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Article

Hog1 MAP Kinase Controls Early Riboflavin Overproduction Under Combined Acidic pH and Salinity in the Yeast *Debaryomyces hansenii*

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Abstract

Riboflavin (vitamin B2) is an essential precursor of flavin cofactors involved in redox metabolism, and its industrial production increasingly relies on microbial fermentation. *Debaryomyces hansenii* (previously as syn. *Candida famata*) is a halotolerant flavinogenic yeast previously exploited for riboflavin biosynthesis; however, its biotechnological application has been limited by genetic instability and poor understanding of its regulatory networks. Here, we uncover a novel role for the High Osmolarity Glycerol (HOG) pathway in riboflavin metabolism of *D. hansenii*. Using the first stable knockout mutant (*Dhhog1Δ*), we demonstrate that loss of *DhHog1* triggers early, premature, and enhanced secretion of riboflavin under acidic and saline conditions, visible as a yellow fluorescent pigment in the culture medium. Accelerated riboflavin accumulation in the mutant was accompanied by altered assimilation of phosphorus, sulfur, and magnesium, but not iron, suggesting that regulation extends beyond classical iron limitation. Gene expression analyses showed consistent up-regulation of *RIB1*, *RIB4*, and *RIB6* genes and derepression of the iron regulator *SEF1* in *Dhhog1Δ*, supporting a model where *DhHog1* negatively controls riboflavin biosynthesis through stress-responsive transcription factors and pseudo iron-starvation signaling. Our findings broaden the functional scope of the HOG pathway by linking osmotic stress adaptation with secondary metabolism and establish *DhHog1* as a key negative regulator of early riboflavin overproduction and secretion. This work provides new insights into yeast stress-metabolism crosstalk and highlights *D. hansenii* as a promising platform for metabolic engineering of industrial riboflavin production.

Keywords: *Debaryomyces hansenii*; riboflavin metabolism HOG pathway; *DhHog1*; flavinogenic yeasts; stress adaptation

1. Introduction

Debaryomyces hansenii (previously as syn. *Candida famata*) is an ascomycetous yeast of the subphylum Saccharomycotina, belonging to the CTG(Ser1) clade, a group of yeasts that predominantly translate the CTG codon as serine instead of leucine (Krassowski, et al., 2018; Ochoa-Gutiérrez et al., 2022; Prista et al., 2016). One of its most studied traits is halotolerance, as it can grow in media containing up to 4 M NaCl (Onishi, 1963), and is considered an extremotolerant yeast (González et al., 2025; Segal-Kischinevsky et al., 2022). This ability is largely mediated by the High Osmolarity Glycerol (HOG) pathway, a phosphorylation cascade that activates Hog1 MAP kinase (Bansal and Mondal, 2000; Sánchez et al., 2020). Phosphorylated Hog1 orchestrates adaptive responses, promoting glycerol accumulation and regulating osmoprotective genes (de la Fuente-Colmenares et al., 2024; Hohmann, 2002, 2009; Posas et al., 1996; Sánchez et al., 2020).

Compared with its close relative, *Candida albicans*, the production of stable mutants in *D. hansenii* has been hindered by a low occurrence of homology-directed repair of its DNA, as well as the scarcity of optimized genetic tools geared towards the modification of this specific yeast, due in part to the ambiguous translation of the CUG codon characteristic of the yeasts belonging to the CTG clade (Gomes et al., 2007; Minhas et al., 2009; Ochoa-Gutiérrez et al., 2022; Richard et al., 2005). The first stable null mutant, *Dhhog1Δ*, was developed for the further study of *D. hansenii*'s HOG pathway (Sánchez et al., 2020).

In addition to halotolerance, *D. hansenii* is a flavinogenic yeast, capable of synthesizing and secreting riboflavin (RF; vitamin B2) (Gadd and Edwards, 1986). RF is a precursor of the flavin coenzymes FAD and FMN, which are essential for multiple physiological processes, including redox homeostasis, protein folding, DNA repair, fatty acid β -oxidation, amino acid oxidation, and choline metabolism (Baron and Haylemon, 1995; Bray et al., 1964; Frerman, 1988; Mansoorabadi et al., 2007; Macheroux et al., 2011; Walsh & Wenciewicz, 2013). Unlike plants, fungi, and most prokaryotes, animals cannot synthesize riboflavin and must obtain it from their diet or, to a lesser extent, gut microbiota (LeBlanc et al., 2013; Nysten & Van Dijck, 2023). Therefore, industrial-scale production of riboflavin is of great importance. Biological synthesis has replaced chemical synthesis due to higher efficiency, reduced waste, lower energy requirements, and use of renewable substrates such as sugars or vegetable oils (Vandamme, 1992; Stahmann et al., 2000; Lim et al., 2001; Abbas & Sibirny, 2011; Schwechheimer et al., 2016). The global riboflavin market is projected to be valued at USD 508.6 million in 2025, with steady growth expected to reach USD 872.24 million by 2033, representing a compound annual growth rate (CAGR) of 6.98% during this period (Sharma, 2025).

The first report of *D. hansenii*'s flavinogenic capacity was made by Gadd and Edwards (1986), when they noticed a yellow pigment in the supernatant of iron-depleted media. Riboflavin (vitamin B2) is a yellow, water-soluble compound whose green fluorescence can be detected within 440/535 nm excitation/emission wavelengths. Since then, iron limitation has become one of the most commonly used conditions in flavinogenic media, among others such as exposure to copper, cobalt, zinc, chromium, and industrial protein-rich wastewaters (Fedorovich et al., 1999; Fedorovych et al., 2001; Seda-Miró et al., 2007).

The biosynthesis pathway of riboflavin has been elucidated in the *D. hansenii* synonym species *C. famata*. The pathway, which includes six *RIB* genes, involves sequential dephosphorylation and reduction reactions that convert GTP and ribulose-5-phosphate into riboflavin through several intermediates (**Figure 1**). Key enzymes include GTP cyclohydrolase II (Rib1), DAcrPP deaminase (Rib2), 6,7-dimethyl-8-ribityllumazine synthase (Rib4), riboflavin synthase (Rib5), 4-dihydroxy-2-butanone-4-phosphate synthase (Rib6), and 5-amino-6-(5-phosphoribosylamino) uracil reductase (Rib7) (Bacher et al., 2001; Voronovsky et al., 2004). In *C. famata*, the overexpression of *RIB1* and *RIB6* has proven effective for the overproduction of riboflavin (Petrovska et al., 2022).

