

Both human and soya bean ferritins highly improve the accumulation of bioavailable iron and contribute to extend the chronological life in budding yeast

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Summary

Ferritin proteins have an enormous capacity to store iron in cells. In search for the best conditions to accumulate and store bioavailable iron, we made use of a double mutant null for the monothiol glutaredoxins GRX3 and GRX4. The strain *grx3grx4* accumulates high iron concentrations in the cytoplasm, making the metal easily available for ferritin chelation. Here, we perform a comparative study between human (L and H) and soya bean ferritins (H1 and H2) function in the eukaryotic system *Saccharomyces cerevisiae*. We demonstrate that the four human and soya bean ferritin chains are successfully expressed in our model system. Upon coexpression of either both human or soya bean ferritin chains, respiratory conditions along with iron supplementation led us to obtain the maximum yields of iron stored in yeast described to date. Human and soya bean ferritin chains are functional and present equivalent properties as promoters of cell survival in iron overload conditions. The best system revealed that the four human and soya bean ferritins possess a novel function as anti-ageing proteins in conditions of iron excess. In this respect, both ferritin chains with

oxidoreductase capacity (human-H and soya bean-H2) bear the highest capacity to extend life suggesting the possibility of an evolutionary conservation.

Introduction

Iron is an essential metal used as an essential cofactor by a variety of proteins involved in multiple processes, such as cellular respiration, lipid biosynthesis, translation and amino acid biogenesis, DNA replication and repair, oxygen transport, photosynthesis or nitrogen fixation (Ramos *et al.*, 2020). Excess or deficiency of iron can have harmful effects to the cells and the consequence of an impaired iron metabolism results in multiple diseases (Ward *et al.*, 2014; Angelova and Brown, 2015; Biasiotto *et al.*, 2016). Excess of free iron can lead to the generation of deleterious reactive oxygen species (ROS) due to their role in Fenton and Haber-Weiss reactions (Koppenol and Hider, 2019).

Iron bioavailability is limited because Fe³⁺ form ferric hydroxides with a tendency to form precipitates at a physiological pH 7.4. Iron supplementation with ferrous salts is one of the common strategies to combat iron deficiency anaemia (IDA) the most frequent nutritional disorder worldwide in humans (Zimmermann and Hurrell, 2007).

However, this treatment can provoke several inconveniences associated to gastric alterations and a poor iron absorption in the intestine (Zimmermann and Hurrell, 2007). In line with this, some studies searching for biotechnological approaches have shown that iron contained in ferritin is soluble and bioavailable to alleviate iron deficiency in humans and animals (Beard *et al.*, 1996; Chang *et al.*, 2005).

Ferritins are expressed in most eubacteria, archaea, plants, animals and humans, with the exception of yeasts (Arosio *et al.*, 2009). Yeast genome in their turn contains a gene, *YFH1* encoding for a small mitochondrial protein involved in iron-sulfur cluster assembly called frataxin. Yfh1p monomers can assemble in an iron-dependent manner to form a macromolecular complex similar to ferritins with iron storage capacity (Park *et al.*, 2003; Seguin *et al.*, 2010).

The main function of ferritins is to store bioavailable iron and at the same time to avoid the oxidative stress

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that free iron accumulation can cause in cells. Ferritins are proteins with antioxidant capacity since they protect cells from the Fenton reaction and from de accumulation of hydroxyl radicals (Arosio and Levi, 2002).

Human ferritins can be divided into two main monomers: the chain H (Heavy) and the chain L (Light) (21 and 19 kDa respectively) (Arosio *et al.*, 2017). The H chain has ferrous oxidase activity and is capable to oxidize Fe^{2+} to Fe^{3+} , whereas the L ferritin is involved in iron nucleation, hence it is capable to store larger iron atoms than the H chain. The H chain contains a dinuclear ferroxidase site located in the four-helix bundle of the subunit and it catalyses the oxidation of ferrous iron by O_2 producing hydrogen peroxide. On the contrary, the L-subunit contains additional glutamate residues on the internal surface of the protein shell which produce a microenvironment that facilitates mineralization and the turnover of Fe^{3+} at the H-subunit ferroxidase centre.

Plant ferritins are similar to animal ferritins (Liu and Theil, 2005). Ferric iron is also in a shell of ferritins subunits as an iron core (Waldo *et al.*, 1995). Plant ferritins exhibit distinctive characteristics, they are nuclear located in plastids and only possess H-type subunits that equivalently to animal ferritins also perform nucleation and oxidation (Laulhere *et al.*, 1989; Li *et al.*, 2009). A more recent study concluded that either animal or plant ferritins can be equivalently used and biotechnologically engineered as iron sources (Lv *et al.*, 2015).

In *Saccharomyces cerevisiae*, external iron is captured and imported into the cells through specific channels integrated by the Fet3/Ftr1 proteins, which are part of the iron high-affinity system (Stearman *et al.*, 1996). These genes along with a wide group related to iron metabolism in general constitute the iron regulon (Outten and Albetel, 2013; Martinez Pastor *et al.*, 2017). This regulon is under the surveillance of Aft1/Aft2 transcriptional factors (Yamaguchi-Iwai *et al.*, 1996; Shakoury-Elizeh *et al.*, 2004). When iron is limiting Aft1 translocates to the nucleus, on the contrary, iron refeeding induces its cytoplasm localization. The two redundant monothiol glutaredoxins, Grx3 and Grx4 regulate Aft1 transcriptional function and also participate in export Aft1 from the nucleus (Pujol *et al.*, 2006). The simultaneous absence of both *GRX3* and *GRX4* causes a constitutive Aft1 localization in the nucleus and hence induction of the iron regulon, as a consequence of that *grx3grx4* double mutant accumulates large quantities of iron (Pujol *et al.*, 2006) in the cytoplasm (Mühlenhoff *et al.*, 2010). The double-mutant *grx3grx4* exhibits endogenous oxidative stress in part provoked by the high concentrations of iron accumulated in the cytoplasm (Pujol *et al.*, 2006) and also to the impairment of other cellular functions such as the interaction with the cell integrity pathway (Pujol and de la Torre-Ruiz, 2017).

Budding yeast are GRAS (generally recognized as safe) microorganisms. They constitute a suitable model system to explore multiple processes conserved in human or superior eukaryotes. Yeasts are also very useful in biotechnology for multiple applications, to obtain a by-product taking advantage of the easiness to expression of heterologous proteins or their simple use as a nutritional supplement. In this respect, there are some examples in the literature illustrating the use of yeast as a cell factory to express ferritins with the aim of supplying bioavailable iron to humans or animals (Kim *et al.*, 2003; Seo *et al.*, 2003; Kyaly *et al.*, 2015; de Llanos *et al.*, 2016).

In this study we show that the double mutant *grx3grx4* of *Saccharomyces cerevisiae* is a suitable model to over-express both heavy and light ferritin chains from humans and soya bean. We present data demonstrating that by using this model we can obtain the maximum yield of bioavailable iron described to date, in special, in respiratory conditions of growth. We also show that either human or soya bean ferritins are equally efficient in iron storage when expressed in budding yeast. In our study all the ferritin chains have a good potential as antioxidants and what is more interesting, they all increase the chronological life span of budding yeast in iron overloaded media. Our study spans the knowledge of ferritins function and opens and improves the conditions for the use of yeast as cell factories for bioavailable iron storage.

