Aeration mitigates endoplasmic reticulum stress in *Saccharomyces cerevisiae* independently of mitochondrial respiration

出芽酵母における通気培養はミトコンドリア呼吸非依存的に小胞体ストレスを緩和 する

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Abstract

The endoplasmic reticulum (ER) is a membrane-bound cellular compartment in which secretory and transmembrane proteins are folded and modified. Impairment or insufficiency of the ER functions is so-called ER stress and damages cells. Because ER client proteins are frequently subjected to the cysteine disulfide-bond formation and the N-glycosylation, ER stress is strongly induced by cellular exposure to the disulfide-reducing reagent dithiothreitol (DTT) or the N-glycosylation-inhibiting antibiotics tunicamycin. Upon ER stress, a number of proteins working for the ER is transcriptionally induced. This protective event is named as the unfolded protein response (UPR) and is conserved throughout eukaryotic species. The ER-located transmembrane endoribonuclease Ire1 is known to act as an ER-stress sensor which provokes the UPR upon ER stress. In ER-stressed yeast *Saccharomyces cerevisiae* cells, Ire1 promotes splicing of the *HAC1* mRNA, which is then translated into the transcription-factor protein that is involved in the UPR gene expression. A wide variety of stressing stimuli other than chemical imposition including DTT and tunicamycin are known to induce ER stress and to provoke the UPR in various organisms. While hypoxia results in ER stress in animal cells, the mechanism of this phenomenon is still obscure. In the present study, I then addressed the relationship between low-oxygen conditions and ER stress by using an easier-to-use organism, yeast *Saccharomyces cerevisiae*, which can proliferate well both under aerated and hypoxic conditions.

In laboratories, yeast cells are usually cultured under aerobically shaken conditions. The *HAC1*-mRNA splicing was not induced by culturing cells at the static condition without shaking in tightly capped tubes, indicating that the UPR is not induced solely by hypoxia. On the other hand, the low-level *HAC1*-mRNA splicing provoked by low concentrations of DTT and tunicamycin was boosted when I set cells at the static condition. A similar observation was obtained by shaking cultures in nitrogen gas-filled flasks. In agreement with this observation, the expression of *BiP/KAR2*, a prominent UPR target gene, was elevated by setting weakly ER-stressed cells in hypoxic conditions. I think that hypoxia aggravates weak ER stress, since the *IRE1*-deletion mutation compromised growth of weakly

ER-stressed cells under hypoxic conditions. Unexpectedly, aeration mitigated ER stress and DTT-induced impairment of ER oxidative protein folding even when mitochondrial respiration was halted by the ρ^0 mutation, which is an entire loss of the mitochondrial genome. Moreover, oxygen uptake was elevated by exposure of a low concentration of DTT to ρ^0 cells. An ER-located protein Ero1 is known to directly consume molecular oxygen to promotes the oxidative protein folding of ER client proteins. Our further study using the *ero1-1* mutant strain without mitochondrial respiration suggested that this Ero1-medaited reaction contributes to the mitigation of ER stress by molecular oxygen. Taken together, here I demonstrate a scenario in which aeration acts beneficially on *S. cerevisiae* cells even under fermentative conditions, in which mitochondrial respiration is not required.

Table of Contents

Chapter I. Introduction

1.1. Endoplasmic reticulum (ER) and ER stress

The endoplasmic reticulum (ER) is a membrane-bound and flat or tubular-shaped sacs, which is carried by almost all eukaryotic cells. The ER is responsible for a wide variety of essential cellular functions such as folding membrane and secretory proteins, making lipidic molecules, and preserving cellular calcium homeostasis (Lavoie and Paiement, 2008). Approximately one-third of eukaryotic proteins traverse the secretory pathway, which starts from the ER (McCaffrey and Braakman, 2016). Newly-synthesized polypeptides are translocated into the ER lumen, where they are folded and modified to take correct conformation. Subsequently, the resulting mature proteins are transported to the Golgi apparatuses for their processing before being delivered to their final destinations. Furthermore, the ER carries quality control systems to maintain protein-folding status. Incorrectly folded proteins are retained in the ER to be refolded or to be digested via the pathway named as the ER-associated protein degradation (ERAD), resulting in protection of cells from protein toxicity (McCaffrey and Braakman, 2016).

The ER provides an optimal environment for oxidative protein folding because it contains specific chaperones and protein folding enzymes (Ziegler and Poulsen, 1977; Hwang et al., 1992; Frand and Kaiser, 1999). The most prominent ER-located chaperone commonly carried by eukaryotic species is the Binding Immunoglobulin Protein chaperone (BiP). In yeast, the gene encoding BiP is named *KAR2*. BiP is an ATP-dependent molecular chaperone belonging to the heat shock protein Hsp70 family. BiP recognizes translocation-ongoing or newly translocated polypeptides and maintains them in a state that is competent to be folded and assembled (Gething, 1999). As mentioned later, protein folding enzymes such as protein disulfide isomerases (PDI) and ER membrane-associated protein Ero1 (ER oxidoreductin 1) are required for the ER-protein oxidative-folding cascade. In yeast, the PDI gene is named as *PDI1*.

The ER functions are often challenged by a wide variety of internal and external cellular stresses. Upon overload of secretory or transmembrane proteins, the ER protein folding machinery exceeds its capacity (Oakes and Papa, 2015). Mutations of genes encoding ER client proteins or toxic chemicals may cause incorrect protein folding in the ER. Deprivation of amino acids or glucose also can impair ER protein folding (Araki and Nagata, 2011). These undesirable environmental stresses lead to accumulation of misfolded protein in the ER, and is named as ER stress (Sitia and Braakman, 2003). Tunicamycin and dithiothreitol (DTT) are typical ER-stressing

chemicals that respectively impair the cysteine disulfide bond formation and the N-glycosylation of ER client proteins, leading to accumulation of misfolded protein in the ER (Ruddock and Molinari, 2006; Labunskyy et al., 2009). Moreover, imbalance or shortage of ER-membrane lipidic components is also known to cause ER stress independently of ER accumulation of unfolded proteins (PromLek et al., 2011; Ho et al., 2020).

1.2. Unfolded protein response (UPR)

Under ER stress conditions, a signaling transduction pathway, which is named as the unfolded protein response (UPR), is triggered to transmit the stress signal from the ER to the nuclei. The outcome of the UPR is a drastic transcriptome change to increase the ER protein folding capacity and to induce the ERAD (Hetz et al., 2020). Ire1 is an ER-located type-I transmembrane protein that functions as an ER-stress sensor to initiate the UPR signaling pathway. While animal cells also carry other ER-stress sensors, namely PERK and ATF6, Ire1 is conserved throughout a wide variety of eukaryotic species including animals, plants, and fungi (Cox et al., 1993; Shen et al., 2001; Yoshida et al., 2001).

The UPR pathway in yeast *Saccharomyces cerevisiae* is illustrated in Fig. 1. It is widely believed that in *S. cerevisiae*, Ire1 acts as the sole ER sensor that provokes the UPR. Ire1 is required for cell survival under ER stress conditions (Cox et al., 1993). The cytosolic domain of Ire1 carries the Ser/Thr protein kinase motif and the RNase motif. ER stress causes self-dimerization or oligomerization of Ire1, leading to autophosphorylation of Ire1, which is then activated as an RNase (Kimata et al., 2007; Le et al., 2021). The target of Ire1 in *S. cerevisiae* cells is the *HAC1* mRNA. Upon ER stress, Ire1 catalyzes splicing of the *HAC1* mRNA, which is then translated into the Hac1 protein. The Hac1 protein acts as a transcriptional factor for the UPR (Mori et al., 1998). The UPR target genes that is transcriptionally induced by the Hac1 proteins include *PDI1, KAR2*, and those encoding *ERAD* factors, the upregulated expression of which alleviates ER stress.

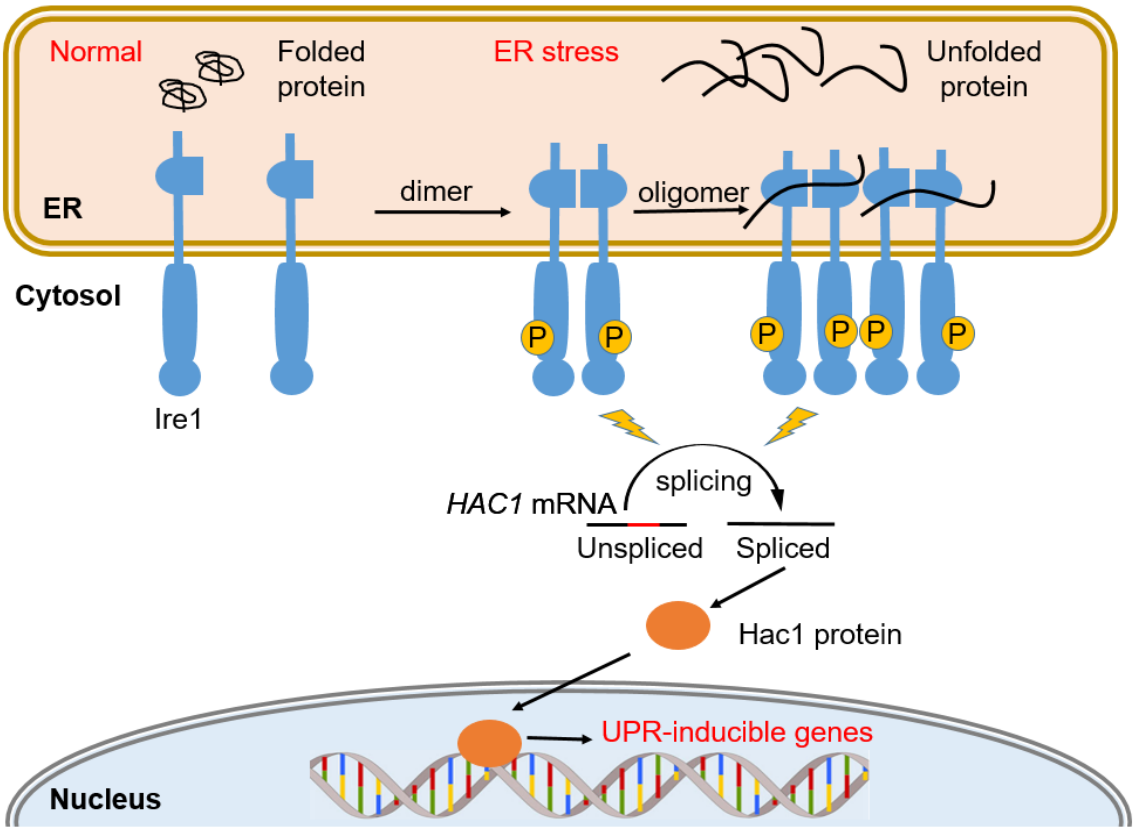

Figure 1. The UPR pathway in yeast *S. cerevisiae*. Under ER stress conditions, Ire1 is self-associated, autophosphorylated, and activated as an RNase, leading to splicing of the *HAC1* mRNA, which is then translated into the transcription factor protein that induces the UPR.

1.3. Structure and function of yeast Ire1

The structure of Ire1 is illustrated in Fig. 2B. According to Kimata *et al.* (Kimata et al., 2003; Kimata et al., 2004), the Ire1 luminal domain can be divided into three sub-regions. The first is the N-terminal unconserved region (NUCR). The second is the core luminal domain (cLD), which carries some evolutionally conserved sequences. The third is the juxtamembrane intrinsically disordered region (JIDR).

BiP is associated with Ire1 in unstressed conditions, and this protein complex dissociates upon ER stress (Okamura et al., 2000). The BiP-binding site on Ire1 is located on the JIDR (Kimata et al., 2004). Because JIDR partial deletion mutations of Ire1 does not result in activation of Ire1, albeit impairing its association to BiP, it is unlikely that the BiP association/dissociation is the principal determinant of Ire1's activity (Kimata et al., 2004).

The cLD is tightly folded and is directly bound to unfolded proteins accumulated in the ER (Credle et al., 2005; Gardner and Walter, 2011). In the presence of

abundant unfolded proteins, the cLD is oligomerized (Gardner and Walter, 2011). This insight well explains the mechanism by which ER accumulation of unfolded proteins leads to self-association and activation of Ire1. A partial mutation of cLD, namely the ΔIII mutation (deletion of a.a. 243-272), is reported to impair the ability of the cLD to recognize unfolded proteins, resulting in compromization of Ire1's activation in response to ER accumulation of unfolded proteins (Kimata et al., 2007; Tran et al., 2019b).

Furthermore, Ire1 is activated by disturbance of ER lipid-bilayer composition, namely lipid bilayer stress (LBS), through a mechanism being different from that for ER-accumulation of unfolded proteins. According to some previous studies (PromLek et al., 2011; Ishiwata-Kimata et al., 2013; Ho et al., 2020), BiP remains associated with Ire1, and Ire1 does not form the high-order oligomer upon LBS. The transmembrane domain, but not the luminal domain, of Ire1 acts as the stress sensory module for LBS. Point mutants, F531R, V535R, and R537Q, on the amphipathic helix sequence of the Ire1 transmembrane domain impairs activation of Ire1 in response to LBS (Fig. 2a) (Halbleib et al., 2017; Tran et al., 2019b); Ho et al. 2020).

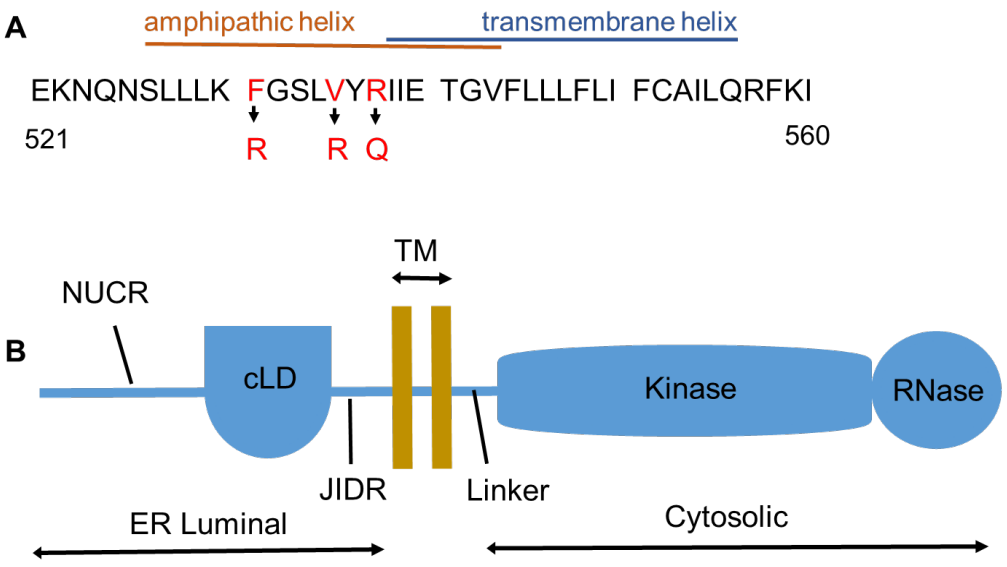

Figure 2. Structure of Ire1 (A) The amino-acid sequence of the transmembrane domain of *S. cerevisiae* Ire1, which is composed of the amphipathic and transmembrane helixes. The red colored letters represent the point mutations that impair activation of Ire1 in response to LBS (Tran et al. 2019b); Ho et al. 2020). (B) Structure of *S. cerevisiae* Ire1. NUCR, the N-terminal unconserved region; cLD, the core luminal domain; JIDR, the juxtamembrane intrinsically disordered region; TM: the transmembrane domain.