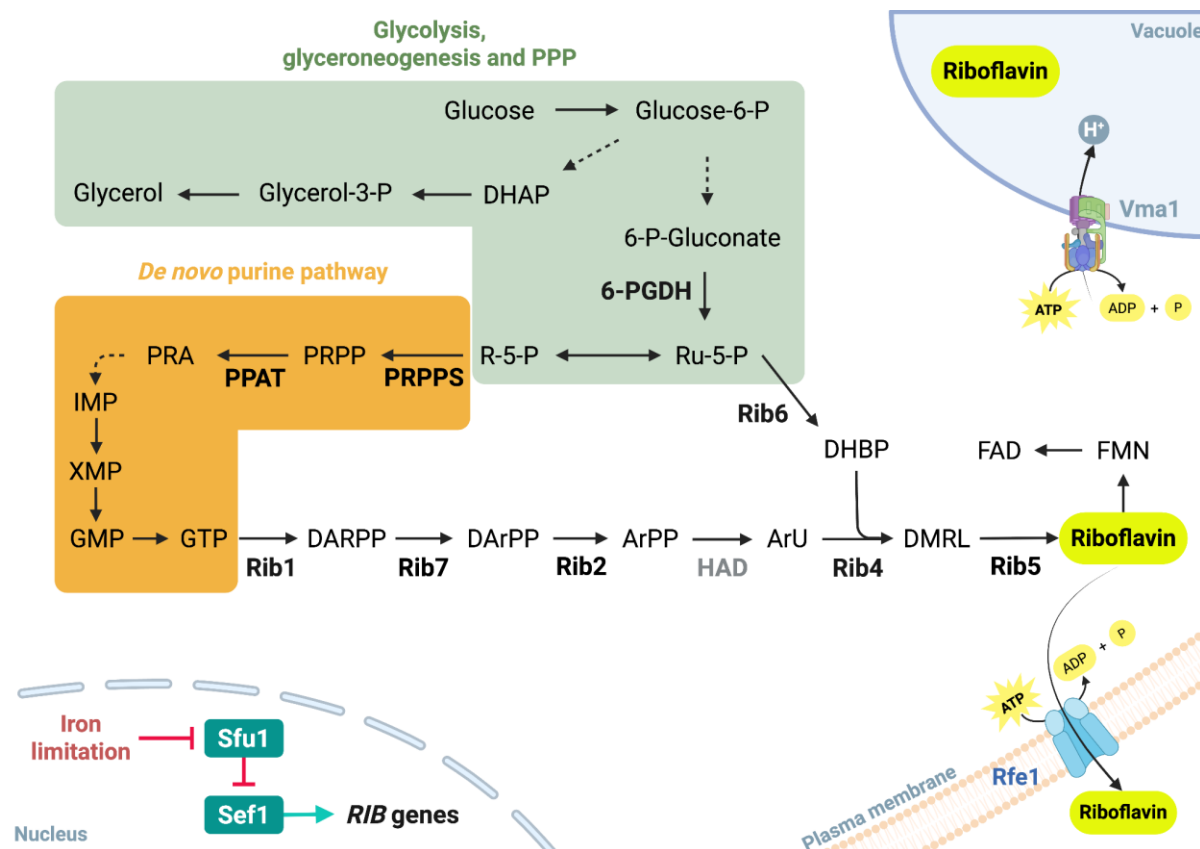


Figure 1. Simplified schematic of the riboflavin biosynthetic pathway in flavinogenic yeasts under iron limitation. The pathway is fueled by glycolysis and pentose phosphate pathway precursors (green square: 6-PGDH, 6-phosphogluconate dehydrogenase; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate). *PRS3* encodes 5-phosphoribosyl-1-pyrophosphate synthetase (PRPPS), responsible for PRPP (5-phosphoribosyl-1-pyrophosphate) production. The *ADE4* gene encodes 5-phosphoribosyl-1-pyrophosphate amidotransferase (PPAT), a key enzyme in purine biosynthesis that catalyzes the first step of the *de novo* pathway. Purine-derived precursors (orange square: PRPP, 5-phosphoribosyl-1-pyrophosphate; PRD, 5-phosphoribosylamine; IMP, inosine monophosphate; XMP, xanthosine monophosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate). Key genes/enzymes of riboflavin pathway include *RIB1*/Rib1 (GTP cyclohydrolase II), *RIB2*/Rib2 (DARPP deaminase), a putative haloacid dehalogenase (HAD), *RIB4*/Rib4 (dimethylribityllumazine synthase), *RIB5*/Rib5 (riboflavin synthase), *RIB6*/Rib6 (4-dihydroxy-2-butanone-4-phosphate synthase), and *RIB7*/Rib7 (5-amino-6-(5-phosphoribosylamino) uracil reductase). The intermediates in the riboflavin pathway include DARPP (2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate), DARPP (5-amino-6-(D-ribitylamino)uracil 5'-phosphate), ArPP (5-amino-6-ribityl-amino-2,4(1H,3H)pyrimidinedione 5'-phosphate), ArU (5-amino-6-(D-ribitylamino)uracil), DHBP (3,4-dihydroxy-2-butanone-4-phosphate), DMRL (6,7-dimethyl-8-ribityllumazine), FMN (flavin mononucleotide), and FAD (flavin adenine dinucleotide). In the vacuole, riboflavin can be stored through a process dependent on Vma1, the vacuolar ATPase subunit A. Riboflavin can also be secreted via Rfe1, a riboflavin excretase. Under iron homeostasis conditions, the GATA-type transcription factor Sfu1 prevents iron toxicity in iron-replete medium by inhibiting Sef1, a transcription factor that activates iron acquisition genes, including *RIB* genes. Blue arrows indicate positive regulation; red blunt arrows indicate negative regulation. Image based on findings reported in the literature (Dmytruk et al., 1999; Voronovsky et al., 2002, 2004; Haase et al., 2013; Sarge et al., 2015; Sa et al., 2016; Ruchala et al., 2022; 2025). Created using BioRender.com, accessed on 29 August 2025.

Unlike *S. cerevisiae*, yeasts belonging to the CTG clade possess a negative feedback loop involved in both iron and riboflavin metabolism. Sef1 is a transcriptional activator of iron acquisition and riboflavin biosynthesis genes that remains repressed by Sfu1 in iron-replete media (Andreieva et al., 2020a, 2020b; Chen et al., 2011; Demuyser et al., 2020).

Iron limitation has been framed as a necessary condition to induce the excretion of riboflavin in most yeasts (Ruchala et al., 2025). In *Ashbya gossypii*, the induction of flavinogenesis has been linked to oxidative stress, UV-light exposure, sporulation, and endoplasmic reticulum stress (Kurabayashi et al., 2025; Nieland and Stahmann, 2013; Ruchala et al., 2025; Silva et al., 2018; Stahmann et al., 2001). In other yeasts, including *Candida albicans*, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae*, Hog1 has been identified as a negative regulator of iron assimilation (Kaba et al., 2013; Lee et al., 2014; Martins et al., 2018). However, a direct connection between Hog1 signaling and riboflavin biosynthesis has not yet been established.

This study aims to elucidate the role of *DhHog1* in riboflavin metabolism under saline stress, integrating genetic, biochemical, and physiological approaches to uncover the interplay between osmotic stress signaling and flavinogenesis in *D. hansenii*.

2. Materials and Methods

2.1. Strains and growth conditions

Precultures of *D. hansenii* wild-type strain CBS767 (WT) and the isogenic mutant *Dhhog1Δ* (generated by Sánchez et al., 2020) were grown and maintained on Yeast Peptone Dextrose agar (YPD agar; 1% yeast extract, 2% peptone, 2% glucose, and 2% agar). Liquid precultures were prepared in YPD medium using a flask-to-medium ratio of 2:5, incubated at 28 °C with shaking at 180 rpm overnight, representing the seed condition (time 0 h). Cells were recovered by centrifugation at 1,100 × g for 5 min, washed twice, and resuspended in sterile distilled water. Subsequently, cultures were grown in a minimal medium based on Yeast Nitrogen Base (YNB) supplemented with 2% glucose, 0.5% ammonium sulfate as the sole nitrogen source, and 0.6 M NaCl (from now on referred to as MM + 0.6 M NaCl).

2.2. Growth curves and pH measurements

Washed preculture cells were used as an inoculum for the minimum medium at an initial optical density of 0.05 (measured at 600 nm, OD_{600nm}). Successive OD_{600nm} measurements were taken using a Beckman Coulter DU® 640 spectrophotometer. For the flavinogenic condition (MM + 0.6 M NaCl, initial pH 4.3), the exponential growth phase (Log) was defined at OD_{600nm} = 0.5 (18–20 h), whereas the stationary growth phase (Stat) was defined at OD_{600nm} = 3.8–4.0 (48 h).

For pH experiments, the basal pH of the minimal medium YNB (initially 4.3) was adjusted to 6.8–7.0 using 2 N NaOH. pH measurements were carried out at defined time intervals with a potentiometer (pH 210, Microprocessor pH Meter, Hanna Instruments), previously calibrated according to the manufacturer's instructions.

2.3. Riboflavin measurements

Extracellular riboflavin was quantified by measuring fluorescence intensity at emission/excitation wavelengths of 440/535 nm. A riboflavin stock solution (20.1 mg/L) was prepared by dissolving riboflavin powder in distilled water under mild heating (< 30 °C). Culture aliquots (15 mL) were centrifuged, and supernatant was filtered through 0.22 µm pore-size filters. Subsequently, 200 µL of the filtered supernatants were transferred to black 96-well Costar® assay plates with clear flat bottoms, and fluorescence was measured with a Synergy H1 Microplate Reader (Biotek), gain = 50. Riboflavin concentrations were determined by interpolation against a standard curve generated from 1:2 serial dilutions of an initial 10.05 mg/L riboflavin solution.

2.4. Riboflavin extraction and RP-HPLC-DAD analysis

Sterile culture supernatants were lyophilized to concentrate the fluorescent compound and remove water. The resulting dried powder was extracted with 50 mL HPLC-grade methanol and vortexed for 5 min at room temperature (25 ± 1.0 °C). Extracts were centrifuged at 3500 rpm for 10

min, and the methanolic phase was recovered and dried at 35 °C for 72 h. Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a Diode Array Detector (DAD), was then carried out, following the method described by Jiménez-Nava et al. (2023). Methanolic residues were resuspended in 10 mL HPLC-grade water, and 2 mL aliquots were loaded in triplicate onto C18 solid-phase extraction (SPE) cartridges (Alltech® Maxi-Clean™, Thermo Fisher Scientific, Waltham, MA, USA). Cartridges were eluted under vacuum with 1.2 mL of HCl-acidified methanol (0.002% v/v). Eluates were analyzed using an Agilent 1260 Infinity Series system equipped with a DAD, monitoring 280 and 440 nm, with a Zorbax SB-Phenyl column (150 mm × 4.6 mm, 5 µm, Agilent Technologies, Santa Clara, CA, USA). The mobile phase was acetonitrile and 0.05% phosphoric acid in water at 0.5 mL/min, applying a linear gradient of 10-100% acetonitrile over 15 min.

Absorption spectra of the HPLC-separated fractions were obtained directly from DAD data and visualized with Agilent ChemStation software, using a riboflavin standard (Sigma-Aldrich, Cat. No. 47861) for reference.

2.5. Element analysis

Supernatants were obtained by centrifuging cultured cells at 18 and 48 h, and 15 mL were collected for analysis. A total of 43 elements and 1 compound were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), including Ag, Al, Ar, B, Ba, Be, Bi, Br, Ca, Cd, Cl, Co, Cr, Cs, Cu, F, Fe, Ga, Hg, I, In, K, La, Li, Mg, Mn, Mo, Na, Ni, P, Pb, PO₄, S, Sb, Sc, Se, Si, Sn, Sr, Te, Ti, V, W, and Zn. Quantification was performed with the proprietary Triton ICP-OES Test, a validated and patented protocol of TRITON Applied Reef Bioscience (<https://www.triton-lab.de/en/icp-oes>).

