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RESEARCH ARTICLE

Management of the endoplasmic reticulum stress by activation of the heat shock response in yeast

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Abstract

In yeast *Saccharomyces cerevisiae*, accumulation of misfolded proteins in the endoplasmic reticulum (ER) causes ER stress and activates the unfolded protein response (UPR), which is mediated by Hac1p. The heat shock response (HSR) mediated by Hsf1p, mainly regulates cytosolic processes and protects the cell from stresses. Here, we find that a constitutive activation of the HSR could increase ER stress resistance in both wild-type and UPR-deficient cells. Activation of HSR decreased UPR activation in the WT (as shown by the decreased *HAC1* mRNA splicing). We analyzed the genome-wide transcriptional response in order to propose regulatory mechanisms that govern the interplay between UPR and HSR and followed up for the hypotheses by experiments *in vivo* and *in vitro*. Interestingly, we found that the regulation of ER stress response via HSR is (1) only partially dependent on over-expression of Kar2p (ER resident chaperone induced by ER stress); (2) does not involve the increase in protein turnover via the proteasome activity; (3) is related to the oxidative stress response. From the transcription data, we also propose that HSR enhances ER stress resistance mainly through facilitation of protein folding and secretion. We also find that HSR coordinates multiple stress–response pathways, including the repression of the overall transcription and translation.

Introduction

In eukaryotic cells, the endoplasmic reticulum (ER) provides an environment for protein folding and quality control of proteins entering the secretory pathway. The accumulation of misfolded proteins in the ER causes ER stress that leads to perturbations. A variety of diseases including diabetes, Alzheimer's, and Parkinson's are related to protein misfolding (Rasheva & Domingos, 2009; Bharadwaj *et al.*, 2010; Brown & Naidoo, 2012). Yeast *Saccharomyces cerevisiae* is an excellent model system for gaining insight into the molecular processes involved in control of protein homeostasis (Bharadwaj *et al.*, 2010; Petranovic *et al.*, 2010; Pineau & Ferreira, 2010).

To ensure proper processing of proteins within the ER, eukaryotic cells employ 'quality control' to recognize and degrade the aberrantly folded proteins that failed re-folding, to prevent potential cellular dysfunction (Plemper & Wolf, 1999). Two processes have often been described to cope with misfolded protein in the ER: ER-associated degradation (ERAD) and the unfolded protein response (UPR; Travers *et al.*, 2000). The ERAD system disposes of the misfolded proteins via degradation in the cytosol. Misfolded proteins are translocated to the cytosol, where they are poly-ubiquitilated and degraded by the 26S proteasome. At least two ERAD pathways have been identified in *S. cerevisiae*, in ER lumen (ERAD-L) and in ER membrane and in cytosol (ERAD-C&M). Besides the ERAD system, UPR is another cellular response which is activated by misfolded protein in the ER. In *S. cerevisiae*, accumulation of misfolded proteins in the ER leads to oligomerization of the transmembrane kinase/nuclease Ire1p, which initiates the splicing of *HAC1* mRNA, resulting in its translation and Hac1p synthesis. The transcription factor (TF) Hac1p up-regulates the transcription of UPR target genes, most of which are involved in the protein folding, vesicle trafficking, and protein degradation (Travers *et al.*, 2000).

Apart from misfolded proteins in the ER, misfolded membrane proteins or cytosolic proteins that do not enter the secretory pathway induce a subset of the heat shock response (HSR) targets genes (called the cytosolic UPR) through regulation by the heat shock TF Hsf1p (Metzger & Michaelis, 2009; Ciplys *et al.*, 2011; Geiler-Samerotte *et al.*, 2011). In *S. cerevisiae*, the heat shock factor Hsf1p is an essential protein, and activates its target genes (coding for heat shock proteins, HSP) through binding to the heat shock element (HSE). Yeast Hsf1p is reported to bind HSEs constitutively, but is transcriptionally silent or bound only to specific HSEs at non-stress conditions (Sewell *et al.*, 1995). In a stress-inducible manner, Hsf1p undergoes a conformational change that leads eventually to up-regulation of the HSP (Sewell *et al.*, 1995; Erkine *et al.*, 1999). An *HSF1* mutation *HSF1-R206S* has been identified to lead to constitutive transcriptional activity of Hsf1p on the consensus HSE-containing promoters without requirement of a temperature shift (Sewell *et al.*, 1995). The targets of Hsf1p include hundreds of genes involved in protein folding and trafficking, detoxification, energy generation, cell wall organization, etc. (Hahn *et al.*, 2004). Originally discovered as a response to heat stress, HSR can also be triggered by other stresses, such as alkaline pH, oxidative stress, or glucose starvation.

Although the HSR mediated by Hsf1p is identified as a cytosolic UPR, a recent study revealed that the HSR activation can rescue the growth deficiency in UPR-deficient *ire1*Δ cells under ER stress (Liu & Chang, 2008). This study showed that constitutively active Hsf1 selectively releases the block of the ER-to-Golgi transport and stimulates ERAD. Among the HSR targets, the induction of Kar2p and COPII cargo receptors such as Erv29p were found to be important for releasing ER stress. The study pointed to an ameliorative effect of HSR on ER stress. However, HSR can activate hundreds of genes and it is not clear how are those genes coordinated to relieve ER stress.

Recent studies collected extensive information on the HSR targets and the interaction of the HSR to the other cellular processes (Eastmond & Nelson, 2006; Bandhakavi *et al.*, 2008; Wu & Li, 2008). However, very few studies have focused on the impact of the HSR on misfolded proteins, and especially the effect of misfolded proteins in the ER. In this study, we observe that the HSR promotes cell survival and decrease the *HAC1* mRNA splicing under ER stress. Over-expression of the target *KAR2* alone could not relieve ER stress, which indicated that multiple pathways were involved, so we performed a genome-wide transcriptome analysis to investigate the biological mechanisms of HSR-mediated suppression of ER stress. We compared the transcriptional responses in a wild-type strain and a UPR-deficient strain, in ER stress conditions. Together with measurements of proteasomal activity and reactive oxygen species (ROS), we found that HSR relieved ER stress through several processes including up-regulating of genes involved in protein folding and the secretory pathway, repressing the overall transcription and translation and coordinating with other stress responses such as the oxidative stress response. Our study hereby obtained new insights of the role of HSR to assist in handling misfolded proteins in the ER.

Materials and methods

Strains and media

Yeast strains used in this study are listed in Table 1. All experiments were performed in the background strain CEN.PK113-5D (*MAT*a *SUC2 MAL2-8*^{c} *ura3-52*; P. Kötter, Frankfurt, Germany). The *HAC1* deletion strain *hac1*Δ was constructed in the previous study (Tyo *et al.*, 2012). For constitutive activation of the heat shock factor, site-directed mutation *HSF1-R206S* was amplified using fusion PCR, digested with appropriate restriction enzymes and cloned. Two plasmids were used to attain different expression levels of *HSF1*, including a 2 μm plasmid pRS426-TEF and a centromere plasmid with pRS416-TEF (Mumberg *et al.*, 1995). The plasmids were also digested with corresponding restriction enzymes and ligated with *HSF1-R206S*. *KAR2* was also amplified from CEN.PK113-5D chromosome DNA, digested and ligated into pRS426-TEF. The resulting plasmid was transformed into CEN.PK113-5D or the *HAC1* deletion strain and the resulting strains are listed in Table 1. For *RPN4* deletion, the *hph* gene from *Klebsiella pneumoniae* encoding hygromycin B phosphotransferase amplified from pAG32 was used as the selection marker and *RPN4* deletion strains *rpn4*Δ, *rpn4*Δ (hsf1), *hac1*Δ*rpn4*Δ, and *hac1*Δ*rpn4*Δ (hsf1) were constructed. The primers used for amplification are listed in Supporting information, Table S1. The media used in this study is SD-URA media and different concentration of dithiothreitol (DTT) or tunicamycin is added into the media.

