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Sphingolipid depletion suppresses UPR activation and promotes galactose hypersensitivity in yeast models of classic galactosemia

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Abstract

Classic galactosemia is an inborn error of metabolism caused by deleterious mutations on the *GALT* gene, which encodes the Leloir pathway enzyme galactose-1-phosphate uridyltransferase. Previous studies have shown that the endoplasmic reticulum unfolded protein response (UPR) is relevant to galactosemia, but the molecular mechanism behind the endoplasmic reticulum stress that triggers this response remains elusive. In the present work, we show that the activation of the UPR in yeast models of galactosemia does not depend on the binding of unfolded proteins to the ER stress sensor protein Ire1p since the protein domain responsible for unfolded protein binding to Ire1p is not necessary for UPR activation. Interestingly, myriocin – an inhibitor of the *de novo* sphingolipid synthesis pathway – inhibits UPR activation and causes galactose hypersensitivity in these models, indicating that myriocin-mediated sphingolipid depletion impairs yeast adaptation to galactose toxicity. Supporting the interpretation that the effects observed after myriocin treatment were due to a reduction in sphingolipid levels, the addition of phytosphingosine to the culture medium reverses all myriocin effects tested. Surprisingly, constitutively active UPR

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Declaration of competing interest

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Felipe S.A. Pimentel: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Caio M. Machado:** Investigation, Formal analysis, Writing – review & editing. **Evandro A. De-Souza:** Investigation, Formal analysis, Writing – review & editing. **Caroline Mota Fernandes:** Investigation, Formal analysis, Writing – review & editing. **Ana Luiza F.V. De-Queiroz:** Investigation, Formal analysis, Writing – review & editing. **Guilherme F.S. Silva:** Investigation, Formal analysis, Writing – review & editing. **Maurizio Del Poeta:** Funding acquisition, Writing – review & editing. **Monica Montero-Lomeli:** Funding acquisition, Writing – review & editing. **Claudio A. Masuda:** Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

signaling did not prevent myriocin-induced galactose hypersensitivity suggesting multiple roles for sphingolipids in the adaptation of yeast cells to galactose toxicity. Therefore, we conclude that sphingolipid homeostasis has an important role in UPR activation and cellular adaptation in yeast models of galactosemia, highlighting the possible role of lipid metabolism in the pathophysiology of this disease.

Keywords

S. cerevisiae ; Galactosemia; Unfolded protein response; Sphingolipids; Inositol

1. Introduction

The intracellular conversion of galactose to glucose-1-phosphate occurs *via* a series of biochemical reactions named the Leloir pathway (Fig. 1). This pathway is conserved from bacteria to mammals [1], and its blockage in humans leads to a set of conditions named galactosemia. Specific loss of function of the enzyme galactose-1-phosphate uridyltransferase (GALT), which catalyses the conversion of galactose-1-phosphate and UDP-glucose to glucose-1-phosphate and UDP-galactose, causes classic (or type I) galactosemia [2]. In humans, this condition has an autosomal recessive nature and is caused by deleterious mutations in the *GALT* gene. If not diagnosed shortly after birth, nursing newborns present acute symptoms such as vomiting, diarrhea, jaundice, hepatosplenomegaly, sepsis and other symptoms that can lead to premature death.

The available treatment for classic galactosemia is dietary restriction of lactose/galactose-containing foods [3,4]. Although this approach prevents acute symptoms in children, long term complications such as cognitive and motor deficits, as well as hypergonadotropic hypogonadism in females, might occur [4–6].

Despite studied for decades, the molecular pathophysiology of classic galactosemia is still elusive. A common feature observed in patients is the intracellular accumulation of galactose-1-phosphate (Gal1P). Although some results suggest that Gal1P accumulation is not the only source of complications [7,8], several pieces of evidence indicate that increased levels of Gal1P are an important feature in the pathophysiology of classic galactosemia [9].

We and others have shown that the endoplasmic reticulum unfolded protein response (UPR) is activated [10] in a Gal1P-dependent manner in yeast models of galactosemia, and that the UPR has a major protective role under these conditions [11]. It was also shown that the UPR is activated by galactose challenge in a human galactosemia cell model [12] and, recently, that tweaking with the PERK branch of the mammalian UPR signaling pathway using salubrinal (an inhibitor of the phosphatase that dephosphorylate the PERK substrate eIF2α) suppresses some of the phenotypes observed in a mouse model of the disease [13,56]. Altogether, these results highlight the importance of the UPR for the pathophysiology of galactosemia.

Different from mammals which present three branches of the UPR - namely Ire1, PERK and ATF6 - *Saccharomyces cerevisiae* presents only the Ire1 branch [14,15]. The yeast UPR

is triggered by the activation of the ER sensor protein Ire1p that, together with the tRNA ligase Trl1p, promotes the splicing of the mRNA of *HAC1*. After the splicing, the *HAC1* mRNA becomes competent for translation and the transcription factor Hac1p induces genes associated with endoplasmic reticulum homeostasis [14,16]. Activation of the Ire1p sensor protein was first described to be dependent on the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum [14]. However, more recent results have shown that Ire1p can also be activated *in vitro* in an unfolded protein-independent manner by flavonol [17], by the kinase inhibitor APY29 [18], and by changes in the lipid composition of membranes [19]. There are also *in vivo* evidences of UPR activation by changes in the lipid composition of membranes [20,21].

Inositol starvation is a known condition of unfolded protein-independent UPR activation in yeast [21,22] that can be prevented by myriocin, an inhibitor of the serine palmitoyltransferase (SPT) complex [21]. These findings suggested a connection between inositol depletion, sphingolipid metabolism and the UPR in yeast. Another link between sphingolipids and UPR in yeast comes from the observation that constitutive activation of the SPT complex in the *orm1Δorm2Δ* strain (Orm1/2p are inhibitors of SPT activity) also activates the UPR [23,50]. Although the molecular mechanism of the unfolded protein-independent Ire1p activation is yet to be determined, a comprehensive review of the lipid-associated UPR activation is available [24].

In the present work, we asked whether sphingolipid homeostasis has a role in UPR activation in classic galactosemia, and whether Ire1p activity is dependent or not on the unfolded protein binding in yeast models of the disease. Our findings indicate that the activation of UPR in galactosemia can be unfolded protein-independent and suggest a role for sphingolipid metabolism in the pathophysiology of this disease.

2. Materials and methods

Yeast strains and media.

