was diluted in an equal volume of water and placed on a microscope slide. The number of cell aggregates with >10 cells per high-power field was counted for 50 high-power fields on three separate days for each strain. Reported values are the mean and SD of the three separate replicates.

Analysis of Cell Wall Proteins

Dithiothreitol (DTT)-extracts of intact cells were prepared according to a previously described protocol (Klis *et al.*, 2007). Briefly, cells were grown to early stationary phase (OD₆₀₀ ~10) overnight in YPD, harvested by centrifugation (300 × g), washed with water, and resuspended (1 OD₆₀₀ equivalent/μl) in extraction buffer (50 mM Tris, pH 7.5, and 5 mM DTT). The cell suspension was shaken in a multi-vortex apparatus for 2 h at 4°C. The supernatant was removed, mixed with 2× Laemmli sample buffer, boiled for 5 min at 100°C, and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (4–15% gradient gel; Bio-Rad, Philadelphia, PA). Fractionated proteins were visualized by silver stain according to the manufacturer's protocol (Silver Snap kit; Bio-Rad). Gels were photographed, and images were processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

NaOH extracts of isolated cell wall material were prepared according to a published procedure (Kaptein *et al.*, 1999). Briefly, cells were cultivated and harvested as described for the DTT extracts. Cell walls were isolated, suspended in β-mercaptoethanol/SDS extraction buffer (50 mM Tris, pH 7.8, 0.1M EDTA, 2% SDS, and 40 mM β-mercaptoethanol), and heated to 100°C for 5 min. The cell walls were pelleted, washed with water three times, and resuspended in 30 mM NaOH (1 mg wet cell wall/μl). The cell wall suspension was incubated overnight at 4°C. The resulting supernatant was removed, mixed with 2× Laemmli sample buffer, and processed as described for the DTT extracts.

Total cell wall protein (Albrecht *et al.*, 2006) and hexose (Francois, 2006) were determined exactly as described in previously reported protocols.

Electron Microscopy

Strains were grown to stationary phase and harvested. Yeast were fixed and processed according to previously published methods but with modifications to the resin type and resin infiltration/embedding steps (Gammie *et al.*, 1998). Briefly, the yeast were fixed 30 min in 40 mM potassium phosphate buffered 2.0% glutaraldehyde containing 1 mM CaCl₂, 1 mM MgCl₂, and 0.2 M sorbitol. The yeast were pelleted, resuspended in buffer, incubated for 4 h in 4.0% potassium permanganate, rinsed four times in water, resuspended in sodium periodate, washed in potassium phosphate buffer and ammonium phosphate, rinsed in water, resuspended in 2.0% uranyl acetate (covered with foil to exclude light), and incubated overnight at 4°C on a rotator plate. The yeast were then washed in water and dehydrated in 60-min steps in a graded series of ethanol:water solutions to 100:0%. Spurr epoxy resin was substituted for the LR White used in the published procedure (Gammie *et al.*, 1998), and the infiltration of the resin involved overnight incubations (on rotator) in an increasing concentration series (1:1, 1:2, 1:3, 1:4, and 100% resin) of Spurr resin diluted in 100% ethanol. The yeast were pelleted into fresh resin in Eppendorf tubes and polymerized overnight at 60°C. Thin sections were cut at 70 nm onto grids (no staining of grids) and examined with a Hitachi 7650 transmission electron microscopy by using a Gatan Erlangshen 1000SW digital camera.

β-Galactosidase Reporter Assay

Overnight cultures of yeast strains harboring pJC104 were diluted to 0.1 OD₆₀₀ in YPD and grown for two-doublings (~3 h). Following previous experiments using this reporter (Cox and Walter, 1996) to assay UPR response, cultures were exposed to stressor for 5 h, harvested, and the pellets flash-frozen in liquid nitrogen. Cell lysates were prepared and β-galactosidase activity determined as described previously (Burke *et al.*, 2000). The specific activity for the extracts is expressed as nanomoles per milligram of protein per minute (Miller units). Each reported value represents the mean of at least two experiments with independent transformants of the strain performed in duplicate or triplicate. Error is expressed as SE of the mean.

Reverse Transcription (RT)-PCR Assay of *HAC1* mRNA Splicing

HAC1 mRNA splicing was assayed using a modification of a reported protocol (Bicknell *et al.*, 2007). Overnight cultures were grown to mid-log phase (0.5–1.0 OD₆₀₀) in YPD and treated with tunicamycin or CFW for 1 h. The cells were harvested, and total RNA was isolated using an RNA Easy kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. cDNA was generated from 2 μg of total RNA using the SuperScript III First Strand Synthesis system (Invitrogen) and oligo(dT) primers according to the manufacturer's protocol. *HAC1*^u and *HAC1*ⁱ cDNA was amplified by PCR using primers flanking the intron (Bicknell *et al.*, 2007): *HAC1*^F, 5'-TAGAGGGATT-TCCAGAGCACG and *HAC1*^R, 5'-TCATTGAAGTGATGAAGAAATC and the following thermocycle conditions: 94°C, 1 min; 25 cycles of [94°C, 1 min; 54°C, 1 min; 65°C 1 min]; 65°C, 7 min. Amplicons were analyzed by electrophoresis on 2% agarose gels. *ACT1* cDNA was amplified as a loading control by using previously reported primers and conditions (Zhong and Greenberg, 2003). All RT-PCR experiments were performed at least three times with independent strain isolates.