Induction of ER stress

DTT or tunicamycin was added to the medium to induce ER stress. Different concentrations were tested to find the appropriate concentrations for different strains. For the qualitative growth assay, 0.5 μg mL^{-1} tunicamycin and 2.5 mM DTT were chosen as concentrations for completely preventing the growth of *hac1*Δ; growth of *hac1*Δ*(hsf1)* was not affected significantly. 1.0 μg mL^{-1} tunicamycin was then used in the comparison of WT and

Table 1. Strains used in this study

Strains	Relevant genotype	Reference
CEN.PK 113-5D	*MATa SUC2 MAL8^C ura3-52*	P. Kötter
CEN.PK 113-5D *hac1*Δ	*MATa SUC2 MAL8^C hac1::KanMX4*	Tyo *et al.* (2012)
WT	CEN.PK 113-5D *pRS426-TEF*	This study
WT (L-hsf1)	CEN.PK 113-5D *pRS416-HSF1*	This study
WT (hsf1)	CEN.PK 113-5D *pRS426-HSF1*	This study
*hac1*Δ	CEN.PK 113-5D *hac1::KanMX4 pRS426-TEF*	This study
*hac1*Δ (L-hsf1)	CEN.PK 113-5D *hac1::KanMX4 pRS416-HSF1*	This study
*hac1*Δ (hsf1)	CEN.PK 113-5D *hac1::KanMX4 pRS426- HSF1*	This study
WT (kar2)	CEN.PK 113-5D *hac1::KanMX4 pRS426-KAR2*	This study
*hac1*Δ (kar2)	CEN.PK 113-5D *hac1::KanMX4 pRS426-KAR2*	This study
*rpn4*Δ	CEN.PK 113-5D *rpn4::hph pRS426-TEF*	This study
*rpn4*Δ (hsf1)	CEN.PK 113-5D *rpn4::hph pRS426-HSF1*	This study
*rpn4*Δ*hac1*Δ	CEN.PK 113-5D *rpn4::hph hac1::KanMX4 pRS426-TEF*	This study
*rpn4*Δ*hac1*Δ (hsf1)	CEN.PK 113-5D *rpn4::hph hac1::KanMX4 pRS426-HSF1*	This study

WT (hsf1), but this condition cannot be used for the *HAC1* deletion strains as they did not grow at all. 0.4 μg mL^{-1} tunicamycin and 2.0 mM DTT was used for *hac1*Δ*rpn4*Δ *(hsf1)*, as higher concentration prevented its growth. For the stress induction in liquid culture, we used 2.0 mM DTT to activate UPR as described previously (Travers *et al.*, 2000). The experiments were performed duplicate or triplicate, and the replicates were compared using Student's *t*-test whenever indicated. A *P*-value < 0.05 was considered statistically significant.

Qualitative growth assays

Survival of the strains was determined by qualitative growth assays on plates containing DTT or tunicamycin. Cells were harvested by centrifugation, washed twice with water and resuspended in water. The cell density was normalized to $OD_{600\ nm} = 1.0$, and diluted four time in 10-fold series ($OD_{600\ nm}$ 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). Aliquots (3.5 μL) of each dilution were spotted onto the SD-Ura plates with or without DTT or tunicamycin. Growth was recorded after 2–3 days of incubation at 30 °C.

RNA extraction and microarrays

The strains were inoculated into SD-Ura medium and grown to $OD_{600\ nm} = 0.4$. 2 mM DTT was added into the medium to induce ER stress. After 2 h induction, samples for total RNA extraction were taken by rapidly transferring 10-mL culture into 30-mL ice in a 50-mL Falcon tube so that the samples were cooled down immediately. The cells were harvested by centrifugation, frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using Qiagen RNeasy Mini Kit (QIAGEN, Valencia, CA). The quality of total RNA was determined using Agilent 2100 Bioanalyzer (Agilent Technologies). The cDNA synthesis, aRNA synthesis, and labeling were performed as described in Affymetrix GeneChip® Expression Analysis Technical Manual. The aRNA was fragmented and hybridized to Yeast Genome 2.0 Arrays (Affymetrix). Washing and straining of the hybridized microarrays were carried out on the Affymetrix Genechip® Fludics Station 450 and scanned using the Affymetrix GeneChip® 3000 7C Scanner. The microarray data are deposited to NCBI Gene expression omnibus (GEO: accession number GSE39311).

Transcriptome data analysis

Affymetrix CEL-data files were analyzed using R suite and Bioconductor packages. The background correction and normalization were processed using the method of Probe Logarithmic Intensity Error (PLIER), perfect match (PM) only, and QSPLINE algorithm. A Singular Value Decomposition was employed to identify the patterns of the transcriptome. The moderated Student's *t*-test and two-way Analysis of Variance (ANOVA) were performed to determine the genes whose expression levels have significantly changed in different conditions. For integrated analysis, the Reporter Features algorithm (Patil & Nielsen, 2005; Oliveira *et al.*, 2008) was applied to identify not only the most significant transcriptional changes, but to find regulations at the level of pathways, and regulations under the same TFs; these two methods use different types of biological networks derived from Kyoto Encyclopedia of Genes and Genomes (KEGG), and TF-DNA interactions from CHIP-chip (TF; Harbison *et al.*, 2004), respectively. The algorithm identifies key biological features in the respective networks around which the most significant transcriptional changes occur. To identify biological features (KEGG pathways and TFs) with neighboring genes that were either up- or down-regulated, the reporter algorithm

was used again with a subset of the original network, containing only the up- or down-regulated genes and their relations with the biological features, as input.

Real-time quantitative PCR (qPCR) and *HAC1* mRNA splicing

Total RNA was isolated using UNIQ-10 spin column RNA extraction kits (Sangon Biological Engineering, China). The first strand of cDNA was synthesized using the PrimeScript™ RT Reagent Kit (TaKaRa, Japan) and then used for qPCR amplification in the Light Cycle PCR System (SYBR Green Real-time PCR Master Mix, Japan). *ACT1* gene was used as a reference gene. Relative transcription level of the genes in mutant strains were normalized by comparing the C_T value to the wild type without DTT treatment using a $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).The gene specific primers are listed in Table S1. The relative transcription level of the targets genes were determined by relating the C_q values of the sample to the reference gene. The *HAC1* mRNA splicing was determined by PCR using synthesized cDNA as templates.

Proteasome activity assays

Proteasome activity was measured by determining the luminescence of the substrates for chymotrypsin-like, trypsin-like or caspase-like 20S protease activity using the Proteasome-Glo™ Cell-Based Assays (Promega GmbH, Germany) according to the manufacturer's instructions. Cells were either treated with 2 mM DTT for 2 h or left untreated before harvest. Different number (20 000, 40 000, and 80 000) of exponential cells were mixed with 100 μL of respective reagent and incubated for 10 min at room temperature (Ruenwai *et al.*, 2011). Luminescence was recorded using a microplate reader (Safire II, TECAN, Switzerland) and every sample was measured in duplicate. The relative luminescence unit in different conditions was normalized by the intensity of wild-type untreated cells.

Detection of ROS

ROS were detected with dihydrorhodamine 123 (DHR123). $OD_{600\ nm}$ = 1.0 cells were harvested, washed, and resuspended in PBS, then DHR123 (50 μM) was added and incubated in the dark for 30 min. Cells were washed and resuspended in 50 mM sodium citrate buffer, and monitored with fluorescence microscope Leica DMI4000 B using DIC and YFP filters. Fluorescence intensity was quantified using the fluorescence microplate reader (FLUOstar Omega, BMG LABTECH, Germany) with an excitation filter 485 nm and an emission filter 520 nm (Ruenwai *et al.*, 2011).