All *S. cerevisiae* strains used in this work (Supplementary Table 1) were haploid, derived from the BY4741 background (MATa, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*). Single mutants were acquired from the *MATa* deletion library (Open Biosystems, USA). Double mutants or plasmid-expressing strains were constructed in our laboratory using the lithium acetate transformation protocol [25]. Expression vectors (pRS315-based plasmids) of *IRE1* constructs were a generous gift from Dr. Yukio Kimata [21,26]. Expression plasmids (YCp50) of *HAC1* constructs were gifts from Dr. Kazutoshi Mori [16]. YPGal medium (2% peptone, 1% yeast extract and 2% galactose) or YPGly medium (2% peptone, 1% yeast extract and 2% glycerol) were used in all experiments. For solid media, 2% agar was added to the mixture. Liquid cultures were grown at 30 °C in 200 RPM shaker. The *lys2Δ* strain was used as a control strain for reasons previously described [11,27].

In the experiments using the centromeric plasmid-based *IRE1* and *HAC1* constructs, cells were initially grown in selective (in regard to the plasmid selection marker) synthetic dextrose (SD) media (2% glucose, 0.67% Yeast nitrogen base without amino acids) containing the appropriate supplements. Cells were then changed to YPGly or YPGal media

for the experimental treatment with either galactose or lithium, respectively. Despite the fact that all plasmids used contain a centromeric region to ensure proper segregation during mitosis, we monitored whether these plasmids had been lost during the experiment by plating (after the experiment had finished) the same number of cells in either non-selective YPD or SD plates, or selective (minus leucine or minus uracil) SD plates. The number of colonies formed on both plates were the same in all tests performed, indicating no significant loss of plasmid during the experimental procedure.

Chemicals and treatments.

Inositol (Ins, I5125), myriocin (Myr, M1177), tunicamycin (TM, T7765), galactose (GAL, G0750) and lithium chloride (LiCl, L9650) were acquired from Sigma Aldrich, and phytosphingosine (PHS, 860499) from Avanti Polar Lipids. For culture treatments of inositol supplementation, the powder was directly added to culture media to a final concentration of 50 mM. Myriocin was dissolved in methanol to a concentration of 2 mg/mL and added to the cultures to a final concentration of 0.6 μg/mL. Phytosphingosine was dissolved in methanol to a concentration of 15 mM and added to the cultures to a final concentration of 15 μM.

RNA preparation and RT-PCR analysis.

Total RNA was extracted as previously described [11]. For *HAC1* mRNA splicing analysis, RT-PCR was performed as described [11]. For UPR induction quantification, pictures of ethidium bromide stained gels under UV light were taken and images processed using the Image J software [28]. In order to obtain optimal signal to noise ratio, the subtract background process was applied to the entire photo. Band intensity was measured according to the Image J user manual. Finally, the percentage of *HAC1* splicing induction is mathematically expressed as:

$$\%\, Splicing = \frac{100 * I_{HAC1i}}{I_{HAC1i} + I_{HAC1u}}$$

where I_{HAC1i} and I_{HAC1u} represent the area under curve of the pixel intensity of the band representing the induced (lower) or uninduced (higher) form of *HAC1* mRNA.

qRT-PCR protocol.

Total RNA extraction, cDNA synthesis and qRT-PCR protocols and analysis were performed as previously described [11].

Growth and viability assays.

For growth assays, yeast strains were grown in YPGly, YPGal or SD medium to stationary phase, and serial dilutions of the cultures were prepared in sterile-distilled water to O. $D._{600\ nm}$ values of 0.3, 0.03 and 0.003. Cell dilutions were spotted onto the medium described in the experiments with a 48-pin replicator from Sigma-Aldrich (R2383). Plates were incubated at 30 °C for 3 days for YPGal medium and 4 days for YPGly medium. Plates were photographed using a Canon 20D camera and images processed using the Adobe

Photoshop software. Representative images of at least three independent experiments are presented. For the viability assays, colony forming units (CFU) counting was conducted as described [11].

Galactose-1-phosphate measurements.

Cultures were grown in the appropriate medium until 0.5–1.0 $O.D._{600nm}$ when cells were collected by filtration, using a 0.45 μm nitrocellulose filters, prior to or after treatment with lithium chloride or galactose (one hour or two hours of treatment, respectively). Immediately after filtration, cells were washed with −20 °C cold 60% methanol in HEPES 10 mM pH 7.1. The filters were transferred to a glass tubes containing 3 mL ethanol 80% in HEPES 10 mM pH 7.1 solutions and heated to 80 °C for 3 min. Metabolites extracts were then transferred to a 2 mL Eppendorf tubes and samples were dried out in SpeedVac (Eppendorf). Dried samples were solubilized in Milli-Q water, in an equivalent volume for a final concentration of 100 $O.D._{600nm}$ per mL, according to the total filtered cell density. For galactose-1-phosphate measurement, a reaction medium containing 50 mM Tris/HCl pH 8.8; 1 mM NAD^+; 0.66 mM EDTA; 3.5 mM $MgCl_2$ was prepared in a final volume of 240 μL in each well of a UV non-absorbing 96-well plate and analysed at 340 nm using a microplate reader (SpectraMax® M5, Molecular Devices). 10 μL of each extract was then added to each well and the reaction was started with the addition of 2 mU of galactose dehydrogenase, the reaction was followed until all the free galactose is consumed. After that, 10 mU of alkaline phosphatase was added to the reaction medium and followed until the end of the reaction. The production of NADH after the addition of alkaline phosphatase is directly proportional to the galactose-1-phosphate content of each extract. The molar extinction coefficient for NADH was calculated experimentally in our experimental conditions to be 4.51 $O.D._{340nm}$/mol.

Sphingolipid levels measurement.

Yeast cells were grown in YPGly medium, in the absence or presence of myriocin, phytosphingosine, or both, until cultures reached approximately 0.2 $O.D._{600nm}$/mL. At time zero, cells were harvested by centrifugation to collect the minus galactose conditions, and the rest of the cultures were challenged with 0.2% galactose. After 2 h, cells were harvested to collect the plus galactose conditions. Aliquots of approximately 10^8 cells were harvested, washed three times with deionized water and then lyophilized before lipid extraction. Lyophilized samples were sent to the Stony Brook lipidomics facility for sphingolipids levels determination as previously described [29].

Statistical analysis.

GraphPad Prism version 6.00 for Windows (GraphPad Software, USA) was used for all statistical analysis in this work. p-values of 0.05 or less were considered as statistically relevant. Specific tests and *post hoc* tests are indicated in figures legends.