1.4. Oxidative protein folding

Concurrently with mRNA translation on the ER membrane, nascent polypeptides of membrane and secretory proteins are translocated into the ER lumen through the translocon channels. Subsequently, the polypeptides undergo folding and maturation, which frequently depend on formation of disulfide bonds between two cysteine residues, namely the oxidative protein folding (Sevier and Kaiser, 2008). The ER provides an optimal environment for the oxidative protein folding. For instance, the ER abundantly carries small molecular oxidants, such as glutathione, which act as redox buffer (Ziegler and Poulsen, 1977; Hwang et al., 1992; Frand and Kaiser, 1999). Ero1 and PDI are essential proteins that facilitate the oxidative protein folding in the ER. Ero1, which was initially found through *S. cerevisiae* studies, is evolutionally conserved and does not exhibit any homology to other proteins. Meanwhile, PDI has four homologs in *S. cerevisiae* (*EUG1, MPD1, MPD2* and *EPS1*) and more than ten homologs in higher eukaryotes (Tu and Weissman, 2004).

The oxidative protein folding is an electron transfer cascade in which PDI oxidizes cysteine free thiols of substrate client protein, which then form disulfide bonds, and accepts electrons to become the reduced PDI form. According to an *in vitro* biochemical study by Tu and Weissman (2002), Ero1 re-oxidizes PDI and transfers

electrons from PDI to molecular oxygen via its cofactor flavin adenine dinucleotide (FAD). In other words, molecular oxygen may function as a terminal electron acceptor and is consumed directly by Ero1 during the ER disulfide formation (fig. 3). However, it is still uncertain if this reaction actually occurs in *S. cerevisiae* cells, since, as described later, they grow well even without molecular oxygen.

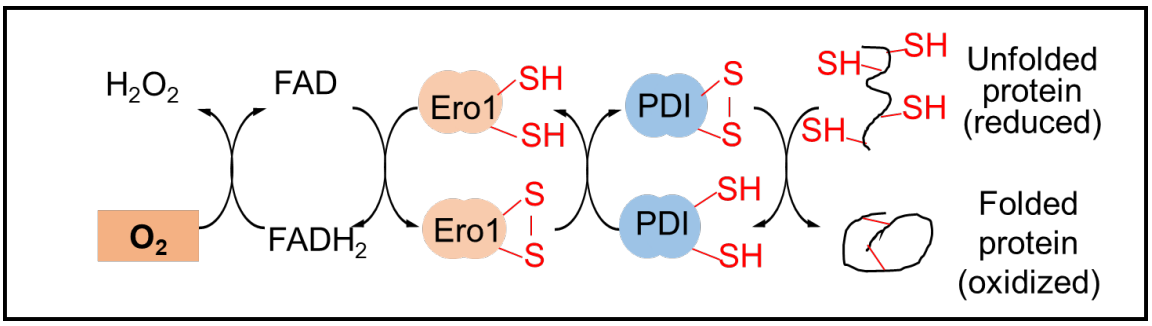

Figure 3. ER oxidative protein folding pathway. Ero1, ER oxidoreductin 1, is a FAD binding protein. The ER protein disulfide-bond formation is driven by Ero1 and generates ROS in the ER lumen. Ero1-FAD is reported to oxidize PDI via consuming molecular oxygen directly, and PDI transfers disulfide bonds to substrate proteins.

1.5. Hypoxia and ER stress

Aerobic organisms, such as animals, require a proper molecular oxygen level to maintain their normal cellular metabolism. Shortage of molecular oxygen, so-called hypoxia, causes ER stress and cell death in healthy tissues (Zhou et al., 2006; McKeown, 2014).

Upon hypoxia, animal cells trigger an adaptive response, namely transcriptional activation of hypoxia response element (HRE)-regulated genes, which encode, for example, factors for metabolic pathways and anti-oxidative responses (Chipurupalli et al., 2019). In mammalian cells, hypoxia-inducible factor-1 (HIF-1) is involved in the transcriptional induction from the HRE-containing promoters in response to hypoxia (Jiang et al., 1996). HIF-1 is a heterodimeric complex that is composed of two subunits, namely oxygen-responsible HIF-1α and constitutively expressed HIF-1β (Salceda and Caro, 1997; Jiang et al., 1996). HIF-1 α is stabilized under hypoxia and is translocated into nucleus to bind with HIF-1 β. According to Chipurupalli et al. (2019), the HIF-1 complex is involved in the transcriptional induction of genes encoding erythropoietin, glycolytic enzymes, factors for vascular growth, apoptosis inhibitors, and factors for cell differentiation. Because constitutively suffering from

hypoxia, cells frequently overexpress HIF-1α in tumors (Bos et al., 2003; Giatromanolaki et al., 2001; Birner et al., 2001; Aebersold et al., 2001).

Moreover, hypoxia is known to induce ER stress, which explains the reason why solid tumor cells are frequently ER-stressed (Akman et al. 2021). The mechanism by which hypoxia leads to ER stress in animal cells is still obscure. Needless to say, the mitochondrial respiration is the most prominent and major oxygen-consuming event. Therefore, it is possible that hypoxia compromises the mitochondrial respiration, leading to energy deprivation, which can impair protein folding in the ER. Alternatively, as a more direct scenario, the oxidative protein folding shown in Fig. 3 can be impaired in the absence of molecular oxygen.

S. cerevisiae cells grow well both under aerated and hypoxic conditions in the presence of fermentable sugar, such as glucose, because they efficiently get energy via glycolysis and perform ethanol fermentation (Diaz-Ruiz et al., 2011). Meanwhile, although not containing HIF-1 orthologues, *S. cerevisiae* cells activate a cellular response against low concentrations of molecular oxygen (Emerling and Chandel, 2005), which induces expression of factors for the respiratory chain, the fatty acid metabolism, and the heme synthesis pathways (Alimardani, et al. 2004; Ferreira, et al. 2007).

Even under the anaerobic condition, *S. cerevisiae* cells should perform disulfide-bond formation of ER client proteins. According to Kim et al. (2018), fumarate acts as a final electron acceptor instead of molecular oxygen for the ER-protein disulfide–bond formation cascade under anaerobic conditions (Fig. 4). The fumarate reductase enzyme Osm1 thus plays a crucial role for anaerobic growth of *S. cerevisiae* (Kim et al., 2018).

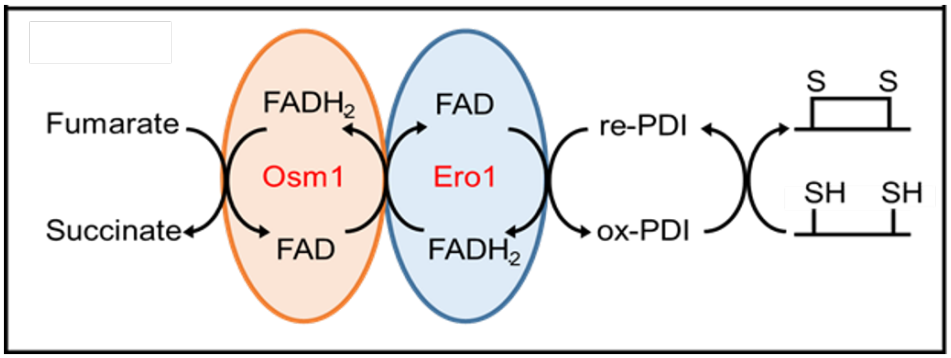

Figure 4. ER oxidative protein folding in *S. cerevisiae* cells without molecular oxygen

1.6. The aim of this study

In the present study, I explored the relationship between ER stress and hypoxia by using *S. cerevisiae* as a model organism. In general, *S. cerevisiae* cells are grown under aerated conditions for laboratory research, while they are cultured under less aerobic conditions in various industrial scenes. *S. cerevisiae* physiology under aerobic and anaerobic conditions thus sounds an intriguing research topic. Moreover, my findings in this study can provide some suggestions for understanding the molecular mechanism by which hypoxia leads to ER stress in animal cells.

Chapter II. Materials and methods

2.1. Chemicals and media

Ethidium bromide stock solution of 1mg/mL is stored at 4°C. Dithiothreitol (DTT, Nakalai Tesque) and tunicamycin (Sigma Aldrich) were prepared as stock solutions of 1 M in water and 2 mg/mL dimethyl sulfoxide (DMSO), respectively.

YPD medium contained 1% yeast extract (Bacto), 2% peptone (Bacto), and 2% glucose. YPG medium contained yeast extract (Bacto), 2% peptone (Bacto), and 3% glycerol. Synthetic dextrose (SD) medium contained 2% glucose and 0.66% yeast nitrogen base without amino acids (YNB w/o A.A., Difco) and was supplemented with appropriate auxotrophic requirements. For preparation of agar plates, the media were solidified with 2% Bacto agar.

2.2. Yeast strains

S. cerevisiae strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) (Brachmann et al. 1998) was used as the wild-type strain. The *ire1Δ (ire1::kanMX4)* derivative of BY4742 was obtained from EUROSCARF (Y11907; http://www.euroscarf.de/). *S. cerevisiae* BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) is congenic with BY4742, and its *ero1-1* derivative (TSA203) was also obtained from EUROSCARF. *S. cerevisiae ire1Δ* strain KMY1015 (*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-901 lys2-801 ire1::TRP1*) was provided by Prof. K. Mori (Kyoto University, Kyoto, Japan) and was used in this study.

To generate the ρ^o mutant strain not containing the mitochondria genome DNA, BY4742 cells were grown at 30°C and *ero1-1* cells were grown at 25°C in YPD medium containing 10 μg/mL ethidium bromide (EtBr) for approximately 15 generations, and colonies not being able to grow on YPG agar plates were selected.

2.3. Plasmids

Plasmid pPM28 carries the *URA3* selectable marker and the eroGFP gene, the expression of which is controlled under the constitutive *TDH3* promoter. It was constructed by Merksamer et al. (2008) and was obtained from Addgene (https://www.addgene.org/).

The single-copy *IRE1* plasmid pRS313-IRE1 was derived from centromeric vector pRS313 (Kimata et al., 2004; Sikorski and Hieter, 1989). The overlap PCR technique and the *in vivo* homologous recombination technique were employed for introduction of a partial-deletion mutation (ΔIII; deletion of aa. 235-272 of Ire1) and a point mutation (V535R) into the *IRE1* gene on pRS313-IRE1. The first PCR fragment was

obtained by the PCR amplification of BamHI-digested pRS313-IRE1 with the forward primer *SalI-300* and the reverse mutagenic primer (*ΔIII-C* or *V535R-R*). The second PCR fragment was obtained by the PCR amplification of BamHI-digested pRS313-IRE1 with the forward mutagenic primer (*ΔIII-F* or *V535R-F*) and the reverse primer *XbaI+300*. For the overlap PCR, the first PCR fragment and the second PCR fragment were mixed and were used as the template of PCR with primers *SalI-50* and *XbaI+50*. Subsequently, the product fragment of the overlap PCR was mixed with SalI/XbaI-digested pRS313-IRE1 and was used for yeast transformation. All primers used here are list in Table 1.

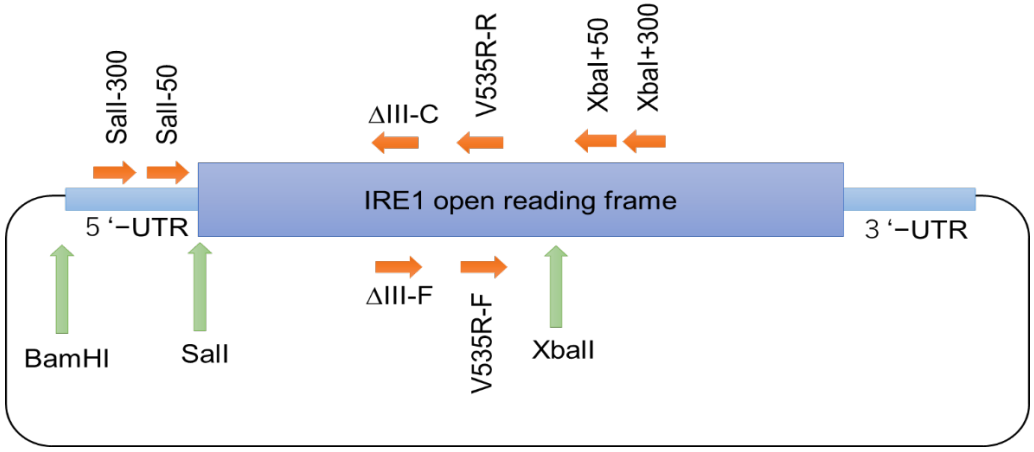

Figure 5. Plasmid pRS313-IRE1. The restriction enzyme sites (green arrows) and the oligonucleotide primers (orange arrows) are shown.

Table 1. Oligonucleotide primers used for *IRE1* mutation

Name	Primer sequences
SalI-300	5'-GAGATTAATCACATAGTAACAAGAA-3'
XbaI+300	5'-TCAGGTTTTCATCTGATACATTCTT-3'
SalI-50	5'-CCATTATCACTTTTCTCCATATC-3'
XbaI+50	5'-CCTTGAAAACTTCCCTGAAAAACT-3'
ΔIII-F	5'CAGCGTTCGGACCTGGTTCAAAAGAAGTGAAAATATGATTGTAATAGGC-3'
ΔIII-C	5'CCTATTACAATCATATTTTCACATTCTTTTGAACCAGGTCCGAACGCTG-3'
V535R-F	5'-TGAAGTTTGGAAGTCTATCGAATTATAGAG-3'
V535R-R	5'-CTCTATAATTCGATAGACTTCCAAACTTCA-3'

2.4. Yeast transformation

Yeast transformation was performed as described by Kaiser et al. (Kaiser et al., 1994). In briefly, *S. cerevisiae* cells were cultured overnight in 1 mL YPD medium by 160 rpm shaking at 30˚C. The cells were then diluted in 20 mL YPD medium and cultured under the same condition for 4 hours. Subsequently, cells were collected by centrifugation at 3000 rpm for 1 min and washed with 5 mL of LiAc/TE solution. After washing, the cells were suspended again in 1 mL of LiAc/TE solution and shaken at 60 rpm/ 30˚C for 1 hour. 100µl of the resulting cell suspension was mixed with 5 µL carrier DNA-Salmon sperm DNA and 1 µg plasmid (or digested plasmid and DNA fragment) and was incubated statically at 30˚C for 30 min. Then, 800µL of 40% Polyethylene Glycol 4000 was added into the cell suspension and was further incubated at 30˚C for 1 hour. Finally, the resulting cell suspension was spread on a SD agar plate containing appropriate amino acids, which was then incubated at 30˚C for 2-4 days.