Results

L and H human and H1 and H2 soya bean ferritin chains are functional to chelate and store iron in conditions of iron overload in Saccharomyces cerevisiae

We focused our efforts in trying to optimize the concentration of bioavailable iron stored in the yeast *Saccharomyces cerevisiae*. To achieve this, we made use of the overexpression of eukaryotic ferritins, more specifically the H and L human chains and the H1 and H2 soya bean chains in several yeast mutants known to abnormally accumulates iron in the cells: *grx3grx4* (Pujol *et al.*, 2006) which accumulates high iron concentrations in the cytoplasm due to the abnormal activation of the iron regulon, *slt2* (Pujol *et al.*, 2021) which also is related to Aft1 dysregulation and the consequent increase in cellular iron content as compared to wild-type cells and the constitutively active allele Aft1-up (Pujol *et al.*, 2021) transformed in each wt and *grx3grx4* strains. This allele has a mutation in Aft1p (C291F), which provokes a constitutive localization in the nucleus and hence a constant activation of the iron regulon stimulating the uptake of Fe (Babcock *et al.*, 1997).

We cloned each of the four ORFs of the ferritins (human H and L chains and soya bean H1 and H2 chains) in the plasmid pUG35 fused to the GFP epitope in the C

terminus, to be subsequently transformed into *S. cerevisiae* strains indicated above. Our first objective was to determine the capacity to store iron that each of the four ferritins conferred to each of the yeast strains described above. With this purpose, we used different concentrations of the metal and different metabolic conditions. Without external iron addition, the overexpression of each of the four ferritins did not substantially increase the iron concentration accumulated in the cells (Fig. 1A). In Fig. 1A, we represent the steady iron levels calculated in conditions of exponential growth in SD medium for the strains mentioned above. We can observe the basal levels of iron in each of the strains tested, being the double-mutant *grx3grx4* and the expression of Aft1-up the strains with in which we could determine the highest iron content (Fig. 1A). The expression of each of the four ferritins was similar in all the strains tested (Fig. 1B).

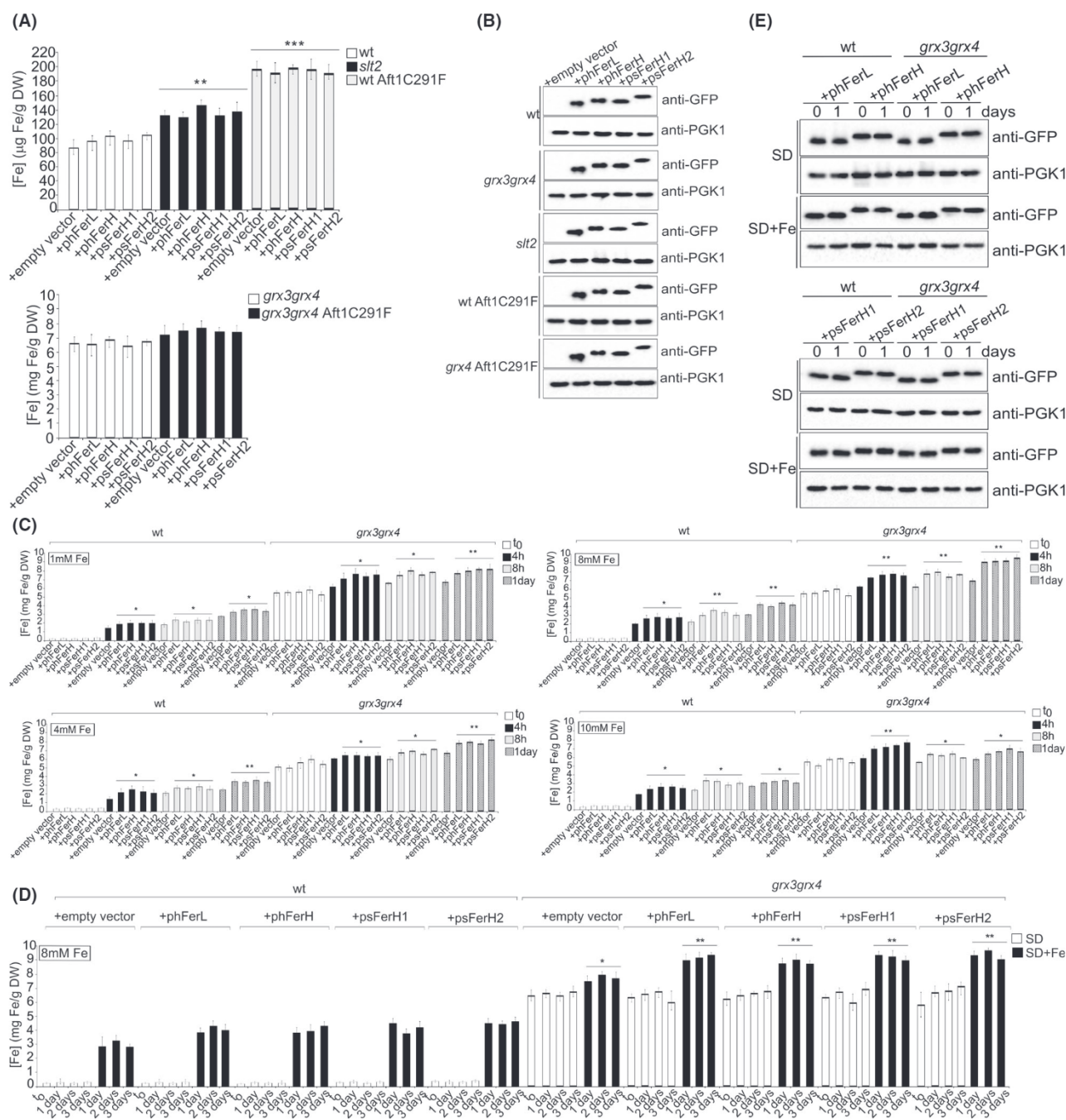
In order to optimize the experimental procedure, we tried different iron concentrations in SD medium: 1, 4, 8 and 10 mM and collected samples at different times of incubation: in logarithmic conditions (O.D.₆₀₀: 0.6) (t_0) and upon 4, 8 h and 1 day (when the cells begin to enter into diauxic shift) to determine iron concentration in the cultures (Fig. 1C). We performed these assays in both wt and *grx3grx4* the strain that contained the highest iron levels from all those tested above, transformed with each of the human and soya bean ferritins. The addition of iron to the different cultures provoked, as expected, a high increase in cellular iron content in all the strains tested. However, and relevant for this study, the overexpression of any of the four ferritins significantly increased the concentration of iron stored in both wt and *grx3grx4* cells (Fig. 1C). The amount of iron accumulated in the cells was proportional to the concentration of iron in the media up to 8 mM. Concentrations in the culture media higher than 8 mM (10 mM) did not improve the yield of iron stored in the cells. We could SHI determine that addition of 8 mM and 1 day of incubation in selective medium (SD) were the best conditions to obtain the highest yields of iron in both wt and *grx3grx4* mutant ($\sim 9 \text{ mg Fe g}^{-1} \text{ DW}$) when any of the four ferritins were used. We tried longer periods of incubation upon the addition of 8 mM iron (Fig. 1D). We observed that 2 or 3 days of culture did not render higher intracellular iron concentrations as compared to 1 day, probably due to the loss of viability that cells experience during ageing (Fig. 1D). In these conditions, the total iron concentration determined in both strains was similar when either of the four ferritins were overexpressed. In addition, it can be observed that the levels of expression of the four human and soya bean ferritin chains were similar in both wt and *grx3grx4* strains and for all the times tested (log phase and 1 day), thus validating that our results can be comparative (Fig. 1E).