2.6. RNA extraction

Total RNA was extracted following the protocol described by Schmitt et al. (1990). Cultures were first grown overnight in rich medium (YPD) to obtain seed cultures (time 0 h), and subsequently inoculated into minimal medium with 0.6 M NaCl. Cells were harvested at exponential phase and stationary phase.

Briefly, cells were washed twice, centrifuged at 1,100 × g, and mechanically disrupted using a vortex mixer with sterile glass microbeads (425–600 µm) previously incubated in phenol (pH 4.5). Samples were incubated at 65 °C for 5 min and vortexed twice for 30 s. The mixture was chilled and centrifuged to separate the aqueous and phenolic phases. The aqueous phase was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). RNA was precipitated by adding 1/10 volumes of sodium acetate (3 M, pH 5.2) and 2.5 volumes of absolute ethanol, followed by incubation at –20 °C for 30 min and centrifugation at 16,000 × g. The resulting pellet was washed, dried, and resuspended in RNase-free water. RNA integrity was assessed by electrophoresis on a 1% denaturing agarose gel.

2.7. Identification of riboflavin biosynthesis genes

The *D. hansenii* genome remains only partially annotated, with most gene assignments based on *in silico* predictions of orthologous genes. To validate these annotations, multiple sequence alignments of selected riboflavin biosynthesis proteins and the transcription factor Sef1 were carried out against their orthologous in *Saccharomyces cerevisiae* and *Candida albicans* (**Supplementary Figures S1-S14**). Amino acid sequences were further compared to confirm that the predicted ORFs corresponded to the expected protein functions (*RIB1*, locus DEHA2A12870g; *RIB2*, locus DEHA2E11374g, *RIB4*, locus DEHA2D04180g; *RIB5*, locus DEHA2D13926g; *RIB6*, locus DEHA2G09504g; *RIB7*, locus DEHA2G10010g; *SEF1*, locus DEHA2C16676g). Protein identifiers (IDs) were retrieved from the NCBI Protein Database. Both analyses were performed within the NCBI BlastP Tool.

2.8. In silico predictions of transcription factor binding sites

The intergenic region spanning from –625 bp upstream to the start codon of each gene was scanned with position-specific scoring matrices (PSSMs) using the Regulatory Sequence Analysis Tools (RSAT) platform (<https://rsat.france-bioinformatique.fr/fungi/>), employing the full matrix-scan tool within the Fungi Server (Turatsinze et al., 2008). Binding motifs for Hog1-regulated transcription factors were identified, including Sef1 (ID: 2900038, locus DEHA2C16676g), Sko1 (ID: 2901375, locus DEHA2D09196g), Skn7 (ID: 2913769, locus DEHA2B08052g), Msn2/4 (ID: 2899670, locus DEHA2A08382g), and Yap1 (ID: 2904514, locus DEHA2G02420g).

Except for Sef1, motif matrices were retrieved from the JASPAR database, specifically from the 2024 core fungi collection: Sko1 (ID: MA0382.1), Skn7 (ID: MA0381.1), Msn2/4 (ID: MA0341.1/MA0342.1), and Yap1 (ID: MA0415.1). Putative Sef1 binding sites were searched manually in each intergenic region based on the DNA recognition motifs identified by Chen et al. (2011) for *C. albicans* Sef1. A *D. hansenii*-specific background model estimation method with a Markov order of 1 was applied, and a significance threshold of *p*-values < 0.0006 was used for significant determination.

2.9. Analysis of gene expression

Total RNA was treated with DNaseI (RQ1 RNase-Free DNase, Promega) to eliminate contaminating genomic DNA. cDNA synthesis reactions were performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, United States) with oligo(dt) primers, following the manufacturer’s recommendations.

Quantitative PCR (RT-qPCR) was performed using the standard curve method with gene-specific deoxyoligonucleotides designed for the genes encoding Hog1 MAPK (*DhHOG1*), sugar transporter-like protein (*DhSTL1*), the transcriptional activator Sef1 (*DhSEF1*), GTP cyclohydrolase II (*DhRIB1*), DArPP deaminase (*DhRIB2*), 6,7-dimethyl-8-ribityllumazine synthase (*DhRIB4*), riboflavin synthase (*DhRIB5*), 4-dihydroxy-2-butanone-4-phosphate synthase (*DhRIB6*), 5-amino-6-(5-phosphoribosyl-amino) uracil reductase (*DhRIB7*). The actin gene (*DhACT1*) was used as the housekeeping control.

Deoxyoligonucleotides were evaluated to ensure the absence of dimer formation and cross-hybridization, and only deoxyoligonucleotides pairs with amplification efficiencies >90% were used (Table 1). RT-qPCR was performed with a Rotor-Gene Q thermocycler (Qiagen) using SYBR Green as the detection dye (KAPA SYBR Fast Kit, Roche). Cycling conditions were: 94 °C for 5 min (1 cycle), followed by 35 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s.

Relative transcript levels were normalized to the WT mean Ct for each gene using the 2^{–ΔΔCt} method, and data were expressed as fold change values. Results represent mean ± SD from three biological replicates, each analyzed in duplicate (technical replicates).

Table 1. Deoxyoligonucleotides used for RT-qPCR relative gene expression analysis.

Gene	ID, ORF	Fw 5′ → 3′	Rv 5′ → 3′
<i>DhACT1</i>	2901278, DEHA2D05412g	CCCAGAAGAACACCCAGTTT	CGGCTTGGATAGAAACGTAGA A
<i>DhRIB1</i>	2899385, DEHA2A12870g	AAGACACCCTGCTGATGGTC	TGTCGGGGTTGTTGGTCAAT
<i>DhRIB2</i>	2902834, DEHA2E11374g	TGGAACCATGCTCCTTGAGA TT	CTGGCTCCACAACACCAACA

<i>DhRIB4</i>	2901083, DEHA2D04180g	TGTTTGACCGATGAGCAAGC	ACACATTTTCGACAGCAGCAG
<i>DhRIB5</i>	2901307, DEHA2D13926g	GCCTGGGTGTAAGTACCAT	GGAGAAGGGGTTTCATTGCCA
<i>DhRIB6</i>	2904849, DEHA2G09504g	TGGTCTTATGAAGTCTACCG GC	TATGCTGATGGCACGACCAC
<i>DhRIB7</i>	2904875, DEHA2G10010g	ACTTGACCTCCTTCAACCA T	GGTGCATTTGTCAGGCTTCC
<i>DhSEF1</i>	2900038, DEHA2C16676g	CCGTTTGCTTCGACCCTTTA	CTGCCAACAATGCTACCGTG
<i>DhSTL1</i>	2902951, DEHA2E01364g	TGGGAATGGCTGACACTTAT G	GCTCTTCTACCCAACCTATCAA TC
<i>DhHOG1</i>	2902985, DEHA2E20944g	AACCGCTCGCTGAATGGAAT	TCTCCACCTCCAGACGTGAT

2.10. Statistical analysis

For all *in vitro* experiments, statistical significance was evaluated using Student’s *t*-test in GraphPad Prism 10.2.3 (GraphPad Software Inc.). A *p*-value < 0.05 was considered statistically significant. For *in silico* predictions of transcription factor binding sites (TFBS), a significance threshold of *p*-values ≤ 0.0006 was considered for the selection of riboflavin-related genes.

3. Results

3.1. Initial low pH induces the excretion and accumulation of riboflavin in *D. hansenii*

The high-osmolarity glycerol (HOG) pathway, with the MAP kinase Hog1 as its terminal effector under salt stress, is recognized as the most conserved signaling cascade for stress adaptation in fungi (de Nadal et al., 2002; Hohmann, 2002, 2009; Hohmann et al., 2007; Saito and Posas, 2012). Given previous observations of riboflavin efflux in acidic media (Perl et al., 1976; Vanetti and Aquarone, 1992), we investigated whether the initial medium pH modulates growth and riboflavin excretion in *D. hansenii* under saline stress, raising the possibility that *DhHog1* signaling may also influence riboflavin metabolism. Comparative assays were conducted between the WT strain and the *Dhhog1Δ* mutant in minimal medium (YNB) with glucose (2%) supplemented with 0.6 M NaCl under either neutral or acidic initial pH conditions. Significant differences in biomass and cell viability were observed during the stationary phase (**Figure 2A–B**), highlighting the role of *DhHog1* in maintaining cellular fitness under osmotic stress.