Results

HSR promotes cell survival and decreases *HAC1* mRNA splicing under ER stress

To ensure a constitutively activation of HSR without a temperature shift, the *HSF1* mutant gene *HSF1-R206S* (Sewell *et al.*, 1995) was over-expressed in a wild-type yeast *S. cerevisiae* strain CEN.PK113-5D (WT background) or a strain *hac1*Δ (UPR deficient background), with either a high-copy 2 μm plasmid [strains WT (hsf1), *hac1*Δ (hsf1)] or a low-copy centromeric CEN plasmid [strains WT (L-hsf1), *hac1*Δ (L-hsf1); Table 1]. ER stress was induced with DTT (inhibitor of disulfide bond formation), and tunicamycin (an inhibitor of *N*-glycosylation). Figure 1(a) shows that *hac1*Δ is very sensitive to ER stress and cannot grow in the presence of DTT or tunicamycin. Over-expression of *HSF1* in the *hac1*Δ can partially restore growth in the presences of 2.5 mM DTT or 0.5 μg mL^{-1} tunicamycin, and higher expression of *HSF1* results in better growth (Fig. 1a). This shows that HSR promotes cell survival and increases the resistance to ER stress in UPR-deficient cells. To determine whether HSR can increase the resistance in wild-type strains, we did qualitative growth assay with higher tunicamycin concentration, and found that WT over-expressing Hsf1p WT (hsf1) grew better than WT in 1.0 μg mL^{-1} tunicamycin (Fig. 1b). ER stress activates UPR which is regulated by *HAC1*, therefore we also determined the *HAC1* mRNA splicing level. In WT there was a small fraction of spliced *HAC1* mRNA even under standard conditions, and DTT or tunicamycin addition increased the level of spliced mRNA (Fig. 1c and Fig. S1). In WT (hsf1), there was no spliced *HAC1* mRNA irrespective of DTT or tunicamycin addition (Fig. 1c and Fig. S1), indicating that HSR activation reduces the UPR induction.

Over-expression of *KAR2* can only partially rescue UPR deficiency

Kar2p/BiP is a HSP70 family chaperone that mediates protein folding in the ER and also plays an important role in ER quality control as it takes part in both ERAD and UPR (Tokunaga *et al.*, 1992; Nishikawa *et al.*, 2005). It is also regulated by the HSR and it is reported that *KAR2* over-expression rescued the translocation defect and facilitated degradation in UPR-deficiency cells (Liu & Chang, 2008). Therefore, to evaluate if Kar2p is the key component of the HSR that is relieving the ER stress, we over-expressed *KAR2* in the WT and *hac1*Δ strains and did qualitative growth assay, with DTT or tunicamycin to induce the ER stress. Figure 2 shows that over-expression of *KAR2* alone cannot fully rescue the growth deficiency

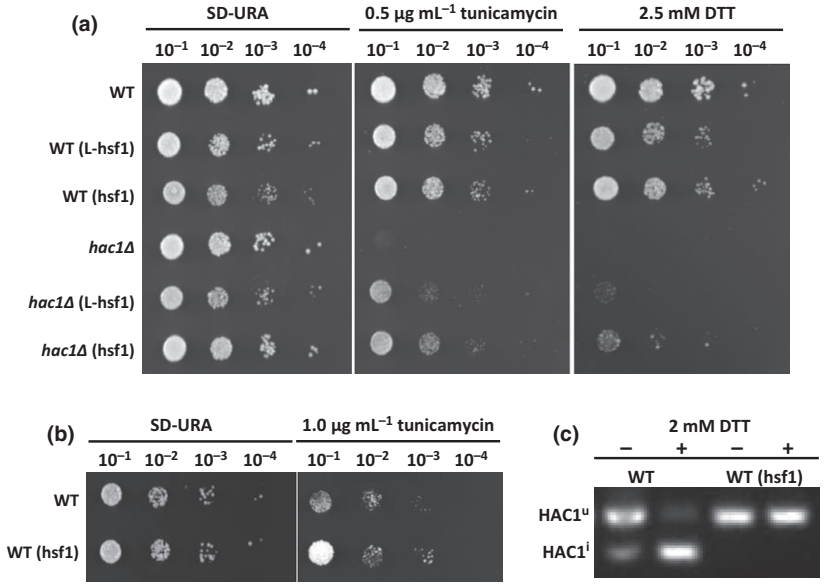

Fig. 1. HSR increases cell survival and decreases the *HAC1* mRNA splicing under ER stress. *HSF1-R206S* was over-expressed in WT (CEN.PK113-5D) and *hac1*Δ backgrounds, with either the centromeric or a 2 μm plasmid, giving rise to four strains WT (L-hsf1), *hac1*Δ (L-hsf1), WT (hsf1) or *hac1*Δ (hsf1). The centromeric plasmid with a *TEF1* promoter (pRS416-TEF) gives lower expression levels of *HSF1-R206S* [strains WT (L-hsf1), *hac1*Δ (L-hsf1)], and the 2 μm plasmid with a *TEF1* promoter (pRS426-TEF) gives higher expression of *HSF1-R206S* [strains WT (hsf1) or hac1Δ (hsf1)]. (a) Strains were spotted on SD-URA plate and SD-URA plates with 2.5 mM DTT or 0.5 μg mL^{-1} tunicamycin. (b) WT and WT (hsf1) strains were spotted on SD-URA plates with 1.0 μg mL^{-1} tunicamycin. (c) The *HAC1* mRNA splicing in WT and WT (hsf1) with or without 2 mM DTT. HAC^{u} stands for unspliced *HAC1* mRNA, and HAC^{i} stands for induced (spliced) *HAC1* mRNA. For concentrations of DTT and tunicamycin please see Materials and methods. Results were from two independent experiments.

of the *hac1*Δ strain, when ER stress is induced with tunicamycin or 2.5 mM DTT. Over-expression of *KAR2* can recover the growth of the *HAC1* deletion mutant *hac1*Δ (kar2) only in less severe ER stress (lower concentrations of DTT). These results clearly demonstrate that even though Kar2p can partially rescue UPR deficiency, it is not the sole component responsible for the observed HSR-mediated suppression of ER stress.

Over-expression of *HSF1* under ER stress induces global transcriptional changes

To get further insight into the mechanisms of the HSR-mediated suppression to ER stress, we performed genome-wide transcriptome analysis and assessed the impact of expressing *HSF1* in wild-type and UPR-deficient cells under ER stress, induced by DTT. Principal component analysis (PCA) was performed in order to visualize the general transcriptional response (based on the expression of all genes) for each sample. The PCA analysis showed a clear separation of the four strains (Fig. S2a) which means that the global transcriptional response is different for the four different strains. Both *HSF1* over-expression and *HAC1* deletion induced global transcriptional responses under ER stress (Fig. S2b). In order to facilitate interpretation of the data, we used the Reporter Features algorithm and performed integrated data analyses (Patil & Nielsen, 2005; Oliveira *et al.*, 2008).

From the identified reporter KEGG pathways (www.kegg.jp; reporter $P < 0.005$), we found that ribosome synthesis, amino acids metabolism, and aminoacyl-tRNA synthesis were down-regulated, when comparing the *HSF1* over-expression strains (in WT or *hac1*Δ background), with the WT or the *hac1*Δ strains (Fig. 3). This repression of translation represents a protective response to stress conditions. Many metabolic pathways, including energy generation, respiration, tricarboxylic acid cycle, and glutathione metabolism were up-regulated (Fig. 3). *HSF1* over-expression also induced up-regulation of genes for protein processing in the ER, which includes induction of many chaperones involved in protein folding. Interestingly, Reporter TFs analysis (Patil & Nielsen, 2005; Oliveira *et al.*, 2008), which aims to identify co-regulated genes that are under the same TF (see also Materials and methods) showed that *HSF1* over-expression activates genes under the regulation of TFs involved in stress response (*MSN2/4*, *YAP1*, and *SKN7*), ribosome synthesis (*IFH1* and *SFP1*), amino acid synthesis (*GCN4*, *BAS1*, *MET4*, and *PPR1*), glucose de-repression and β-oxidation (*SNF4*, *OAF1*, and *PIP2*), and cell cycle (*SFP1* and *SIT4*; Table 2).