Western Blotting

Hac1p levels were determined with cells harboring pMB4 following the procedure described previously (Bonilla and Cunningham, 2003). Briefly, logarithmic phase cultures in synthetic dextrose medium lacking leucine were treated with either tunicamycin or CFW, incubated for 2 h, harvested, and flash frozen. Cell extracts (25–50 OD₆₀₀ equivalents) were prepared by glass beads lysis in sorbitol breaking buffer (300 mM sorbitol, 100 mM NaCl, 5 mM MgCl₂, 10 mM Tris pH 7.5, mini-complete protease inhibitors [Roche Diagnostics, Indianapolis, IN]). Extracts were normalized by protein concentration (Bradford assay; Bio-Rad), fractionated by SDS-PAGE electrophoresis (10% gel), and transferred to nitrocellulose membranes. The membranes were blocked in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat dry milk for 2 h at room temperature and then incubated overnight at 4°C in the same buffer containing a 1:5000 dilution of mouse anti-HA antibody (12CA5; GE healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The TBST-washed membranes were then incubated with a 1:5000 dilution horseradish peroxidase-conjugated anti-mouse secondary antibody (GE Healthcare) in TBST with 5% nonfat dry milk, washed with TBST and developed for chemiluminescence with an ECL-Plus detection kit (GE Healthcare).

RESULTS

Deletion of *IRE1* or Expression of Misfolded Proteins Causes Cell Wall Defects

To test the hypothesis that ER quality control mechanisms affect cell wall biosynthesis, we compared the growth of an *ire1Δ* mutant to WT in the presence of the cell wall perturbing agents CFW and Congo red. *ire1Δ* (W303 background) is hypersensitive to CFW (Figure 1A) and Congo red (Supplemental Figure S1A). Because the phenotypes of *ire1Δ* and cell wall-related mutants can vary with strain background (Larson *et al.*, 2002), we also tested an *ire1Δ* mutant constructed in the S288c background. Although the S288c-derived *ire1Δ* strain was less sensitive to CFW than the W303 *ire1Δ* mutant, it showed a growth defect when exposed to the combined stress of CFW and elevated growth temperature (Figure 1B).

A well known difference between the W303 and S288c strain backgrounds is a polymorphism at the *SSD1* locus. *SSD1* is involved in cell wall integrity (Kaerberlein and Guarente, 2002) and W303 has a hypofunctional, truncated allele (*ssd1-d*), whereas S288c has a functional, full-length allele (*ssd1-v*). As shown in Figure 1B, the S288c-

Figure 1. Null mutation of *IRE1* or expression of CPY* causes cell wall defects. (A) *ire1Δ* mutants in both W303 and S288c backgrounds are hypersensitive to CFW by spot dilution assay. (B) In the S288c background, *ire1Δ* and *ssd1Δ* show synthetic hypersensitivity to CFW. (C) Expression of CPY* causes hypersensitivity to CFW. (D) The indicated strains were grown to stationary phase in yeast peptone galactose (YPG), harvested, adjusted to an OD₆₀₀ of 1 in 10 mM Tris buffer containing Zymolyase 20T (1 mg/ml), and incubated for 1 h at which time the OD₆₀₀ was determined. Data are mean percentages of initial OD₆₀₀ based on three independent experiments performed in triplicate. Error bars denote SD.

derived *ssd1Δire1Δ* mutant recapitulates the CFW phenotype of W303-derived *ire1Δ*, suggesting that the background dependence of *ire1Δ* CFW sensitivity is related to differences in the functionality of *SSD1*. The hypersensitivity of *ire1Δ* mutants to cell wall perturbation and the genetic interaction of *IRE1* with *SSD1*, a gene involved in cell wall integrity, support our hypothesis that *IRE1* is required for proper yeast cell wall biosynthesis.

Because Ire1p is required for the cell to effectively degrade unfolded proteins during periods of stress (Casagrande *et al.*, 2000), it seemed likely that the cell wall defects associated with *ire1Δ* mutants may be due to the accumulation of misfolded or misprocessed proteins. If that was correct, then expression of a misfolded protein would be expected to also cause cell wall defects. To test this hypothesis, we examined the growth of strains expressing the well-studied misfolded protein, carboxypeptidase Y (CPY*) from a galactose-inducible promoter on plates containing CFW. Spear and Ng have shown that expression of CPY* at this level saturates ER quality control mechanisms and causes severe ER stress (Spear and Ng, 2003). In wild-type (WT) cells, high level expression of CPY* causes hypersensitivity to CFW (Figure 1C), indicating that saturation of ER quality control mechanisms with a misfolded protein compromises cell wall integrity. It is important to note that, although *ire1Δ* mutants in some strain backgrounds grow poorly on galactose medium, the growth of *ire1Δ* on YPG is only slightly reduced relative to WT cells and that its growth is clearly decreased by the addition of CFW to YPG, indicating that the phenotype is due to the presence of CFW (Larson *et al.*, 2002).