3. Results

3.1. UPR activation is independent of the binding of unfolded proteins to Ire1p in yeast models of classic galactosemia

Previous results from our group showed that the chemical chaperone 4-PBA did not protect *gal7*Δ cells from galactose toxicity [11]. Similar results were observed in experiments using WT cells treated with galactose and lithium [11], another yeast model of galactosemia [30,31]. These results raised the question of whether the activation of UPR under galactosemic conditions is dependent or not on the binding of unfolded proteins to the UPR sensor protein Ire1p. To address this question, we transformed the *gal7*Δ*ire1*Δ strain with expression vectors containing either the wildtype allele (WT) or a mutant (ΔIII) allele of Ire1p lacking the domain responsible for binding to unfolded proteins [26]. We observed that *gal7*Δ*ire1*Δ mutants expressing both forms of Ire1p were able to activate UPR with similar efficiency after galactose treatment, as measured by *HAC1* mRNA splicing (Fig. 2A, central columns) and by the transcriptional response of the Hac1p target genes *KAR2* and *ERO1* (Supplementary Fig. S1A). In contrast, only cells expressing the WT form of Ire1p efficiently activated UPR when challenged with tunicamycin (Fig. 2A, right columns), a condition in which unfolded proteins accumulate in the lumen of the ER and UPR activation is dependent on the binding of unfolded proteins to the domain III of Ire1p [19,21,26,32]. Importantly, the kinetics of UPR activation is strikingly similar between cells expressing WT or ΔIII Ire1p (Fig. 2B) when challenged with galactose indicating that, under this condition, the binding of unfolded proteins to the Ire1p sensor protein is not required for activation of the UPR.

It had been previously shown [21] that myriocin inhibits UPR activation under an ER stress condition (inositol depletion) that does not depend on unfolded protein-binding to Ire1p to activate UPR, while it does not affect UPR activation under conditions that unfolded protein-binding to Ire1p is relevant (DTT or Tm treatment). We observed that myriocin treatment suppressed UPR activation (*HAC1* mRNA splicing (Fig. 2C) and transcriptional response (Supplementary Fig. S1C)) when *gal7*Δ cells were exposed to galactose, further supporting the hypothesis of an unfolded protein-independent activation of UPR under galactosemic conditions.

We had previously shown that the deletion of the *IRE1* gene decreased the tolerance to galactose treatment of the *gal7*Δ strain [11]. The expression of the ΔIII form of Ire1p was as able to rescue the decreased galactose tolerance of the *gal7*Δ*ire1*Δ strain as the WT form of Ire1p (Fig. 2D). Altogether, these results support the hypothesis that the trigger of UPR activation in this model of galactosemia is not unfolded protein accumulation in the ER.

3.2. Inositol supplementation has a minor effect on UPR activation in yeast models of galactosemia

Inositol depletion from the culture media is a known condition that leads to the UPR activation independent of unfolded protein binding to Ire1p in yeast [21,33]. Because Gal1P is a known alternative substrate for inositol monophosphatases [34], it has been proposed that a possible mechanism of Gal1P toxicity could be *via* promoting inositol depletion in

cells due to decreased rates of inositol phosphate recycling [35,36]. To address the relevance of this mechanism, we tested whether inositol supplementation in the culture medium would be sufficient to reduce galactose toxicity. Surprisingly, the addition of high concentrations of inositol to the culture media did not affect galactose toxicity in *gal7Δ* yeast cells, even in the *gal7Δire1Δ* and *gal7Δhac1Δ* strains that are incompetent for UPR activation in response to ER stress (Fig. 3A). Inositol supplementation caused a small decrease in *HAC1* mRNA splicing at the 2-hour time-point when *gal7Δ* cells were challenged with galactose, but full activation was reached 4 h after the galactose challenge (Fig. 3B). Analysis of the transcriptional response of UPR-target genes supports these results (Supplementary Fig. S1B). These results indicate that changes in inositol homeostasis may contribute, but do not have a major role on the galactose-induced UPR activation in this model.

3.3. Sphingolipid depletion suppresses UPR activation and exacerbates galactose toxicity

It had been suggested that the mechanism of UPR activation under inositol deprivation, which is also independent of unfolded protein binding to Ire1p [21], involves the *de novo* synthesis of sphingolipids [22]. We have already observed that myriocin suppressed UPR activation in *gal7*Δ cells challenged with galactose (Fig. 2C). To further test the relevance of the *de novo* synthesis of sphingolipids in the context of classic galactosemia, we pre-treated *gal7*Δ cells with different concentrations of myriocin and observed a dose-dependent reduction in UPR activation when *gal7Δ* cells were challenged with galactose for 2 h (Fig. 4A). The impairment in the UPR activation induced by myriocin was sustained even after longer periods (Fig. 4B). We also observed that myriocin decreased the galactose tolerance of *gal7Δ* cells (Fig. 4C). The decreased galactose tolerance induced by myriocin is dependent on Gal1P accumulation, since the galactokinase-null *gal7*Δ*gal1*Δ strain - which does not accumulate Gal1p even in the presence of galactose - grows normally regardless of galactose and/or myriocin presence (Fig. 4C). Effects of myriocin pre-treatment cannot be explained by increased levels of Gal1P since myriocin did not significantly change Gal1p levels in *gal7Δ* cells treated with galactose (Fig. 4D).

As a control of the myriocin treatment, we quantified several sphingolipid species through mass spectrometry and observed the expected reduction in sphingolipid content (*e.g.* sphingoid bases, ceramides and inositolphosphorylceramides) induced by myriocin (Fig. 4E and Supplementary Table 1). We have previously described that UPR-incompetent *gal7Δ* cells lose their viability when challenged with galactose [11]. We tested whether the decreased activation of UPR in the presence of myriocin could promote loss of viability in galactose-treated *gal7Δ* cells. The viability of *gal7Δ* cells challenged with galactose was reduced by 28% in cultures pre-treated with myriocin (Fig. 4F). Altogether, these results show that myriocin is not increasing the galactose-induced stress *via* an increase of Gal1p levels. Instead, it is probably decreasing the adaptive capacity of cells to respond to galactose toxicity.

