IgMin Research STEM ISSN: 2995-8067 A Multidisciplinary Open Access Journal

SCIENCE SUBJECT
Molecular Biology TOPIC(S)

Article Information

Submitted: January 11, 2024
Approved: January 29, 2024
Published: January 30, 2024

How to cite this article: Monguchi M, Kimata Y. Enforcement and Enlargement of the *Saccharomyces cerevisiae* Endoplasmic Reticulum through Artificial Evocation of the Unfolded Protein Response. IgMin Res. Jan 30, 2024; 2(1): 036-038. IgMin ID: igmin142; DOI: 10.61927/igmin142; Available at: www.igminresearch.com/articles/pdf/igmin142.pdf

Keywords: Organelle; Yeast; Stress response; Biotechnology; Microbiology

Mini Review

Enforcement and Enlargement of the *Saccharomyces cerevisiae* Endoplasmic Reticulum through Artificial Evocation of the Unfolded Protein Response

Masaki Monguchi and Yukio Kimata*

Division of Biological Science, Department of Science and Technology, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan

***Correspondence:** Yukio Kimata, Ph.D. Associate Professor, Division of Biological Science, Department of Science and Technology, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan, Email: kimata@bs.naist.jp

Abstract

Upon dysfunction of the Endoplasmic Reticulum (ER), eukaryotic cells provoke a gene expression program, namely, the Unfolded Protein Response (UPR), leading to an increase in the size and function of the ER. In the yeast *Saccharomyces cerevisiae*, the UPR is modulated by the Hac1i protein, which is a transcription factor produced by ER stress. When the UPR is artificially triggered under non-stress conditions by artificial expression of the Hac1i protein, *S. cerevisiae* cells carry an enforced and enlarged ER, which allows us to obtain commercially valuable materials such as secretory proteins and functional lipids abundantly.

Introduction

Owing to their rapid and cost-effective growth characteristics, various species of yeast are frequently employed as platforms for the production of commercially valuable substances. *Saccharomyces cerevisiae*, also known as baker's yeast, is used not only for ethanol fermentation but also for basic and applied molecular biology, partly because a large number of genetic engineering techniques have been developed for this organism. A notable example of yeast application lies in the realm of production of human secretory proteins such as antibodies and interferons, which are widely used as biopharmaceutical agents [1]. Moreover, yeasts can potentially serve as catalysts for the production of lipidic compounds. Through the utilization of genetically modified yeasts, it is now possible to synthesize plant-derived functional lipids [2].

The ER and UPR in *Saccharomyces cerevisiae*

The cellular compartment known as the Endoplasmic Reticulum (ER) is the site at which these molecules are synthesized through various biological reactions. In the ER, secretory proteins undergo complicated processes of folding and modification processes, involving the acquisition of cysteine disulfide bonds and N-linked glycosylation chains, which are essential for their subsequent transport to the cell surface. Additionally, a number of enzymes that facilitate lipid biosynthesis are located in the ER membrane. In the ER, lipid droplets, which act as storage sites for neutral lipids, such as triglycerides, emerge as budding structures [3,4]. The ER in higher eukaryotes also serves as a site in which calcium ions are stored. On the other hand, the calcium ion concentration in the yeast ER is reportedly fairly low [5].

The ER, a sac surrounded by a single biological membrane, can take either flat or tubular shapes. The tubular ER often undergoes branching and fusion processes, resulting in the formation of a network-like structure. Notably, the ER occupies a significant portion of the cytoplasm in a wide variety of animal and plant cells. However, in yeast cells, the ER seems to be less developed, forming only two distinct regions: the peripheral ER and the inner ER [6]. The peripheral ER, which can be found adjacent to the plasma membrane, is composed of a complex network of interconnected tubules within yeast cells [7]. On the other hand, the inner ER is an alias of the nuclear envelope, a structure that envelops chromosomal DNA, thus forming the nucleus. Therefore, it is reasonable to hypothesize that by expanding this organelle in yeast cells, the capacity of the yeast cells to generate valuable substances can be considerably enhanced.

Applying the Unfolded Protein Response (UPR) is a possible method for enhancing the size and function of the ER. ER stress, which is triggered by dysfunction or deficiency in ER function, is often accompanied by the accumulation of unfolded proteins in the ER. Eukaryotic cells commonly have the ability to provoke a protective response known as the Unfolded Protein Response

ISSN 2995-8067 DOI: 10.61927/igmin142

(UPR) to counteract ER stress and ensure the robustness of the ER [8].