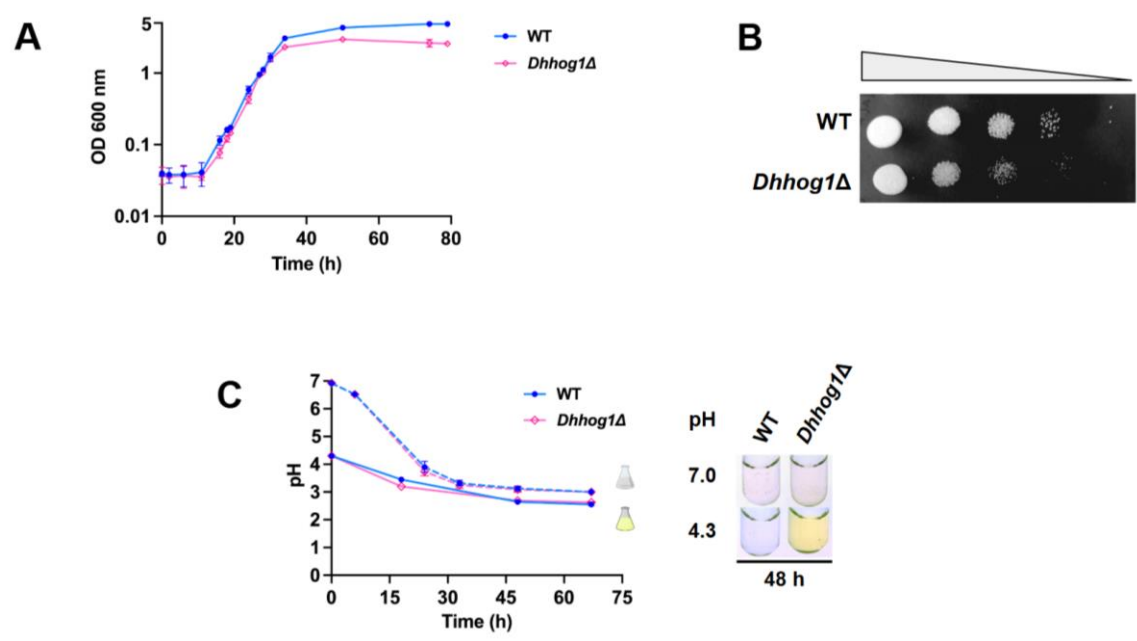


Figure 2. Initial low pH induces the secretion and accumulation of a yellow pigment in the culture supernatant of *D. hansenii*. (A) Growth curves of wild type (WT, blue) and the *Dhhog1Δ* mutant (pink) were determined in MM + 0.6 M NaCl at an initial low pH (pH 4.3). (B) Cells of WT and the *Dhhog1Δ* mutant were grown in MM + 0.6 M NaCl to the stationary phase (74 h), followed by 10-fold serial dilutions (up to 10⁻⁴). A 10 μ L aliquot of each dilution was spotted onto YPD agar plates containing 0.6 M NaCl and incubated at 28 $^{\circ}$ C for 3 days. (C) The pH of cultures grown in MM supplemented with 0.6 M NaCl, initiated under neutral (pH 6.8–7.0, dotted lines) and acidic (pH 4.3, solid lines) conditions, was monitored over a 70 h period. Representative images from three independent experiments are shown. Data represent the mean $\pm$ standard deviation (SD) of three independent experiments (n = 3).

Remarkably, cultures initiated at low pH exhibited secretion of a yellow pigment as early as the stationary phase in the *Dhhog1Δ* mutant (48 h) (**Figure 2C**), whereas in the WT strain, pigment accumulation became evident only at the late stationary phase (122–172 h) (**Figure 4A**). By contrast, no pigment secretion was detected in either strain when cultures were initiated at neutral pH (**Figure 2C**), even after 5 days of growth. These findings indicate that initial acidic conditions are a key determinant for pigment production and excretion in *D. hansenii*, with an accelerated onset in the absence of *HOG1*.

Previous studies have reported that *D. hansenii* is capable of producing riboflavin, a metabolite responsible for the characteristic yellow coloration (Gadd and Edwards, 1986, Seda-Miró et al., 2007). Based on this evidence, we hypothesized that the yellow pigment observed under our culture conditions could correspond to riboflavin. To test this, we identify the pigment by RP-HPLC-DAD analyses in the culture supernatants from the *Dhhog1Δ* mutant, revealing the presence of a major fluorescent compound. Retention times closely matched those of a riboflavin standard (8.450 ± 0.003 min). The *Dhhog1Δ* mutant exhibited clearer chromatographic separation and UV-Vis absorption peaks characteristic of flavins ($\lambda_{\text{max}} = 223, 268, 370, 445$ nm), consistent with riboflavin identity. In contrast, WT supernatants displayed weaker signals with partially overlapping absorption peaks. Representative chromatograms and UV-Vis spectra are shown in **Figure 3A–F**.

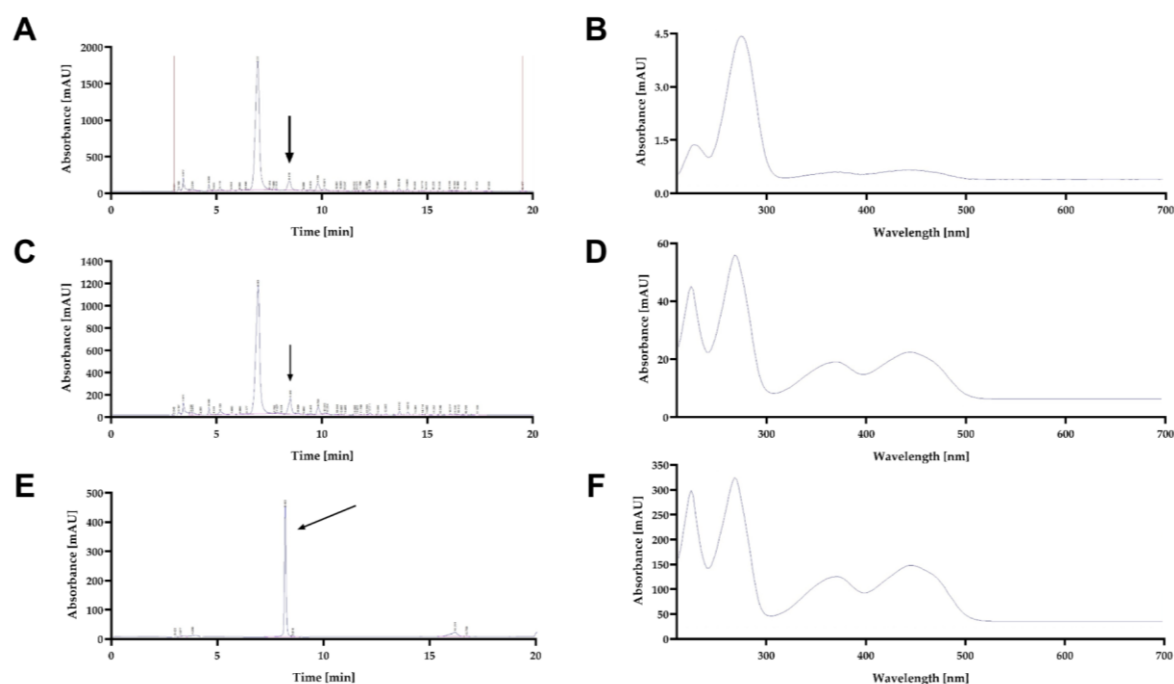


Figure 3. RP-HPLC-DAD analysis and UV-Vis spectra of riboflavin in *D. hansenii* WT and *Dhhog1Δ*. Chromatograms at 280 nm obtained by RP-HPLC-DAD for WT (A), *Dhhog1Δ* (C), and riboflavin standard (E), with arrows indicating the HPLC-separated fraction whose UV-Vis spectra are shown in panels B, D, and F. Samples were collected during stationary phase from strains grown in MM + 0.6 M NaCl at initial pH 4.3. Riboflavin accumulation was higher in the *Dhhog1Δ* mutant compared with WT. Values represent the mean of at least three independent measurements.

Furthermore, we examined the fluorescence properties of the riboflavin by recording excitation and emission spectra at the characteristic wavelengths (excitation: 440 nm; emission: 520–535 nm). Supernatants were collected at multiple time points (24–122 h, **Figure 4A**), and riboflavin concentrations were quantified by fluorimetry using a standard calibration curve (**Figure 4B–D**). These experiments confirm that the accumulation of riboflavin in the supernatant of cultures initiated at low pH, accumulates more rapidly over time in the *Dhhog1Δ* mutant. This observation indicates, for the first time, that *DhHog1* likely plays a role in the riboflavin biosynthesis in *D. hansenii*.