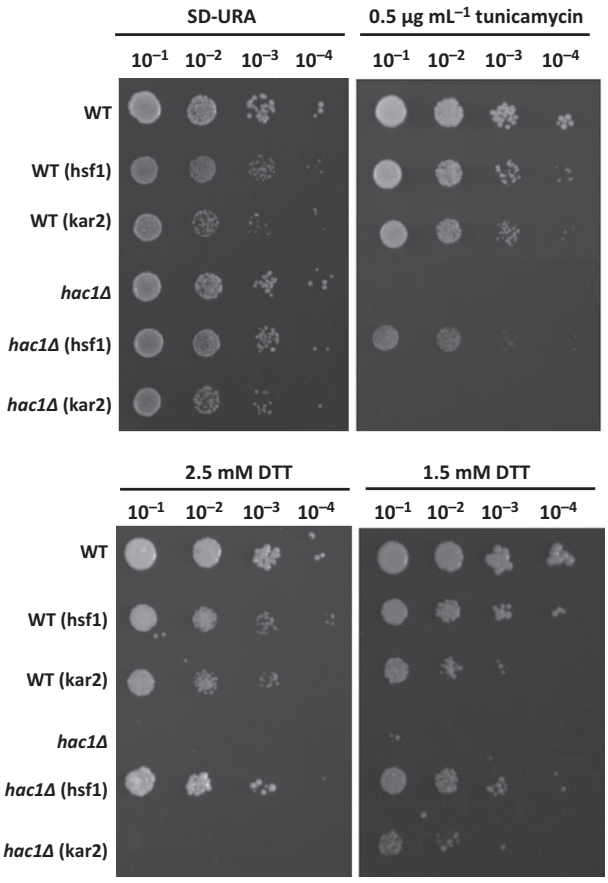

Fig. 2. Over-expression of *KAR2* cannot fully restore the growth deficiency of *hac1*Δ strain under ER stress. WT, WT (hsf1), WT (kar2), *hac1*Δ, *hac1*Δ (hsf1), and *hac1*Δ (kar2) were spotted on SD-URA plates and SD-URA plates with 2.5 mM DTT, 1.5 mM DTT, or 0.5 μg mL^{-1} Tunicamycin. Two independent experiments were performed.

Identification of these TFs reporters suggested that the HSR coordinates other pathways to protect the cells from different types of stress (Wu & Li, 2008).

Addition of DTT induced UPR in the wild-type strain but not the *HAC1* deletion strain, and the *hac1*Δ strain had lower expression of the genes involved in protein process in ER, protein export, and amino acids synthesis than the wild-type strain (Fig. 3). *HAC1* deletion also led to the repression of ribosome and RNA biosynthesis, but the proteasome-related process was significantly up-regulated in the *hac1*Δ strain (Fig. 3 and Table S2).

HSR up-regulates the genes in the secretory pathway

In order to compare how HSR and UPR manage ER stress, we identified significantly changed genes ($P < 0.001$) that are involved in protein folding, translocation, secretion, and degradation processes, and compared the effects of *HSF1* over-expression (HSR+ vs. HSR−) and *HAC1* deletion (UPR+ vs. UPR−; Table 3). Over-expressing *HSF1* up-regulated many genes involved in both cytosolic and ER protein folding (Table 3). Among these targets, ER chaperones like *KAR2*, *SIL1*, and disulfide bond formation enzymes such as *ERO1* are up-regulated in response to both HSR and UPR. Some vesicle trafficking related genes including ER-Golgi trafficking, Golgi-endosome trafficking, and endosomal-vacuolar trafficking were also up-regulated when over-expressing *HSF1*. Several genes such as *ERV29*, *SAR1*, and *NUS1* were significantly changed in both comparisons. These results suggest overlapping of UPR and HSR in regulating protein folding and protein trafficking. Differently from UPR, HSR also up-regulated many genes encoding cytosolic HSP such as *SSA1/3/4*, *SSE2*, *FES1*, *HLJ1*, and *HSP30/42* which have chaperone activity and can suppress aggregation of unfolded proteins in the cytosol. We observed significant up-regulation of the proteasomal system in the *HAC1* deletion strains but not in *HSF1* over-expression strains from our reporter KEGG pathway analysis (Fig. 3). Indeed, although the well-known ERAD genes *DER1* and *DFM1* were up-regulated in wild type strains by UPR activation [WT and WT (hsf1) vs. *hac1*Δ and *hac1*Δ (hsf1)], the expression of other genes in ubiquitin and proteasome system (UPS) were much higher in the *HAC1* deletion strains. In the *HSF1* over-expression strains, several ubiquitin and proteasome-related genes were also up-regulated. We also determined the transcription level of several HSR and UPR targets with or without DTT using qPCR, to verify the transcriptome data (Fig. 4) and the obtained results were consistent.

The role of proteasome in HSR-mediated suppression of ER stress

From the transcriptome analysis, we observed that *HAC1* deletion results in the increase in the expression of some genes involved in ubiquitination and proteasomal processes. *HSF1* over-expression also up-regulated a few genes involved in UPS such as *RPN4* (Fig. 3 and Table 3). To investigate the role of the proteasome in relieving ER stress, we measured the 20S proteasomal activities (the chymotrypsin-, caspase-, and trypsin-like proteases) in exponentially grown cells of WT, WT (hsf1), *hac1*Δ, and *hac1*Δ (hsf1) (Fig. 5a). All three proteolytic activities followed the same trend in different strains and conditions. DTT addition increased all the proteolytic activities in all the strains, and this shows that induction of misfolded proteins in the ER results in the