The expression of human or soya bean ferritins in the absence of both glutaredoxins Grx3 and Grx4 provokes the maximum intracellular iron accumulation in respiratory conditions during the diauxic shift

Once established the optimal conditions regarding iron concentration and times of incubation, we directed our efforts to try to obtain the best yields of intracellular iron chelated by either human (L and H) or soya bean (H1 and H2) ferritin chains in the four strains mentioned above: *grx3grx4*, *slt2*, wtAft1C291F and *grx3grx4Aft1C291F*.

In all cases, the highest levels of intracellular iron chelated by either of the four ferritin chains were obtained in the double mutant *grx3grx4*, upon 1 day of culture in SD medium supplemented with 8 mM iron (Table 1). The presence of Aft1-up in wt cells provoked a significant increase in iron concentration, although the absolute values were lower than those determined in *grx3grx4* (Table 1). Curiously, the presence of the allele Aft1-up in *grx3grx4* containing each of the four ferritins did not further improve the values of iron concentration determined in that strain as compared to *grx3grx4* only transformed with any of the human or soya bean ferritins (Table 1). The mutant *slt2* accumulated higher intracellular iron concentrations as compared to wt cells, as a consequence of a higher Aft1 activity as described in (Pujol *et al.*, 2021) (Table 2). We did not observe a significant difference between wt and the *slt2* mutant with regard to intracellular iron levels when H and L or H1 and H2 ferritins were expressed 1 day upon iron addition to SD medium. The absence of the MAPK Slr2 provokes cell wall problems that can be at least partly alleviated upon the addition of sorbitol to the culture medium. When we grew *slt2* strain containing each of the four ferritins in SD medium containing 0.8 M sorbitol, we observed a small but significant increase in iron storage caused by human ferritins L and H as compared to that determined in wt cells (Table 3). Nevertheless, the absolute values of iron concentration determined in *slt2* carrying each of the four ferritins and cultured in SD medium plus sorbitol were lower than those observed in the double mutant *grx3grx4* (Table 3). In conclusion and supporting the findings shown above, when external iron is supplied, both human and soya bean ferritins are similarly efficient in the capacity to store intracellular iron in all the strains tested, except in the mutant *slt2*. In these conditions, the highest values of iron stored in cells were determined in the double mutant *grx3grx4* of the yeast *Saccharomyces cerevisiae*.

In view of the former results, we continued our analyses with both wt and *grx3grx4* strains expressing each of the four ferritins mentioned in this study. We also cloned both ferritins in pUG35 without GFP epitope



fused in the C terminus and in the plasmid pCM265 under the *tetO₇* promoter (Garí *et al.*, 1997) fused or not to HA epitope, with the object to check whether the epitope would impair the capacity of the ferritins to store iron. We did not observe any significant difference in the capacity to store intracellular iron between these plasmids and between proteins fused or not to each of both epitopes GFP or HA (not shown).

The observation that the highest levels of iron were determined upon 1 and 2 days of culture in glucose SD

made us to consider the possibility that a respiratory metabolism would favour iron storage in ferritins, since at this moment nearly all the glucose has been metabolized to ethanol and cells have entered into the diauxic shift with the consequent onset of a respiratory metabolism. In order to prove this hypothesis, we used glycerol as a respiratory carbon source and incubated both wt and *grx3grx4* strains for several periods of time as depicted in Table 4. The highest levels of iron accumulated in both wt and *grx3grx4* ($\sim 12 \text{ mg Fe g}^{-1} \text{ DW}$) strains were

Fig. 1. Study of the most optimal iron concentrations and times of incubations to obtain the highest yields of iron stored in yeast cells overexpressing either human or soya bean ferritin chains.

A. Determination of total iron content in yeast cells exponentially growing in SD medium. wt, *slt2*, wtAft1C291F, *grx3grx4* and *grx3-grx4Aft1C291F* strains were transformed with empty plasmid pUG35 (+empty vector) and with plasmids pUG35 containing human ferritin chains (+phFerL and +phFerH) or soya bean ferritin chains (+psFerH1 and +psFerH2) (see Material and Methods). Cultures were logarithmically grown (O.D.₆₀₀:0.6) in SD medium plus amino acids at 30°C to determine the total iron concentration as described in Materials and Methods. Endogenous iron content displayed as weight of iron per yeast dry weight (DW) (iron concentration was expressed in µg Fe per gram of dry weight for wt, *slt2* and wtAft1C291F strains and in mg Fe per gram of dry weight for *grx3grx4* and *grx3grx4C291F* strains). Histograms represent the average values from three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, 0.001 > *P* > 0.0001).

B. Protein expression of both human (L and H) and soya bean (H1 and H2) ferritin chains in yeast cells exponentially growing in SD medium. Samples from exponentially growing cultures described in A were collected for protein extraction and western blot analyses to identify the expression of both human (L and H) and soya bean (H1 and H2) ferritin chains. Ferritin chains were detected with the anti-GFP antibody. Anti-Pgk1 antibody was used as a loading control to detect the protein Pgk1.

C. Optimization of iron concentrations and times of incubation in yeast cells growing in SD medium. Wt and *grx3grx4* cells transformed with each of the human or soya bean ferritin chains and the empty vector as in (A) were exponentially grown in SD medium plus amino acids to O.D.₆₀₀:0.6. At this moment, each culture was split into five parts and 1, 4, 8 or 10 mM iron were, respectively, added to four of them. Samples were collected at the indicated times (4 h, 8 h and 1 day) to determine the total iron concentration as in (A). In this case, iron concentration was expressed in mg Fe g⁻¹ DW. Histograms represent the average values obtained from three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001).

D. Exponentially growing cells described in (A) were treated (SD + Fe) or not (SD) with 8 mM of iron. Samples were collected upon 1, 2 and 3 days of culture to determine the total iron content as in (A). Iron concentration was expressed in mg Fe g⁻¹ DW as in (C). Histograms represent the average values obtained from three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001).