To establish a causal relationship between the inhibition of the *de novo* sphingolipid synthesis (not an unspecific toxicity) by myriocin and its effect on the galactose-induced UPR activation on *gal7Δ* cells, we performed phenotypic reversion experiments

co-supplementing yeast cultures with myriocin and phytosphingosine (PHS) to restore sphingolipids species in myriocin-treated cells [23]. The sphingolipidomic analysis showed that PHS co-supplementation partially suppresses the general decrease in sphingolipids levels caused by myriocin (Fig. 5A and Supplementary Table 2). PHS co-supplementation was also sufficient to suppress myriocin effects on both galactose-induced UPR activation (as measured both by *HAC1* mRNA splicing (Fig. 5B) and transcriptional response (Supplementary Fig. S1C)) and galactose tolerance (Fig. 5C) in *gal7Δ* cells. PHS supplementation alone did not produce any noticeable effect on neither UPR activation nor galactose tolerance (Fig. 5B and C). These results indicate that myriocin effects on UPR and galactose tolerance are indeed due to the myriocin-induced changes in sphingolipid levels. Altogether, these results implicate sphingolipid metabolism in the UPR-mediated cellular adaptation to galactose toxicity.

Since the modulation of sphingolipid levels affected the UPR activation and the adaptation to galactose-1-phosphate accumulation, we reasoned that galactose treatment in *gal7*Δ cells could change the sphingolipid levels, and that this change could be the cause of the activation of the UPR. However, our lipidomic analyses indicated only small, non-statistically significant changes on sphingolipids levels in the *gal7*Δ cells after a 2-hour treatment with galactose (Supplementary Fig. S2 and Supplementary Table 2). Therefore, these results do not support a model in which Gal1P-induced changes in sphingolipid composition would cause the UPR activation and galactose toxicity. Instead, they indicate that proper sphingolipid homeostasis is necessary for yeast cells to adapt to galactose toxicity, enabling cells to respond to this stress *via*, for example, UPR activation.

3.4. Constitutive expression of Hac1p does not restore normal galactose tolerance in the presence of myriocin

Finally, we investigated whether the constitutive activation of the UPR *via* the expression of the *HAC1* Δintron construct – an allele of the *HAC1* gene which encodes an active form of *HAC1* independent of Ire1p processing activity [16] – would suppress myriocin effects on galactose tolerance of *gal7Δ* cells. Surprisingly, the expression of the *HAC1* Δintron construct was not sufficient to suppress the growth defect caused by myriocin treatment under galactosemic conditions (Fig. 6A), although it was able to recover growth in the presence of tunicamycin (Fig. 6B).

One possible explanation for this result would be that myriocin could regulate both *HAC1* mRNA splicing and Hac1p transcriptional activity. To test whether myriocin has any effect on Hac1p transcriptional activity, we monitored the expression of Hac1p target genes *ERO1* and *KAR2* in *gal7Δhac1Δ* cells expressing the *HAC1* Δintron allele, treated or not with myriocin. Quantitative RT-PCR experiments showed that myriocin was unable to inhibit (on the contrary, it may even increase) the transcriptional activity of the constitutively active form of Hac1p (Fig. 6C). These results indicate that sphingolipids are involved not only in the cellular adaptation to galactose toxicity *via* the inhibition of UPR, but also *via* at least one other unidentified mechanism.

4. Discussion

4.1. Unfolded protein-independent UPR activation in galactosemia

The results presented here using the *gal7*Δ yeast model show that UPR activation by Gal1p is not dependent on unfolded protein-binding to Ire1p. Our group had previously described another model of galactosemia which consists of the treatment of galactose-growing yeast cells with lithium – an inhibitor of phosphoglucomutase – which also leads to the accumulation of Leloir pathway metabolites including Gal1P [30,31]. Similar to what was observed in the *gal7*Δ model, the treatment with lithium and galactose also caused yeast cells to activate the UPR [11,27,37]. The expression of the ΔIII Ire1p mutant was also sufficient to restore UPR activation (Supplementary Fig. S3A) and lithium tolerance in galactose growing *ire1*Δ cells (Supplementary Fig. S3B) as well as the WT form of Ire1p. The results obtained using this second model of galactosemia further support the hypothesis that the main trigger of UPR activation is not the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum when Gal1P accumulates. Our interpretation is in accordance with models previously described in the literature to discern unfolded protein-dependent from unfolded protein-independent UPR activation in other conditions [19,21,32].

The glycosylation of proteins and lipids is different from yeast to mammals. For example, *S. cerevisiae* does not galactosylate neither proteins nor lipids as mammalian cells do [51]. Many reports show changes in the glycosylation of proteins and lipids in samples derived from galactosemia patients or models of the disease (reviewed in [38]), even leading to a comparison between galactosemia and other congenital disorders of glycosylation [39]. These changes in glycosylation patterns could lead to misfolding of proteins and ER stress in patients' cells. Therefore, we cannot state that unfolded protein-independent is the only UPR/Ire1p activation mechanism in galactosemia patients. Interestingly, Balakrishnan and collaborators recently discussed the inability of the chemical chaperone 4-PBA to suppress the increased BiP expression – an indicator of UPR activation in mammals – in a mouse model of classic galactosemia [13]. This observation suggests that the unfolded protein-independent mode of UPR activation we are proposing using yeast models may also occur in mammalian models and patients. We believe that both unfolded-protein dependent and independent mechanisms could be relevant in the pathophysiology of classic galactosemia, but the relative importance of each mechanism needs to be addressed in future studies.

4.2. Inositol depletion in galactosemia

A role for inositol homeostasis in the pathophysiology of galactosemia is still under investigation. A decrease in the inositol levels on the brains of galactosemic patients have been reported [40–43]. Two mechanisms have been proposed to explain this decrease: inhibition of inositol transport due to galactitol accumulation, and decreased recycling of inositol phosphates due to Gal1P accumulation [35]. Because in yeast models all phenotypes identified so far are Gal1P-dependent, we will focus the discussion on the second mechanism.

It has been shown that inositol monophosphatases could also dephosphorylate Gal1P [34]. Because the same enzyme can use two different substrates, it was proposed that

accumulation of Gal1P could negatively impact on inositol dephosphorylation reactions in cells, and thus decrease free inositol levels [34,35]. Later, Mehta and co-workers showed that the overexpression of the human inositol monophosphatase increases tolerance to galactose in *gal7Δ* cells [36], and we have shown that deletion of both inositol monophosphatase-encoding genes in yeast - *INM1* and *INM2* - decreased tolerance to lithium and galactose treatment [30]. The increase in galactose tolerance due to the overexpression of inositol monophosphatase [36] could be explained by two different mechanisms: 1) decrease in Gal1P levels, and/or 2) normalization of inositol levels. In this work, we observed that inositol supplementation did not restore the growth defect of the *gal7Δ* model under galactosemic conditions (Fig. 3A). It did promote, however, a small delay in UPR activation (Fig. 3B).