The molecular mechanism regulating the UPR was initially elucidated through pioneering investigations using *S. cerevisiae* as an exemplary model organism [9]. Ire1, a transmembrane ribonuclease located in the ER, is evolutionarily conserved across various eukaryotic species and serves as a sensor of ER stress. In fungal species belonging to the phylum Ascomycetes, which includes *S. cerevisiae*, Ire1 facilitates the splicing of the *HAC1* gene transcript in response to ER stress (Figure 1). In *S. cerevisiae* cells, the unspliced variant of *HAC1* mRNA (referred to as *HAC1*u, with the letter u representing "uninduced") exhibits poor translational efficiency. Moreover, the Hac1u protein is extremely unstable. Conversely, the spliced form of *HAC1* mRNA (designated HAC1i, with the letter i representing "induced") undergoes translation to yield a transcription factor known as the Hac1i protein, which plays the main role in the transcriptional induction on the UPR (Figure 1).

Enhancement and enlargement of the *S. cerevisiae* ER by artificial induction of the UPR

Transcriptome analyses conducted by our research group and other researchers have provided evidence demonstrating that a number of genes are induced by the UPR in a manner that is controlled by Ire1 and *HAC1* in *S. cerevisiae* cells [10-12]. The Hac1i-target genes encompass a wide range of functional categories, including those involved in ER-located molecular chaperones and enzymes that facilitate the formation of cysteine disulfide bonds, N-linked sugar chains, and lipidic molecules. Consequently, when the Hac1i protein was expressed in an unregulated manner from a constitutive promoter, protein secretion increased, although the magnitude of this elevation was relatively modest, as demonstrated in the pioneering work of Valkonen, et al. [13]. More recently, Lin, et al. [14] reported that heterologous protein secretion from *S. cerevisiae* cells was elevated by some genetic mutations that led to an increase in cellular Hac1i protein abundance. The Hac1i protein also increased protein secretion from another yeast species *Pichia pastoris* [15,16]. Furthermore, some investigations have revealed that *S. cerevisiae* cells engineered to artificially express the Hac1i protein exhibit upregulation of the production of lipidic molecules (Figure 2A) [17,18]. It should also be noted that the ER in *S. cerevisiae* cells artificially expressing the Hac1i protein is highly expanded (Figure 2B) [18].

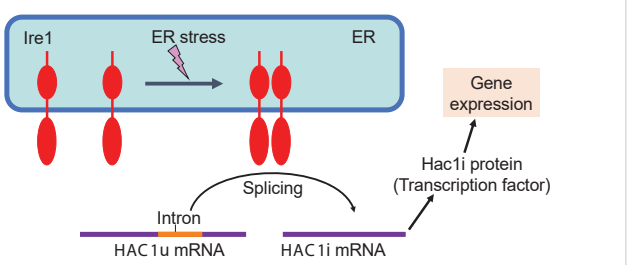

Figure 1: The UPR in *S. cerevisiae* cells
Under non-stress conditions, the *HAC1* mRNA (*HAC1*u) contains the intron sequence and is functionless. Upon ER stress, Ire1 is homo-associated to splice the *HAC1*u mRNA, yielding the *HAC1*i mRNA, which is translated to the Hac1i protein to induce gene expression for the UPR.