2.5. Yeast culturing and stress imposition

For pre-culturing, *S. cerevisiae* strains were inoculated into YPD medium, and were aerobically shaken at 30 °C for overnight duration. Cultures were then diluted with YPD to OD600 0.30 (or to OD600 0.50 for the ρo mutant strain) and were further cultured at 30 °C for 4 hrs before stress imposition and harvest. For shaking condition, 50 mL conical tubes (polypropylene; Nichiryo) containing 5 mL of cultures were slantwise shaken in the respire shaker Taitech Personal Lt-10F (30 mm stroke, 160 rpm). For static condition, 15 mL conical tubes (polypropylene; Nichiryo) containing 5 mL of cultures were stood without shaking. Alternatively, 5 mL of cultures were stirred in 100 mL Erlenmeyer flasks (Iwaki) by magnetic stir bars (approximately 120 rpm) with the Scinics Milti-stirrer. For deaeration of stirring cultures, nitrogen gas was filled in the Erlenmeyer flasks, which were then tightly plugged with butyl rubber plugs. In the experiments shown in Fig. 16 and Fig. 17, cells were cultured in SD medium. See Promlek et al. (2011) for culturing cells under inositol depletion conditions. Culture density was monitored using the spectrophotometer Smartspec 3000 (BioRad).

Dithiothreitol (DTT; Nakarai tesque; 1 M stock solution in water), tunicamycin (Sigma-Aldrich; 2 mg/mL stock solution in dimethylsulfoxide) and ethanol were added into media 4 hr after culture start, and the culturing was continued without changing aeration conditions. Just after addition of ER-stressing agents into static cultures, the conical tubes were gently inverted 5 times for mixing.

2.6. RNA analyses

Total RNA samples were extracted from *S. cerevisiae* cells using the hot-phenol method (Collart and Oliviero 1993). 1 µg of total RNA was subjected to reverse transcription (RT)-PCR analysis using ReverTra Ace kit (Toyobo) and the poly(dT) RT primer. Subsequently, the PCR reaction was performed in a mixture containing 2 µL of cDNA, 1 µL each of 10 µM *HAC1*-specific PCR primers (Table 2), 1X PCR buffer, 2 µL of 2.5 mM dNTP and 0.13 µL of KAPA taq DNA polymerase (5 U/µL) (Kapa Biosystem). The PCR amplification was performed through 25-repeat thermal cycle reactions of 94°C for 30 s, 54°C 30 s, and 72°C for 60 s (PromLek et al. 2011). Because the *HAC1* specific PCR primers interposes the *HAC1* intron sequence, the unspliced (*HAC1*^{u}) and spliced (*HAC1*^{s}) variants of the *HAC1* mRNA yielded different-sized RT-PCR products, which were separated by electrophoresis on 2% agarose gel. Fluorescent images of the EtBr-stained gels were analyzed by the Image J image-processing software (https://imagej.nih.gov/ij/) for calculating the "*HAC1*-mRNA splicing efficiency" through the following formula:

$$\text{*HAC1* mRNA-splicing efficiency (\%)} = \frac{100 \times (\textit{HAC1}^{s}\text{ band intensity})}{(\textit{HAC1}^{s}\text{ band intensity}) + (\textit{HAC1}^{u}\text{ band intensity})}$$

For RT-qPCR analysis, residual genomic DNA in the total RNA samples was removed by treatment with DNase I enzyme (RNase-free; Takara). Subsequently, the total RNA samples were subjected to RT reaction using the PrimeScript II Reverse Transcriptase kit (Takara) and the oligo $(dT)_{18}$ primer. RT-qPCR were analyzed with SYBR Premix Ex Taq II (Tli RNAeseH Plus; Takara) and the real-time PCR machine LightCycler 96 (Roche). The qPCR was performed through pre-incubation at 95^{o}C for 5 mins, which was followed by the amplification cycles of 95^{o}C/10 seconds, 60^{o}C/10 seconds, 72^{o}C/10 seconds. *TAF10* gene was used as a housekeeping-gene control for the RT-qPCR analysis. The primers using in these experiments were listed in Table 2.

Table 2. Primers used for RT-PCR and RT-qPCR

Name	Primer sequences
HAC1-F	5'-TACAGGGATTTCCAGAGCACG-3'
HAC1-R	5'-TGAAGTGATGAAGAAATCATTCAATTC-3'
KAR2-F	5'-TCTGAAGGTGTCTGCCACAG-3'
KAR2-R	5'-TTAGTGATGGTGATAGATTCGGATT-3'
TAF10-F	5'-ATATTCCAGGATCAGGTCTTCCGTAGC-3'
TAF10-R	5'-GTAGTCTTCTCATTCTGTTGATGTTGTTGTTG-3'

2.7. Fluorescence microscopy

Cells carrying the eroGFP-expression plasmid pPM28 were grown in YPD medium at 30°C under the aerobic shaking condition, and DTT (at final concentration of 0.5 mM) was added (or not added) into cultures. Subsequently, the shaking incubation was additionally continued at 30°C for 30 min, or the cultures were set into argon gas-filled glass-bottom dishes and statically incubated at 30°C for 30 min. The eroGFP-fluorescent images of *S. cerevisiae* cells were then observed under the laser scanning microscopy SP8 FALCON (Leica) with the 63x/1.40 HC PL APO CS2 objective lens. For excitation, the 405 nm diode laser (UV/violet-light excitation, 25% output) and the 487 nm white light laser (blue-light excitation, 70% output) were employed. For detection, the hybrid detector (gating 492-527 nm) was employed. The pinhole size was 1.00 AU. Fluorescence intensities of a cell illuminated by these two lights were respectively quantified using the Image J software (50 cells were analyzed from each sample), and were used to calculate the "eroGFP value" through the following formula:

eroGPF value = (Fluorescence intensity with blue-light excitation)/(Fluorescence intensity with UV/violet-light excitation)

2.8. Oxygen consumption assay

YPD cultures were aerobically agitated until their OD_{600} reached approximately 2.0. Then, 10 mL of the cultures were transferred to 50 mL polypropylene conical tubes, which were subsequently filled with argon gas and were statically incubated in a 30 °C water bath. Dissolved oxygen concentration of the cultures was monitored for 3 min by inserting a dissolved oxygen electrode (Dissolved Oxygen Meter AR8210 (Smart Sensor)) into the conical tubes. For DTT exposure, DTT (final concentration

of 0.5 mM) was added into the cultures 30-min before the measurement. The "oxygen uptake rate" value is normalized against the culture optical density (OD600).

2.9. Statistics

The culture optical density, the *HAC1* mRNA-splicing efficiency, and the oxygen uptake rate were determined from triplicate cultures, and are subjected to calculation of averages and standard deviations, which are presented in the figures. In order to obtain p values, I performed two-tail unpaired tTest using Microsoft Excel.

Chapter III. Results

3.1. The *HAC1*-mRNA splicing is compromised by aerobic shaking of cultures

At the beginning of this study, I examined how aerobic shaking of cultures affects ER-stressing status of *S. cerevisiae* cells. Under ER stress conditions, the ER transmembrane sensor Ire1 of *S. cerevisiae* is activated to trigger the UPR via splicing of the *HAC1* mRNA (Kimata et al., 2003). In other words, the *HAC1* mRNA splicing efficiency represents ER-stressing situation of *S. cerevisiae* cells. The culturing protocol was showed in the Fig. 6. After overnight pre-culturing under aerobic shaking condition in YPD medium at 30°C, the cultures were diluted to 0.3 of OD_{600} and further incubated under the aerobically shaken condition or the static condition in conical tubes. The cultures were then exposed to ER-stressors DTT, tunicamycin, or ethanol and were checked for the *HAC1*-mRNA splicing.

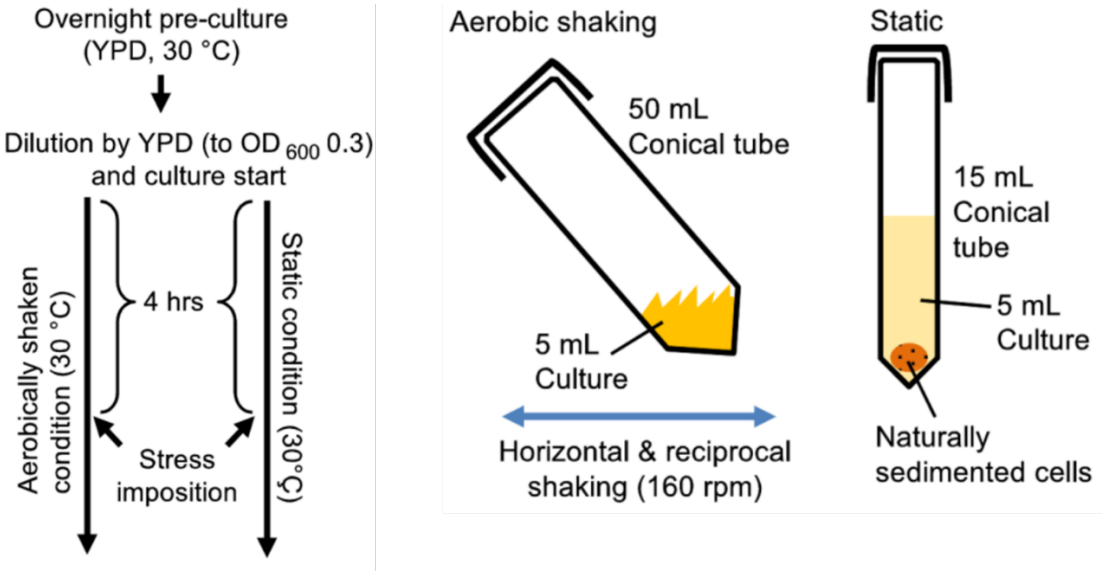

Figure 6. Culturing procedure for the aerobic shaking or static condition. After overnight pre-culture, *S. cerevisiae* cells were diluted to 0.3 of OD_{600} of cultures, which were then aerobically shaken or incubated statically for 4 hr before stress imposition.

Firstly, I checked how the static culturing affects the growth of *S. cerevisiae* wild-type BY4742 cells. In the experiment shown Fig. 7, cells were cultured as indicated in Fig. 6 without stress imposition, and the culture optical density was monitored, showing that the cellular growth under the static condition was slightly slower than that under the aerobically shaken condition.

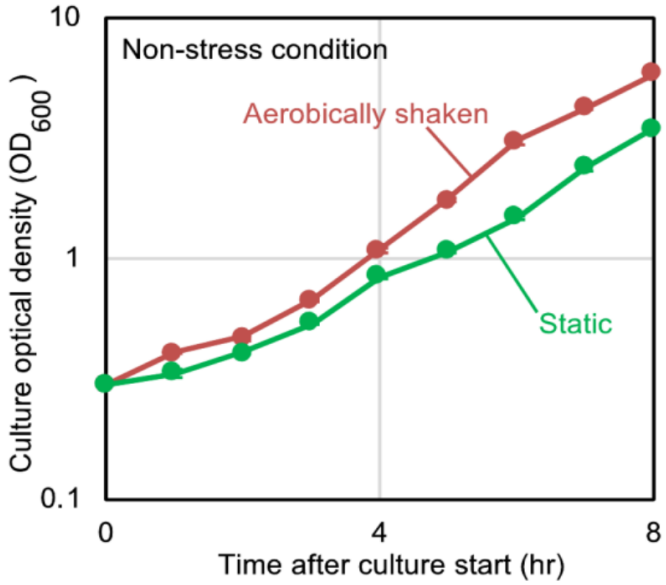

Figure 7. Cellular growth under aerobically shaken and incubated statically conditions. *S. cerevisiae* wild-type BY4742 cells were cultured as shown in Fig. 6 without stress imposition, and the culture optical density was monitored.

Subsequently, we checked the UPR level of cells cultured and stressed as indicated in Fig. 6. Throughout this study, splicing of the *HAC1* mRNA was monitored by RT-PCR analysis of total RNA samples, and the splicing efficiency was quantitatively measured as described previously (PromLek et al., 2011). The *HAC1*-mRNA splicing was almost negligible in the absence of ER-stress stimuli under both the aerobically shaken and static conditions (non-stress samples in Fig. 8A and Fig. 9, and time 0 samples in Fig. 8B), this result may be caused by either aeration or culturing method that would be ascertained by another method in the next part.

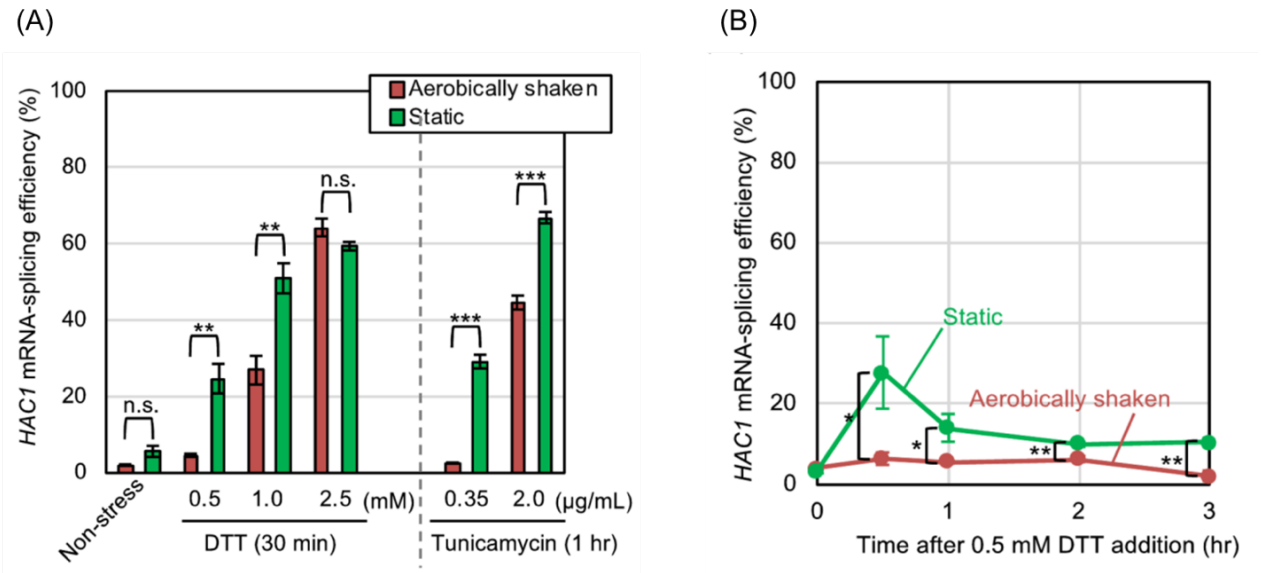

Figure 8. Enhancement of DTT or tunicamycin-induced *HAC1*-mRNA splicing by static culturing. *S. cerevisiae* wild-type BY4742 cells were cultured as shown in Fig. 6 and were stressed as indicated. n.s. (not significant): $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

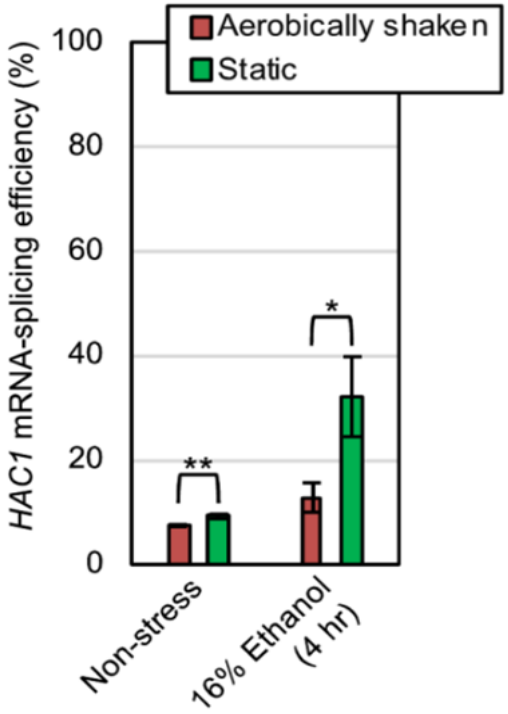

Figure 9. Enhancement of ethanol-induced *HAC1*-mRNA splicing by static culturing. *S. cerevisiae* wild-type BY4742 cells were cultured as shown in Fig. 6 and stressed by addition of ethanol into culture. *: $p < 0.05$, **: $p < 0.01$.