Interestingly, one of lithium's proposed mode of action is also *via* inositol depletion [52] due to its inhibitory effect on inositol phosphatases [53,54,57], including on yeast enzymes [55]. Even with this possible additional effect, inositol supplementation did not increase lithium/galactose tolerance (Supplementary Fig. S4A) on this second model of galactosemia but, similar to what was observed with the *gal7*Δ model (Fig. 2), promoted a small delay in the UPR activation (Supplementary Fig. S4B). These results further support the conclusion that inositol depletion is not the main cause of toxicity in yeast models of galactosemia. In patients, however, if we assume that galactose-derived metabolites could induce both a decrease in inositol uptake, as well as a decrease in inositol phosphate recycling, changes in inositol homeostasis could have a more prominent effect. Further work will be necessary to shed more light into this question.

4.3. UPR-dependent and UPR-independent roles of sphingolipids in the pathophysiology of galactosemia

The observation that myriocin drastically reduced UPR activation when *gal7Δ* cells were challenged with galactose (Fig. 4A and B), and that this effect was completely abolished when PHS was concomitantly added to the cultures indicated a causal relationship between sphingolipid synthesis inhibition and UPR silencing (Fig. 5B). Myriocin also increased galactose sensitivity, and this effect was also rescued by PHS co-supplementation (Fig. 5C).

It is known that UPR signaling regulates *de novo* sphingolipid synthesis, and that sphingolipid levels, in turn, affect ER function and stress signaling [44,45]. We addressed the possibility that galactose treatment could lead to changes in sphingolipids in a *gal7*Δ cell, based on the hypothesis that these putative changes in sphingolipids would be the trigger of UPR activation under this condition. However, we have observed only minor (non-statistically significant) changes in sphingolipid levels in *gal7*Δ cells treated for two hours with galactose (Supplementary Fig. S2). Similar results were observed on the *gal7*Δ*gal1*Δ cells (Supplementary Fig. S5), cells in which UPR is not activated after treatment with galactose, further supporting that minor galactose-induced changes in sphingolipid levels are not responsible for the observed UPR activation.

Still, there is the possibility that the overall cellular levels of sphingolipids are not altered, but the subcellular distribution of these lipids is, causing significant changes in sphingolipids on the ER membrane that could contribute to the activation of UPR. Unfortunately, we were

unable to analyse organelle-specific sphingolipid composition. Another possibility is that we have missed in the sphingolipidomic analysis a lipid specie that could be specifically relevant in this context. For example, long-tailed C14 and C16 ceramides were specifically implicated in ER homeostasis and ER stress in other contexts [46,47]. Differential effects on UPR were also reported for different species of other lipid classes as well. For example, it was shown that exposure of rat intestinal epithelial cells to the saturated fatty acid myristate (C14:0) activates UPR. However, this cell line does not activate UPR when exposed to palmitate (C16:0), which is a known inducer of UPR in other cell types such as hepatocytes and pancreatic β cells [49]. These examples highlight the complexity of this process, showing that UPR activation could be both lipid species-specific and cell type-dependent. So, the fact that we were unable to identify significant differences in any of sphingolipid species measured in a condition that UPR is already fully active does not completely rule out the possibility that a specific species not measured in our analysis could be involved in UPR activation. That said, based on the results available, our current proposal is that sphingolipids are not acting as the trigger of the UPR activation. Instead, a proper sphingolipid homeostasis seems to be necessary for yeast cells to engage an effective UPR under galactose toxicity condition.

Curiously, although UPR activation is essential for galactose tolerance in these models [11], constitutive UPR activation was not sufficient to restore normal sensitivity to galactose of *gal7*Δ cells pre-treated with myriocin (Fig. 6A). This result suggests that myriocin either inhibits the protective effect of UPR activation further downstream of the transcriptional activity of Hac1p, or that it affects another cellular process relevant to the adaptation of cells to Gal1P accumulation. We are currently investigating other cellular processes that could be affected by the inhibition of sphingolipid synthesis, thus explaining the fact that restoration of UPR was not enough to restore galactose tolerance in the presence of myriocin.

In summary, this work indicates that galactose-induced UPR activation occurs independent of unfolded-protein binding to Ire1p, and implicate for the first-time sphingolipid metabolism in the pathophysiology of galactosemia. Both findings suggest that lipid homeostasis may have a more prominent role in the pathophysiology of galactosemia than previously anticipated. If similar processes occur in patient, monitoring lipid levels during treatment may be a relevant parameter to follow. These results also suggest that drugs or supplements that affect lipid metabolism may be useful for treating this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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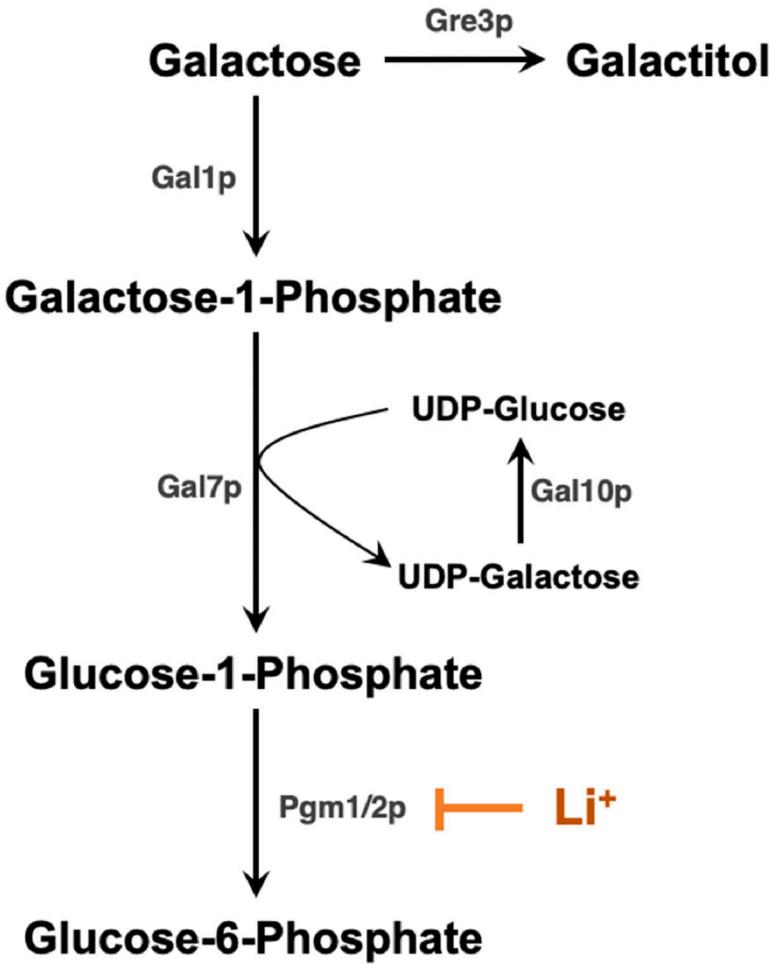

Fig. 1.