To confirm the effect of misfolded protein accumulation on cell wall integrity, we also tested a second misfolded protein, the ERAD substrate referred to as KWW. KWW is a chimeric fusion of the luminal portion of the simian virus 5 hemagglutinin-neuraminidase ectodomain (KHN) with the transmembrane and cytosolic domains of Wsc1p (KWW = KHN/Wsc1p transmembrane domain/Wsc1p cytosolic domain; Vashit and Ng, 2004). Indeed, expression of KWW from the *ACT1* promoter also increased the CFW sensitivity of both WT and *ire1Δ* cells, demonstrating that this effect is

not unique to CPY* (Supplemental Figure S1B). These results suggest that uncompensated ER stress generated by either loss of UPR or the accumulation of a misfolded protein leads to defects in cell wall integrity.

Next, we examined the stability of *ire1Δ* and CPY*-expressing strains to treatment with the cell wall-degrading enzyme, zymolyase to confirm the cell wall effects of ER stress by using a second standard cell wall phenotype (de Nobel *et al.*, 2000). Consistent with the spot dilution assays, *ire1Δ*, GAL-CPY*, and *ire1Δ* GAL-CPY* are more easily lysed by zymolyase than WT (Figure 1D). We note that *ire1Δ* GAL-CPY* strains do not grow on galactose-containing agar plates (Spear and Ng, 2003) but, in our hands, slowly grow for four to five doublings in liquid medium, permitting the assays shown here and below.

Although the ER is involved in a variety of cellular processes that could, in principle, affect cell wall integrity, it seemed likely that cell wall protein homeostasis might be most directly affected by uncompensated ER stress. To test this hypothesis, we carried out a set of experiments to characterize the protein fraction of the cell wall in ER-stressed strains. As shown in Figure 2A, the total amount of protein per milligram of cell wall is increased in these mutants; indeed, twice as much protein is present in the cell wall of the *ire1Δ*GAL-CPY* strain relative to WT. Consistent with this finding, *ire1Δ*GAL-CPY* also stained much more intensely than WT when treated with fluorescein isothiocyanate-labeled concanavalin A, a lectin that binds mannoprotein (Supplemental Figure S2A). Furthermore, ER-stressed strains formed large cellular aggregates (Figure 2B) and displayed slightly increased flocculation (Supplemental Figure S2B). Because intracellular interactions responsible for aggregation are mediated by the outermost, mannoprotein layer of the cell wall (Ferreira *et al.*, 2006), this result suggests that the surface properties of ER-stressed cells are quite different from WT cells.

To begin to characterize the cell walls of ER-stressed mutants, we extracted whole cells with 5 mM DTT, a procedure that liberates noncovalently and dithiol-linked cell wall proteins and that yields increased amounts of extractable pro-

Figure 2. Null mutation of *IRE1* and expression of *CPY** causes alterations in cell wall composition and architecture. (A) Cell wall protein composition of cell walls from the indicated strains was determined (micrograms of protein per milligram of cell wall dry weight) as described in *Materials and methods*. Data represent three independent experiments done in triplicate; error bars denote SD. (B) The indicated strains were grown to stationary phase in YPG and analyzed by light microscopy. The number of multicellular aggregates (>10 cells) in 50 high-power fields (hpf) was counted for three independent cultures for each strain. Data are mean number of multicellular aggregates per 50 hpf for the three experiments and error bars indicate SD. (C) Equivalent numbers of whole cells (1 OD₆₀₀ equivalent/μl) of the indicated strains were extracted with 5 mM DTT for 2 h at 4°C. Extracts corresponding to equivalent cell numbers (20 OD₆₀₀ equivalent/lane) were fractionated by SDS-PAGE on a gradient gel (4–15%), and proteins were visualized by silver stain. (D) Spent medium from stationary phase cultures of the indicated strains was treated with trichloroacetic acid/deoxycholate to precipitate proteins. Precipitates corresponding to equivalent cell numbers (150 OD₆₀₀ equivalents/lane) were analyzed as described in C. (E) Cell walls were isolated from the indicated strains and extracted with 30 mM NaOH at 4°C. Extracts were normalized by cell wall wet weight (extracts from 30 mg wet weight of walls per lane) and analyzed as described for C.

teins when the cell wall is defective (Hagen *et al.*, 2004). SDS-PAGE fractionation of DTT extracts from equivalent numbers of cells (20 OD₆₀₀ equivalents/lane) showed that increased amounts of protein are released by DTT in ER-

stressed mutants as detected by silver staining (Figure 2C). In addition, the spent media from cultures of *ire1Δ*, *GAL*-CPY*, and *ire1Δ**GAL*-CPY* contains more protein than that from WT cultures (Figure 2D). Increased release of protein

Figure 3. Uncompensated ER stress causes alterations in cell wall ultrastructure. The indicated strains were grown to stationary phase in YPG, harvested, and processed for electron microscopy as described in *Materials and Methods*. Bars, 0.5 μ m.

into the culture medium is also a phenotype of strains with mutations that affect cell wall proteins (Richard *et al.*, 2002). Thus, these two experiments indicate that uncompensated ER stress causes changes to the protein component of the cell wall that are similar to those observed when cell wall proteins are mutated, supporting the idea that ER quality control mechanisms may be required for the proper function of cell wall proteins.