Both DTT and tunicamycin are the two most widely used ER stressors not only for *S. cerevisiae* but also for other eukaryotic species. DTT is known to prevent the ER oxidative protein folding through cleavage of the cysteine disulfide bonds, while tunicamycin inhibits the protein N-glycosylation in the ER (Ruddock and Molinari, 2006; Labunskyy et al., 2009). Therefore, treatment of cells with DTT or tunicamycin is likely to evoke the UPR via the accumulation of misfolded proteins in the ER. According to Fig. 8A, the *HAC1*-mRNA splicing that was weakly induced by low concentrations of DTT or tunicamycin was substantially amplified by culturing cells under the static condition. The difference of *HAC1*-mRNA splicing between the aerobically shaken and static conditions was most obviously shown under the condition of 0.5 mM DTT or 0.35 μg/mL tunicamycin, and was compressed when cells were strongly ER-stressed by higher concentrations of DTT or tunicamycin (Fig. 8A). The result shown Fig. 8A was confirmed by a time-course experiment shown in Fig. 8B. Because the difference of *HAC1*-mRNA splicing between the aerobically shaken and static conditions under the condition of 0.5 mM DTT was most obviously shown at 30 min after the stress onset, I hereafter employed this condition, namely 0.5 mM DTT for 30 min, for further studies.

High concentrations of ethanol was reported to induce ER stress in *S. cerevisiae* cultures (Miyagawa et al., 2014; Navarro-Tapia et al., 2017). A similar observation as shown in Fig. 8 was obtained in the case of the ethanol-induced ER stress. According to my observation shown in Fig. 9, the *HAC1*-mRNA splicing induced by ethanol imposition was lowered by the aerobic shaking.

3.2. Deaeration boosts the DTT-induced *HAC1* mRNA splicing

I next explored the relationship between the UPR and the aeration status more deeply using DTT as a model ER-stress inducer. By using the culturing method in Fig. 6, the *HAC1*-mRNA splicing levels of *S. cerevisiae* cells were mostly not induced under static condition alone but were boosted by low-dose DTT treatment under static condition (Fig. 8A). In order to ascertain that this observation is actually due to their aeration status, I employed another culturing procedure. As shown in Fig. 10, after pre-cultured under aerobically shaking conditions, *S. cerevisiae* wild-type BY4742 cells were diluted in YPD to OD_{600} of 0.3, were further cultured under the aerated condition or with agitation in nitrogen-gas filled conical flasks, and were chemically stressed.

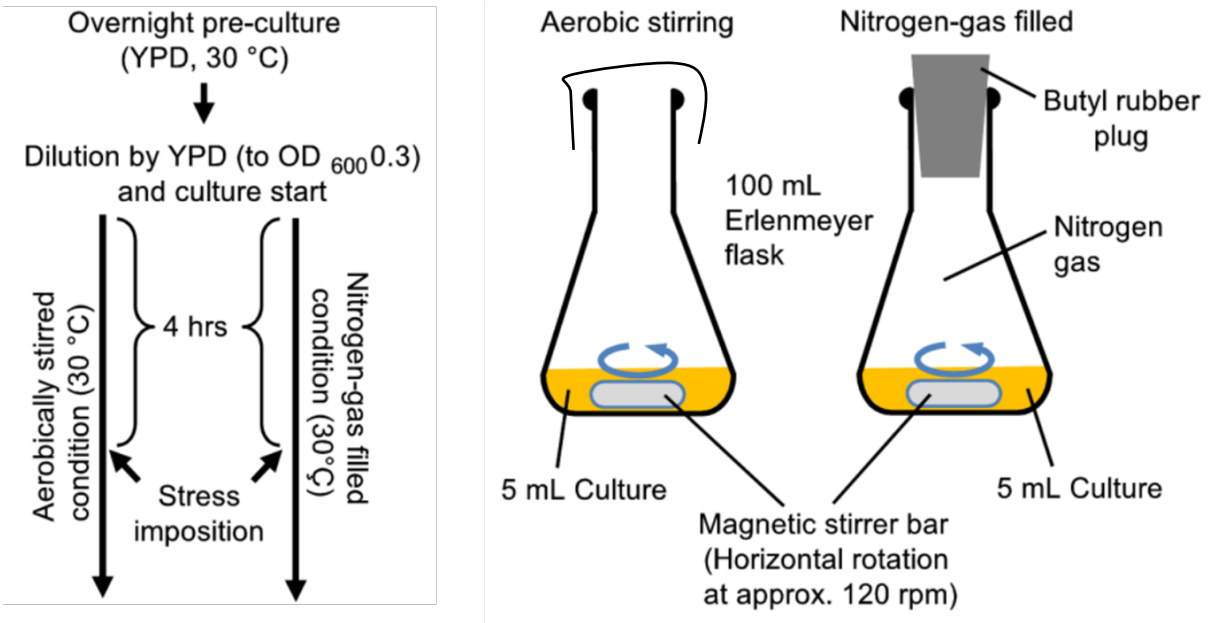

Figure 10. Culturing procedure under aerobically stirred and nitrogen gas-filled (and stirred) conditions. After the overnight pre-culture, *S. cerevisiae* cells were diluted to 0.3 (or to 0.5 in the case ρ^0 cells) of OD_{600} of cultures. The culture flasks were cover loosely by an aluminum foil or tightly capped by butyl rubber plugs in the case of the nitrogen-gas filled condition, and the cultures were agitated by magnetic stirrer bars.

The Fig. 11 shows that the growth rates of *S. cerevisiae* cells were almost the same under these two conditions. Similarly previous culturing procedure (Fig. 6), the *HAC1*-mRNA splicing was almost not induced by hypoxic condition without ER-stress stimuli. In other words, aeration alone does not change the ER-stressing situation in *S. cerevisiae*. Meanwhile, the *HAC1*-mRNA splicing induced by low-dose DTT was boosted by the anaerobic culturing (Fig. 12). To further ascertain that the static culturing and the inert-gas filling enhance the *HAC1*-mRNA splicing for the same reason, I performed the experiment shown in Fig. 13. In agreement with the aforementioned observations, the *HAC1*-mRNA splicing in cells treated with 0.5 mM DTT was almost equally boosted by culturing cells under the static condition or the inert gas-filled conditions. Importantly, the static culturing and the inert-gas fill did not seem to act additively to boost the *HAC1*-mRNA splicing. I thus assume that these conditions commonly enhance the *HAC1*-mRNA splicing via de-aeration.

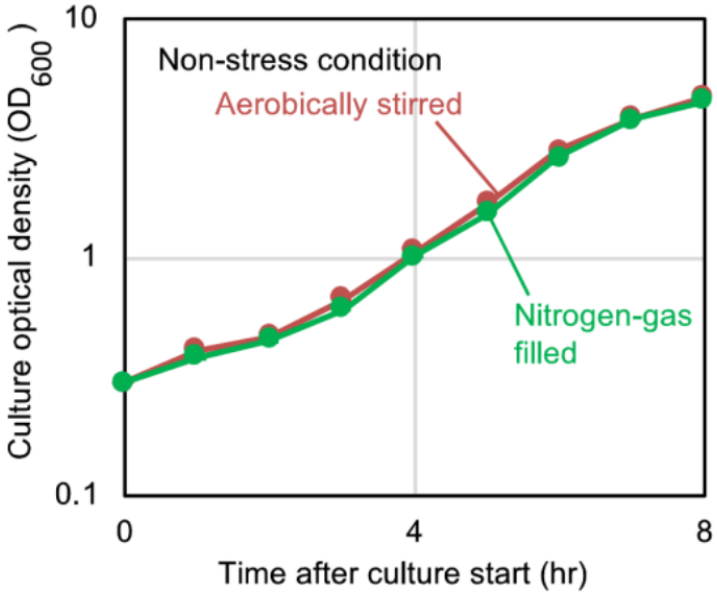

Figure 11. Cellular growth under aerobically stirred and nitrogen gas-filled (and stirred) conditions. *S. cerevisiae* wild-type BY4742 cells were cultured as shown in Fig. 10 without stress imposition, and the culture optical density was monitored.

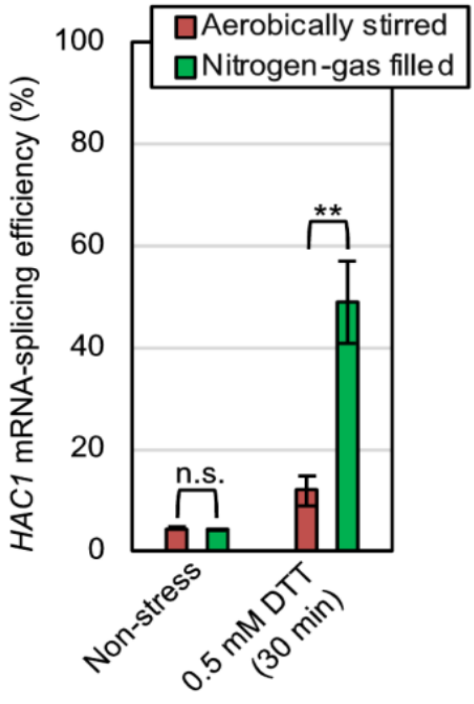

Figure 12. The DTT-induced *HAC1*-mRNA splicing was boosted under the nitrogen gas-filled (and stirred) condition. *S. cerevisiae* wild-type BY4742 cells was cultured and stressed by DTT as shown in Fig. 10. n.s. (not significant): $p > 0.05$, **: $p < 0.01$.

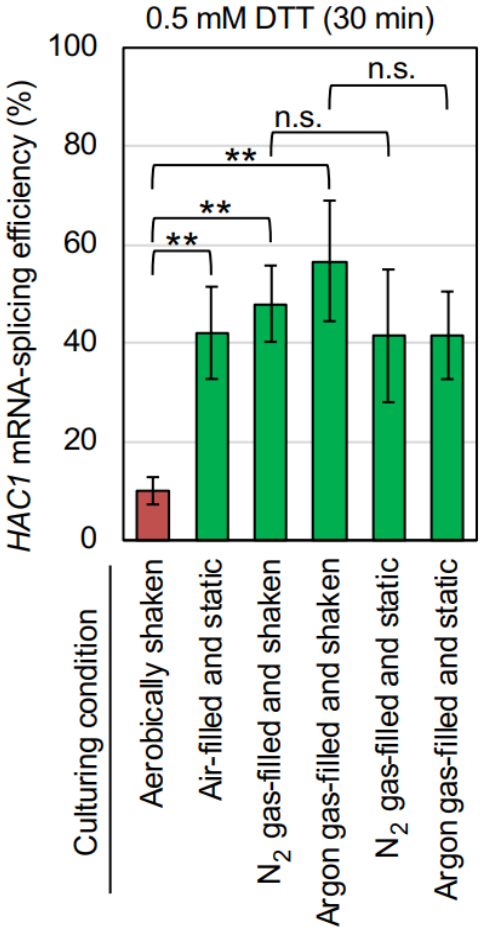

Figure 13. Hypoxic conditions commonly and non-additively boost DTT-induced ER stress. YPD cultures of *S. cerevisiae* wild-type BY4742 cells (5 mL) were incubated at 30°C statically or with shaking (160 rpm) in 50 mL conical tubes filled with the indicated gases. DTT (0.5 mM final concentration) was added into all cultures 30-min before harvest.

KAR2 is the most prominent UPR-target gene that is transcriptionally induced by the Hac1 protein. In order to confirm my finding shown in Fig. 12, I checked the *KAR2* mRNA-expression level using the RT-qPCR technique. As expected, the *KAR2* mRNA-expression level induced by the low-DTT was boosted by setting cells in the nitrogen gas-filled condition (Fig. 14).

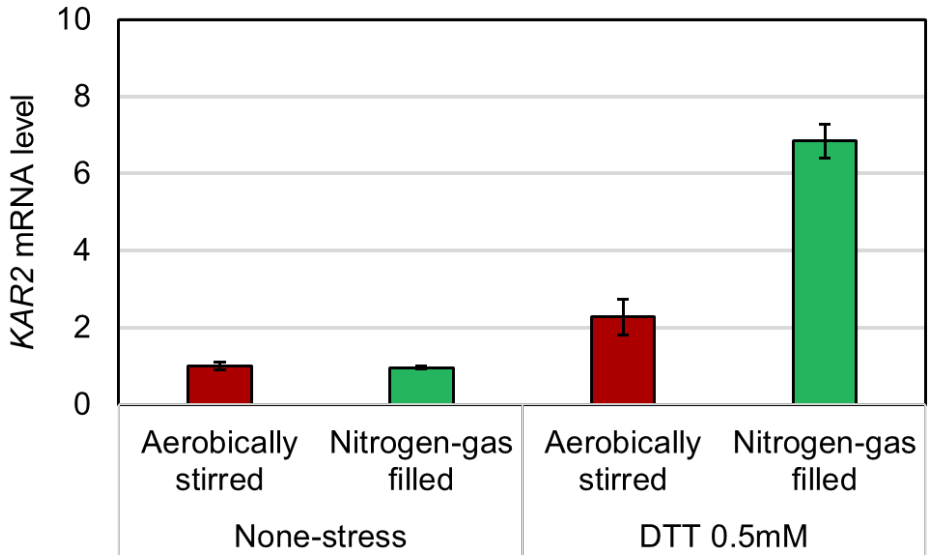

Figure 14. Anaerobic culturing increases the *KAR2* expression under the DTT-stress condition. *S. cerevisiae* wild-type BY4742 cells was cultured and stressed by DTT (0.5 mM, 30 min) as shown in Fig. 10. The *KAR2* expression level was checked by RT-qPCR using the *TAF10*-mRNA level as the internal control, and is normalized against that of the aerobically stirred and non-stressed samples.