The Leloir Pathway in *Saccharomyces cerevisiae*. The Leloir Pathway is conserved between yeast and humans. Represented are the names of the yeast enzymes, and the step inhibited by lithium is indicated. Once inside yeast cells, β-D-galactose is converted to α-D-galactose by the galactose mutarotase (*GAL10* in yeast/*GALM* gene in humans - step not shown in the scheme for clarity). Galactose can either be reduced to galactitol in a reaction catalysed by the aldose reductase Gre3p (*AKR1B1* in humans), or receives a phosphate group linked to the carbon 1′ by the action of the galactokinase Gal1p (*GALK* in humans), which uses ATP as the phosphate donor. Galactose-1-phosphate and UDP-glucose molecules are substrates of the galactose-1-phosphate uridyltransferase Gal7p (*GALT* in humans), which produces glucose-1-phosphate and UDP-galactose. UDP-glucose and UDP-galactose levels are maintained by the action of the UDP-galactose 4′-epimerase Gal10p (*GALE* in humans). The final product of the Leloir pathway glucose-1-phosphate is then converted to glucose-6-phosphate by the phosphoglucomutase enzymes Pgm1/2p (*PGM1/2/5* in humans).

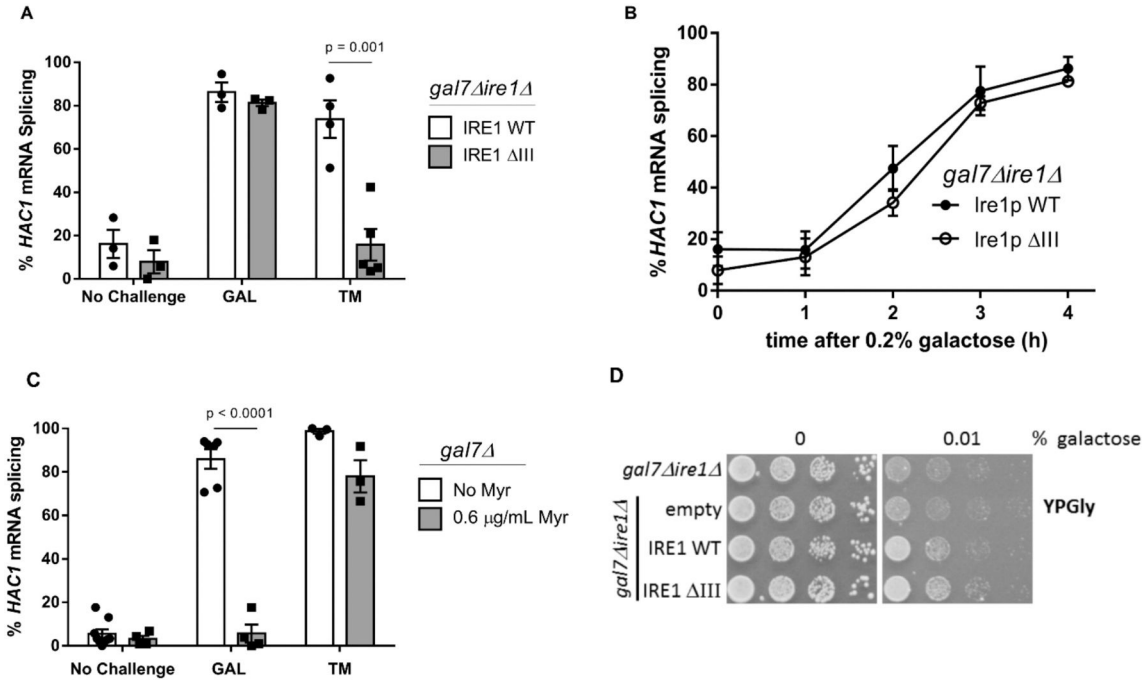

Fig. 2.

Unfolded protein-independent UPR activation in yeast model of classic galactosemia. **(A)** Yeast *gal7Δire1Δ* cells were transformed with the pRS315 plasmid containing wild type (WT) or ΔIII form of the *IRE1* gene. Cells were grown in synthetic defined (SD) medium without leucine, and then transferred to fresh YPGly medium. When cultures reached approximately 0.2 O.D./mL, cells were challenged with 0.2% galactose and samples were harvested 2 h after the galactose challenge for total RNA extraction and *HAC1* mRNA splicing analysis. Data are represented as mean ± SEM. 2way ANOVA was applied to the data set and the Sidak *post hoc* test was used for multiple comparisons between indicated groups. **(B)** The indicated yeast strains were grown as in (A) and samples were collected prior to, or at the indicated times after galactose challenge (0.2%). Data are represented as mean ± SEM (n = 5). *t*-Test analysis was applied to the data set, indicating absence of statistical differences between the groups. **(C)** Yeast *gal7*Δ cells were grown in YPGly overnight and then transferred to fresh YPGly media, containing or not 0.6 μg/mL myriocin. At times 0 or 2 h after galactose challenge (0 and 1.5 h for tunicamycin (TM)), cells were collected for RNA extraction as in (A). Data are represented as mean ± SEM. 2way ANOVA was applied to the data set and the Sidak *post hoc* test was used for multiple comparisons between indicated groups. **(D)** A ten-fold serial dilution of the indicated strains were plated in solid YPGly medium in the presence or absence of galactose, using a replica plater as described in Materials and methods section. Plates were incubated for 4 days at 30 °C before they were photographed. Figure is representative of three independent experiments.

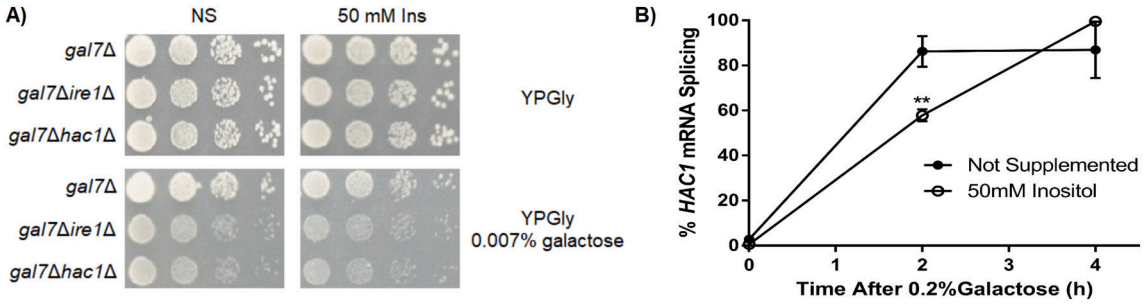

Fig. 3.