To further explore the effects of uncompensated ER stress on the protein component of the cell wall, we examined the fraction of proteins covalently attached to the cell wall through an alkali-sensitive ester linkage with 1,3- β -glucan (Kapteyn *et al.*, 1999) in ER-stressed strains. In contrast to the noncovalently bound cell wall protein fraction, ER stress seems to decrease the amount of protein that is released from cell walls by alkali extraction (Figure 2E). An important caveat to the cell wall extraction and protein release experiments is that no internal protein loading control is available; thus, the samples were normalized by cell number or cell wall weight. Consequently, we cannot rule out the possibility that differences in the ratio of cell wall material to cell number may contribute to some of the observed changes in protein levels. With these limitations in mind, our results are consistent with similar experiments performed with cell wall mutants (Kapteyn *et al.*, 1999; Hager *et al.*, 2004). Therefore, the data support the notion that ER stress has a variety of effects on cell wall protein homeostasis. Furthermore, the fact that multiple fractions of cell wall protein are altered by ER stress suggests that these cell wall defects are unlikely to be due to misprocessing of a single class of cell wall proteins or components.

As a final means to characterize the cell wall of ER-stressed mutant strains, we examined the cells by electron microscopy (Figure 3). WT, *ire1* Δ , GAL-CPY*, and *ire1* Δ GAL-CPY* strains all displayed the characteristic bilayer cell wall with a translucent, carbohydrate region sandwiched between two electron-dense mannoprotein layers (Osumi, 1998). Although no gross, general morphological defects were observed in the mutant strains, two more subtle differences between WT and ER-stressed strains are apparent. First, the outer mannoprotein layer of *ire1* Δ and *ire1* Δ GAL-CPY*, in particular, seems less electron dense. Additionally, fewer electron dense mannoprotein fibrils emanate from the cell surface of these strains (Figure 3).

Second, the distance between the electron-dense layers of the cell wall of the *ire1* Δ and *ire1* Δ GAL-CPY* cells is decreased in comparison with either WT or GAL-CPY*. The distance between electron-dense layers in WT cells was similar to that reported for the same W303-WT strain (105 nm [SD 20], Wright *et al.*, 2007), whereas the translucent layer in the *ire1* Δ and *ire1* Δ GAL-CPY* strains was thinner (75 nm [SD 19] and 46 nm [SD 5], respectively). Because the translucent layer of the cell wall is mainly carbohydrate, we measured the total carbohydrate content of cell walls isolated from the mutants and, consistent with the morphological observations, there is less carbohydrate in the cell wall of *ire1* Δ (25% reduced) and *ire1* Δ GAL-CPY* (48% reduced) relative to WT and GAL-CPY* (Supplemental Figure S2C). Thinning of the cell wall has also been observed in *Candida albicans* mutants lacking glycosylphosphatidylinositol (GPI)-linked cell wall proteins, indicating that our findings are likely to represent significant alterations to cell wall structure (Plaine *et al.*, 2008). Together, our data support the notion that uncompensated ER stress causes a variety of changes to the cell wall and, hence, seems to affect several processes involved in cell wall biosynthesis.

ire1 Δ and ERAD Pathway Mutants Display Synthetic Cell Wall Phenotypes

The genes involved in ERAD are up-regulated by UPR and are also required for maintaining ER quality control during unstressed, vegetative growth. We, therefore, wondered whether mutations of ERAD components would cause cell wall defects. Because ERAD and *ire1* Δ mutants display synthetic phenotypes (Friedlander *et al.*, 2000; Travers *et al.*, 2000), we also constructed ERAD/UPR double mutants to determine whether loss of both quality control mechanisms would cause cell wall defects. ERAD has three subtypes that are distinguished by whether the lesion is in the luminal (ERAD-L), membrane (ERAD-M), or cytosolic (ERAD-C) portion of the misfolded protein (Carvalho *et al.*, 2006; Ismail and Ng, 2006). To test the effects of these different subtypes, we constructed three ERAD single and ERAD/*ire1* Δ double mutants in the S288c background: *hrd1* Δ /*ire1* Δ (ERAD-L and -M); *doa10* Δ /*ire1* Δ (ERAD-C), and *ubc7* Δ /*ire1* Δ (common to ERAD-L, -M, and -C).

As shown in Figure 4, A and B, none of the single ERAD mutants was hypersensitive to Congo red or zymolyase digestion. However, *ire1* Δ *hrd1* Δ and *ire1* Δ *ubc7* Δ were hypersensitive to all three conditions, with *ire1* Δ *hrd1* Δ being affected most severely. *hrd1* Δ *ire1* Δ and *ubc7* Δ *ire1* Δ also showed increased DTT-extractable cell wall protein (Figure 4C) in a pattern similar to either CPY* expression or *ire1* Δ mutation in the W303 background (see Figure 2C). *ire1* Δ *doa10* Δ , in contrast, showed no increased sensitivity to cell wall perturbation and no changes in DTT-extractable protein.