E. Samples exponentially growing and upon 1 day of culture from (D) were taken and processed for protein extraction and Western blot analyses to observe the expression of each of the human and soya bean ferritin chains as in (B).

achieved between upon 1 day of incubation in glycerol. Again, values were equivalent when any of the human (L and H) or soya bean (H1 or H2) ferritins were overexpressed in these strains (Table 4). The expression of the four ferritins in the respiratory medium SGly was similar to that determined in the fermentative medium SD in all the times tested (Fig. 2). In this context, we determined and increase of 34.3% in iron concentration in cultures of *grx3grx4* and of 32.2% in wt cells as compared to the use of glucose as a carbon source. We conclude that the use of carbon sources that activate a respiratory metabolism and avoid fermentation in budding yeast favours higher yields of iron stored in cells upon overexpression of human or soya bean ferritins.

Coexpression of both human (L and H) or soya bean (H1 and H2) ferritin chains enhances the amount of iron accumulated in yeast cells

We next decided to assay the coexpression of both ferritin chains, L and H and H1 and H2 in both wt and *grx3-grx4* strains. In Fig. 3A, we show that L and H ferritins and also the couple H1-H2 are expressed at similar levels in both wt and *grx3grx4* and during the time of the experiment. The coexpression of both human or soya bean ferritins significantly increased the accumulation of iron in both strains, in both media, SD (~ 11 mg Fe g⁻¹ DW) and SGly (~ 13 mg Fe g⁻¹ DW) with respect to the single chains (Fig. 3B). We concluded that the highest concentrations of iron accumulated in cells were determined in *grx3grx4* co-expressing either soya bean or human ferritins and growing in SGly for 1 day upon

addition of 8 mM of iron (Fig. 3B). Our results show that both human and soya bean ferritins are equivalently functional with respect to the capacity to chelate and store iron in budding yeast.

Ferritins suppress oxidative stress and iron toxicity in the absence of both Grx3 and Grx4 glutaredoxins

In order to start to analyse the effects that both human and soya bean ferritins have on viability, we first checked that neither of the four ferritin chains did not alter the pattern of the survival curves of any of the strains under all tested conditions (Table 5).

We have previously reported that *grx3grx4* double mutant presents a high level of endogenous oxidative stress (Pujol *et al.*, 2006) as compared to wt cells, consequence of cytoplasmic iron accumulation caused by the abnormal activity of the iron regulon (Mühlenhoff *et al.*, 2010). We plated wt and *grx3grx4* strains transformed with each of the four ferritins, human and soya bean, into SD plates containing different concentrations of iron (Fig. 4A). The four ferritins suppressed the toxicity that iron provoked in the double mutant although the L human ferritin and the H2 soya bean ferritin were more efficient than their corresponding partners at lower iron concentrations, 15 and 20 mM. Above those values, 25 and 30 mM, there were no observable differences between ferritins (Fig. 4A). Moreover, we also observed that both human and soya bean ferritins equivalently reduced the oxidative stress caused by iron overload in the absence of both Grx3 and Grx4 glutaredoxins (Fig. 4B). In conclusion, the use of a budding yeast mutant with endogenous

Table 1. Average values of total iron content ([Fe] mg Fe g⁻¹ DW) and standard deviations (SD) calculated for the following strains: wt, wtAft1C291F, *grx3grx4Aft1C291F*. Each strain was transformed with the empty vector pUG35 (-empty vector) and with each of the human ferritin chains (+phFerL or +phFerH) or soya bean ferritin chains (+psFerH1 or +psFerH2) cloned in pUG35 vector.

		[Fe] (mg Fe g ⁻¹ DW)	SD	t-Student value	[Fe] (mg Fe g ⁻¹ DW)	SD	t-Student value
wt	-empty vector	t ₀	±0.01		t ₀	±0.13	
		1 day	±0.01		1 day	±0.14	
	SD+Fe	1 day	±0.12		SD+Fe	±0.12	
	SD	t ₀	±0.01		SD	±0.13	
	+phFerL	1 day	±0.01		+phFerL	±0.14	
	SD+Fe	1 day	±0.13		SD+Fe	±0.12	0.00043**
	SD	t ₀	±0.01		SD	±0.14	
	+phFerH	1 day	±0.01		+phFerH	±0.14	
	SD+Fe	1 day	±0.12		SD+Fe	±0.13	0.00051**
	SD	t ₀	±0.02		SD	±0.12	
wtAft1C291F	-empty vector	t ₀	±0.01		t ₀	±0.13	
		1 day	±0.01		1 day	±0.13	
	SD+Fe	1 day	±0.13		SD+Fe	±0.14	0.00053**
	SD	t ₀	±0.01		SD	±0.12	
	+psFerH2	1 day	±0.02		+psFerH2	±0.15	
	SD+Fe	1 day	±0.12		SD+Fe	±0.14	0.00038**
	SD	t ₀	±0.01		SD	±0.14	
	+empty vector	t ₀	±0.01		t ₀	±0.12	
		1 day	±0.01		1 day	±0.13	
	SD+Fe	1 day	±0.11		SD+Fe	±0.15	
wtAft1C291F	-empty vector	t ₀	±0.01		t ₀	±0.13	
		1 day	±0.01		1 day	±0.13	
	SD+Fe	1 day	±0.12		SD+Fe	±0.13	0.00054**
	SD	t ₀	±0.02		SD	±0.13	
	+phFerH	1 day	±0.11		+phFerH	±0.14	
	SD+Fe	1 day	±0.11		SD+Fe	±0.13	0.00050**
	SD	t ₀	±0.01		SD	±0.12	
	+psFerH1	1 day	±0.01		+psFerH1	±0.14	
	SD+Fe	1 day	±0.11		SD+Fe	±0.13	0.00039**
	SD	t ₀	±0.01		SD	±0.13	
wtAft1C291F	-empty vector	t ₀	±0.01		t ₀	±0.12	
		1 day	±0.01		1 day	±0.12	
	SD+Fe	1 day	±0.11		SD+Fe	±0.13	0.00048**
	SD	t ₀	±0.01		SD	±0.11	
	+empty vector	t ₀	±0.01		t ₀	±0.11	
		1 day	±0.01		1 day	±0.11	
	SD+Fe	1 day	±0.11		SD+Fe	±0.11	
	SD	t ₀	±0.01		SD	±0.11	
	+phFerL	1 day	±0.01		+phFerL	±0.11	
	SD+Fe	1 day	±0.01		SD+Fe	±0.11	

Strains were grown to exponential phase (t₀) and to diauxic shift (1 day) in SD and SD fortified with 8 mM of iron (SD+Fe), plus amino acids at 30°C. Samples were collected to determine the total iron concentration as described in Fig. 1A. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, 0.001 > *P* > 0.0001).

Table 2. Average values of total iron content ([Fe] mg Fe g⁻¹ DW) and standard deviations (SD) calculated for wt, *slt2* and *grx3grx4* strains expressing the empty vector and each of the human and soya bean ferritins, as in Table 1, were grown to exponential phase (*t*₀) and to diauxic shift (1 day) in SD and SD supplemented with 8 mM of iron (SD+Fe) plus amino acids.