Inositol supplementation has a minor effect in yeast adaptation and UPR activation in response to galactose challenge. **(A)** A ten-fold serial dilution of the indicated strains were plated in solid YPGly medium in the presence or absence of galactose, supplemented or not with 50 mM inositol, using a replica plater as described in Materials and methods section. Plates were incubated for 4 days at 30 °C before they were photographed. Figure is representative of three independent experiments. **(B)** Yeast *gal7*Δ cells were grown in YPGly overnight and then transferred to fresh YPGly media, containing or not 50 mM inositol. When cultures reached approximately 0.2 O.D./mL, cells were challenged with 0.2% galactose and samples were harvested for total RNA extraction and *HAC1* mRNA splicing analysis at the indicated time points. Data are represented as mean ± SEM (n = 3). 2way ANOVA was applied to the data set and Sidak *post hoc* test was used for multiple comparisons. ** $p < 0.01$.

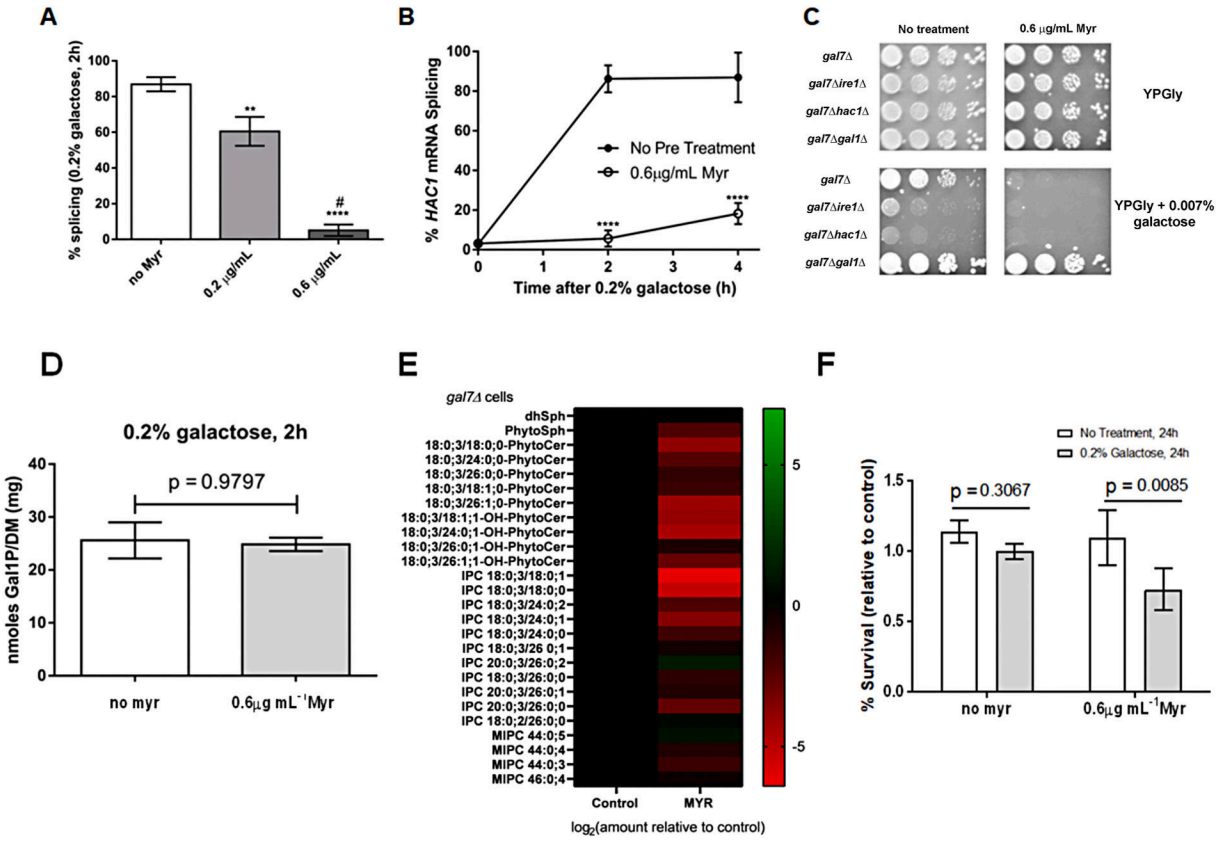

Fig. 4.

Sphingolipid depletion hampers UPR activation and promotes galactose hypersensitivity. **(A)** Cells were grown in the presence or absence of increasing doses of myriocin, and then were challenged with 0.2% galactose. After 2 h of galactose challenge, cells were harvested for RNA isolation and RT-PCR analysis. Data are represented as mean ± SEM (n = 3). One-way ANOVA was applied to the dataset for multiple comparisons, followed by the Bonferroni *post hoc* test. ** $p < 0.01$, **** $p < 0.0001$, # $p < 0.01$. **(B)** *gal7*Δ cells were grown as described and RNA collected for RT-PCR analysis. Data are represented as mean ± SEM (n = 5). One-way ANOVA was applied to the dataset for multiple comparisons, followed by the Bonferroni *post hoc* test. **** $p < 0.0001$. **(C)** A ten-fold serial dilution of the indicated strains was plated in solid YPGly medium in the presence or absence of galactose or myriocin. Plates were incubated for 4 days at 30 °C before they were photographed. Figure is representative of three independent experiments. **(D)** *gal7*Δ cells were grown as detailed above and treated with galactose for 2 h. Galactose-1-phosphate measurements were performed as described in the Materials and methods section. **(E)** Yeast *gal7*Δ cells were grown in YPGly medium, in the presence or absence of myriocin. Cells were harvested, washed, lyophilized and the lipid fraction isolated for LC-MS/MS analyses. The heatmap represents the mean of the $\log_2$-transformed relative values of sphingolipids from myriocin treated cultures *versus* control cultures. n = 3. **(F)** *gal7*Δ cells were grown in YPGly, in the presence or absence of myriocin, and then challenged with galactose for 24 h. Samples prior to and after the 24 h galactose treatment were plated in fresh YPD medium, and after 2 days,

colony forming units were counted. Data represented as mean ± SEM (n = 5). 2way ANOVA was applied to the dataset, followed by the Sidak *post hoc* multiple comparisons test.