We also examined the cell wall ultrastructure of the *ire1* Δ *hrd1* Δ mutant (Supplemental Figure S3). Although the cell wall thickness was not decreased appreciably, the outer cell wall layer of *ire1* Δ *hrd1* Δ cells is less electron dense than WT, and, similar to the mutants shown in Figure 3, the hair-like mannoprotein fibrils decorating the outer surface of the cells are less prominent in the *ire1* Δ *hrd1* Δ cell wall. Together, the cell wall defects shown by double mutants involving specific ERAD components and *ire1* Δ provide additional evidence that ER quality control mechanisms are required to preserve cell wall integrity. Ire1p, however, seems to be more important for cell wall integrity than any of the single ERAD components examined.

Figure 4. Double mutants of *ire1Δ* and ERAD mutants cause cell wall defects. (A) *ire1Δ hrd1Δ* and *ire1Δ ubc7Δ* mutants are hypersensitive to CFW and Congo red by spot dilution assay. (B) Zymolyase sensitivity of *ire1Δ* and ERAD mutants was determined as described in Figure 1D. (C) Whole cells of the indicated strains were extracted with 5 mM DTT and extracts corresponding to 20 OD₆₀₀ equivalents were analyzed by SDS-PAGE/silver stain as described in Figure 2C.

Cell Wall Stress Activates UPR

Because our data clearly demonstrate UPR is involved in the maintenance of cell wall integrity, it seemed plausible that cell wall stress may induce UPR. Although ER stress is

known to activate the CWI pathway (Bonilla and Cunningham, 2003; Chen *et al.*, 2005), to our knowledge, cell wall stress has not been shown to trigger UPR. To test this hypothesis, we used a sensitive reporter of UPR-mediated transcription that contains four copies of the unfolded protein response element (UPRE) fused to the *E. coli* β -galactosidase gene (*lacZ*) on a centromeric plasmid (Cox and Walter, 1996). Treatment of WT cells harboring the 4X-UPRE reporter with cell wall perturbing agents CFW and caffeine induced reporter activity six- and ninefold, respectively (Figure 5A). Although this level of UPR activation is three- to fourfold below that induced by DTT (a compound that generates misfolded proteins; Back *et al.*, 2005), UPR is clearly induced by exposure of cells to cell wall stress. The UPR reporter activity induced by both CFW and caffeine is abolished by null mutation of *IRE1* (Figure 5A), indicating that cell wall stress activates the reporter through UPR and not via an alternate, Ire1p-independent pathway.

To confirm these observations, we next examined the effect of other cell wall stressors on UPR activity. Incubation at 37°C is a mild cell wall stress that activates CWI signaling, and, accordingly, causes a modest induction of UPR. *gas1Δ* is a mutant with significant cell defects that result in constitutive activation CWI signaling pathway as a compensatory response (de Nobel *et al.*, 2000; Popolo and Vai, 1999); the fact that *gas1Δ* also shows increased basal UPR, strongly supports the notion UPR is induced by cell wall stress.

The cellular effects of cell wall stress are frequently suppressed by the addition of an osmotic support such as 1 M sorbitol to the growth medium of cell-wall-stressed cells (Levin, 2005). Indeed, CFW treatment of cells in YPD + 1 M sorbitol induced less UPR activity compared with standard YPD, providing further support for the notion that cell wall damage, and not some other cellular CFW effect, triggers UPR (Figure 5B).

To confirm that cell wall stress activates UPR, we next examined the effect of CFW on *HAC1^u* splicing by using a sensitive RT-PCR assay (Bicknell *et al.*, 2007). As shown in Figure 5C, CFW treatment results in an increase in spliced *HAC1ⁱ*, the key molecular event mediated by Ire1p. CFW treatment also leads to the translation of hemagglutinin-tagged Hac1p as demonstrated by the Western blot shown in Figure 5D. Together, these data demonstrate for the first time that cell wall stress induces UPR.

UPR Activation during Cell Wall Stress and Vegetative Growth Is Dependent on Mpk1p

The CWI MAP kinase pathway is the most important regulator of the cell wall stress response in *Saccharomyces cerevisiae*; therefore, it seemed a likely candidate to mediate cell wall stress-induced UPR (Levin, 2005). Consistent with this hypothesis, treatment of a strain lacking *MPK1*, the MAP kinase of the CWI pathway, with sublethal CFW (20 μg/ml) did not increase UPR activity (Figure 6A). In addition, the basal level of UPR activity during unstressed vegetative growth was decreased twofold. To confirm that Mpk1p contributes to the regulation of basal UPR activity, we compared the extent of *HAC1^u* splicing in unstressed, logarithmic phase WT and *mpk1Δ* cells by RT-PCR (Figure 6B). *HAC1* splicing under these conditions was reduced by one-half in *mpk1Δ* cells compared with WT. Bicknell *et al.* (2007) recently showed that vegetative cells express low levels of basal UPR activity and that it is required for efficient cytokinesis. The CWI pathway is activated during new bud formation; hence, a low level of Mpk1p activity is present during vegetative growth (Zarzov *et al.*, 1996). Thus, the