				[Fe] (mg Fe g ⁻¹ DW)	SD	t-Student value
wt	+empty vector	SD	<i>t</i> ₀	0.1	±0.01	
			1 day	0.1	±0.01	
	+phFerL	SD+Fe	1 day	3.0	±0.20	
		SD	<i>t</i> ₀	0.1	±0.01	
	+phFerH	SD+Fe	1 day	4.1	±0.13	
		SD	<i>t</i> ₀	0.1	±0.01	
	+psFeH1	SD+Fe	1 day	4.2	±0.12	
		SD	<i>t</i> ₀	0.1	±0.01	
	+psFerH2	SD+Fe	1 day	4.0	±0.13	
		SD	<i>t</i> ₀	0.1	±0.01	
		SD+Fe	1 day	4.1	±0.12	
		SD	<i>t</i> ₀	0.2	±0.01	0.0016**
<i>slt2</i>	+empty vector		1 day	0.2	±0.01	0.0021**
		SD+Fe	1 day	3.0	±0.11	
	+phFerL	SD	<i>t</i> ₀	0.2	±0.01	0.0018**
			1 day	0.2	±0.01	0.0024**
	+phFerH	SD+Fe	1 day	4.0	±0.12	
		SD	<i>t</i> ₀	0.2	±0.02	0.0027**
	+psFeH1		1 day	0.2	±0.02	0.0020**
		SD+Fe	1 day	4.2	±0.12	
	+psFerH2	SD	<i>t</i> ₀	0.2	±0.01	0.0023**
			1 day	0.2	±0.01	0.0026**
		SD+Fe	1 day	4.0	±0.13	
		SD	<i>t</i> ₀	0.2	±0.01	0.0021**
<i>grx3grx4</i>	+empty vector		1 day	0.2	±0.01	0.0023**
		SD+Fe	1 day	4.1	±0.12	
	+phFerL	SD	<i>t</i> ₀	6.3	±0.11	
			1 day	6.4	±0.13	
	+phFerH	SD+Fe	1 day	7.4	±0.12	
		SD	<i>t</i> ₀	6.4	±0.13	
	+psFeH1		1 day	6.5	±0.11	
		SD+Fe	1 day	9.1	±0.13	0.0045**
	+psFerH2	SD	<i>t</i> ₀	6.3	±0.14	
			1 day	6.8	±0.13	
		SD+Fe	1 day	9.1	±0.12	0.0040**
		SD	<i>t</i> ₀	6.3	±0.13	
			1 day	6.4	±0.14	
		SD+Fe	1 day	9.3	±0.14	0.0051**
		SD	<i>t</i> ₀	6.4	±0.12	
			1 day	6.7	±0.12	
		SD+Fe	1 day	9.0	±0.13	0.0032**

Samples were collected to determine the total iron content as described in Fig. 1A. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001).

oxidative levels and that anomalously hyper-accumulates iron in the cytoplasm has revealed the functionality as prosurvival and antioxidant proteins of the four ferritin chains, human L and H and soya bean H1 and H2. However, neither human nor soya bean ferritins suppressed the lack of viability that *slt2* mutant showed in response to iron overload (not shown). This observation suggests that ferritins function in yeast in certain metabolic conditions related to iron storage.

We repeated these experiments in plates with glycerol as the sole carbon source to force cells to perform a

respiratory metabolism (Fig. 4C). We obtained similar results at those described above. Again, the four ferritins H, L, H1 and H2 equally rescued both the lack of viability of *grx3grx4* mutant caused by iron overload (Fig. 4C), and as observed in SD cultures (Fig. 4A) again human L and ferritin H2 were the most efficient at lower iron concentrations. Consequently, human and soya bean ferritins are all functional in budding yeast as iron chelators and as detoxifiers of the oxidative stress caused by iron overload, thus promoting cell survival in both fermentative and respiratory conditions.

Table 3. Average values of total iron content ([Fe] mg Fe g⁻¹ DW) and standard deviations (SD) calculated for wt, *slt2* and *grx3grx4* strains expressing the empty vector and each of the human and soya bean ferritins, as in Table 2, except that sorbitol was added to each culture (SD + 0.8 M Sorbitol and SD + 0.8 M Sorbitol +8 mM Fe).

				[Fe] (mg Fe g ⁻¹ DW)	SD	t-Student value
wt	+ empty vector	SD+Sorbitol	<i>t</i> ₀	0.1	±0.01	
			1 day	0.1	±0.01	
	+phFerL	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	3.0	±0.12	
			1 day	0.1	±0.01	
	+phFerH	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	4.0	±0.11	
			1 day	0.1	±0.01	
	+ ps F eH1	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	4.2	±0.12	
			1 day	0.1	±0.01	
	+psFerH2	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	4.0	±0.13	
			1 day	0.1	±0.01	
<i>slt2</i>	+empty vector	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	4.0	±0.13	
			1 day	0.2	±0.01	
	+phFerL	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	0.2	±0.02	
			1 day	3.0	±0.12	
	+phFerH	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	0.2	±0.01	
			1 day	5.5	±0.12	0.015*
	+psFeH1	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	0.2	±0.02	
			1 day	0.2	±0.01	
	+psFerH2	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	5.7	±0.12	0.018*
			1 day	0.2	±0.01	
<i>grx3grx4</i>	+empty vector	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	4.0	±0.13	
			1 day	0.2	±0.01	
	+phFerL	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	4.1	±0.13	
			1 day	6.3	±0.11	
	+phFerH	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	6.4	±0.13	
			1 day	7.5	±0.12	
	+psFeH1	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	6.4	±0.12	
			1 day	6.5	±0.13	
	+psFerH2	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	9.1	±0.12	0.0041**
			1 day	6.2	±0.13	
	+psFerH2	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	6.9	±0.14	
			1 day	9.1	±0.13	0.0038**
	+psFerH2	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	6.2	±0.13	
			1 day	6.4	±0.13	
	+psFerH2	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	9.0	±0.14	0.0030**
			1 day	6.3	±0.14	
	+psFerH2	SD Sorbitol+Fe SD+Sorbitol	<i>t</i> ₀	6.6	±0.12	
			1 day	9.1	±0.13	0.0036**

Samples were collected to determine the total iron content as described in Fig. 1A. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001).

Coexpression of the two ferritin chains from humans or soya bean provoked an additional increment in *grx3-grx4* survival in both SD (Fig. 4D) and SGly (Fig. 4F) plates containing high and low iron concentrations as compared to the values obtained upon the expression of each single ferritin (Fig. 4D). Nevertheless, the simultaneous expression of both human or soya bean ferritin chains did not provoke a further reduction in the *grx3-grx4* oxidative stress caused by iron overload as compared to the effects caused by the expression of each single ferritin chain (Fig. 4E).

Both human and soya bean ferritins play a role in chronological life span in conditions of iron overload in budding yeast

It has been reported that the L human chain ferritin presented certain capacity to increment life extension in a budding yeast mutant (Desmyter *et al.*, 2004). We decided to explore the potential function that each of the four human or soya bean ferritins and also their coexpression could play in both wt and *grx3grx4* life extension, both under normal conditions or upon iron

Table 4. Average values of total iron content ([Fe] mg Fe g⁻¹ DW) and standard deviations (SD) calculated for wt and *grx3grx4* strains transformed with the empty vector or each of the four ferritin chains (human and soya bean) respectively, as in Table 1, were exponentially grown in SD, SD plus 8 mM of iron (SD+Fe), SGly (containing glycerol as unique carbon source) and SGly plus 8 mM of iron (SGly+Fe) media plus amino acids.