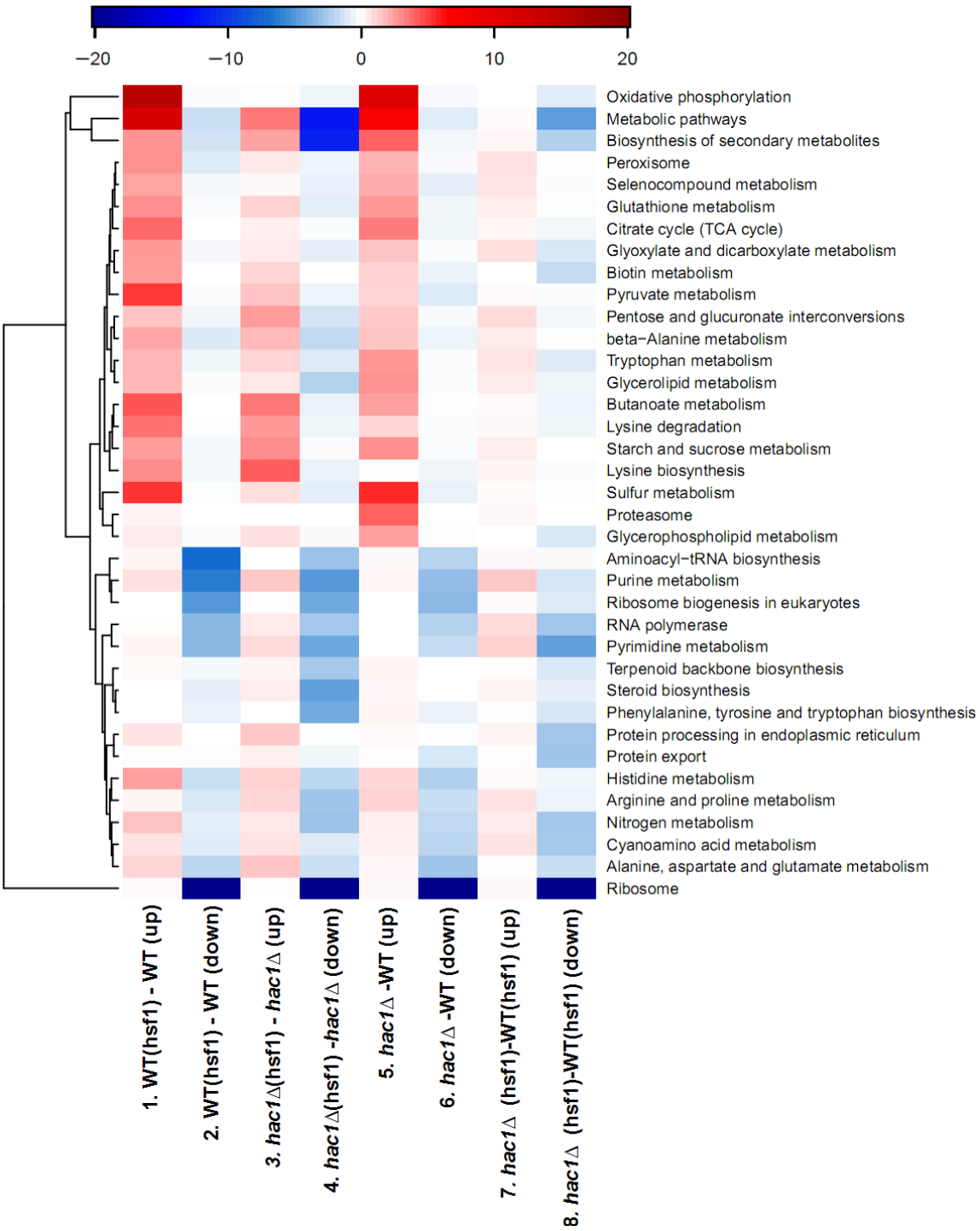

Fig. 3. Identification of the reporter KEGG pathways in different strains under ER stress. For integrated analysis of transcriptome, the reporter KEGG pathway ($P < 0.005$) was plotted by heatmap plot. The comparisons are WT (hsf1) vs. WT (up and down, lanes 1 and 2), *hac1*Δ (hsf1) vs. *hac1*Δ (up and down, lanes 3 and 4), *hac1*Δ vs. WT (up and down, lanes 5 and 6), and *hac1*Δ (hsf1) vs. hsf1 (up and down, lanes 7 and 8). The color key represents $\log_{10}$ (*P*-values), red represents up-regulation and blue represents down-regulation.

increase of the 20S proteasome activity and the increase of protein degradation. In the *hac1*Δ strain, there was overall higher proteolytic activity, probably because misfolded protein cannot be refolded in the ER by processes normally induced by the UPR and therefore have to be degraded by the proteasome. Interestingly, in the *HSF1*

Table 2. Reporter TFs for *HSF1* over-expression

Regulation	TF Name	TF Class
HSF1 over-expression in WT [WT (hsf1) vs. WT]		
↑	*MSN2/4*	General stress response
↑	*YAP1*	Oxidative stress response
↑	*HAP2/3/4/5*	Respiratory regulation
↓	*BAS1*	Purine/histidine biosynthesis
↓	*IFH1*	Ribosome synthesis
↑	*RAS2*	Nitrogen starvation response
↑	*MET4*	Sulfur amino acid synthesis
↓	*TPK2*	Vegetative growth
↓	*SNF4*	Glucose derepression
↑	*SKN7*	Oxidative stress response
HSF1 over-expression in *hac1*Δ [*hac1*Δ (hsf1) vs. *hac1*Δ]		
↑	*HSF1*	Thermal stress response
↓	*IFH1*	Ribosome synthesis
↑	*OAF1*	Beta-oxidation and peroxisome
↓	*GCN4*	Amino acid synthesis
↑	*PPR1*	Uracil biosynthesis
↑	*PIP2*	Oleate response and beta-oxidation
↓	*RAP1*	Telomere maintenance
↓	*SFP1*	Cell cycle and ribosome biosynthesis
↓	*BAS1*	Purine/histidine biosynthesis
↓	*SIT4*	Cell cycle

over-expression strains proteasomal activity was decreased (in both the WT and *hac1*Δ background strains; Fig. 5a), despite of transcriptional induction of proteasome genes *RPN4* and *RPN14* (Table 3). Therefore, to evaluate the role of proteasome in HSR-mediated suppression of ER stress, *RPN4*, a TF that activates the transcription of proteasome genes, was deleted in WT, WT (hsf1), *hac1*Δ and *hac1*Δ (hsf1) and the growth and proteasomal activity was tested under ER stress conditions (Fig. 5b and c). *RPN4* gene deletion decreased the proteasome activity significantly (Fig. 5c), and it also affected the growth of the cells in SD medium (Fig. 5b). Interestingly, *RPN4* gene deletion reduced the cell viability slightly in WT under ER stress, which suggested that the decreased proteasome activity could affect the resistance to ER stress (Fig. S3). However, *HSF1* over-expression could again recover the growth deficiency of *hac1*Δ*rpn4*Δ even in strains with relatively low proteasome activity (Fig. 5b). The above results indicate that up-regulating *RPN4* genes may contribute to the suppression to ER stress to some extent, but because the protein folding, refolding, and secretion are much more efficient when induced by the HSR, the over-expression of *HSF1* under ER stress reduces the requirement for the proteasome.

HSR decreases ROS

The integrated transcriptome analysis also revealed up-regulation of the oxidative stress response in *HSF1* over-expression strains. We therefore measured ROS accumulation by staining the cells with DHR123 (Fig. 6). Without DTT addition, ROS positive cells could not be detected by microscopy (Fig. 6a). After induction for 2 h with 2 mM DTT, we observed ROS accumulation in all strains, but to less extent in strains over-expressing *HSF1* [*hac1*Δ vs. *hac1*Δ (hsf1), and WT vs. WT (hsf1); Fig. 6b]. We also quantified the rhodamine fluorescence in a fluorescence microplate reader. In the presence of 2 mM DTT, *hac1*Δ showed 2–3 times higher ROS accumulation than the wild type (Fig. 6c). *HSF1* over-expression up-regulated the oxidative stress response genes and in consequence decreased the ROS level in the *HAC1* deletion strain under ER stress. These results show that a strain with a *HAC1* deletion cannot manage ER stress efficiently leading to increased ROS, due to accumulation of misfolded proteins, whereas *HSF1* over-expression decreased ROS accumulation presumably due to the increase of the protein folding capacity and the induction of oxidative stress response.

Table 3. Transcriptionally significantly changed genes in protein folding, translocation, secretion, and degradation process in the comparison of UPR induced (WT) vs. UPR non-induced (*HAC1* deletion) strains, and HSR-induced (*HSF1* over-expression) vs. HSR non-induced strains (WT)

Processing	*HAC1* deletion[1]	*HSF1* over-expression[2]
Protein Folding		
Chaperones	***KAR2***, ***SIL1***, LHS1, ERJ5, *JEM1*, *SCJ1*	***KAR2***, ***SIL1***, *FES1*, *HLJ1*, *SSA1/3/4*, *HSP30/42*, *SSE2*
Disulfide bond formation	*MPD1*, *EUG1*, ***ERO1***	***ERO1***
Vesicle Trafficking	***ERV29***, ***NUS1***, ***SAR1***, *ERP1*, *ERP2*, *RET3*	***ERV29***, ***NUS1***, ***SAR1***, *EMP46*, *BTN2*, *RCR2*, *GRH1*,*SEC15*
Translocation	*SEC61/62/63/65/66/72*	*SEC63/66*, *SRP72*
Protein Degradation		
ER-associated degradation	*DER1*, *DFM1*	
Ubiquitination/proteasome	*DOA1*, *BLM10*, *PRE10*, *RPN9/12*, *RPT3*, *UFD1*, *UMP1*, *UBC5/8*, *HRT1*, *HUL5*	*UFD4*, *RPN4*, *RPN14*, *UIP4*, *UBA2*

Normal: up-regulated genes; Underline: down-regulated genes; Bold: common genes up-regulated in both comparisons.
[1]WT and WT (hsf1) vs. *hac1*Δ and *hac1*Δ (hsf1) (UPR+ vs. UPR−).
[2]WT (hsf1) and *hac1*Δ (hsf1) vs. WT and *hac1*Δ (HSR+ vs. HSR−).