Figure 5. Cell wall stress activates UPR. (A) WT, *ire1Δ*, *gas1Δ*, or *ire1Δ gas1Δ* cells (S288c background) containing the UPR reporter plasmid pJC104 (CEN, URA, 4XUPRE-lacZ; Cox and Walter, 1996) were grown to logarithmic phase and exposed to the indicated conditions for 5 h at 30°C (except as indicated) before processing for β -galactosidase activity (Burke *et al.*, 2000). DTT, 5 mM DTT; CFW, 25 μ g/ml CFW; CAF, 10 mM caffeine. (B) WT cells containing pJC104 were exposed to CFW (25 μ g/ml) for 5 h in either YPD or YPD supplemented with 1 M sorbitol. The cells were processed as described for A. For A and B, bars indicate the mean of two to three independent experiments performed in duplicate, and error bars denote SEM. (C) The indicated strains (S288c) were grown to logarithmic phase, treated with CFW (25 μ g/ml) or TM (tunicamycin; 2 μ g/ml) for 1 h, harvested, and RNA isolated as described in *Materials and Methods*. RT-PCR analysis of *HAC1* mRNA splicing was performed as described previously (Bicknell *et al.*, 2007) by using 2 μ g of total RNA. (D) Hac1p translation was assayed using BY4741 containing pMB4 (CEN, LEU, *HAC1*-6XHA; Bonilla and Cunningham, 2003). Cells containing empty vector or pMB4 were grown to logarithmic phase, treated with either CFW (25 μ g/ml) or TM (2 μ g/ml) for 2 h, and harvested. Cell lysates were analyzed by Western blotting with anti-HA antibodies.

partial dependence of basal UPR on Mpk1p provides further support for the notion that the CWI pathway regulates UPR.

The fact that basal and cell wall stress-activated UPR requires both Mpk1p and Ire1p suggested that these two

proteins may function in a common regulatory pathway. Consistent with this model, the *ire1Δmpk1Δ* double mutant is as sensitive to CFW as the *mpk1Δ* single mutant, indicating that the *ire1Δ* and *mpk1Δ* mutations are epistatic (Figure 6C). Furthermore, these results also indicate that a linear pathway may connect CWI signaling to UPR during cell wall stress and vegetative growth.

Mid2p and Swi6p Are Involved in CWI Pathway-mediated UPR Activation

To determine which components of the CWI pathway are involved in cell wall stress-induced UPR, we measured CFW-induced 4XUPRE-lacZ reporter activity in a set of CWI-pathway mutants (Table 2). Initially, we tested strains lacking the putative cell wall stress receptors Mid2p and Wsc1p (Figure 7A). One difference between Mid2p and Wsc1p is that Mid2p mediates CFW-induced CWI pathway activity, whereas Wsc1p does not (de Nobel *et al.*, 2000). As shown in Table 2, CFW-induced UPR activation was decreased in the *mid2Δ* mutant, but WT activity was retained in the *wsc1Δ* mutant. RT-PCR assays confirmed that CFW-induced splicing was decreased in the *mid2Δ* mutant but not in *wsc1Δ* (Figure 7B). That Mid2p mediates both CFW-induced cell wall gene expression and UPR supports the idea that these two transcriptional responses represent separate outputs of CWI signaling.

Three transcription factors/regulators are controlled in part by the CWI pathway: Rlm1p, Swi4p, and Swi6p (Levin, 2005). Basal UPR activity was decreased twofold in the absence of Rlm1p (Table 2). In contrast, CFW treatment of *rlm1Δ* induced the same relative increase in UPR as observed with WT (~5-fold), indicating that Rlm1p may play a role in CWI-dependent basal UPR, but that it does not seem to mediate cell wall-stress-induced UPR. Swi4p and Swi6p form a dimeric transcription factor called SBF that plays a role in CWI and cell cycle signaling (Levin, 2005; Madden *et al.*, 1997). The *swi4Δ* mutant displayed basal and CFW-induced UPR activity similar to WT, whereas UPR activation was significantly lower in the *swi6Δ* mutant under both conditions (Table 2 and Figure 7C). In addition, introduction of a multicopy plasmid expressing *SWI6* from its endogenous promoter into a *swi6Δ* strain complemented the HAC1 splicing defect and, in fact, increased basal and CFW-induced UPR by RT-PCR (Figure 7C). These results strongly suggest that Swi6p is required for basal and cell wall stress-induced UPR in a manner that is independent of the Swi4p and hence SBF.

In addition to Swi4p, Swi6p has two other well-characterized binding partners, Mbp1p and Stb1p. Mbp1p interacts with Swi6p to form a second dimeric transcription factor known as the MBF complex (Koch *et al.*, 1993), whereas Stb1p regulates the timing of transcription at Start through its interaction with Swi6p (Ho *et al.*, 1999). As shown in Table 2, CFW treatment of either *mbp1Δ* or *stb1Δ* activated the UPR reporter to the same extent as WT strains, indicating that the role of Swi6p in cell wall stress-induced UPR is independent of its three best characterized binding partners (Swi4p, Mbp1p, and Stb1p).

DISCUSSION

Uncompensated ER Stress Adversely Affects Cell Wall Integrity in Yeast

We have demonstrated that uncompensated ER stress leads to cell wall defects in yeast. Protein glycosylation, GPI-anchor biosynthesis, and 1,6- β -glucan synthesis (Lesage and

Figure 6. CFW-induced UPR activity is dependent on Mpk1p. (A) Wt and *mpk1Δ* (S288c) cells containing pJC104 were exposed to CFW (25 μg/ml) and processed for β-galactosidase activity as described in Figure 5A. Bars indicate the mean of two-three independent experiments performed in duplicate and error bars denote SEM (B) The indicated strains (S288c) were grown to logarithmic phase and processed for RT-PCR analysis of *HAC1^u* mRNA splicing as described in Figure 5C. Percentage apparent splicing was determined by densitometry and represents the ratio of spliced *HAC1* to unspliced *HAC1*. The experiment was repeated three times with similar results and a representative example is shown. (C) The indicated strains were grown on YPD plates supplemented with the indicated amounts of CFW at 37°C for 2 d and photographed.