							[Fe] (mg Fe g ⁻¹ DW)		t-Student value		[Fe] (mg Fe g ⁻¹ DW)		SD	t-Student value
							t ₀	1 day			t ₀	1 day		
wt	+empty vector	SD					t ₀	0.1	±0.01		wt	t ₀	0.1	±0.01
							1 day	0.1	±0.01			1 day	0.1	±0.02
	+phFerL	SD+Fe					t ₀	2.9	±0.11			t ₀	4.1	±0.11
		SD					1 day	0.1	±0.01			1 day	0.1	±0.02
	+phFerH	SD+Fe					t ₀	0.1	±0.01			t ₀	0.1	±0.01
		SD					1 day	4.1	±0.13			1 day	5.2	±0.12
	+psFerH1	SD+Fe					t ₀	0.1	±0.01			t ₀	0.1	±0.02
		SD					1 day	0.1	±0.01			1 day	0.1	±0.02
	+psFerH2	SD+Fe					t ₀	4.3	±0.12			t ₀	5.6	±0.13
		SD					1 day	0.1	±0.01			1 day	0.1	±0.01
<i>grx3grx4</i>	+phFerL	SD+Fe					t ₀	0.1	±0.01			t ₀	0.1	±0.01
		SD					1 day	0.1	±0.01			1 day	0.1	±0.01
	+psFerH1	SD+Fe					t ₀	4.0	±0.11			t ₀	5.4	±0.13
		SD					1 day	0.1	±0.01			1 day	0.1	±0.01
	+psFerH2	SD+Fe					t ₀	0.1	±0.01			t ₀	0.1	±0.01
		SD					1 day	0.1	±0.01			1 day	0.1	±0.01
	+empty vector	SD+Fe					t ₀	4.3	±0.12		<i>grx3grx4</i>	t ₀	5.4	±0.13
		SD					1 day	6.4	±0.13			1 day	7.7	±0.13
	+phFerL	SD+Fe					t ₀	6.5	±0.14			t ₀	7.4	±0.14
		SD					1 day	7.3	±0.12			1 day	97	±0.13
	+phFerH	SD+Fe					t ₀	6.5	±0.14			t ₀	8.4	±0.13
		SD					1 day	6.6	±0.14			1 day	8.9	±0.13
	+psFerH1	SD+Fe					t ₀	9.1	±0.14	0.0043**		t ₀	12.0	±0.13
		SD					1 day	6.4	±0.14			1 day	8.5	±0.13
	+psFerH2	SD+Fe					t ₀	6.3	±0.13	0.0051**		t ₀	12.0	±0.14
		SD					1 day	6.5	±0.15			1 day	8.3	±0.12
	+empty vector	SD+Fe					t ₀	9.2	±0.14	0.0053**		t ₀	12.0	±0.12
		SD					1 day	6.4	±0.12			1 day	8.0	±0.12
	+phFerL	SD+Fe					t ₀	7.0	±0.15			t ₀	8.4	±0.13
		SD					1 day	9.0	±0.14	0.0039**		1 day	12.1	±0.14

Samples were collected at the indicated times to determine total iron accumulated in cells as described in Fig. 1A. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001).

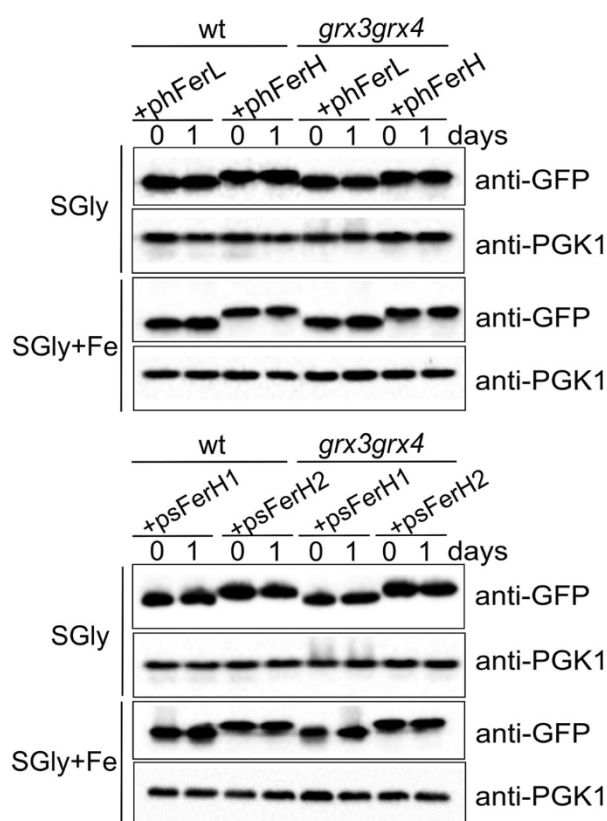


Fig. 2. Protein expression of both human (L and H) and soya bean (H1 and H2) ferritin chains in yeast cells growing in SGly medium. Protein expression of each of the human (L and H) or soya bean (H1 and H2) ferritin chains were determined in wt and *grx3grx4* cells cultures growing exponentially or upon 1 day in SGly and in SGly supplemented with 8 mM of iron (SGly+Fe) plus amino acids. Samples were collected and processed for Western blot. Ferritin chains were detected with the anti-GFP antibody. Pgk1, detected with anti-PGK1 antibody was used as a loading control.

overload. We have performed an extensive comparative analysis of human and soya bean ferritins in conditions of iron overload and found that the four ferritins, human H and L and soya bean H1 and H2, significantly contributed to extend life in both wt and *grx3grx4* strains (Fig. 5A and B). In these conditions, heavy chains, H2 from soya bean and H from human ferritin were more efficient in this function that their corresponding partners, either H1 or L chains. Coexpression of L and H and H1 and H2, however, did not increase chronological life span as compared to the expression of single H or H2 ferritin chains (Fig. 5A and B). However, when culture media did not contain excess of iron, in standard SD medium, only the heavy chains, H and H2, from human and soya bean ferritins, respectively, significantly extended chronological life as compared to the CLS values calculated in both wt and *grx3grx4* strains transformed with the empty plasmid. In this situation the coexpression of both heavy or light chains from human

or soya bean ferritins did not cause any additional effect in life extension neither in wt nor in *grx3grx4* mutant as compared to the observed with the single chains H and H2 (Fig. 5A and B).

In conclusion, we have demonstrated that human and soya bean ferritins are fully functional to extend life in a wt strain and in *grx3grx4* double mutant that accumulated abnormal concentrations of iron in the cytoplasm when iron is overloaded in the culture media. Our results indicate that coexpression increases cell survival upon iron overload, as compared to the single expression of each heavy and light ferritin chains, by oxidizing and chelating iron in the yeast *Saccharomyces cerevisiae*. It is really interesting that both human and soya ferritins played an equivalent role regarding life extension in budding yeast. We also present evidence demonstrating that is the heavy chain with oxidoreductase capacity the ferritin that possess a conserved function in either soya bean or humans to extend life in standard or iron overload conditions of growth.