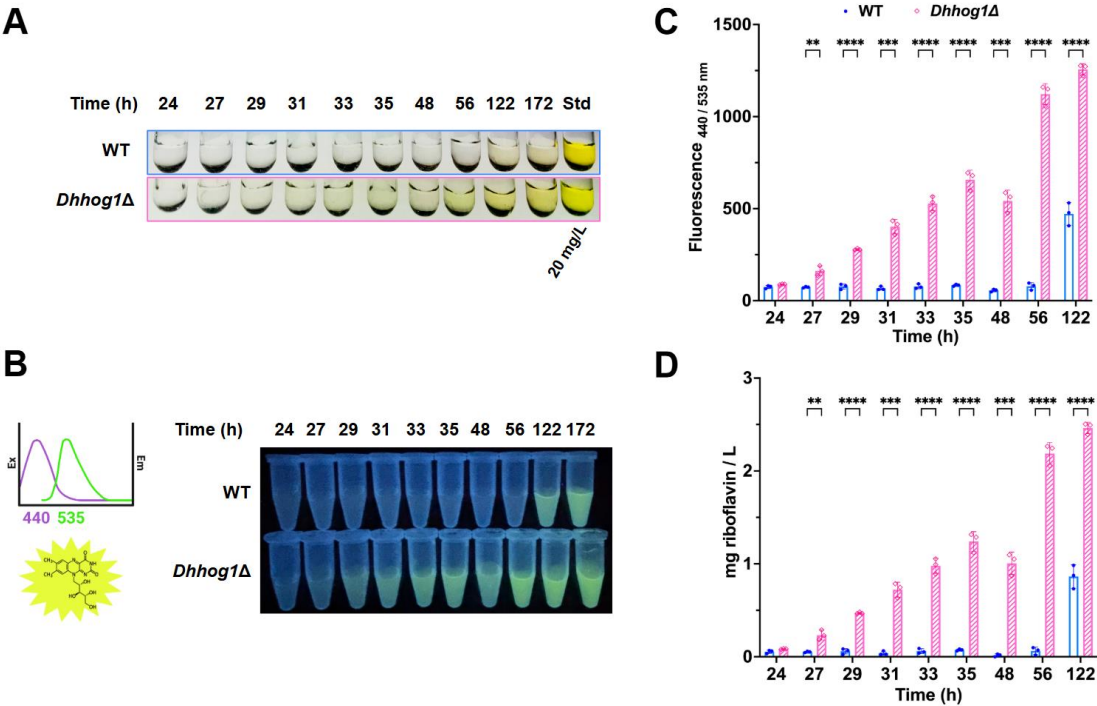


Figure 4. Quantification of riboflavin in the culture supernatant of *D. hansenii*. (A) A yellow pigment was observed in the supernatants from the WT and *Dhhog1Δ* cultures; the color appeared earlier in the culture time in the *Dhhog1Δ* strain. (B) Samples were collected at defined time points (24–172 h) from WT and *Dhhog1Δ* strains grown in MM + 0.6 M NaCl at an initial low pH (4.3) and excited with a UV light transilluminator. (C–D) Quantification was performed by measuring fluorescence (440/535 nm) and comparing with a calibration curve generated from a riboflavin standard in a 96-well spectrofluorometer. Data represent the mean ± standard deviation (SD) of three independent experiments (n = 3). Significant differences: *p*-value ≤ 0.05 (*), ≤ 0.005 (**), ≤ 0.0005 (***), ≤ 0.00005 (****).

3.2. Accelerated uptake of essential elements in *Dhhog1Δ* cells

Iron limitation and the availability of other essential elements have been reported as key triggers for riboflavin excretion in riboflavin-producing yeasts (Averianova et al., 2020; Ruchala et al., 2025). To determine whether differences in elemental uptake contribute to the enhanced riboflavin accumulation observed in the *Dhhog1Δ* mutant, culture supernatants from 18 and 48 h under saline conditions and initial low pH were analyzed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Figure 5).

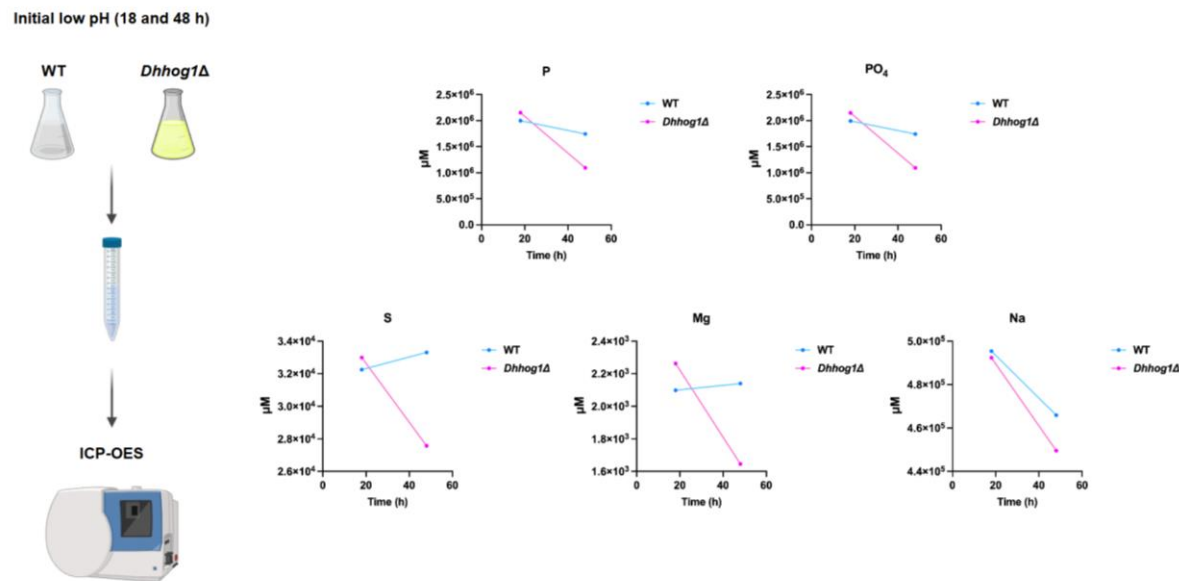


Figure 5. Identification and quantification of macro nutrients and trace elements in the culture supernatant of *D. hansenii* WT and *Dhhog1Δ* strains. Supernatants were collected from WT and *Dhhog1Δ* cultures grown in MM + 0.6 M NaCl at an initial low pH (4.3) at 18 and 48 h, and analyzed by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). A total of 43 elements (plus phosphate) were measured; the profiles of phosphorus (P), phosphates (PO₄), sulfur (S), magnesium (Mg), and sodium (Na), are shown. Quantification was performed using the Triton ICP-OES Test.

By 48 h, the *Dhhog1Δ* mutant displayed more rapid assimilation of several essential elements, including phosphorus, phosphates, sulfur, and magnesium, compared to the WT strain. In contrast, uptake of iron and other elements was similar in both strains (Table S1), indicating that mechanisms other than iron limitation are likely responsible for stimulating riboflavin excretion in *D. hansenii*. These results suggest that accelerated assimilation of specific elements (P, S, Mg, and Na) in the *Dhhog1Δ* mutant may contribute to the earlier and higher riboflavin production observed under acidic and saline conditions.

3.3. Riboflavin biosynthesis genes are upregulated in the *Dhhog1Δ* mutant

The riboflavin biosynthesis genes *RIB1*, *RIB2*, *RIB5*, *RIB6*, and *RIB7* were previously cloned from the synonymous species *Candida famata* (Dmytruk et al., 1999; Voronovsky et al., 2002, 2004), and their sequences are available in NCBI GenBank as part of the Genolevures Consortium’s annotated reference genome for *D. hansenii* (ASM644v2). The *RIB4* gene sequence was inferred from its homologs in *S. cerevisiae* and *C. albicans* and incorporated into the Genolevures Consortium reference genome (Morgunova et al., 2007; Skrzypek et al., 2017). Amino acid sequence alignments confirmed the identity of the main riboflavin biosynthetic enzymes and revealed the degree of sequence conservation among them (Table 2).

Table 2. Sequence similarity and query coverage of selected *D. hansenii* riboflavin biosynthesis proteins compared with *S. cerevisiae* and *C. albicans*.

Protein name, Identifier	<i>S. cerevisiae</i>		<i>C. albicans</i>	
	Similarity (%)	Query cover (%)	Similarity (%)	Query cover (%)

<i>DhRib1</i> , DEHA2A12870p	77	80	85	92
<i>DhRib2</i> , DEHA2E11374p	73	86	80	100
<i>DhRib4</i> , DEHA2D04180p	83	99	95	100
<i>DhRib5</i> , DEHA2D13926p	73	98	82	100
<i>DhRib6</i> , DEHA2G09504p	73	98	88	100
<i>DhRib7</i> , DEHA2G10010p	62	100	65	100
<i>DhSef1</i> , DEHA2C16676p	67	76	70	100

To investigate the potential regulation of riboflavin biosynthesis genes by *DhHog1* in *D. hansenii*, we performed an *in silico* analysis to identify putative stress-related transcription factor (TF) binding sites within the promoters of *RIB* genes and the transcription factor *SEF1* (**Figure 6A**). Previous work by de la Fuente-Colmenares validated the likely conservation of function *in silico* of Sko1, Skn7, Msn2/4, and Yap1 proteins in *D. hansenii* by comparing their sequences with homologs from *S. cerevisiae* and *C. albicans*, revealing conserved or semi-conserved residues within critical domains (de la Fuente-Colmenares et al., 2024). This suggests that these TFs perform similar regulatory roles across these yeast species.