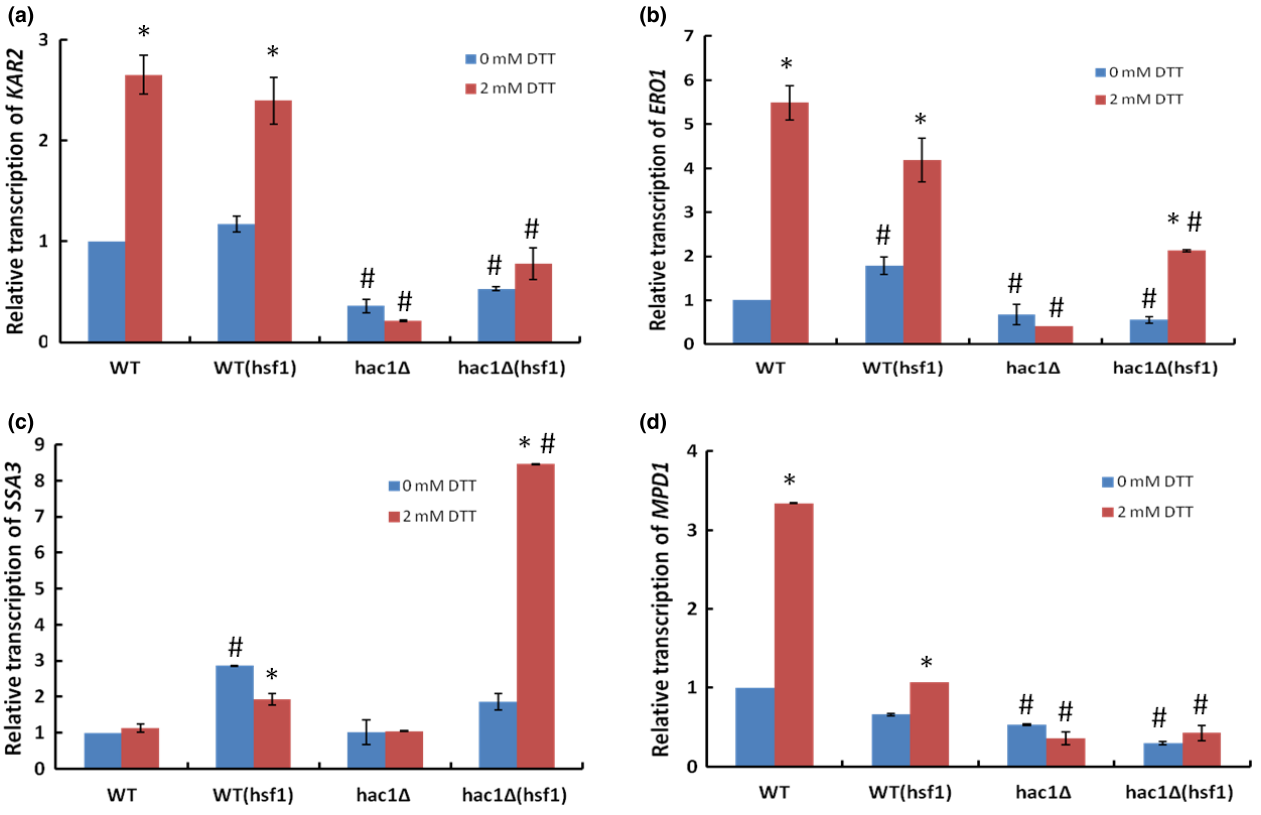

Fig. 4. The relative transcription level of HSR and/or UPR targets in WT, WT (hsf1), *hac1*Δ and *hac1*Δ (hsf1) with or without 2 mM DTT. (a) The relative transcription level of *KAR2*; (b) The relative transcription level of *ERO1*; (c) The relative transcription level of *SSA3*; and (d) The relative transcription level of *MPD1*. Blue: the relative transcription level without DTT; Red: the relative transcription level with 2 mM DTT addition. Data are presented as means ± standard errors from three independent experiments. *$P < 0.05$ (the marked strain without DTT treatment vs. the same strain with DTT treatment); #$P < 0.05$ (the marked strain vs. the wild type strain with the same treatment).

Discussion

In this study, we over-expressed the constitutively active TF Hsp1p (induces HSR) in the wild-type and UPR-deficient background (by deleting the gene encoding the TF HAC1p) and studied the mechanisms of ER stress management. We combined several analyses of the genome-wide transcriptional responses, verified transcription of targets using qPCR, and combined qualitative growth assays with measurements of the UPS activation (proteasomal activities) and oxidative stress (ROS levels). We demonstrated that (1) in addition to its role in cytoplasmic stress responses, HSR has overlapping roles with the UPR in terms of protein folding and secretion; (2) at the transcription level, HSR decreases the overall transcription and translation in the cell, and also induces other response pathways, like the oxidative stress response, and (3) HSR reduces proteasomal activities under ER stress. This leads us to conclude that induction of HSR under ER stress promotes cell survival mainly through facilitating the protein folding and secretion and management of the oxidative stress (resulting from misfolded protein accumulation) and not through degradation of proteins via the UPS.

HSR and UPR jointly control the protein folding and secretion under ER stress

The genome-wide transcription analysis revealed that under ER stress HSR activated the expression of genes encoding for ER and cytosolic chaperones, vesicle trafficking proteins and also a few UPS-related proteins. Therefore, the HSR clearly facilitates protein folding, simulates protein trafficking, and regulates ERAD (Fig. 7). Among the HSR targets, several genes such as *KAR2*, *SIL1*, *ERO1*, *ERV29*, *NUS1*, and *SAR1* are also regulated by the UPR (Table 3). Kar2p is an ER luminal chaperone that mediates protein folding and the translocation of ER soluble