Discussion

In this study, we show that the yeast mutant, *grx3grx4*, with special characteristics related to iron accumulation in the cytoplasm, is an excellent system to express both human and soya bean ferritins to render the highest iron levels accumulated per cell described in the literature. Ferritin superfamily has an extraordinary capacity to store iron from bacteria to humans. On despite yeast do not encode ferritins in their genome, their capacity to successfully express heterologous proteins makes them a suitable eukaryotic system to assay different mutants and conditions in order to obtain the highest concentrations of bioavailable iron.

In model organisms, as occurs in yeast, human ferritins have been coexpressed in a specific strain rendering maximum levels of iron of 1 mg g⁻¹ dry weight (Kim *et al.*, 2003). We have improved the concentration of iron chelated by the simultaneous overexpression of both L and H human ferritin chains in the double mutant *grx3grx4* to nearly 11 mg g⁻¹ dry weight in glucose media and to 13 mg g⁻¹ dry weight in glycerol minimum media. When we expressed single ferritins each of them, L or H human ferritin chains were equally efficient in iron storage in wt or in the double-mutant *grx3grx4*. These results disagreed with the study of (Seo *et al.*, 2003). These authors claim that only H chain had a function in iron storage in *Saccharomyces cerevisiae*. In our study, L chain is also capable to induce the storage of a high concentration of iron, at the same level as observed upon H chain expression. We cannot rule out these discrepancies, it is possible that part of the reason is the efficiency of the overexpression and the yeast

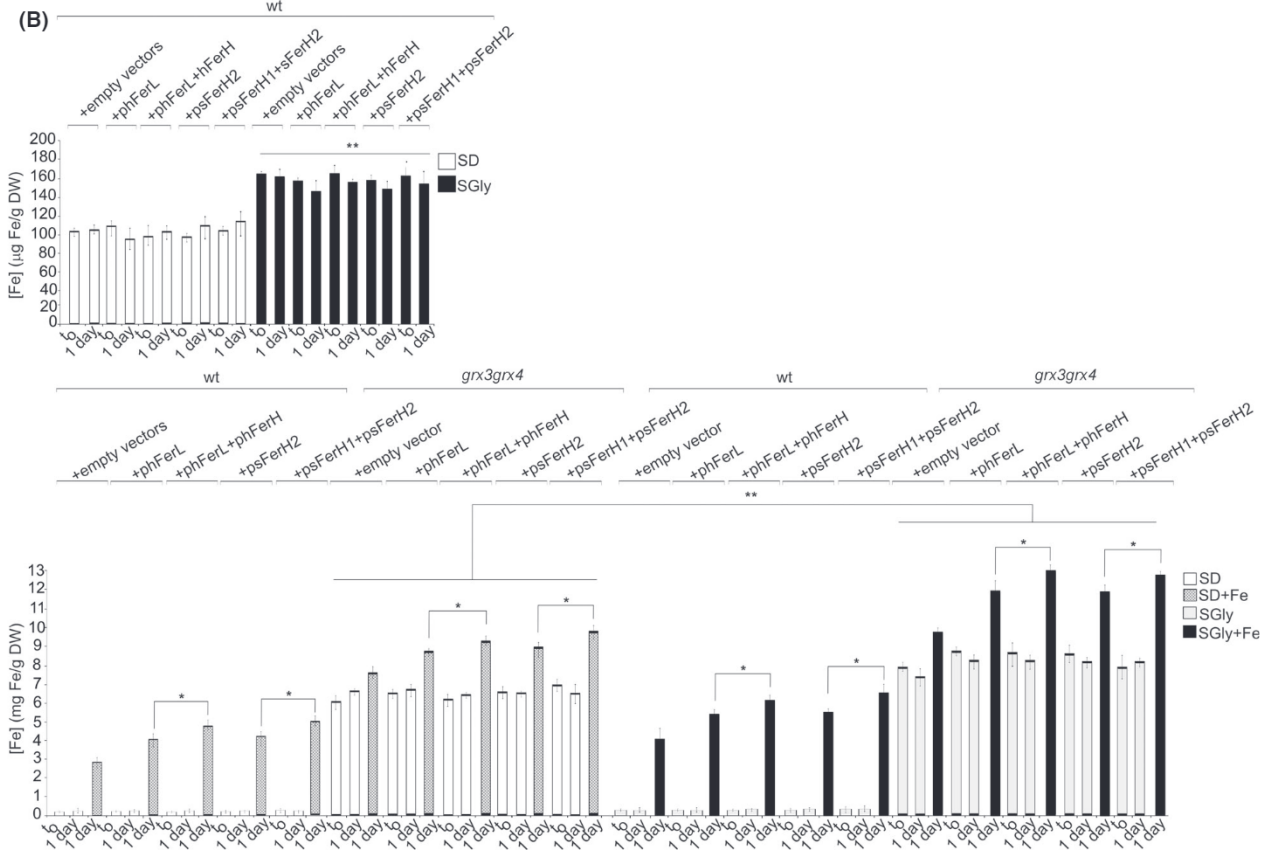
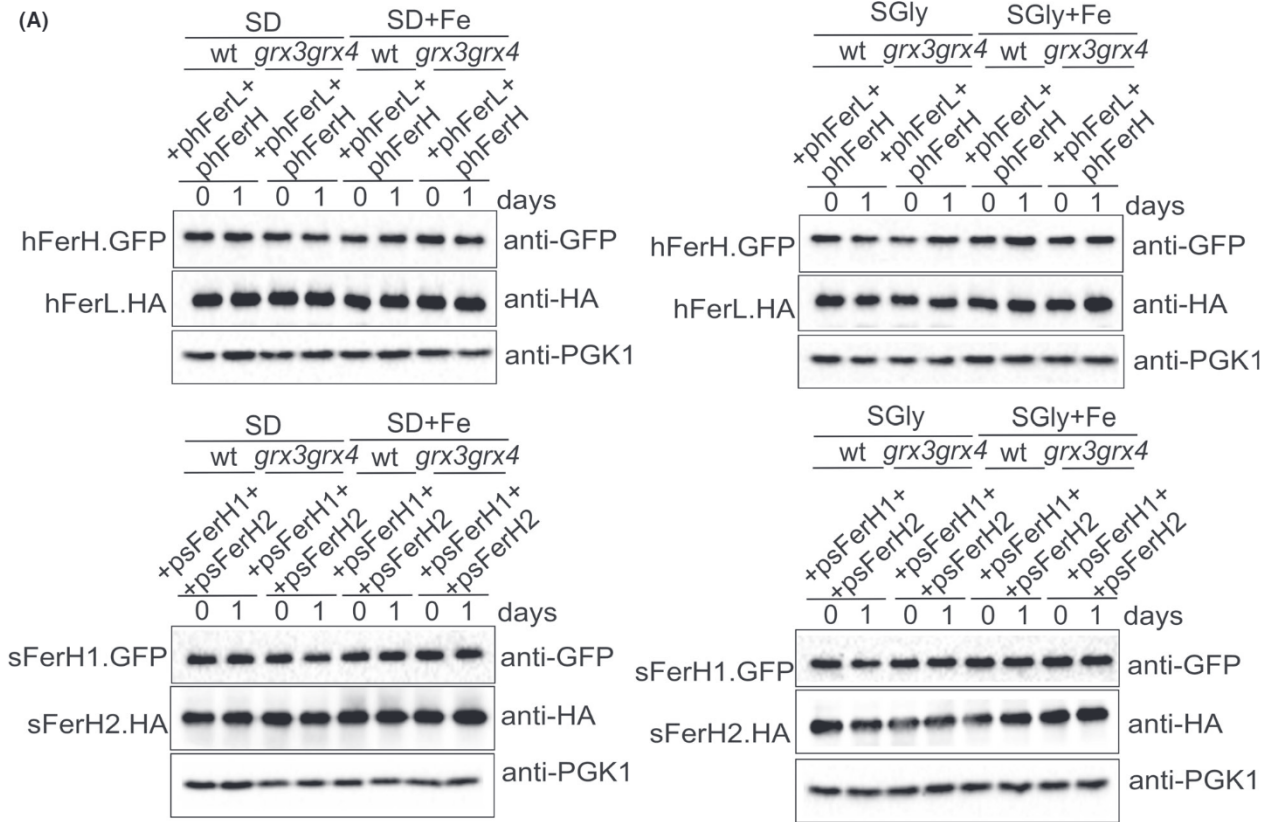