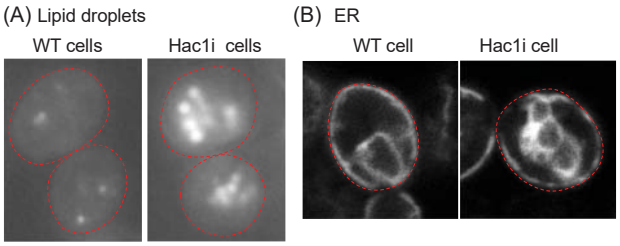

Figure 2: Fluorescence microscopic analysis of *S. cerevisiae* cells
Wild-type *S. cerevisiae* cells (BY4742 (*MATα, ura3, leu2, his3, lys2*): WT cell) and their derivative carrying the *HAC1*i expression plasmid (Hac1i cell) were cultured in yeast standard synthetic complete (SC) medium at 30 °C and fluorescence microscopically observed. To generate the *HAC1*i expression plasmid, the *HAC1*i gene was cloned into the Tet-off vector pCM190 [20], leading to inducible expression of the Hac1i protein upon culturing cells in the SC medium. (A) Cells were stained with BODIPY 493/503 to visualize neutral lipids. Hac1i cells carried larger and more abundant lipid droplets than WT cells. (B) The fluorescent ER marker protein, Elo2-mCherry, was expressed from a plasmid that had been created by insertion of the *ELO2* gene, which encodes an ER membrane protein Elo2, into the mCherry expression plasmid pYT-TDH3p-PMA1-mCherry [21]. The ER in Hac1i cells was more expanded than in WT cells. Red dashed lines represent the cell outline.

However, artificial and constitutive expression of the Hac1i protein has a significant inhibitory effect on the growth of *S. cerevisiae* cells, as demonstrated by studies conducted by Nguyen, et al. [18] and Mori, et al. [19]. This inhibitory effect leads to various undesired outcomes, such as a reduction and/or delay in the production of biomass, as well as the occurrence of fast-growing mutants with a low UPR. As a result of these issues, achieving stable growth of *S. cerevisiae* cells constitutively and highly producing the Hac1i protein has become a challenging task.

Nevertheless, in the case of Valkonen, et al. [13] and Qu, et al. [17], *S. cerevisiae* cells that continuously produced the Hac1i protein did not exhibit significant retardation of growth. In this particular scenario, it is postulated that the UPR was not potent enough to impede cell growth, presumably due to the low expression level of the Hac1i protein. Indeed, the level of expression of the prominent UPR target gene *KAR2*, as reported by Valkonen, et al. [13], was not as high as that documented by Nguyen, et al. [18], where the Hac1i protein was synthesized from the endogenous *HAC1* promoter. It is plausible that the growth retardation and the ER productivity resulting from the artificial expression of *HAC1i* are contradictory issues. In Valkonen, et al. [13], when expressed in *S. cerevisiae* cells, the Hac1i protein derived from *Trichoderma reesei* led to more severe growth retardation, greater UPR activation, and more secreted protein production than did the authentic *S. cerevisiae* Hac1i protein.

As a potential solution to this antinomic predicament, we previously developed a methodology wherein ER stress is induced in *S. cerevisiae* cells expressing the Hac1i protein [18]. Intriguingly, the retarded growth of *S. cerevisiae* cells expressing the Hac1i protein was partially ameliorated, albeit without any reduction in the UPR level, when these cells were grown in the presence of weak ER stress stimuli, such as low concentrations of tunicamycin. Using this new methodology, we successfully demonstrated considerable enhancement in the generation of lipidic molecules from *S. cerevisiae cells* [18]. Nonetheless, it should also be noted that this technique has a disadvantageous point as ER stress, even at a mild level, can be toxic. In fact, *S. cerevisiae* cells subjected to

mild ER stress exhibited a significant decrease in viability upon continuous culturing.

Conclusion and future perspective

The ER is a cellular compartment in which a number of materials, some of which are commercially valuable, are biosynthesized. As a biotechnological technique to increase the productivity of the ER, we and others enforced and enlarged the ER through artificial and constative evocation of the UPR, which, in nature, is a cytoprotective response induced upon ER stress in *S. cerevisiae* cells. One disadvantage of this methodology is that the unregulated UPR induced by the strong and constitutive Hac1i-protein expression severely retards cellular growth.

One possible solution to this problem is to express the Hac1i protein in a regulated manner using an inducible promoter system. Upon the growth phase to yield a large cell mass, the *S. cerevisiae* cells proliferate rapidly by repressing the Hac1i protein expression. Subsequently, the expression of the Hac1i protein is induced to increase the capacity of the ER to produce desired materials. Another intriguing approach is to identify genes responsible for growth retardation upon artificial induction of the UPR. We anticipate that we can generate rapid-growing and high-UPR yeast strains through genetic modifications to delete such genes.

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How to cite this article: Monguchi M, Kimata Y. Enforcement and Enlargement of the *Saccharomyces cerevisiae* Endoplasmic Reticulum through Artificial Evocation of the Unfolded Protein Response. IgMin Res. Jan 30, 2024; 2(1): 036-038. IgMin ID: igmin142; DOI: 10.61927/igmin142; Available at: www.igminresearch.com/articles/pdf/igmin142.pdf