3.3. *S. cerevisiae* cells are more damaged by ER stress under hypoxia

I subsequently investigated whether *S. cerevisiae* cells are more severely damaged by ER stress with the low-dose DTT under hypoxic conditions than under aerated conditions. As described in the introduction section, Ire1 triggers the UPR, which then subdues ER stress. The *IRE1*-gene knockout mutation (*ire1Δ*) thus causes hypersensitivity of *S. cerevisiae* cells to ER stress (Cox et al., 1993). In this experiment, I monitored the culture density 14 hours after DTT imposition (18 hours after culture start), wherein the cultures had reached the stationary phase (Fig. 15). *S. cerevisiae* cells being weakly ER-stressed by 0.5 mM DTT poorly grown only when carrying the *ire1Δ* mutation and cultured under the static condition or under the nitrogen gas-filled condition.

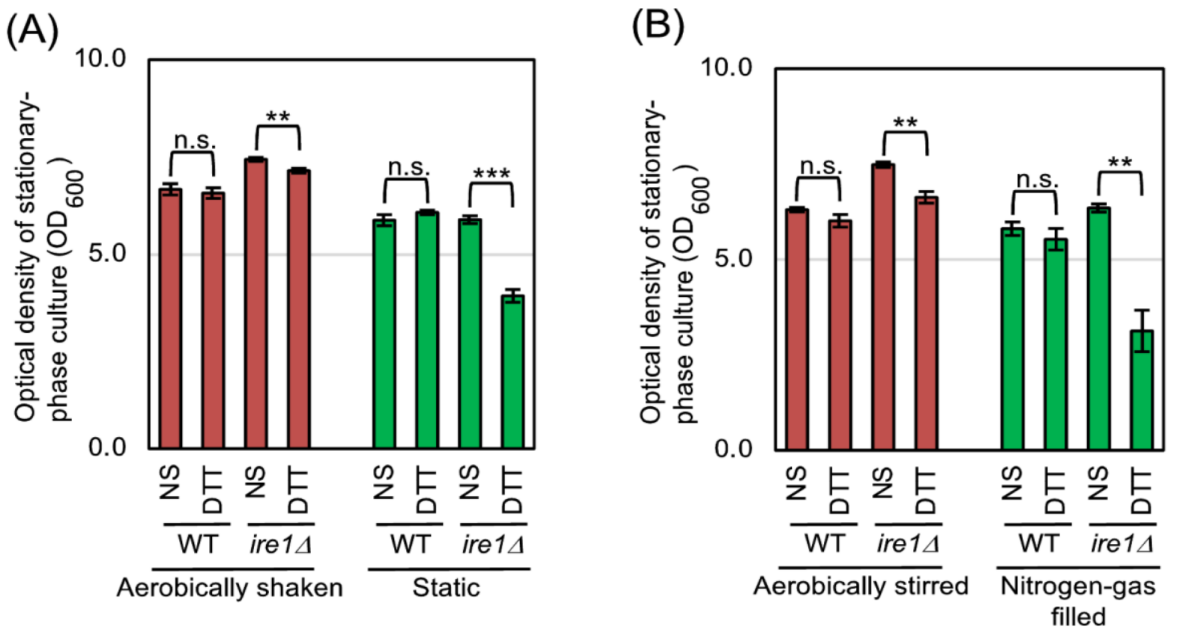

Figure 15. Hypoxic treatment aggravates ER stress-induced cellular damage. (A) Wild-type BY4742 cells and the congenic *ire1Δ* mutant cells were cultured as shown in Fig. 6, and optical density of the cultures was measured 18-hr after culture start. For DTT treatment, we added DTT (0.5 mM final conc.) into media 4-hr after culture start, and further performed the culturing for 14 hr. (B) The same strains used in panel A were cultured as shown in Fig. 10, and optical density of the cultures was measured 18-hr after culture start. For DTT treatment, we added DTT (0.5 mM final conc.) into media 4-hr after culture start, and further performed the culturing for 14 hr. n.s. (not significant): $p > 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.4. Hypoxia does not boost ER stress induced by lipid bilayer stress (LBS)

Besides accumulation of unfolded proteins in the ER, namely proteotoxic stress, ER stress is also induced by perturbation of ER membrane phospholipid metabolism, namely LBS. In yeast cells, LBS is induced, for instance, by depletion of the membrane lipid component inositol or mutations of genes involved in lipid homeostasis. As described in the Introduction section, Ire1 senses proteotoxic stress and LBS in different manners. The luminal-domain partial-deletion mutant of Ire1, ΔIII Ire1, is impaired in its activation upon proteotoxic stress, while the transmembrane-domain point mutant V535R Ire1 is poorly activated by LBS (Tran et al., 2019b). In the experiment shown in Fig. 16, I confirmed this observation. The *HAC1* mRNA splicing in cells producing ΔIII Ire1 was only moderately induced by DTT or tunicamycin, while the V535R mutation was compromised activation of Ire1 in response to inositol depletion.

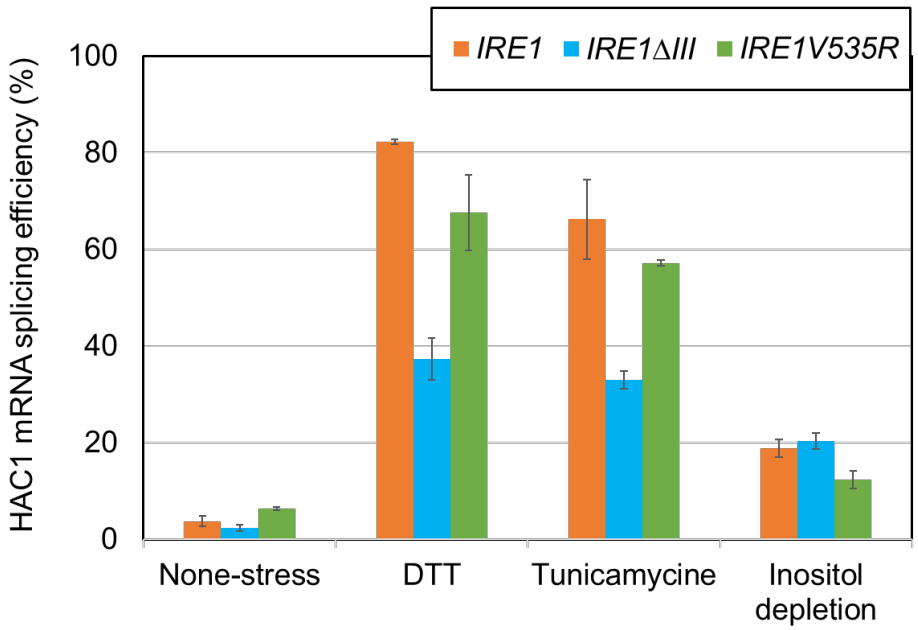

Figure 16. *HAC1*-mRNA of *S. cerevisiae* cells carrying *IRE1* wild-type, *IRE1V535R*, and *IRE1ΔIII* induced by proteotoxic stressors or lipid bilayer stressor. *S. cerevisiae* KMY1015 (*ire1Δ)* cells were transformed with pRS313-*IRE1* (wild-type *IRE1*) or its mutants. After being cultured for 4hr in SD medium under shaking condition at 30°C, yeast cells remained non-stressed, or were treated with proteotoxic stressors (5mM DTT for 30 min or 2 μg/ml tunicamycin for 1hr), or were cultured under LBS condition (inositol-depleted medium for 4hr) for checking the *HAC1*-mRNA splicing efficiency.

Fig. 17 showed that the *HAC1*-mRNA splicing induced by low-dose DTT is boosted by statically culturing in both wild-type *IRE1* cells and V535R mutant Ire1 cells but almost not in ΔIII mutant Ire1 cells. This observation suggests that hypoxia aggravates ER stress via strengthening proteotoxic stress.

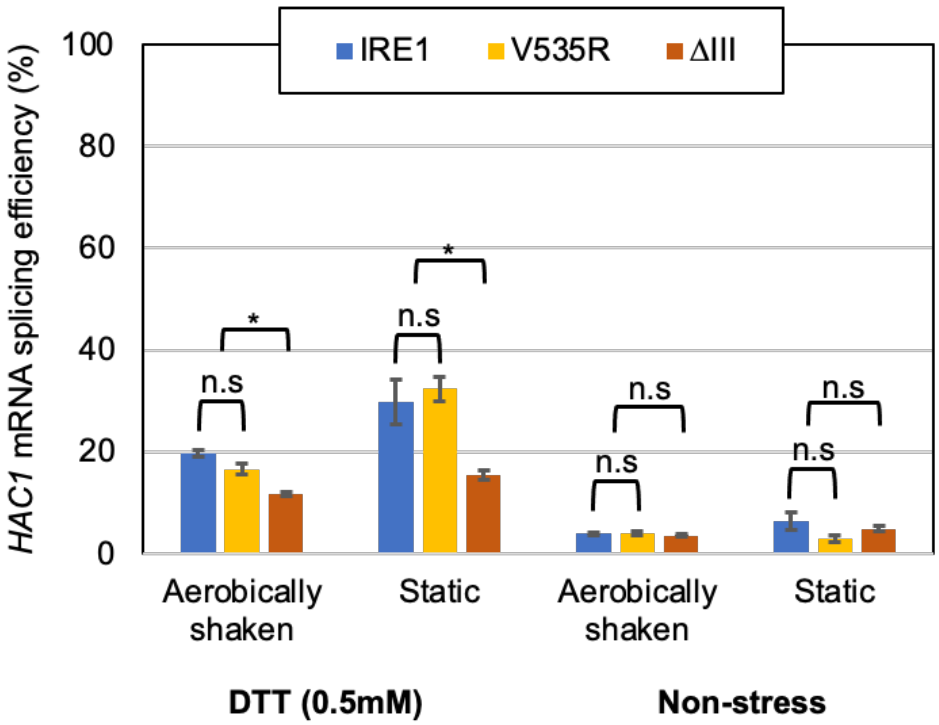

Figure 17. Under hypoxia, the DTT-induced *HAC1*-mRNA splicing was lower in ΔIII-Ire1 cells than Ire1 wild-type and Ire1 V535R mutation. *S. cerevisiae* Δ*ire1* strain KMY1015 was transformed with plasmids carrying the wild-type *IRE1* gene or its mutants. The transformants were then cultured under aerobically shaken or static condition in SD medium for 4hr before DTT exposure (0.5 mM, 30 min) or remained non-stressed, and were checked for the *HAC1* mRNA splicing as shown in Fig. 6. n.s. (not significant): $p > 0.05$, *: $p < 0.05$.

In order to exam if ER stress induced by LBS is also boosted by hypoxia, I next monitored the *HAC1*-mRNA splicing upon inositol depletion under aerobically shaken condition and static condition. Fig. 18 shows that the *HAC1*-mRNA splicing induced by inositol depletion was almost equal under these two conditions. In other words, ER stress induced by LBS is not boosted by hypoxia.

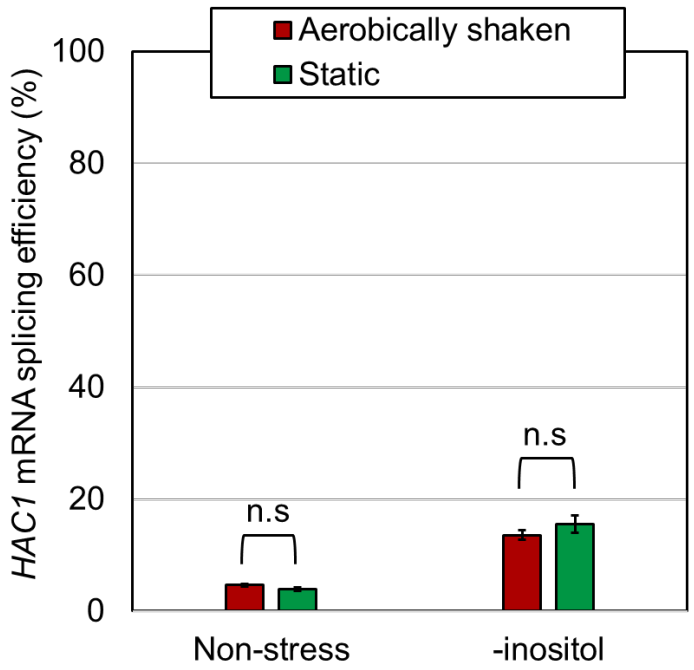

Figure 18. *HAC1* mRNA splicing induced by inositol depletion was not boosted by hypoxia. *S. cerevisiae* BY4742 cells were cultured under aerobically shaken and static conditions in inositol depleted medium or non-stress SD medium. After culture for 4hr, cells were harvested to check *HAC1*-mRNA splicing. n.s. (not significant): $p > 0.05$.

3.5. Aeration mitigates ER stress even without mitochondrial respiration

In eukaryotic organisms, molecular oxygen is mainly consumed by mitochondrial respiration. I then asked if the mitochondrial function is involved in the mitigation of ER stress by aeration. The mitochondrial DNA of *S. cerevisiae* was removed by ethidium bromide treatment as described in the Material and Method section. The resulting ρ^0 mutant cells, therefore, are unable to perform mitochondrial respiration. In the experiments shown in Fig. 18 and Fig. 19, the ρ^0 cells and congenic wild-type cells were cultured under the aerobically shaken condition. Probably because of impairment of mitochondrial functions, the ρ^0 cells grew slower than wild-type cells (Fig. 19). Meanwhile, the *HAC1*-mRNA splicing that was induced by DTT was not boosted, but was somewhat compromised by the ρ^0 mutation (Fig. 20). I thus presume that the *HAC1* mRNA-splicing in the ρ^0 cells is lower than that in equally ER-stressed wild-type cells possibly because of the difference in their growth rates.

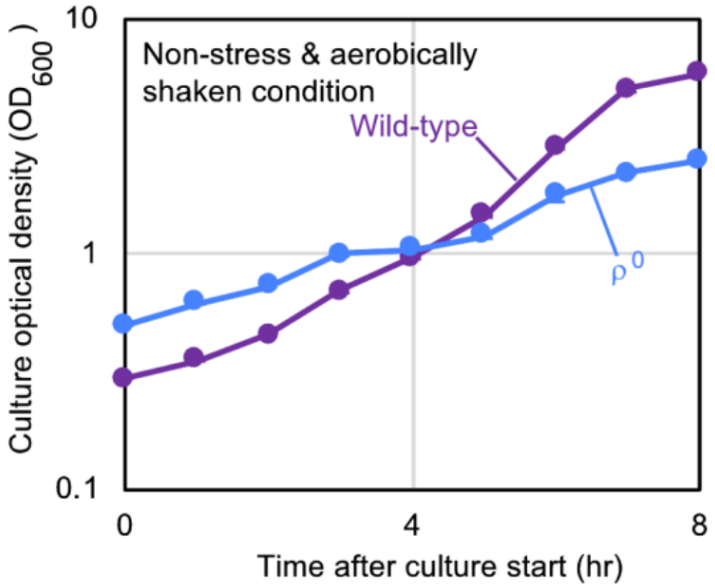

Figure 19. Growth retardation of *S. cerevisiae* cells by the ρ^0 mutation. Wild-type BY4742 cells and its ρ^0 mutant were cultured at 30°C under the aerobically shaking condition in YPD, and were checked for culture optical density.