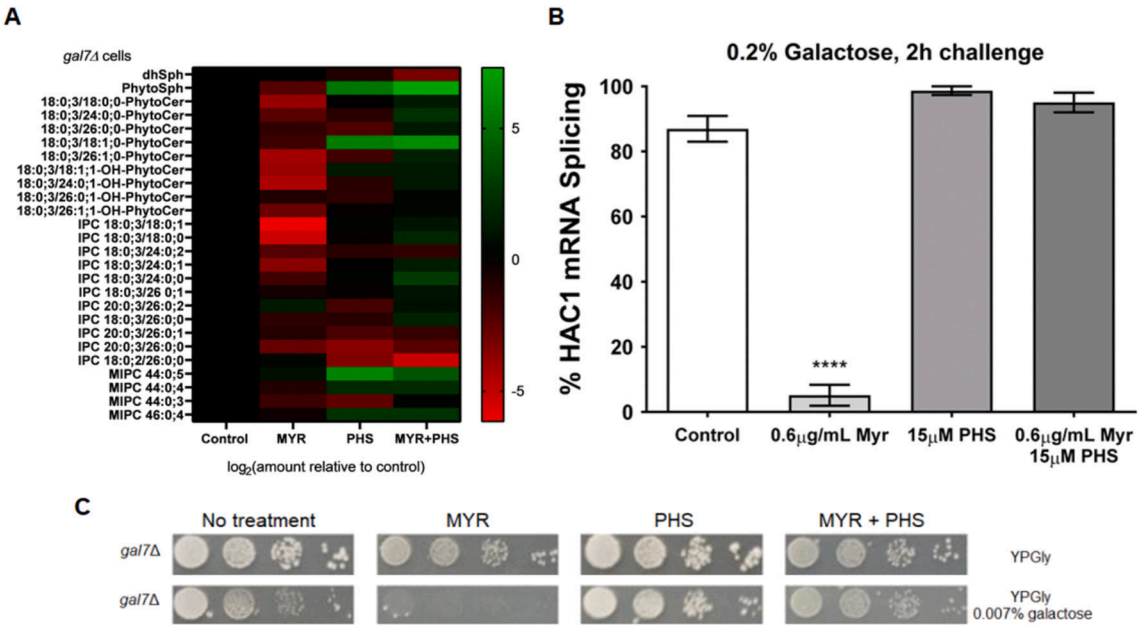

Fig. 5.

Causal relationships between sphingolipid depletion and UPR activation and between sphingolipid depletion and galactose hypersensitivity. **(A)** Yeast *gal7*Δ cells were grown in YPGly medium, in the presence or absence of myriocin, supplemented or not with 15 μM PHS. Cells were harvested, washed, lyophilized and the lipid fraction isolated for LC-MS/MS analyses. The heatmap represents the mean of the $\log_2$-transformed relative values of sphingolipids from myriocin and/or PHS treated cultures *versus* control cultures. n = 3. **(B)** Yeast *gal7*Δ cells were grown in YPGly overnight and then transferred to fresh YPGly media, containing or not 0.6 μg/mL MYR, or 15 μM PHS or both. When cultures reached approximately 0.2 O.D./mL, cells were challenged with 0.2% galactose and samples were harvested for total RNA extraction and *HAC1* mRNA splicing analysis after 2 h. Data are represented as mean ± SEM (n = 3). One-way ANOVA was applied to the data set and Bonferroni *post hoc* test was used for multiple comparisons. **** $p < 0.0001$. **(C)** A ten-fold serial dilution of the *gal7*Δ strain was plated in solid YPGly medium in the presence or not of myriocin, PHS or galactose as indicated. Plates were incubated for 4 days at 30 °C before they were photographed. Figure is representative of three independent experiments.

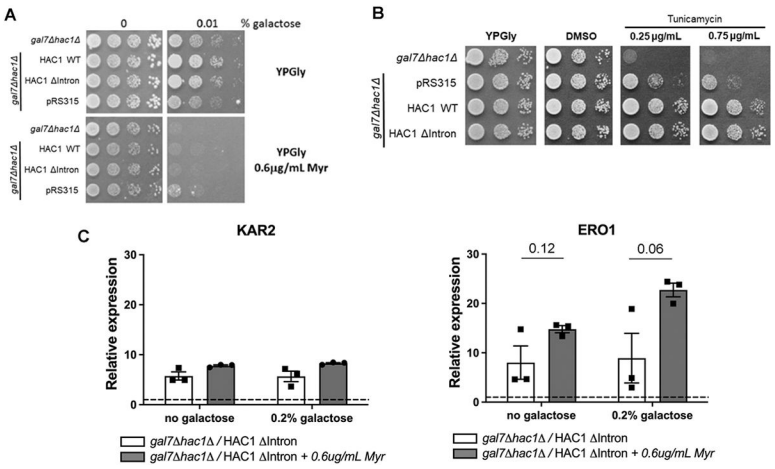

Fig. 6.

Constitutive activation of the UPR is not sufficient to restore galactose toxicity in sphingolipid-depleted galactosemic cells. **(A)** A ten-fold serial dilution of the indicated strains were plated in solid YPGly medium in the presence or absence of galactose, myriocin or both as indicated. Plates were incubated for 4 days at 30 °C before they were photographed. Figure is representative of three independent experiments. **(B)** The indicated strains were plated in solid YPGly medium containing or not 0.1% DMSO or the indicated concentrations of tunicamycin. Plates were incubated for 4 days at 30 °C before they were photographed. Figure is representative of three independent experiments. **(C)** Cells of the *gal7*Δ strain expressing the constitutively active allele of *HAC1* gene (HAC1 Δintron) were grown in YPGly medium in the presence or absence of myriocin, and then challenged with 0.2% galactose for 2 h. Samples were processed for qRT-PCR analysis. Myriocin administration *per se* did not inhibit Hac1p activity as a transcription factor, since mRNA levels of known Hac1p target genes (*ERO1* and *KAR2*) does not decrease in samples treated with myriocin. Dashed line indicates the expression level of target genes in the *gal7*Δ strain not treated with galactose (non-activated UPR). n = 3. Multiple *t*-test (myriocin treated *vs* untreated group) was used for the analysis, followed by Holm-Sidak *post hoc* test to determine statistical significance.