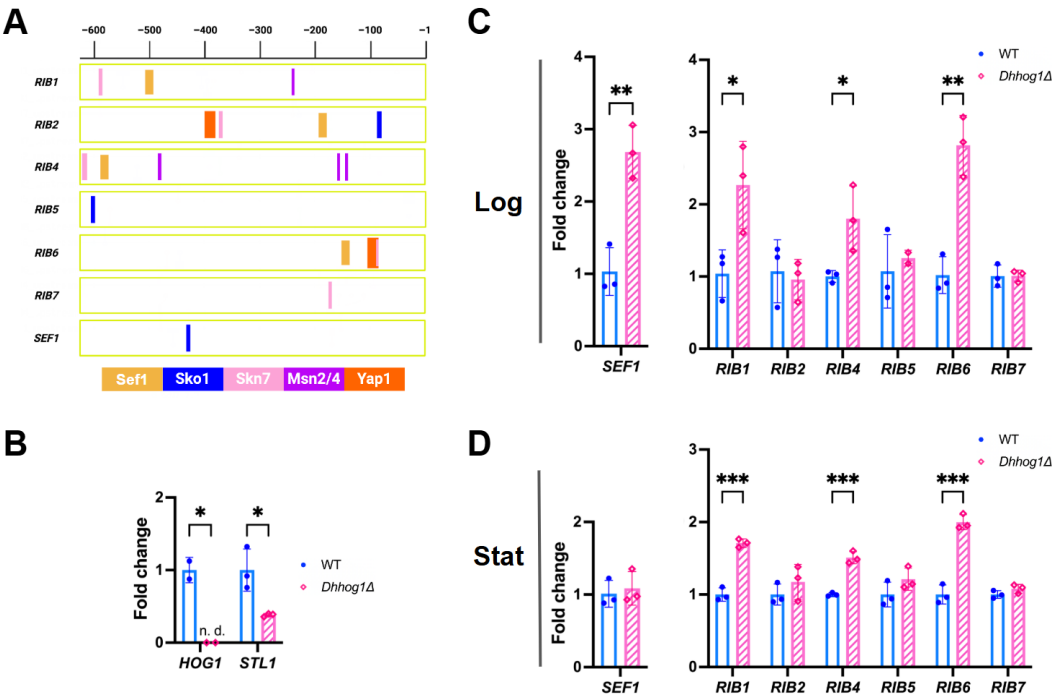


Figure 6. Predicted stress-related transcription factor binding sites in the promoter regions of riboflavin biosynthesis genes and corresponding gene expression in WT and *Dhhog1Δ* strains. (A) Schematic representation of intergenic region from -625 bp upstream to the +1 ATG; predicted motifs are highlighted for *SEF1* (yellow), *SKO1* (blue), *SKN7* (pink), *MSN2/4* (purple), and *YAP1* (orange). (B) As an initial control, *HOG1* and *STL1* expression were detected in WT (blue) and *Dhhog1Δ* (pink) strains. As expected, *HOG1* expression was not detected (n.d.) in the mutant, whereas partial *STL1* expression was observed, consistent with its role as a positive regulator previously described (Sánchez et al., 2020). (C–D) Gene expression analysis of *SEF1*, *RIB1*, *RIB2*, *RIB4*, *RIB6*, and *RIB7* under MM + 0.6 M NaCl at an initial low pH in WT and *Dhhog1Δ* strains. Total RNA was extracted from WT and mutant cells during the mid-log phase (Log, 18 h) and stationary phase (Stat, 48 h) and analyzed by RT-qPCR. Bars represent fold changes in gene expression, with transcript levels normalized to *ACT1*. Values are presented as the mean of at least three independent measurements ± (SD). Significant differences: p -value ≤ 0.05 (*), ≤ 0.005 (**), ≤ 0.0005 (***).

Our intergenic region analysis revealed that the *RIB* gene promoters contain binding motifs for Hog1-controlled transcription factors Sko1, Skn7, Msn2/4, and Yap1, as well as for the iron metabolism regulator Sef1 (**Figure 6A**). Specifically, *SEF1*-binding sequences were found in the promoters of *RIB1*, *RIB2*, *RIB4*, and *RIB6*, while *SKO1* motifs were present in *RIB2*, *RIB5*, and *SEF1* promoters. *SKN7*-binding sequences were detected in *RIB1*, *RIB2*, *RIB4*, and *RIB7*. *MSN2/4* motifs in *RIB1* and *RIB4*, and *YAP1*-binding sites in *RIB2* and *RIB6*. It is worth noting that *RIB5* and *SEF1* each contain a single *SKO1*-binding site, whereas *RIB7* harbors a single *SKN7*-binding site, suggesting that their transcription may be predominantly regulated by Sko1 or Skn7.

The absence of *HOG1* expression was confirmed in the *Dhhog1Δ* mutant, accompanied by reduced expression of *STL1*, a downstream *HOG* pathway gene involved in glycerol symport (**Figure 6B**). Expression profiles further reveal that *DhHog1* acts as a negative regulator of *SEF1*, *RIB1*, *RIB4*, and *RIB6* genes during the logarithmic phase (Log), with upregulation of *RIB1*, *RIB4*, and *RIB6* genes persisting into the stationary phase (Stat) in the *Dhhog1Δ* mutant (**Figure 6C–D**). These findings suggest a model in which *DhHog1* negatively modulates riboflavin biosynthesis in *D. hansenii*, likely through the coordinated control of stress-responsive transcription factors such as Sko1, Skn7, Msn2/4, Yap1, and together with the iron metabolism regulator Sef1.

4. Discussion

The results of this study broaden the functional scope of the *HOG* pathway in *Debaryomyces hansenii*, revealing that the MAP kinase *DhHog1* participates not only in classical osmoadaptation but also in the regulation of riboflavin biosynthesis. Previous work has established *DhHog1* as a central effector of the high-osmolarity glycerol response, orchestrating glycerol accumulation and catalase expression in response to saline and oxidative stress (de la Fuente-Colmenares et al., 2024; Sánchez et al., 2020). Our findings add a new layer to this paradigm by showing that *DhHog1* acts as a negative regulator of riboflavin secretion, delaying its onset until stationary phase. The premature yellow pigment accumulation observed in the *Dhhog1Δ* mutant exposed to NaCl under acidic conditions suggests that *DhHog1* integrates extracellular initial pH and osmotic cues that prevent riboflavin overproduction during active growth. This role highlights the versatility of the *HOG* pathway as a global integrator of environmental signals beyond osmostress.

4.1. Conserved riboflavinogenesis in *Debaryomyces* species and potential repression by *DhHog1*

The identification of riboflavin as the fluorescent compound is consistent with previous reports of riboflavin production by *D. hansenii* and closely related species, including *D. fabryi*, *D. subglobosus*, and *D. prosopidis* (Averianova et al., 2020; Nguyen et al., 2009; Ruchala et al., 2025). Historically, taxonomic heterogeneity in the *Debaryomyces* clade complicated the assignment of riboflavin-producing strains, as exemplified by the reclassification of *Candida famata* VKM Y-9 (source of industrial riboflavin overproducers) as *D. subglobosus* (Dmytruk et al., 2011, 2014, 2020; Nguyen et al., 2009).

Beyond *Debaryomyces*, riboflavin biosynthesis has been reported across *Debaryomycetaceae*, including *Candida albicans*, *Meyerozyma guilliermondii*, *C. membranifaciens*, *C. tropicalis*, *Schwanniomyces occidentalis*, and *Hyphopichia wangnamkhiaoensis* (Amornrattanapan, 2013; Demuyser et al., 2020; Fedorovych et al., 2001, 2020; Jiménez-Nava et al., 2024; Palma et al., 2022). The higher riboflavin levels observed in the *Dhhog1Δ* mutant compared to the WT strain indicate that *DhHog1* acts as a negative regulator of *RIB1*, *RIB4*, *RIB6*, and *SEF1*. This observation further implies that similar regulatory mechanisms may be conserved and could operate in a comparable manner across other riboflavin-producing yeast species.

4.2. HOG-dependent effects of pH and NaCl on vacuolar dynamics and riboflavin secretion

The vacuolar H⁺/ATPase in yeast, whose catalytic subunit is encoded by *VMA1*, actively pumps protons from the cytosol into the vacuole (Kakinuma et al., 1981; Anraku et al., 1989; Hirata et al., 1990). *Vma1* activity relies on the regulated assembly and disassembly of its subunits in response to metabolic cues such as glucose availability and cytosolic pH (Deprez et al., 2018). The assembly of *Vma1* is reduced when yeast cells are grown in an acidic medium compared with neutral pH (Diakov and Kane, 2010). Therefore, under our growth conditions, the initially low extracellular pH (4.3) may have impaired *Vma1* activity. Disassembly of *Vma1* results in cytosolic acidification, thereby altering the protonation state of many biomolecules (Orij et al., 2011).

Yeast flavin-containing monooxygenase (yFMO) catalyzes the oxidation of thiols provided by cysteine, cysteamine, and glutathione (Suh et al., 1996). Oxidized thiols are essential for the proper assembly of functional iron-sulfur clusters (Bandyopadhyay and Outten, 2022). While some flavoproteins contain iron-sulfur clusters, the biogenesis of these cofactors itself depends on a flavin-dependent ferredoxin reductase (Lange et al., 2000; Webert et al., 2014). Under our experimental conditions, the pH range of 4.3–2.5 lies below the pK_a of many biological thiols (Ferrer-Sueta, 2022). Such an acidic environment is likely to favor thiol protonation, thereby limiting their reactivity and availability for iron-sulfur cluster assembly. A shortage of iron-sulfur clusters is, in turn, known to trigger iron-deficiency response pathways through the activation and nuclear retention of the transcription factor *Sef1* (Ror and Panwar, 2019).

These circumstances may lead to: 1) excess flavin production relative to the number of functional sulfur-iron proteins in which they can be incorporated; moreover, the redox potential of the isoalloxazine ring in flavins may be reduced in a protonic environment, making them less effective for redox homeostasis; 2) increased demand for flavoproteins involved in thiols oxidation and iron-sulfur cluster assembly; or, 3) sustained expression of the *Sef1*-regulated iron regulon caused by defective biogenesis of mitochondrial iron-sulfur clusters, thereby mimicking an iron-starved state.