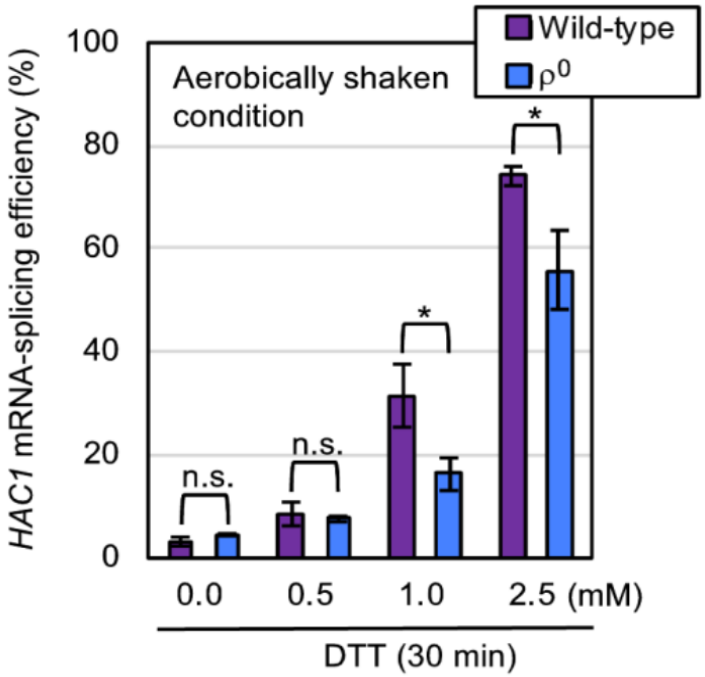

Figure 20. The DTT-induced *HAC1*-mRNA splicing is not boosted by the ρ^0 mutation. Wild-type BY4742 cells and its ρ^0 mutant were cultured at 30°C under the aerobically shaking condition in YPD, were stressed by DTT as indicated, and were checked for the *HAC1*-mRNA splicing. n.s. (not significant): $p > 0.05$, *: $p < 0.05$.

I next cultured ρ^0 cells with stirring under the aerated or nitrogen gas-filled condition as shown in Fig. 10. Similar to the case of wild-type cells (Fig. 12), the *HAC1*-mRNA splicing of ρ^0 cells induced by low-dose DTT was boosted by culturing under the nitrogen gas-filled condition (Fig. 21). This observation indicates that aeration mitigates ER stress, at least partially, via a mechanism(s) independent of mitochondrial respiration. This insight was confirmed by using tunicamycin as the ER stressor. In the Fig. 22B, a similar experiment was done as Fig. 8A using 0.35 μg/mL tunicamycin was performed using ρ^0 cells. According to my result shown in Fig. 22, the aerobic agitation mitigated the tunicamycin-induced *HAC1*-mRNA splicing in both wild-type cells and ρ^0 mutant cells.

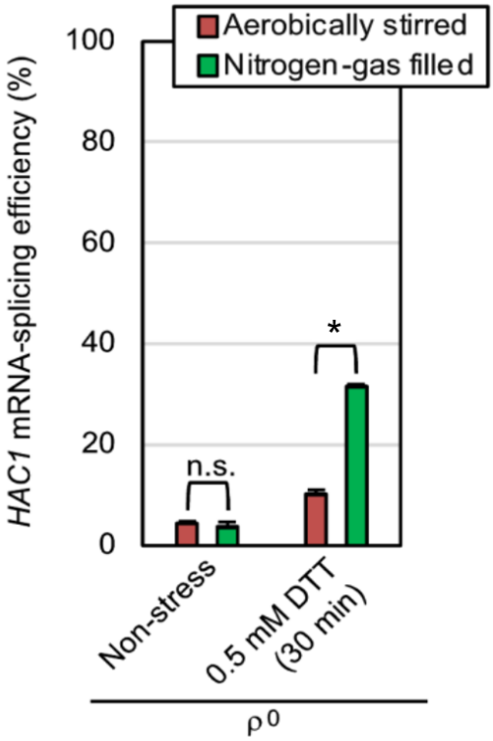

Figure 21. Hypoxic treatment boosts the DTT-induced *HAC1*-mRNA splicing even in ρ^0 cells. The ρ^0 mutant of *S. cerevisiae* wild-type BY4742 was cultured and stressed by DTT as shown in Fig. 10, and was checked for the *HAC1*-mRNA splicing. n.s. (not significant): $p > 0.05$, *: $p < 0.05$.

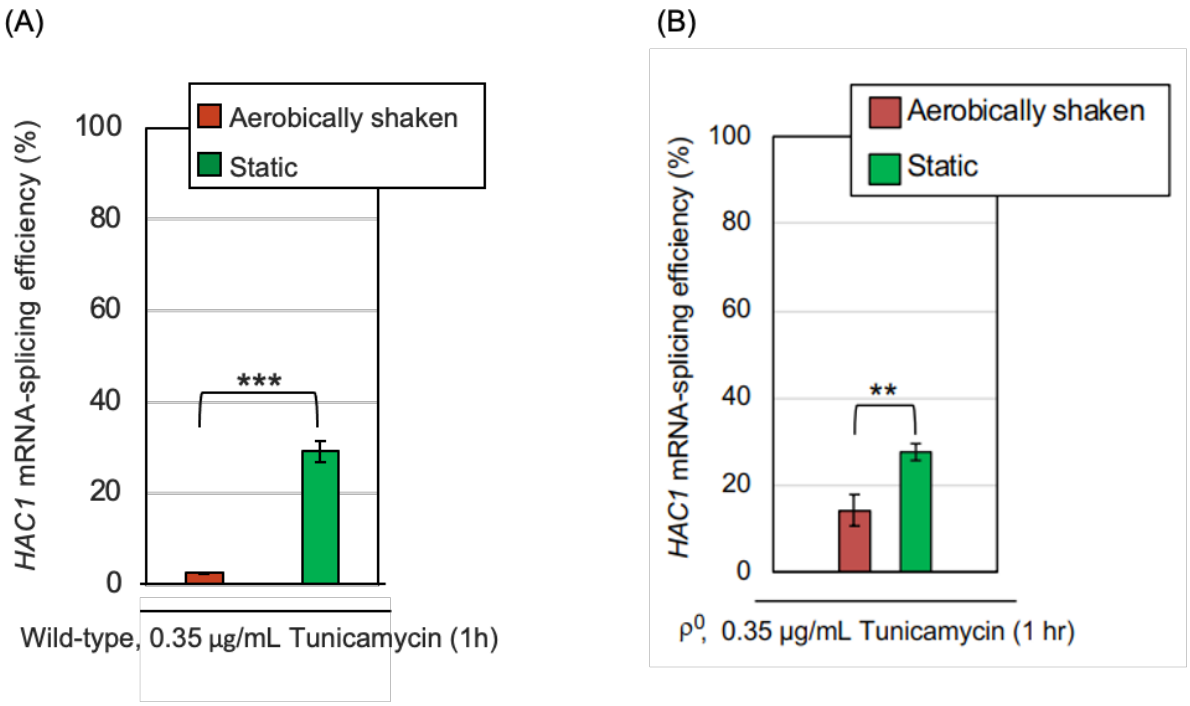

Figure 22. Static condition boosts the tunicamycin-induced *HAC1*-mRNA splicing in both wild-type cells (this was showed in Fig.8A) and ρ^0 cells. The ρ^0 mutant of *S. cerevisiae* wild-type BY4742 was cultured as shown in Fig. 6 but stressed by 0.35 µg/mL tunicamycin for 1hr. Yeast cells then were collected and checked for the *HAC1*-mRNA splicing. **: $p < 0.01$, ***:$P<0.001$

Disulfide-bond formation is an important step in protein folding in the ER, which is thus referred to as oxidative protein folding. As described later, it is highly likely that molecular oxygen is directly involved in this cellular event. In order to determine the cellular ability to form cysteine disulfide bonds in the ER, I used the eroGFP reporter, which is an ER-located GFP variant and changes its fluorescence excitation spectrum dependent on the formation of an intramolecular cysteine disulfide bond (Merksamer et al., 2008). In the experiments shown in Fig. 23, *S. cerevisiae* cells expressing the eroGFP reporter were observed under a fluorescence microscope with two different excitation lights. Cells emit bright fluorescence only upon excitation with blue light (high eroGFP ratio) when the disulfide-bond formation in eroGFP is inhibited, whereas oxidatively folded eroGFP is efficiently excited by UV/violet light. For this assay, cells were aerobically cultured in order to allow maturation of the eroGFP fluorophore, and were set into the aerobic shaking condition or the argon gas-filled static condition immediately after the ER-stress onset. The treatment of wild-type cells with diluted DTT significantly increased the eroGFP value only under the argon gas-filled hypoxic condition (Fig. 23). Moreover, a similar result was obtained

when cells carried the ρ^0 mutation (Fig. 23). I therefore postulate a mitochondrial respiration-independent role(s) of molecular oxygen to mitigate ER stress and to promote the oxidative protein folding.

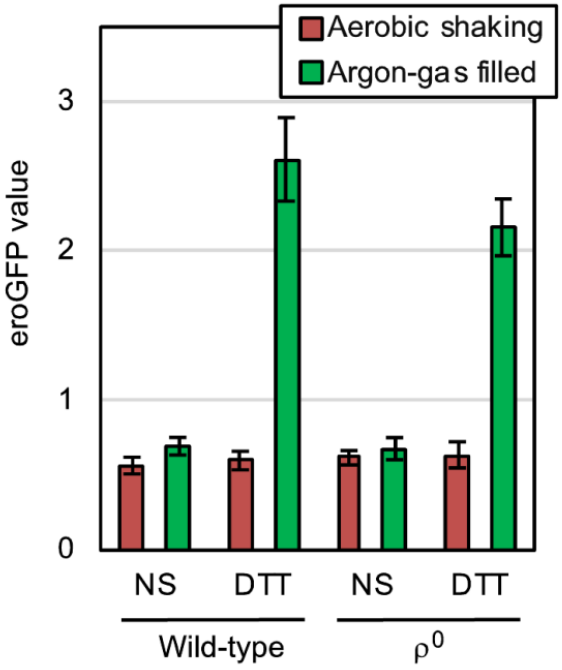

Figure 23. Hypoxic condition potentiates ER stress-induced impairment of the cysteine disulfide-bond formation in the ER of both wild-type and ρ^0 mutant cells. At 30°C in YPD, wild-type BY4742 cells and the congenic ρ^0 mutant carrying the pPM28 eroGFP-expression plasmid were aerobically grown, and were stressed by 0.5 mM DTT (or remained non-stressed (NS)) under the aerobic shaking or argon gas-filled condition for 30 min. The cells were then fluorescence-microscopically observed using two different excitation lights for measurement of the eroGFP values, the calculation method of which is described in the Materials and Methods section.

I next monitored molecular oxygen consumption rate of *S. cerevisiae* BY4742 wild-type cells and its congenic ρ^0 mutation cultures. Fig. 24 shows that oxygen consumption of non-stressed *S. cerevisiae* cultures was nearly completely abolished by the ρ^0 mutation (compare the third column to the leftmost column). This observation is consistent with the commonly accepted idea that molecular oxygen is mainly consumed by mitochondrial respiration in eukaryotic cells. According to a previous report by others (Knupp at al. 2019), ER stress stimulates oxygen consumption of *S. cerevisiae* cultures. However, in my hands, the oxygen consumption of the wild-type cells was decelerated by DTT exposure (Fig. 24; compare the second column to the leftmost column). In contrast, DTT accelerated the oxygen

consumption of ρ^0 cells (Fig. 24; compare the rightmost column to the third column). This observation suggests that yeast cells consume molecular oxygen under ER stress conditions through a way in which the mitochondrial respiration is not involved.

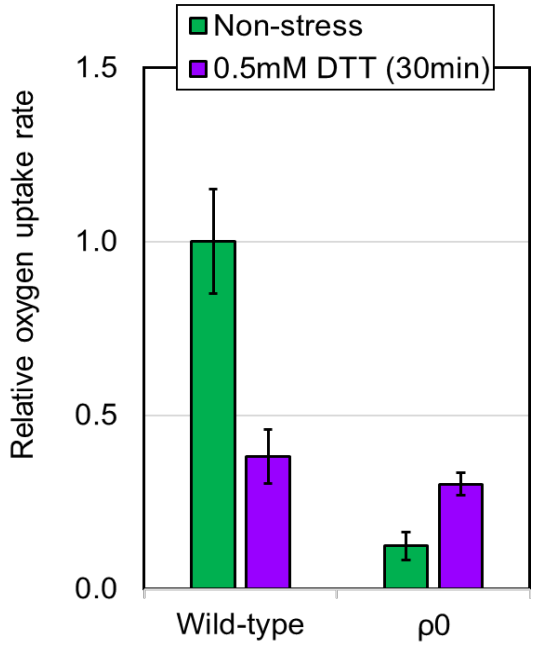

Figure 24. Oxygen consumption of yeast cells under non-stress and ER stress conditions. Wild-type BY4742 cells and theirs congenic ρ^0 mutant were cultured in aerobically shaken at 30°C, YPD cultures of yeast cells were subjected to measurement of the oxygen-consumption rate in the presence or absence of 0.5 mM DTT. The oxygen uptake ratios were the calculated as described in the Materials and Methods section, and are normalized against that of non-stressed wild-type cells or non-stress condition, which is set at 1.0. *: $p < 0.05$, **: $p < 0.01$.

3.6. The Ero1-mediated oxidative protein folding is probably involved in mitigation of ER stress by aeration

How do aeration and oxygen consumption lead to the mitigation of ER stress independently of mitochondrial respiration? For ergosterol biogenesis, lipidic intermediates are subjected to multi-step oxidization, in which molecular oxygen is involved, on the ER membrane of *S. cerevisiae* cells (Jordá and Puig, 2020). One possible scenario hypothesized from these insights is that hypoxia causes ergosterol deficiency, leading to induction or aggravation of ER stress. However, the *HAC1*-mRNA splicing induced by cellular treatment with low-dose DTT under the anaerobic condition was not mitigated, but was slightly enhanced by addition of ergosterol into culturing medium (Fig. 25).