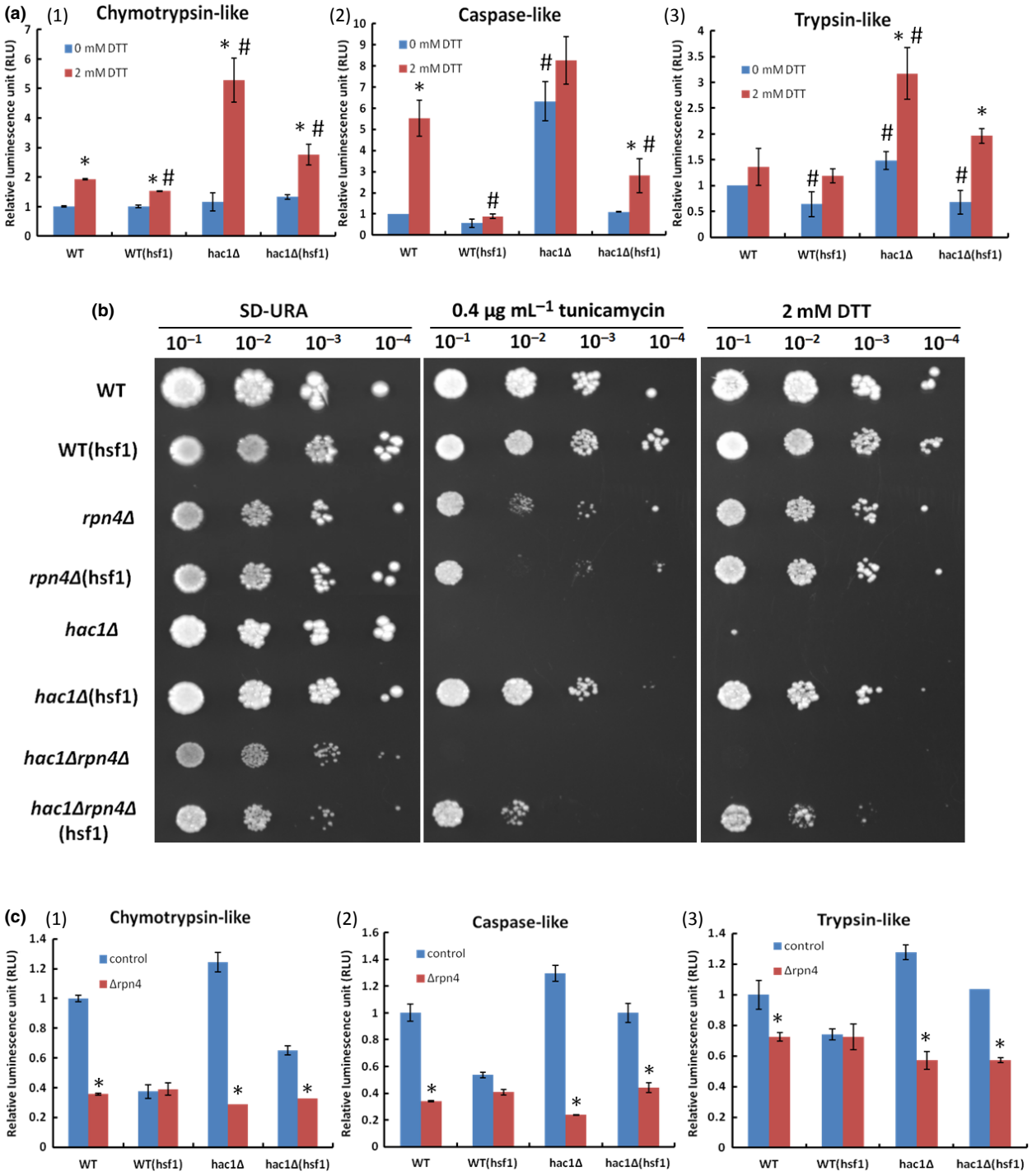

Fig. 5. The role of proteasome in HSR-mediated suppression of ER stress. (a) Proteasome activities in WT, WT (hsf1), *hac1*Δ and *hac1*Δ (hsf1). Relative luminescence units of chymotrypsin- (1), caspase- (2) and trypsin-like (3) proteolytic activities of the 20S proteasome are measured. **P* < 0.05 (the marked strain without DTT treatment vs. the same strain with DTT treatment); #*P* < 0.05 (the marked strain vs. the wild type strain with the same treatment). (b) Qualitative growth assay for *RPN4* gene deletion. The strains were spotted on SD-URA plates and SD-URA plates with 2 mM DTT or 0.4 μg mL^{-1} Tunicamycin. (c) The proteasome activities in WT, WT (hsf1), *hac1*Δ and *hac1*Δ (hsf1) with or without *RPN4* deletion. Relative luminescence unit of chymotrypsin- (1), caspase- (2) and trypsin-like (3) proteolytic activities of the 20S proteasome are measured. **P* < 0.05 (the *RPN4* deletion strain vs. the same background strain without *RPN4* deletion). The luminescence signal was detected from three independent experiments and the relative unit was normalized by dividing the signal with the luminescence of wild type without DTT treatment. Data are presented as means ± standard errors from three independent experiments.

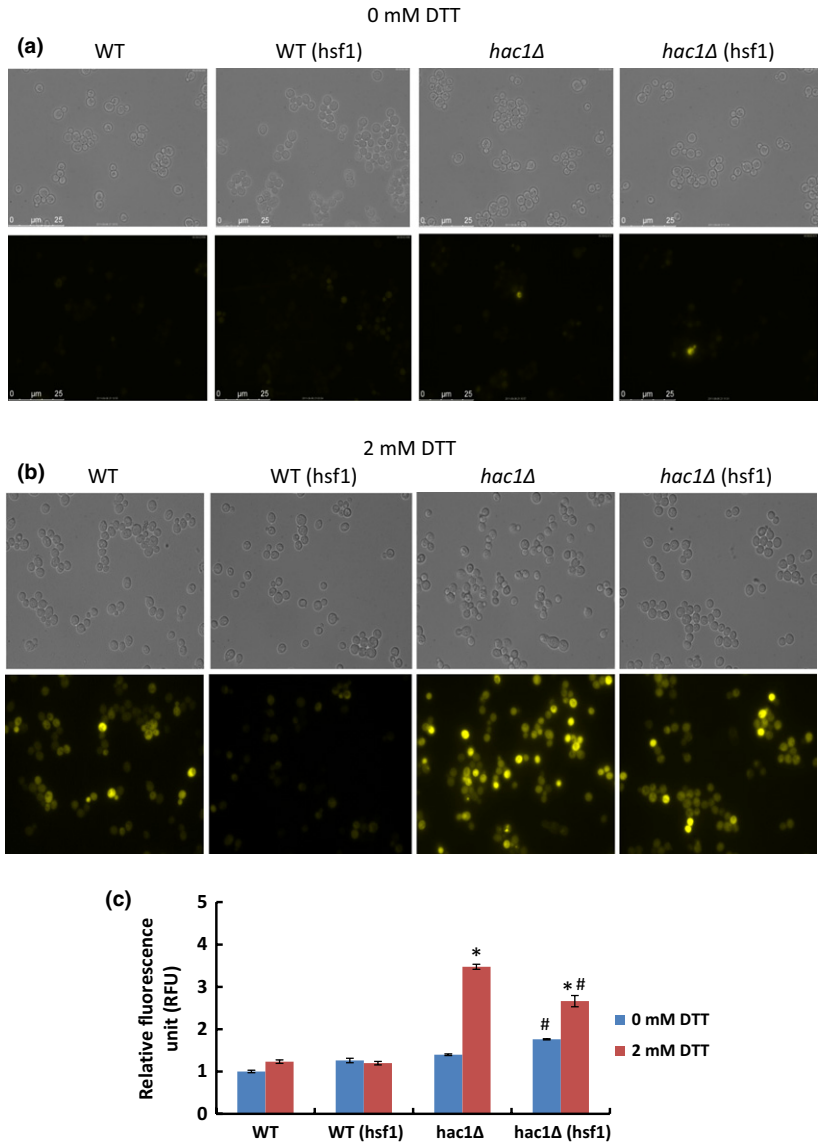

Fig. 6. HSR decreases the ROS accumulation under ER stress. (a) WT, WT (hsf1), *hac1*Δ and *hac1*Δ (hsf1) strains, without DTT treatment, were stained by DHR123 and ROS was detected using fluorescence microscopy. (b) WT, WT (hsf1), *hac1*Δ and *hac1*Δ (hsf1) were stained by DHR123 after treating cells with 2 mM DTT for 2 h and ROS was detected using fluorescence microscopy. (c) Quantification of ROS. Relative fluorescent intensities were measured using fluorescence microplate reader after staining DTT-pretreated cells with DHR123. Three independent experiments were performed.

proteins. It has a critical role in facilitating protein translocation and ERAD in connection with the HSR (Liu & Chang, 2008). Ero1p is crucial for oxidative protein folding in the ER. Moreover, cytosolic chaperone Hsp70 Ssa1p, and its co-chaperones Hsp40s including Ydj1p and Hlj1p are also required for the degradation of ERAD-M substrates (Fig. 7; Nishikawa *et al.*, 2005). One interesting feature of the ERAD-L pathway is the requirement of transport of proteins between the ER and Golgi (Caldwell *et al.*, 2001; Taxis *et al.*, 2002). Caldwell *et al.* found that there was stabilization of misfolded soluble proteins in cells lacking Erv29p, a cargo receptor involved in COPII vesicles formation or cells having blocked ER and Golgi transport as a consequence of mutation of specific *SEC* genes (Caldwell *et al.*, 2001). Therefore, HSR targets like Erv29p, Emp46p, and Sar1p seem to provide a role in facilitating ER luminal protein export and promoting ERAD-L (Fig. 7). We propose that UPR relieves ER stress through the induction of the protein folding, protein translocation, vesicle trafficking, and ERAD-L, while HSR suppresses ER stress and decreases the UPR activation through facilitating protein folding, vesicle trafficking and ERAD-L&M.