Besides the intrinsic effects of low pH, the parallel upregulation of *SEF1* and *RIB1*, *RIB4*, and *RIB6* genes in the *Dhhog1Δ* mutant suggests that riboflavin overproduction reflects a pseudo iron-starvation state caused by defective iron-sulfur homeostasis rather than extracellular iron limitation.

Another intriguing possibility, not directly tested here, is that exposure to NaCl could enhance vacuolar fission in the *Dhhog1Δ* mutant, releasing riboflavin stored in vacuoles into the cytosol and facilitating secretion via plasma membrane riboflavin excretase *Rfe1* (Tsyrlunyk et al., 2021). In *S. cerevisiae*, vacuolar fission has been reported in conditions of hyperosmotic, oxidative and endoplasmic reticulum stress (Gokbayrak et al., 2022). These stressors are expected to be exacerbated in a *hog1Δ* mutant, as suggested by previous reports in *D. hansenii*, *S. cerevisiae*, and *C. albicans* (Bicknell et al., 2010; De la Fuente-Colmenares et al., 2024; Husain et al., 2022; Sánchez et al., 2020; Torres-Quiroz et al., 2010). On the other hand, reduced *Vma1* activity due to low extracellular pH (Diakov and Kane, 2010) could impair the vacuolar proton gradient and, consequently, diminish riboflavin influx into the vacuoles. The hypothesis that halted *Vma1* activity contributes to riboflavin secretion is further supported by the riboflavin excretion phenotype observed in *C. famata* null *VMA1* mutants (Andreieva et al., 2020a, 2020b).

D. hansenii possesses Na⁺/H⁺ antiporters that mediate both Na⁺ efflux from the cytosol and Na⁺ sequestration into the vacuole (Velkova and Sychrova, 2006). In acidic medium, cytosolic proton

accumulation can be accelerated by the presence of NaCl (Sychrová et al., 1999). Furthermore, a more protonic environment could increase the required energy expenditure to pump protons out of the cell. Consequently, maintaining cytosolic pH homeostasis may require further ATP-dependent activity of the plasma membrane H⁺/ATPase (Pma1) and Vma1, which mediate proton efflux and vacuolar sequestration, respectively. Under such conditions, Na⁺ sequestration into the vacuole via vacuolar Na/H⁺ antiporters may be hindered by a reduced Vma1 activity in an initially low extracellular pH, leading to further hyperosmotic stress and promoting vacuolar fragmentation.

Cytosolic acidification may also partially explain the increased demand for phosphorus and oxidative phosphorylation-related elements required for ATP production, as well as Mg²⁺ for ATP hydrolysis, to sustain proton transport into the vacuole or out of the cell in the *Dhhog1Δ* mutant. However, unlike *S. cerevisiae*, a direct regulation of Na/H⁺ antiporters by phosphorylated Hog1 has not been demonstrated in *D. hansenii*. Nonetheless, the upregulation of cation transporters expression remains one of the major adaptative responses of *D. hansenii* to saline stress, together with glycerol accumulation (Almagro et al., 2001; Proft and Struhl, 2004; Velkova and Sychrova, 2006; Carcía-Salcedo et al., 2007; Montiel and Ramos, 2007).

Another possible explanation for the accelerated element uptake observed in the *Dhhog1Δ* mutant may be the impairment of autophagic processes. In *S. cerevisiae*, Hog1 is required for the activation of mitophagy (Mao et al., 2011). The absence of Hog1 could lead to the accumulation of dysfunctional mitochondria that keep consuming cellular resources for ATP synthesis. Yet, Hog1-dependent regulation of autophagy has not been established in *D. hansenii*.

4.3. HOG signaling and multi-element homeostasis in riboflavin secretion

Riboflavin secretion has historically been associated with nutritional stress, particularly iron limitation (Demain, 1972; Gadd & Edwards, 1986; Fedorovich et al., 1999; Knight et al., 2002). Riboflavin and its derivatives can act as siderophore-like molecules, enhancing iron solubility and uptake (David et al., 2025). In *C. albicans*, *C. glabrata*, *S. cerevisiae*, and *Cryptococcus neoformans*, Hog1 has been implicated in repressing iron acquisition systems under iron sufficiency, while *hog1Δ* mutants show derepression of iron uptake, increased intracellular iron, and activation of iron-sulfur enzymes (Kaba et al., 2013; Lee et al., 2014; Martins et al., 2018; Thomas et al., 2013; Srivastava et al., 2015; Sahu et al., 2024). Surprisingly, our ICP-OES results revealed no differences in iron assimilation between WT and mutant strains. Instead, we observed accelerated uptake of phosphorus, sulfur, and magnesium. The comparable sodium uptake observed between strains may suggest the involvement of sodium transporters operating independently of Hog1.

These findings indicate that riboflavin accumulation in *D. hansenii* involves a broader reconfiguration of elemental homeostasis rather than being exclusively dependent on iron availability.

4.4. Sulfur and phosphate metabolism as key contributors to riboflavin overproduction in the *Dhhog1Δ* mutant

Sulfur metabolism may provide a mechanistic link between *DhHog1* function, oxidative stress adaptation, and riboflavin overproduction. Sulfate assimilation fuels the synthesis of methionine and cysteine, which in turn support glutathione biosynthesis, one of the main antioxidant systems in yeasts (Penninckx, 2002). *D. hansenii* contains lower glutathione pools than *S. cerevisiae* under both basal and oxidative stress conditions (Navarrete et al., 2009). Yeast glutathione reductase (GR) and flavin-containing monooxygenase (yFMO) are both flavin-dependent enzymes. Additionally, glycine, an indispensable precursor for the synthesis of GTP and riboflavin, is also required for glutathione synthesis (Meister, 1988; Santos et al., 2022). Compared with the non-riboflavin-overproducing yeast *S. cerevisiae*, the lower glutathione content reported in *D. hansenii* may reduce the cellular demand for flavins and glycine within this antioxidant system, thereby increasing the availability of these metabolites for riboflavin accumulation.

Methionine biosynthesis is positively regulated by Hog1 in *C. albicans* (Enjalbert et al., 2006). In *S. cerevisiae*, Hog1 also activates the histone methyltransferase Dot1, which requires S-adenosylmethionine (SAM) as a methyl donor (Separovich et al., 2024). In the absence of Hog1, a reduced methionine pool combined with impaired Hog1-mediated Dot1 activation could lead to the derepression of multiple genes not directly regulated by the HOG pathway.

We hypothesize that this mechanism is consistent with reports in *C. famata*, where mutants blocked in methionine biosynthesis (*MET2*) overproduced riboflavin, and sulfate limitation induced a pseudo iron-starvation phenotype characterized by enhanced intracellular iron accumulation (Dmytruk et al., 2006). Although riboflavin secretion under sulfate limitation has not been systematically studied in this system, the parallels with our findings are striking.

An additional connection between sulfur and phosphate metabolism may arise from tRNA thiolation. The availability of sulfur amino acids determines the amount of tRNA thiolation (Gupta et al., 2019; Laxman et al., 2013). Reduced tRNA thiolation activates methionine *de novo* and salvage pathways (Laxman et al., 2013); thus, if methionine biosynthesis is impaired in the *Dhhog1Δ* mutant, this could account for the increased sulfur demand observed. Moreover, tRNA thiolation has been shown to connect sulfur amino acid availability with phosphate assimilation via the *PHO* regulon (Gupta et al., 2019). In this context, inorganic phosphate is required for sugar phosphorylation in the pentose phosphate pathway, for pyridoxal-5-phosphate-dependent transamination reactions, and purine biosynthesis. This metabolic convergence positions phosphate and amino acids as integral precursors for both purines and flavins. Given that methionine availability promotes the synthesis of nucleotides and multiple amino acids (Walvekar et al., 2018), it remains possible that upregulation of the methionine regulon in the *Dhhog1Δ* mutant activates *PHO*-regulated phosphate assimilation pathways, thereby providing the purine precursors required for riboflavin biosynthesis.

The faster uptake of magnesium may be coupled with the accelerated assimilation of phosphorus, as Mg^{2+} associates with several phosphometabolites and is an essential cofactor for ATP-dependent enzymes, including proton pumps (Walker, 1994).

Regarding the potential role of riboflavin in oxidative stress, studies in *A. gossypii* have shown that intracellular riboflavin accumulation increases susceptibility to sunlight-induced ROS production and genotoxicity, whereas extracellular riboflavin can exert a protective effect (Stahmann et al., 2001; Silva et al., 2018). Thus, depending on the intracellular redox balance and UV exposure, riboflavin may act either as an antioxidant or as a pro-oxidant, owing to its different oxidation and protonation states.

4.5. *DhHog1-mediated negative regulation of riboflavin biosynthesis via stress- and iron-responsive transcription factors*

Promoter analysis revealed potential binding sites for Hog1-regulated transcription factors, including Sko1, Skn7, Msn2/4, Yap1, and the iron regulator Sef1, suggesting that riboflavin biosynthesis is under multifactorial control by stress- and iron-responsive transcriptional networks. Our expression analysis demonstrates that *RIB1*, *RIB4*, and *RIB6* were consistently upregulated in the *Dhhog1Δ* mutant. These results support the conclusion that *DhHog1* represses riboflavin biosynthesis through the modulation of stress-responsive transcription factors. The induction of *SEF1* reinforces the hypothesis that riboflavin overproduction is driven by a pseudo iron-starvation response, consistent with previous transcriptomic reports showing enrichment of riboflavin biosynthetic genes under salt stress in *D. hansenii* and *D. subglobosus* (Navarrete et al., 2021; Weintraub et al., 2025). Importantly, our study adds the novel observation that initial acidification of the medium is required for riboflavin secretion, highlighting the importance of combined stress factors in controlling this metabolic output.