Bussey, 2006) are examples of the wide range of essential cell wall-related processes localized to the secretory pathway. Therefore, it seems most likely that the cell wall consequences of ER stress are the result of defects in multiple steps in cell wall biosynthesis as opposed to a single defect in a specific step. Among the many possible causes of ER stress-related cell wall defects, we suggest that at least some of the defects may be due to the delivery of misfolded or misprocessed mannoproteins to the cell wall.

At a very simple level, delivery of normally degraded misfolded proteins to the cell wall would explain the in-

creased amount of cell wall protein observed in ER-stressed mutants (Figure 2A). Furthermore, misfolded or misprocessed cell wall proteins may not be suitable substrates for enzymes that incorporate cell wall proteins into the carbohydrate lattice of the cell wall. Similarly, misfolded cell wall proteins may not be able to carry out key structural functions. Consistent with this model, mutants under uncompensated ER stress display cell wall phenotypes that mimic mutants lacking cell wall proteins (e.g., increased DTT extractable proteins and excessive protein release into culture medium (Figure 2, B and C). Obviously, more detailed analyses of the cell wall of these mutants is required to characterize the specific mechanisms by which compromised ER quality control causes cell wall defects.

Table 2. UPR induced by CFW in cell wall integrity MAP kinase signaling pathway mutants

Strain	CFW ^a	UPR activity ^b	-Fold change ^c
WT	—	82 (4)	1.0
WT	+	520 (13)	6.3
<i>wsc1Δ</i>	—	96 (10)	1.2
<i>wsc1Δ</i>	+	407 (14)	4.2
<i>mid2Δ</i>	—	64 (5)	0.75
<i>mid2Δ</i>	+	88 (7)	1.4
<i>rlm1Δ</i>	—	38 (1)	0.46
<i>rlm1Δ</i>	+	290 (22)	7.6
<i>swi4Δ</i>	—	65 (5)	0.79
<i>swi4Δ</i>	+	490 (20)	7.5
<i>swi6Δ</i>	—	9 (1)	0.11
<i>swi6Δ</i>	+	16 (1)	1.8
<i>mbp1Δ</i>	—	50 (3)	0.61
<i>mbp1Δ</i>	+	310 (12)	6.2
<i>stb1Δ</i>	—	61 (6)	0.74
<i>stb1Δ</i>	+	326 (30)	5.3

^a CFW, Calcofluor white, logarithmic phase cells in YPD were treated with CFW (25 μg/ml) for 5 h at 30°C, harvested, and processed for β-galactosidase activity as described in *Materials and Methods*.

^b β-Galactosidase activity of cells containing 4X-UPRE-*lacZ* reporter (pJC104) Values are Miller units (nanomoles of o-nitrophenol per milligram of protein per minute) and are means of two to three independent experiments performed in duplicate-triplicate. SE of the mean is shown in parentheses.

^c For untreated cells, -fold change represents ratio of untreated mutant strain to untreated wild-type strain. For CFW-treated cells, -fold change represents treated mutant strain to untreated mutant strain.

ERAD-L, M but Not ERAD-C Affects Cell Wall Integrity

Although UPR seems to be the most important protein quality control mechanism for cell wall integrity, loss of specific ERAD components affects cell wall integrity when combined with loss of UPR function. Specifically, mutation of ERAD components involved in luminal or membrane proteins quality control (ERAD-L, M) cause cell wall defects, whereas loss of cytosolic ERAD (ERAD-C) does not affect the cell wall. This indicates that misfolded protein stress within the secretory pathway is most damaging to cell wall integrity.

Because ERAD/UPR double mutants (*ire1Δhrd1Δ* and *ire1Δubc7Δ*) also display synthetic noncell wall phenotypes (Friedlander *et al.*, 2000; Travers *et al.*, 2000), an alternate explanation for the synthetic cell wall phenotypes is that they represent a general fitness defect. However, other groups have shown that *hrd1Δ*, *ubc7Δ*, and *doa10Δ* display similar levels of basal UPR activation (Friedlander *et al.*, 2000; Bicknell *et al.*, 2007), indicating that the mutations cause comparable levels of ER stress. Because these ERAD mutants activate UPR similarly yet have quite different effects on the cell wall, we propose that the cell wall phenotypes of ERAD/UPR double mutants are the result of effects on cell wall biosynthesis rather than due to a decreased global fitness. If this model is correct, then the cellular consequences of misfolded protein stress are determined, in part, by the cellular location (ER vs. cytoplasm) subjected to stress.