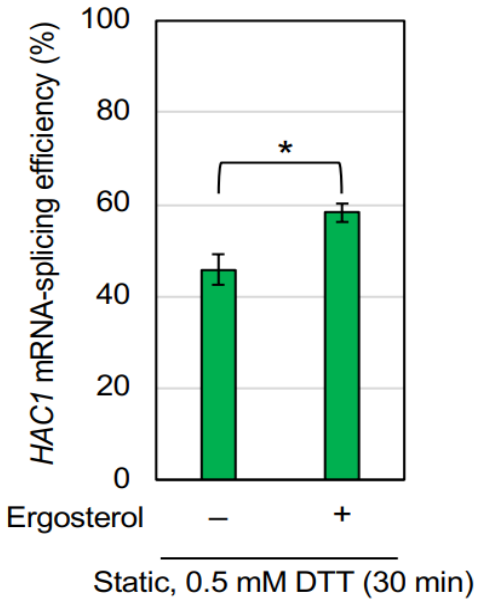

Figure 25. Ergosterol does not mitigate ER stress boosted by de-aeration. Wild-type BY4742 cells were cultured at 30ºC in YPD under the static condition. DTT (0.5 mM final concentration) was added into all cultures 30-min before harvested. For the "+Ergosterol" samples, YPD was supplemented with ergosterol (10 µg/mL final concentration). *: $p < 0.05$.

According to an *in vitro* investigation using purified Ero1-protein samples (Tu and Weissman, 2002), molecular oxygen works to directly oxidize Ero1 and start the ER oxidation cascade. If the mitochondrial respiration-independent mitigation of ER stress by aeration is due to this Ero1-mediated reaction, it may not be observed when Ero1 is deactivated in ρ^0 cells. The *ERO1* is an essential gene, and cells carrying the temperature-sensitive *ero1-1* allele suffer from ER stress when cultured at high temperatures even without external stress stimuli (Frand and Kaiser, 1998). This observation was reproduced in my experiment shown in Fig. 26, which indicate that *ero1-1*/ρ^0 cells exhibited an enhancement of the *HAC1*-mRNA splicing upon the shift of culturing temperature from the permissive temperature of 25°C to the semi-permissive temperature of 30°C although not strongly. Importantly, the *HAC1*-mRNA splicing induced by culturing the *ero1-1*/ρ^0 cells at 30°C was not mitigated, but rather slightly enhanced, by aeration (Fig. 26).

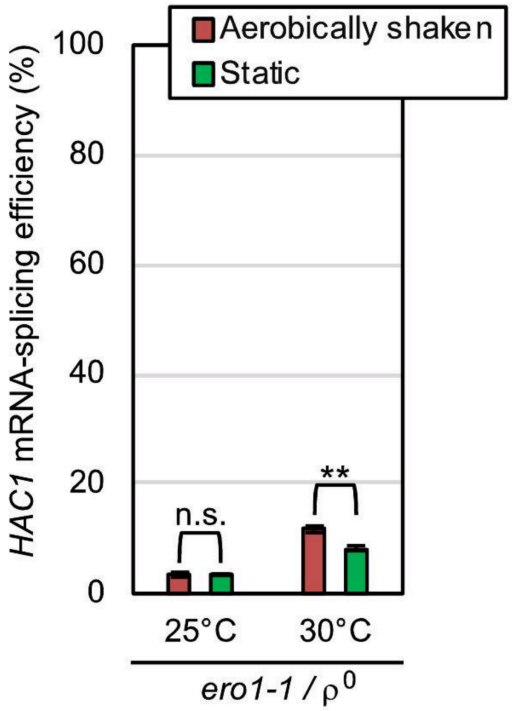

Figure 26. The *HAC1*-mRNA splicing in *ero1-1/ρ⁰* cells cultured at the semi-permissive temperature was not compromised by aeration. After being pre-cultured at 25°C in YPD, *ero1-1* mutant TSA203 cells were cultured at 25°C or 30°C under the indicated conditions in YPD for 4 hr, and were checked for the *HAC1*-mRNA splicing. n.s. (not significant): $p > 0.05$, *: $p < 0.05$.

In order to check if the DTT-induced oxygen uptake in ρ^0 cells is due to Ero1, I next measured the oxygen uptake rate of *ero1-1/ρ⁰* cells. Unlike the case of *ERO1+/ρ⁰* cells (Fig. 24), the oxygen consumption was not increased but rather slightly decreased by treatment of *ero1-1/ρ⁰* cells with 0.5 mM DTT (Fig. 27).

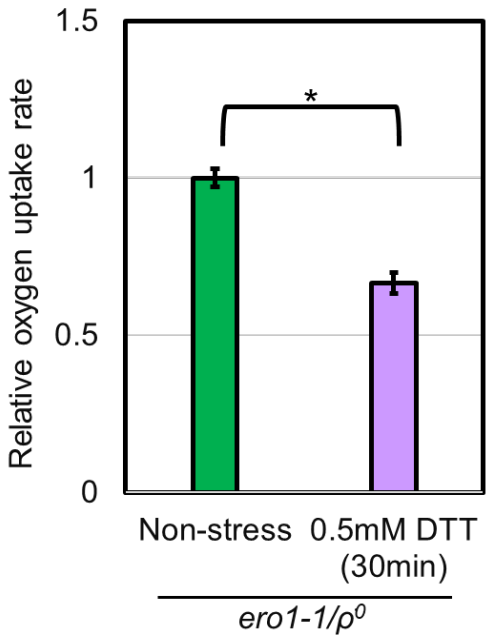

Figure 27: Oxygen consumption of *ero1-1/ρ⁰* cells under non-stress and ER stress conditions. The ρ^0 progeny of *ero1-1* mutant TSA203 cells were cultured under the aerobically shaken condition at 30°C in YPD medium, and were subjected to measurement of the oxygen uptake in the presence or absence of 0.5 mM DTT. The oxygen uptake ratio was the calculated as described in the Materials and Methods section, and are normalized against the value of non-stress condition, which is set at 1.0. *: $p < 0.05$.

Chapter IV. Discussion

The initial aim of this study was to elucidate the effect of aerobic agitation on the physiology of *S. cerevisiae*. Here I focused on ER stress, and monitored the *HAC1* mRNA splicing efficiency in static cultures and in aerobically shaken cultures. According to my results shown in Fig. 8, static culturing alone did not induce the splicing of *HAC1* mRNA. Meanwhile, the *HAC1*-mRNA splicing induced by low-dose DTT, low-dose tunicamycin, or ethanol was boosted when cells were cultured under the static condition (Fig. 8 and 9). This observation implies a benefit of aerobic agitation for industrial ethanol fermentation even though this process usually occurs under less aerobic condition. Nevertheless, when cells were subjected to strong ER stress by high concentrations of DTT or tunicamycin, *HAC1* mRNA was highly spliced both under the static condition and the aerobically shaken condition (Fig. 8). I think that, under these situations, ER stress was too strong to be mitigated by aeration. I then continued my study using DTT as the representative ER stressor. In the experiments shown in Fig. 10, cells were cultured with agitation either in open flasks or in nitrogen gas-filled flasks. This culturing method confirmed my proposal that the *HAC1*-mRNA splicing that is triggered by weak ER stress is boosted by hypoxic conditions, while hypoxia alone does not induce ER stress in *S. cerevisiae* cells (Fig. 12). Our observation in Fig, 14, in which the cellular *KAR2*/BiP-mRNA level was monitored, is consistent with this idea. Moreover, according to the cell-growth assay shown in Fig. 15, deficient aeration aggravates cellular damage being induced by ER stress.

According to previous studies, ER stress is induced either by proteotoxic stress or lipid bilayer stress, which is sensed by Ire1 in different manners (Hetz et al., 2020; Bartoszewska and Collawn, 2020; Promlek et al., 2011). Fig. 17 shows that the DTT-induced *HAC1* mRNA splicing was poorly boosted by hypoxic culturing when Ire1 carried the ΔIII mutation. Moreover, unlike the case of DTT, tunicamycin, or ethanol, aeration was not mitigated mild ER stress induced by inositol depletion (Fig. 18). I thus think that the mechanism by which hypoxia boosts ER stress is related not to lipid bilayer stress but to proteotoxic stress.

The mitochondria are the cellular compartments in which molecular oxygen is highly consumed via respiration. Some previous reports by others have touched on the involvement of mitochondria and mitochondrial respiration in cellular responses against ER stress. According to Haynes et al. (2004), ER stress induces the production of reactive oxygen species (ROS), in which the mitochondria are involved. In agreement with this finding, Knupp et al. (2019) proposed that, in *S. cerevisiae*, ER

stress increases mitochondrial oxygen consumption, which contributes to cellular survival upon ER stress. It is likely that some intracellular signaling pathways mediate the mitochondrial response to ER stress (Hijazi et al., 2020). In mammalian cells, the ER-mitochondrial spatial connection is increased, leading to enhancement of mitochondrial respiration, in response to ER stress (Bravo et al., 2011). However, at least under my experimental conditions, DTT severely attenuated the oxygen uptake of wild-type *S. cerevisiae* cells (Fig. 24). In agreement with this observation, the mitochondrial gene expression in wild-type *S. cerevisiae* cells was drastically decreased by cellular treatment with DTT or tunicamycin (Tran et al., 2019a). Contrary to the previous observations by others (Knupp et al., 2019); Haynes et al., 2004; Hijazi et al., 2020; Bravo et al., 2011), ER stress may damage the mitochondria under certain situations. The reason for this discrepancy should be addressed in future.

Meanwhile, here I also show that oxygen deprivation aggravates ER stress not only in wild-type $\rho+$ cells but also in ρ^0 mutant cells (Fig. 12, 21 and 22). I therefore propose that, in contrast to the aforementioned previous thoughts (Knupp et al., 2019); Hijazi et al., 2020; Bravo et al., 2011), ER stress is alleviated by aeration, at least partly, in a manner(s) in which mitochondrial respiration is not involved. Intriguingly, DTT accelerated the oxygen uptake of ρ^0 mutant cells (Fig. 24). This data implied that, in *S. cerevisiae* cells, molecular oxygen can be consumed in a mechanism(s) independently of mitochondrial respiration.

In agreement with these observations, the eroGFP reporter assay shown in Fig. 23 indicated that aeration mitigates the DTT-induced impairment of ER oxidative protein folding both in wild-type cells and in ρ^0 cells. Importantly, also in ρ^0 cells, ER stress that was induced by the impaired Ero1 function was not compromised by aeration (Fig. 26). In addition, DTT did not increase oxygen uptake of ρ^0 cells carrying the *ero1-1* mutation (Fig. 27). I therefore postulate that molecular oxygen works for mitigation of ER stress in wild-type *S. cerevisiae* cells through being consumed in the ER to initiate the Ero1-mediated oxidation cascade. It is likely that this scenario is applicable not only to DTT-induced ER stress but also to other ER-stress stimuli, such as tunicamycin, the primary effects of which are not related to the formation of disulfide bonds in ER client proteins. This is because, according to Merksamer et al. (2008), ER-stress stimuli collectively damage the protein disulfide-bond formation directly or indirectly.

As aforementioned, the function of molecular oxygen in the ER has been initially proposed through *in vitro* biochemical studies. According to Tu and Weissman (2004), molecular oxygen directly contributes to oxidization of Ero1. My results from this

study provide an *in vivo* cell-biological evidence that argues for the physiological importance of the direct involvement of molecular oxygen in the oxidative protein folding in the ER. Nevertheless, as described in the Introduction section, Ero1 functions to initiate the ER-protein oxidative-folding cascade even without molecular oxygen through the Osm1-dependent Ero1 oxidation in *S. cerevisiae* cells. I assume that the Osm1-dependent Ero1 oxidation is insufficient in ER-stressed cells, in which Ero1 works robustly. In other words, molecular oxygen is required to accelerate the ER-protein oxidative-folding cascade in ER-stressed cells through boosting the Ero1 oxidation.

S. cerevisiae is a facultative anaerobic organism, which grows well under both aerobic and hypoxic conditions in the presence of fermentable carbon sources. In conclusion, here I demonstrate a scene in which aeration is beneficial for *S. cerevisiae* cells even under glucose-rich fermentative conditions. As described in the introduction section, it is widely known that oxygen deprivation causes ER stress and induces the UPR in mammalian cells (Chipurupalli et al. 2019; Bartoszewska and Collawn 2020). This is frequently observed in cancer cells, which stay under hypoxic conditions and proliferate independently of mitochondrial respiration (Diaz-Ruiz et al., 2011; Chipurupalli et al., 2019). Based on my findings on the present *S. cerevisiae* study, I speculate that, also in mammalian cells, hypoxia induces or aggravates ER stress via impairment of the Ero1 function.

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References

1. Aebersold, Daniel M., Philipp Burri, Karl T. Beer, Jean Laissue, Valentin Djonov, Richard H. Greiner, and Gregg L. Semenza. 2001. "Expression of Hypoxia-Inducible Factor-1α: A Novel Predictive and Prognostic Parameter in the Radiotherapy of Oropharyngeal Cancer." *Cancer Research* 61 (7): 2911–16.

2. Akman, Muhlis, Dimas Carolina Belisario, Iris Chiara Salaroglio, Joanna Kopecka, Massimo Donadelli, Enrico De Smaele, and Chiara Riganti. 2021. "Hypoxia, Endoplasmic Reticulum Stress and Chemoresistance: Dangerous Liaisons." *Journal of Experimental & Clinical Cancer Research: CR* 40 (1): 28.

3. Alimardani, Parissa, Matthieu RéGNACQ, Carole Moreau-Vauzelle, Thierry Ferreira, Tristan Rossignol, Bruno Blondin, and Thierry BERGèS. 2004. "SUT1-Promoted Sterol Uptake Involves the ABC Transporter Aus1 and the Mannoprotein Dan1 Whose Synergistic Action Is Sufficient for This Process." *Biochemical Journal* 381 (Pt 1): 195–202.

4. Araki, Kazutaka, and Kazuhiro Nagata. 2011. "Protein Folding and Quality Control in the ER." *Cold Spring Harbor Perspectives in Biology* 3 (11): a007526.

5. Bartoszewska, Sylwia, and James F. Collawn. 2020. "Unfolded Protein Response (UPR) Integrated Signaling Networks Determine Cell Fate during Hypoxia." *Cellular & Molecular Biology Letters* 25 (1): 18.

6. Birner, Peter, Monika Schindl, Andreas Obermair, Gerhard Breitenecker, and Georg Oberhuber. 2001. "Expression of Hypoxia-Inducible Factor 1α in Epithelial Ovarian Tumors: Its Impact on Prognosis and on Response to Chemotherapy." *Clinical Cancer Research* 7 (6): 1661–68.

7. Bos, Reinhard, Petra van der Groep, Astrid E. Greijer, Avi Shvarts, Sybren Meijer, Herbert M. Pinedo, Gregg L. Semenza, Paul J. van Diest, and Elsken van der Wall. 2003. "Levels of Hypoxia-Inducible Factor-1α Independently Predict Prognosis in Patients with Lymph Node Negative Breast Carcinoma." *Cancer* 97 (6): 1573–81.