In *S. cerevisiae*, Yap1- and Skn7-mediated pathways are specifically involved in responses to oxidative and osmotic stresses, respectively (Auesukaree 2017; Yaakoub 2022). Msn2/4 regulate a broad set of genes implicated in general stress adaptation (Mat Nanyan and Takagi 2020; Hasan et al., 2002). Sko1 functions primarily as a repressor that, once phosphorylated by Hog1, recruits the

SAGA histone acetylase and SWI/SNF nucleosome-remodeling complexes to activate the expression of stress-responsive genes (Ikner and Shiozaki 2005; Proft and Struhl 2002). Based on these parallels, we propose that in *D. hansenii* *DhHog1* may modulate riboflavin biosynthesis at least in part through the action of these transcription factors, thereby representing a conserved regulatory mechanism.

4.6. *DhHog1* signaling as a metabolic switch linking carbon and phosphate fluxes to riboflavin biosynthesis

In *D. hansenii*, phosphorylated Hog1 promotes the synthesis of glycerol-3-phosphate from glycolysis-derived dihydroxyacetone-phosphate through the upregulation of *GPD1* (Sánchez et al., 2020). In the absence of *DhHog1*, a larger pool of glucose-6-phosphate may become available for the synthesis of ribulose-5-phosphate through the oxidative branch of the pentose phosphate pathway.

At non-stressful concentrations of NaCl (0.5 M), the glyoxylate shunt is activated in *D. hansenii* (Ruiz-Pérez et al., 2023). Since glyoxylate is an important precursor for glycine biosynthesis (Schlösser et al., 2004; Takada and Noguchi, 1985), this pathway could serve as a relevant source of intermediates for GTP and riboflavin biosynthesis under our experimental conditions. The NaCl-induced activation of glyoxylate and respiration-related enzymes in *D. hansenii* is usually accompanied by intracellular phosphate accumulation, probably enabled by the phosphate/ Na^+ symporter Pho89 (Calahorra et al., 2009; Navarrete et al., 2021; Ruiz-Pérez et al., 2023; Sánchez et al., 2008). In acidic medium, phosphate uptake could be further enhanced by the phosphate/ H^+ symporter Pho84 (Lau et al., 1998; Eskes et al., 2018).

The faster phosphate assimilation observed in the *Dhhog1* Δ mutant suggests an increased ATP demand for proton extrusion across the plasma membrane or sequestration into vacuoles, as well as an enhanced activation of the *PHO*-regulon, possibly triggered by a pseudo-methionine starvation response, as discussed earlier. Furthermore, the biosynthesis of glycine from glyoxylate also requires phosphate, since it depends on pyridoxal-5-phosphate as a cofactor (Schlösser et al., 2004; Takada and Noguchi, 1985).

4.7. Concluding remarks and perspectives

Taken together, our results support a model in which *DhHog1* coordinates extracellular initial low pH sensing, elemental assimilation, and riboflavin metabolism (**Figure 7**). In WT *D. hansenii*, we found that *DhHog1* prevents premature riboflavin production by repressing *RIB* gene activation and secretion until the stationary phase. Loss of *DhHog1* led to derepression, earlier riboflavin secretion, *SEF1* upregulation, and accelerated sulfur, phosphorus and magnesium assimilation.

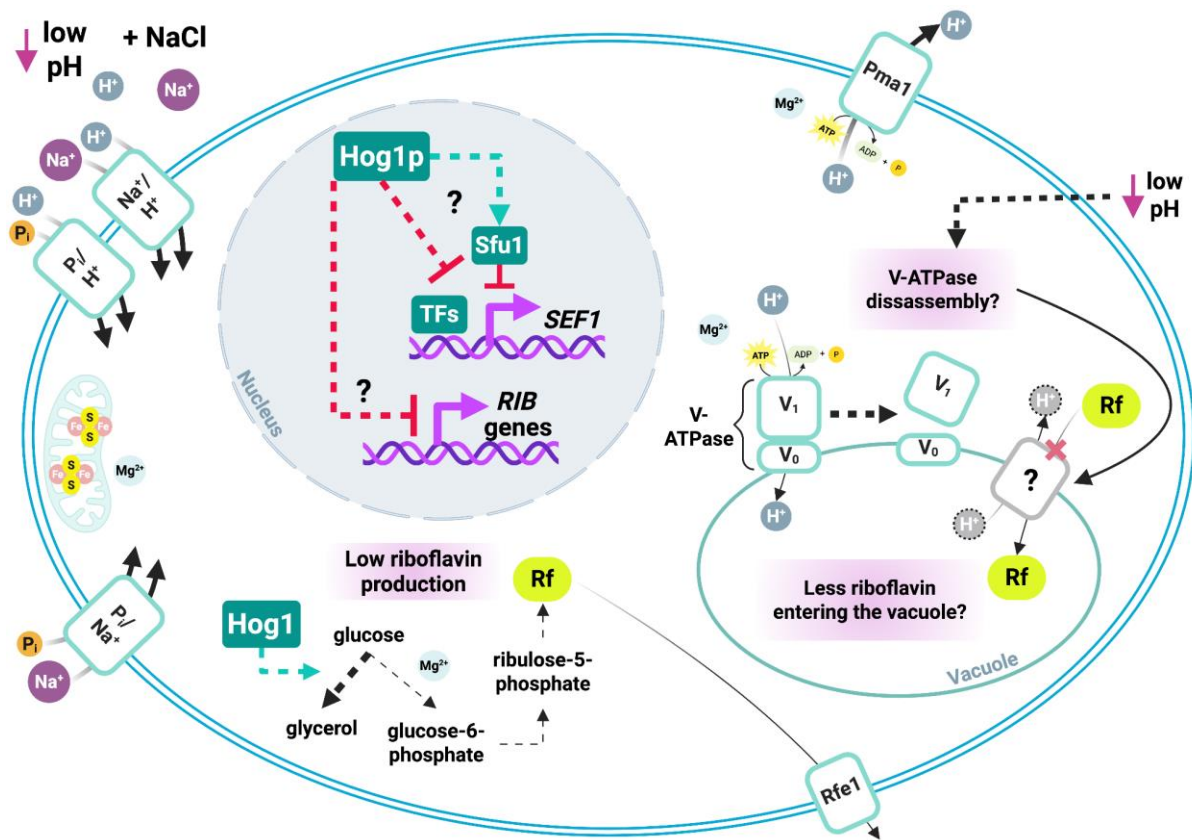


Figure 7. Hypothetical model of riboflavin regulation under acidic and saline conditions. Hog1 negatively regulates *RIB1*, *RIB4*, and *RIB6* by downregulating transcriptional activators such as *SEF1*, and possibly via *SFU1* or other transcription factors (TFs). Low pH and Na⁺ enhance assimilation of essential elements, including inorganic phosphate (P_i) via H⁺- and Na⁺-mediated antiporters, and increases ATP demand for proton extrusion through Pma1 and Vma1. Low pH-induced V-ATPase disassembly may impair the vacuolar proton gradient, limiting riboflavin influx and promoting cytosolic accumulation. In WT, Hog1 channels glucose into glycerol, reducing riboflavin precursors. In *Dhhog1Δ*, absence of Hog1 may trigger a pseudo iron- or sulfur-starvation state, accelerating the assimilation of P_i, S, and Mg²⁺, and cytosolic riboflavin is secreted via Rfe1. Direct interactions of Hog1 with transcriptional activators or repressors mediating *RIB* and *SEF1* repression remain unknown. Rf: Riboflavin. Legend for arrows: thick arrows, upregulated in WT; thin arrows, downregulated in WT; dashed arrows, indirect mechanisms; green dashed arrows, positive Hog1 regulation; red blunt arrows, negative Hog1 regulation. Created using BioRender.com, accessed on 7 September 2025.

Although this work provides novel evidence linking *DhHog1* to riboflavin regulation under acidic and saline stress, the underlying mechanisms remain only partially resolved. The precise contribution of sulfur assimilation, iron–sulfur cluster biogenesis, and vacuolar dynamics could not be directly addressed, as transporter activity, vacuolar morphology, and protein–DNA interactions were not experimentally tested. Likewise, whether *DhHog1* represses *RIB* promoters through direct modulation of transcription factors (e.g., Sef1, Sko1, Msn2/4, Yap1, or Sfu1) or indirectly through metabolic adjustments remains to be clarified. Future studies combining promoter occupancy analyses, phosphoproteomics, and metabolic flux profiling will be essential to disentangle these alternatives. Beyond mechanistic questions, it will also be relevant to determine whether riboflavin secretion represents a mere metabolic overflow or a bona fide adaptive antioxidant strategy. Addressing these points will not only refine our understanding of stress integration in yeasts but may also uncover opportunities to exploit Hog1-dependent pathways for biotechnological riboflavin production.

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