The CWI MAPK Signaling Pathway Modulates UPR

Consistent with the dependence of cell wall integrity on ER quality control, we have shown that cell wall stress activates

Figure 7. CFW-induced UPR activity is dependent on components of the CWI pathway. (A) Schematic of the CWI MAPK signaling cascade. (B) Null mutation of *mid2Δ* but not *wsc1Δ* decreases CFW-induced *HAC1* splicing. (C) *swi6Δ* cells transformed with empty vector or 2μ plasmid encoding *SWI6* (pBD1265) were assayed for CFW-induced *HAC1* mRNA splicing as described in Figure 5C. *ACT1* loading controls were generated by RT-PCR using 2 μg of total RNA from the same sample as that used to perform *HAC1* RT-PCR.

UPR, the pathway that regulates the main compensatory response to secretory stress. Although the effect of cell wall stress on UPR has not been reported previously, Pal *et al.* (2007) have recently shown that CWI pathway activation by hypotonic stress increases the UPR activity of cells that are concomitantly exposed to ER stress (Pal *et al.*, 2007). The synergistic increase in UPR during the simultaneous application of ER and hypotonic stress is the result of an Mpk1p-dependent lengthening of the lifetime of Hac1p. Hypotonic stress alone, however, is not sufficient to induce UPR, probably because hypotonic stress causes only a transient CWI pathway activation (Davenport *et al.*, 1995). Our results indicate that more severe cell wall stress activates UPR through increased Ire1p-mediated *HAC1^u* splicing; thus, cell wall stress seems to modulate UPR activity by directly inducing *HAC1^u* splicing and by increasing the lifetime of Hac1p.

The CWI pathway is also activated in vegetative cells during periods of polarized growth in a process that is partially dependent on Cdc28p. Therefore, it would follow that UPR might also be activated during polarized growth if it was regulated by the CWI pathway. Although the function of UPR has traditionally thought to be limited to periods of cellular stress, Bicknell *et al.* have recently shown that UPR is active at low, basal levels during unstressed vegetative growth (Bicknell *et al.*, 2007). Consequently, our observation that constitutive UPR activity is partially dependent on Mpk1p suggests a potential mechanism by which basal UPR is regulated and implies that this regulation may be related to the cell cycle in yeast.

Cell wall stress signals activate UPR through components of the CWI MAPK pathway, including Mid2p, a plasma membrane-localized CWI pathway stress receptor; Mpk1p, the MAPK for the CWI pathway; and Swi6p, a transcriptional regulator modulated by the CWI pathway. Interestingly, Swi6p has also been shown to interact genetically under cell wall stress with the cytoplasmic Hsp110 chaperone Sse1p, suggesting that it could play a broader role in the cellular response to misfolded protein stress (Shaner *et al.*, 2008). However, it is not clear whether the cell wall defects associated with loss of cytoplasmic chaperone function are due to defective signaling through the CWI pathway (Shaner *et al.*, 2008) or are, alternatively, the result of more direct effects on cell wall structure (Wright *et al.*, 2007).

At present, it is also unclear how the signal to activate UPR is transmitted from Swi6p to Ire1p. Swi6p-mediated UPR activation is not dependent on any of its previously

characterized binding partners (Swi4p, Mbp1p, or Stb1p). Because Swi6p does not seem to possess DNA binding domains (Sedgwick *et al.*, 1998), a transcriptionally mediated mechanism for Swi6p-induced UPR activation would seem to require a previously unidentified Swi6p-dependent DNA binding protein. Alternatively, Swi6p could activate UPR through a nontranscriptionally mediated mechanism involving interactions with other proteins or, even, through direct binding to Ire1p.

Cell Wall and Secretory Stress Initiate Complimentary Compensatory Responses

In this report, we have demonstrated that secretory stress has significant effects on cell wall integrity, particularly in the absence of an appropriate compensatory response. These observations provide a mechanistic rationale for previous reports indicating that secretory stress activates both UPR and CWI stress pathways. Recently, Cohen *et al.* (2008) have shown that the Hos2p/Set3p deacetylase complex mediates transmission of secretory stress signals to the CWI pathway in a process that is parallel to the initiation of UPR. This parallel relationship is consistent with the fact that mutants deficient in UPR and CWI signaling (e.g., *ire1Δmpk1Δ*) show synthetic hypersensitivity to ER stress.

We have shown that cell wall stress also initiates both a secretory pathway stress response (UPR) and a cell wall stress response. In contrast to the parallel activation of these responses by secretory stress, our data indicate that cell wall stress-mediated activation CWI signaling and UPR occurs via linear pathway connecting the CWI pathway to Ire1p-mediated signaling. As such, *ire1Δmpk1Δ* mutants show epistatic cell wall defects relative to the corresponding single mutants.

Combining our results with previous studies on the role of the CWI pathway in secretory stress responses, we propose the following model to describe the interrelated nature of the cellular response to secretory pathway stress and cell wall stress. During secretory pathway stress, the resulting ER dysfunction could lead to cell wall damage; consequently, the cell wall compensatory response is triggered to reinforce cell wall integrity. Conversely, during cell wall stress, any breakdown in ER quality control could further exacerbate cell wall damage and, thus, the folding capacity of the secretory pathway is increased through UPR activation, lessening the chances of further cell wall damage as a result of misfolded proteins. Studies on the molecular mechanisms responsible for this complex interplay between these

two major cellular signaling pathways are in progress and will be reported in due course.

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