8. Brachmann, Carrie Baker, Adrian Davies, Gregory J. Cost, Emerita Caputo, Joachim Li, Philip Hieter, and Jef D. Boeke. 1998. "Designer Deletion Strains Derived from Saccharomyces Cerevisiae S288C: A Useful Set of Strains and Plasmids for PCR-Mediated Gene Disruption and Other Applications." *Yeast* 14 (2): 115–32.

9. Bravo, Roberto, Jose Miguel Vicencio, Valentina Parra, Rodrigo Troncoso, Juan Pablo Munoz, Michael Bui, Clara Quiroga, et al. 2011. "Increased ER-Mitochondrial Coupling Promotes Mitochondrial Respiration and Bioenergetics during Early Phases of ER Stress." *Journal of Cell Science* 124 (Pt 13): 2143–52.

10. Chipurupalli, Sandhya, Elango Kannan, Vinay Tergaonkar, Richard D'Andrea, and Nirmal Robinson. 2019. "Hypoxia Induced ER Stress Response as an Adaptive Mechanism in Cancer." *International Journal of Molecular Sciences* 20 (3): 749.

11. Collart, Martine A., and Salvatore Oliviero. 1993. "Preparation of Yeast RNA." *Current Protocols in Molecular Biology* 23 (1): 13.12.1-13.12.5.

12. Cox, J. S., C. E. Shamu, and P. Walter. 1993. "Transcriptional Induction of Genes Encoding Endoplasmic Reticulum Resident Proteins Requires a Transmembrane Protein Kinase." *Cell* 73 (6): 1197–1206.

13. Credle, Joel J., Janet S. Finer-Moore, Feroz R. Papa, Robert M. Stroud, and Peter Walter. 2005. "On the Mechanism of Sensing Unfolded Protein in the Endoplasmic Reticulum." *Proceedings of the National Academy of Sciences* 102 (52): 18773–84.

14. Diaz-Ruiz, Rodrigo, Michel Rigoulet, and Anne Devin. 2011. "The Warburg and Crabtree Effects: On the Origin of Cancer Cell Energy Metabolism and of Yeast Glucose Repression." *Biochimica Et Biophysica Acta* 1807 (6): 568–76.

15. Emerling, Brooke M., and Navdeep S. Chandel. 2005. "Oxygen Sensing: Getting Pumped by Sterols." *Science's STKE* 2005 (289): pe30–pe30.

16. Ferreira, Túlio César, Libi Hertzberg, Max Gassmann, and Élida Geralda Campos. 2007. "The Yeast Genome May Harbor Hypoxia Response Elements (HRE)." *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP* 146 (1–2): 255–63.

17. Frand, A. R., and C. A. Kaiser. 1998. "The ERO1 Gene of Yeast Is Required for Oxidation of Protein Dithiols in the Endoplasmic Reticulum." *Molecular Cell* 1 (2): 161–70.

18. Frand, A. R., and C. A. Kaiser. 1999. "Ero1p Oxidizes Protein Disulfide Isomerase in a Pathway for Disulfide Bond Formation in the Endoplasmic Reticulum." *Molecular Cell* 4 (4): 469–77.

19. Gardner, Brooke M., and Peter Walter. 2011. "Unfolded Proteins Are Ire1-Activating Ligands That Directly Induce the Unfolded Protein Response." *Science (New York, N.Y.)* 333 (6051): 1891.

20. Gething, M. J. 1999. "Role and Regulation of the ER Chaperone BiP." *Seminars in Cell & Developmental Biology* 10 (5): 465–72.

21. Giatromanolaki, A., M. I. Koukourakis, E. Sivridis, H. Turley, K. Talks, F. Pezzella, K. C. Gatter, and A. L. Harris. 2001. "Relation of Hypoxia Inducible Factor 1α and 2α in Operable Non-Small Cell Lung Cancer to Angiogenic/Molecular Profile of Tumours and Survival." *British Journal of Cancer* 85 (6): 881–90.

22. Halbleib, Kristina, Kristina Pesek, Roberto Covino, Harald F. Hofbauer, Dorith Wunnicke, Inga Hänelt, Gerhard Hummer, and Robert Ernst. 2017. "Activation of the Unfolded Protein Response by Lipid Bilayer Stress." *Molecular Cell* 67 (4): 673-684.e8.

23. Haynes, Cole M., Eric A. Titus, and Antony A. Cooper. 2004. "Degradation of Misfolded Proteins Prevents ER-Derived Oxidative Stress and Cell Death." *Molecular Cell* 15 (5): 767–76.

24. Hetz, Claudio, Kezhong Zhang, and Randal J. Kaufman. 2020. "Mechanisms, Regulation and Functions of the Unfolded Protein Response." *Nature Reviews Molecular Cell Biology* 21 (8): 421–38.

25. Hijazi, Imadeddin, Jeffrey Knupp, and Amy Chang. 2020. "Retrograde Signaling Mediates an Adaptive Survival Response to Endoplasmic Reticulum Stress in Saccharomyces Cerevisiae." *Journal of Cell Science* 133 (6): jcs241539.

26. Ho, Nurulain, Wei Sheng Yap, Jiaming Xu, Haoxi Wu, Jhee Hong Koh, Wilson Wen Bin Goh, Bhawana George, Shu Chen Chong, Stefan Taubert, and Guillaume Thibault. 2020. "Stress Sensor Ire1 Deploys a Divergent Transcriptional Program in Response to Lipid Bilayer Stress." *The Journal of Cell Biology* 219 (7).

27. Hwang, C., A. J. Sinskey, and H. F. Lodish. 1992. "Oxidized Redox State of Glutathione in the Endoplasmic Reticulum." *Science (New York, N.Y.)* 257 (5076): 1496–1502.

28. Ishiwata-Kimata, Yuki, Thanyarat Promlek, Kenji Kohno, and Yukio Kimata. 2013. "BiP-Bound and Nonclustered Mode of Ire1 Evokes a Weak but Sustained Unfolded Protein Response." *Genes to Cells* 18 (4): 288–301.

29. Jiang, B. H., G. L. Semenza, C. Bauer, and H. H. Marti. 1996. "Hypoxia-Inducible Factor 1 Levels Vary Exponentially over a Physiologically Relevant Range of O2 Tension." *The American Journal of Physiology* 271 (4 Pt 1): C1172-1180.

30. Jordá, Tania, and Sergi Puig. 2020. "Regulation of Ergosterol Biosynthesis in Saccharomyces Cerevisiae." *Genes* 11 (7): E795.

31. Kaiser, C., S. Michaelis, and A. Mitchell. 1994. "Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual." *Undefined*.

32. Kim, Sunghwan, Chang Min Kim, Young-Jin Son, Jae Young Choi, Rahel K. Siegenthaler, Younho Lee, Tae-Ho Jang, et al. 2018. "Molecular Basis of Maintaining an Oxidizing Environment under Anaerobiosis by Soluble Fumarate Reductase." *Nature Communications* 9 (1): 4867.

33. Kimata, Yukio, Yuki Ishiwata-Kimata, Tatsuhiko Ito, Aiko Hirata, Tomohide Suzuki, Daisuke Oikawa, Masato Takeuchi, and Kenji Kohno. 2007. "Two Regulatory Steps of ER-Stress Sensor Ire1 Involving Its Cluster Formation and Interaction with Unfolded Proteins." *The Journal of Cell Biology* 179 (1): 75–86.

34. Kimata, Yukio, Yuki I. Kimata, Yusuke Shimizu, Hiroshi Abe, Ileana C. Farcasanu, Masato Takeuchi, Mark D. Rose, and Kenji Kohno. 2003. "Genetic Evidence for a Role of BiP/Kar2 That Regulates Ire1 in Response to Accumulation of Unfolded Proteins." *Molecular Biology of the Cell* 14 (6): 2559–69.

35. Kimata, Yukio, Daisuke Oikawa, Yusuke Shimizu, Yuki Ishiwata-Kimata, and Kenji Kohno. 2004. "A Role for BiP as an Adjustor for the Endoplasmic Reticulum Stress-Sensing Protein Ire1." *The Journal of Cell Biology* 167 (3): 445–56.

36. Knupp, Jeffrey, Peter Arvan, and Amy Chang. 2019. "Increased Mitochondrial Respiration Promotes Survival from Endoplasmic Reticulum Stress." *Cell Death and Differentiation* 26 (3): 487–501.

37. Labunskyy, Vyacheslav M., Min-Hyuk Yoo, Dolph L. Hatfield, and Vadim N. Gladyshev. 2009. "Sep15, a Thioredoxin-like Selenoprotein, Is Involved in the Unfolded Protein Response and Differentially Regulated by Adaptive and Acute ER Stresses." *Biochemistry* 48 (35): 8458–65.

38. Lavoie, Christine, and Jacques Paiement. 2008. "Topology of Molecular Machines of the Endoplasmic Reticulum: A Compilation of Proteomics and Cytological Data." *Histochemistry and Cell Biology* 129 (2): 117–28.

39. Le, Quynh Giang, Yuki Ishiwata-Kimata, Thi Huong Phuong, Shigeto Fukunaka, Kenji Kohno, and Yukio Kimata. 2021. "The ADP-Binding Kinase Region of Ire1 Directly Contributes to Its Responsiveness to Endoplasmic Reticulum Stress." *Scientific Reports* 11 (February).

40. McCaffrey, Kathleen, and Ineke Braakman. 2016. "Protein Quality Control at the Endoplasmic Reticulum." *Essays in Biochemistry* 60 (2): 227–35.

41. McKeown, S. R. 2014. "Defining Normoxia, Physoxia and Hypoxia in Tumours-Implications for Treatment Response." *The British Journal of Radiology* 87 (1035): 20130676.

42. Merksamer, Philip I., Ala Trusina, and Feroz R. Papa. 2008. "Real-Time Redox Measurements during Endoplasmic Reticulum Stress Reveal Interlinked Protein Folding Functions." *Cell* 135 (5): 933–47.

43. Miyagawa, Ken-Ichi, Yuki Ishiwata-Kimata, Kenji Kohno, and Yukio Kimata. 2014. "Ethanol Stress Impairs Protein Folding in the Endoplasmic Reticulum and Activates Ire1 in Saccharomyces Cerevisiae." *Bioscience, Biotechnology, and Biochemistry* 78 (8): 1389–91.

44. Mori, Kazutoshi, Naoki Ogawa, Tetsushi Kawahara, Hideki Yanagi, and Takashi Yura. 1998. "Palindrome with Spacer of One Nucleotide Is Characteristic of the Cis-Acting Unfolded Protein Response Element InSaccharomyces Cerevisiae." *Journal of Biological Chemistry* 273 (16): 9912–20.

45. Navarro-Tapia, Elisabet, Roberto Pérez-Torrado, and Amparo Querol. 2017. "Ethanol Effects Involve Non-Canonical Unfolded Protein Response Activation in Yeast Cells." *Frontiers in Microbiology* 8: 383.

46. Oakes, Scott A., and Feroz R. Papa. 2015. "The Role of Endoplasmic Reticulum Stress in Human Pathology." *Annual Review of Pathology* 10: 173–94.

47. Okamura, K., Y. Kimata, H. Higashio, A. Tsuru, and K. Kohno. 2000. "Dissociation of Kar2p/BiP from an ER Sensory Molecule, Ire1p, Triggers the Unfolded Protein Response in Yeast." *Biochemical and Biophysical Research Communications* 279 (2): 445–50.

48. Promlek, Thanyarat, Yuki Ishiwata-Kimata, Masahiro Shido, Mitsuru Sakuramoto, Kenji Kohno, and Yukio Kimata. 2011. "Membrane Aberrancy and Unfolded Proteins Activate the Endoplasmic Reticulum Stress Sensor Ire1 in Different Ways." *Molecular Biology of the Cell* 22 (18): 3520–32.

49. Ruddock, Lloyd W., and Maurizio Molinari. 2006. “N-Glycan Processing in ER Quality Control.” *Journal of Cell Science* 119 (Pt 21): 4373–80.

50. Salceda, Susana, and Jaime Caro. 1997. “Hypoxia-Inducible Factor 1α (HIF-1α) Protein Is Rapidly Degraded by the Ubiquitin-Proteasome System under Normoxic Conditions: ITS STABILIZATION BY HYPOXIA DEPENDS ON REDOX-INDUCED CHANGES *.” *Journal of Biological Chemistry* 272 (36): 22642–47.

51. Sevier, Carolyn S., and Chris A. Kaiser. 2008. “Ero1 and Redox Homeostasis in the Endoplasmic Reticulum.” *Biochimica Et Biophysica Acta* 1783 (4): 549–56.

52. Shen, Xiaohua, Ronald E. Ellis, Kyungho Lee, Chuan-Yin Liu, Kun Yang, Aaron Solomon, Hiderou Yoshida, et al. 2001. “Complementary Signaling Pathways Regulate the Unfolded Protein Response and Are Required for C. Elegans Development.” *Cell* 107 (7): 893–903.

53. Sikorski, R. S., and P. Hieter. 1989. “A System of Shuttle Vectors and Yeast Host Strains Designed for Efficient Manipulation of DNA in Saccharomyces Cerevisiae.” *Genetics* 122 (1): 19–27.

54. Sitia, Roberto, and Ineke Braakman. 2003. “Quality Control in the Endoplasmic Reticulum Protein Factory.” *Nature* 426 (6968): 891–94.

55. Tran, Duc Minh, Yuki Ishiwata-Kimata, Thanh Chi Mai, Minoru Kubo, and Yukio Kimata. 2019a. “The Unfolded Protein Response alongside the Diauxic Shift of Yeast Cells and Its Involvement in Mitochondria Enlargement.” *Scientific Reports* 9 (1): 12780.

56. Tran, Duc Minh, Hiroshi Takagi, and Yukio Kimata. 2019b. “Categorization of Endoplasmic Reticulum Stress as Accumulation of Unfolded Proteins or Membrane Lipid Aberrancy Using Yeast Ire1 Mutants.” *Bioscience, Biotechnology, and Biochemistry* 83 (2): 326–29.

57. Tu, Benjamin P., and Jonathan S. Weissman. 2002. “The FAD- and O(2)-Dependent Reaction Cycle of Ero1-Mediated Oxidative Protein Folding in the Endoplasmic Reticulum.” *Molecular Cell* 10 (5): 983–94.

58. Tu, Benjamin P., and Jonathan S. Weissman. 2004. “Oxidative Protein Folding in Eukaryotes.” *The Journal of Cell Biology* 164 (3): 341–46.

59. Yoshida, H., T. Matsui, A. Yamamoto, T. Okada, and K. Mori. 2001. "XBP1 MRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor." *Cell* 107 (7): 881–91.

60. Zhou, Jie, Tobias Schmid, Steffen Schnitzer, and Bernhard Brüne. 2006. "Tumor Hypoxia and Cancer Progression." *Cancer Letters* 237 (1): 10–21.

61. Ziegler, D. M., and L. L. Poulsen. 1977. "Protein Disulfide Bond Synthesis: A Possible Intracellular Mechanism." *Trends in Biochemical Sciences* 2 (4): 79–81.