HSR and the regulation of the proteasome system

RPN4 gene deletion in the *hac1*Δ (hsf1) strain did not abolish the resistance to ER stress, although it reduced the cell viability in WT slightly. From the transcriptome data, we observed that the HSR induced the expression of *RPN4*, a TF that stimulates expression of proteasome

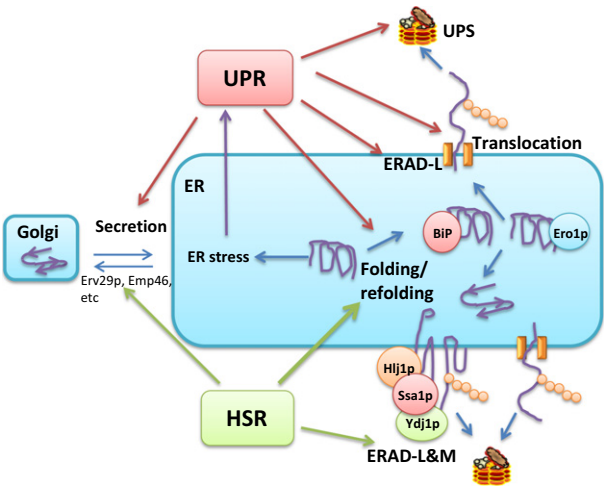

Fig. 7. Proposed model how HSR and UPR control the secretory pathway under ER stress. Accumulation of misfolded proteins induces UPR which activates ER chaperones. It also induces proteins translocation, ERAD-L and UPS to degrade misfolded proteins. Components of the secretory pathway are also up-regulated following the UPR. HSR induces chaperones like Kar2p to promote the protein refolding and translocation, Erv29p, Emp46, and Sar1p to facilitate ER-to-Golgi trafficking and Ssa1p to regulate ERAD-L&M substrate selection and degradation.

genes. However, the proteasome activities in *HSF1* over-expression strain were found to be lower than in the control strains, under ER stress. In addition, *RPN4* gene deletion decreased the proteasome activities, but over-expression of *HSF1* could still recover the growth deficiency of *rpn4*Δ*hac1*Δ, in presence of DTT or tunicamycin. This indicates that proteasome up-regulation may not be crucial for HSR-mediated suppression of ER stress. Induction of *RPN4* by *HSF1* has a temporal controlling mechanism for proteasome synthesis. The proteasome homeostasis in *S. cerevisiae* has a negative feedback regulation in which Rpn4p up-regulates the proteasome genes but the regulator is then rapidly degraded by the assembled proteasome (Hahn *et al.*, 2006). The Rpn4p negative feedback circuit provides a sensitive mechanism to control the proteasome abundance. Therefore, HSR may transcriptionally up-regulate the *RPN4* gene in order to be prepared for the degradation of misfolded proteins under stress conditions; however, due to the sufficient protein refolding and secretion capacity that largely decrease the amount of misfolded proteins, there is less need for proteins to be degraded in the *HSF1* over-expression strains. Although in *hac1*Δ strain there is no activation of UPR, the large accumulation of misfolded proteins induces up-regulation of yeast autophagy genes (such as *ATG1*, *ATG4*, *ATG5*, *ATG8*, and *ATG34*) to possibly decrease the stress load from damaged proteins (Sampaio-Marques *et al.*, 2012).

HSR interacts with other response pathways to protect cells from stresses

TFs involved in stress response (*MSN2/4*, *YAP1*, and *SKN7*), ribosome synthesis (*IFH1* and *SFP1*), amino acid synthesis (*GCN4*, *BAS1*, *MET4*, and *PPR1*), glucose de-repression and β-oxidation (*SNF4*, *OAF1*, and *PIP2*), and cell cycle (*SFP1* and *SIT4*) were all identified by the Reporter TF algorithm in HSR-induced cells (Table 2). The Reporter TFs have identified cellular mechanisms in response to the induction of HSR, including repression of overall transcription and translation, induction of energy generation, and up-regulation of other stress response pathways.

Transcription and translation were repressed under HSR to adjust the cellular processes to cope with stress: repression of ribosome generation, decreased amino acid synthesis, and transiently arresting cells in the cell cycle, which are mechanisms that were also identified in previous studies (Rowley *et al.*, 1993; Eastmond & Nelson, 2006; Castells-Roca *et al.*, 2011).

The cooperation between two transcriptional control systems Hsf1p and Msn2/4p is very important for thermal stress protection in yeast. The zinc-finger TFs Msn2 and Msn4p mediate the response to a variety of cellular and environmental stresses including heat shock and the partial overlapping regulation between Hsf1p and Msn2/4 pathways have been described previously (Castells-Roca *et al.*, 2011). It is therefore not surprising that Msn2/4p targets were activated in response to over-expression of *HSF1*.

In strains with constitutive HSR induction, up-regulation of Yap1p and Skn7p-regulated genes has been observed (Raitt *et al.*, 2000; Wu & Li, 2008). ER stress has been shown to be associated with ROS generation and cell death (Hahn *et al.*, 2004; Hauptmann *et al.*, 2006; Tan *et al.*, 2009). During disulfide bond formation in folding proteins (by the ER oxidoreductase Ero1p), the use of molecular oxygen as the terminal electron acceptor can lead to ROS production and oxidative stress. It has been proposed that 25% of cellular ROS originates from ER, and that ER-derived oxidative stress could results in cell damage and death (Tu & Weissman, 2004). Anti-oxidative stress pathways have also been found to be activated as a consequence of the UPR (Kimata *et al.*, 2006). In our study, we observed that HSR up-regulates the expression of oxidative stress defense genes such as *TSA1*, *SOD1/2*, *TRX2/3*, *GSH1*, and *CTT1*, which are under control of Yap1p or Skn7p, and results in a decrease of ROS accumulation. It is reported that decreasing ROS accumulation by the addition of reduced glutathione into the medium can increase the growth of the cells even in sustained ER stress condition (Haynes *et al.*, 2004). Therefore, our observed decrease of ROS

accumulation by *HSF1* over-expression could also contribute to the restoration of growth of WT (hsf1) and *hac1*Δ (hsf1) strains, under ER stress.

Protein misfolding and aggregation have been identified as a major contributor or cause in many diseases, such as neurological disorders including Parkinson's, Huntington's, and Alzheimer's (Travers *et al.*, 2000; Bharadwaj *et al.*, 2010). Recently, HSP were identified as useful therapeutic targets (Neef *et al.*, 2010), and yeast as a model can surely serve as an important system to study the interactions between proteostasis and cell stress that link protein folding and disease (Bharadwaj *et al.*, 2010). HSR mediated by Hsf1 has been studied widely for decades, but only recently it has been characterized as an important player for misfolded proteins in the cytosol or membrane (Metzger & Michaelis, 2009; Ciplys *et al.*, 2011; Geiler-Samerotte *et al.*, 2011). Thus, our study highlights the important role of HSR in handling misfolded proteins both in the cytosol and the ER, which has implications for our understanding of how eukaryotic cells handle misfolded proteins.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. HSR decreases the *HAC1* mRNA splicing under ER stress.
Fig. S2. PCA (a) and radar plot with adjusted *P*-value (b) of the transcriptome data.
Fig. S3. Qualitative growth assays for *RPN4* gene deletion.
Table S1. Primers used in this study.
Table S2. Reporter TFs for *hac1* deletion.