Fig. 3. Simultaneous coexpression of both human and both soya bean ferritin chains significantly in a double-mutant *grx3grx4* growing in SGly medium for 1 day upon addition of 8 mM of iron is the best condition to obtain the highest concentration of iron accumulated in cells.

A. WthFerL.HA and *grx3grx4*hFerL.HA strains were each transformed with phFerH.GFP, whereas the strains wtsFerH2.HA and *grx3grx4*sFerH2 were transformed with psFerH1.GFP plasmid. Cultures from the four resultant strains co-expressing both human and soya bean ferritin chains, respectively, were grown to logarithmic phase and until 1 day in SD, SGly, SD+Fe and SGly+Fe (8 mM of iron). Samples were collected for protein extraction and subsequent detection of both ferritin chains by Western blot. Human L ferritin chain (phFerL.HA) and soya bean H2 ferritin chain (psFerH2.HA) were detected by means of the anti-HA antibody. Human H ferritin chain (phFerH.GFP) and soya bean H1 ferritin chain (psFerH1.GFP) were detected by using the anti-GFP antibody. In all cases, Pgk1 was used as a loading control.

B. Total iron content in exponentially growing cells and upon 1 day of culture (diauxic shift) in the strains and cultures described in (A). Histograms represent the average values between three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001).

Table 5. Average values of generation time (h) and standard deviations (SD) calculated for wt and *grx3grx4* cultures expressing the empty vector pUG35 (+empty vector) and each of single or combined human (+ phFerL and +phFerH) or soya bean ferritin chains (+psFerH1 and +psFerH2) growing either in SD or SD+Fe (8 mM of iron) plus amino acids at 30°C with continuous shaking (170 rpm).

			Generation time (h)	SD
wt	SD	+empty vector	2.5	±0.1
		+psFeH1	2.4	±0.1
		+psFerH2	2.5	±0.1
		+psFerH1+psFerH2	2.4	±0.1
		+phFerL	2.3	±0.1
		+phFerH	2.6	±0.1
		+phFerL+phFerH	2.4	±0.1
	SD+Fe	+empty vector	2.6	±0.1
		+psFeH1	2.4	±0.1
		+psFerH2	2.5	±0.1
		+psFerH1+psFerH2	2.5	±0.1
		+phFerL	2.4	±0.1
		+phFerH	2.6	±0.1
		+phFerL+phFerH	2.5	±0.1
<i>grx3grx4</i>	SD	+empty vector	3.0	±0.1
		+psFeH1	3.1	±0.1
		+psFerH2	3.0	±0.1
		+psFerH1+psFerH2	3.0	±0.1
		+phFerL	2.9	±0.1
		+phFerH	3.1	±0.1
		+phFerL+phFerH	3.2	±0.1
	SD+Fe	+empty vector	3.2	±0.1
		+psFeH1	3.2	±0.1
		+psFerH2	3.0	±0.1
		+psFerH1+psFerH2	3.1	±0.1
		+phFerL	3.0	±0.1
		+phFerH	3.2	±0.1
		+phFerL+phFerH	3.0	±0.1

background used in those analyses. Iron contained in soya bean ferritin is well absorbed by humans, as demonstrated in certain studies (Lönnerdal, 2009). Recently, the single and simultaneous expression of soya bean ferritins in a budding yeast mutant has been demonstrated to provoke an iron-enriched strain of potential use as an iron source (de Llanos *et al.*, 2016). The iron yields in our preferred yeast mutant system *grx3grx4* has been improved by simultaneous overexpression of both H1 and H2 soya bean ferritins, producing around 11 mg Fe g⁻¹ dry weight in SD medium and 13 mg Fe g⁻¹ dry weight in SGly.

To date, we could not find any data on human or soya bean ferritins that showed higher iron levels than those obtained in this study. Moreover, from a biotechnological point of view, the advantage of using SD medium is that the absence of dropout is an important saving.

We have found that respiratory metabolism improves the ability of both human and soya bean ferritins to chelate and store iron in cells. This is basically the main explanation to the observation that upon 1 day of culture in SD medium we observed the maximum levels of intracellular iron since at that time of incubation the cultures enter into diauxic shift, glucose concentration is dramatically reduced and the Crab tree effect disappears in favour of a respiratory metabolism (Klein *et al.*, 2017). Consequently, glycerol is the preferred carbon source to induce either human or soya bean ferritins to accumulate the highest iron concentrations per dry weight. One interpretation could be that the change from fermentative to respiratory metabolism would augment the iron cellular requirements and consequently the mechanisms to internalize iron. In this context, the presence of overexpressed ferritins would favour the capture of iron leading to cells containing higher iron concentrations. Ferritin-like iron storage proteins (ISPs) are also tightly upregulated when *Escherichia coli* cells enter into stationary phase to ensure iron availability (Pourciau *et al.*, 2019).

The high yield of iron obtained in the double-mutant *grx3grx4* is explained by the fact that the constitutive activation of the iron regulon driven by the nuclear activity of Aft1 causes high levels of iron accumulated in cells (Pujol *et al.*, 2006). Moreover, in this double mutant, iron is stored in the cytoplasm (Mühlenhoff *et al.*, 2010), as opposed to vacuoles and mitochondria as occurs in wt cells (Miao *et al.*, 2011). This condition probably makes iron more accessible to ferritins. This interpretation is consistent with the observation that the overexpression of the allele Aft1-up, which constitutively expresses iron regulon genes (Babcock *et al.*, 1997), did not improve the iron chelated by ferritins when transformed in the *grx3grx4* double mutant and did not provoke that wt cells reached values of iron chelated by ferritins similar to those calculated in *grx3grx4*. It has been published that this allele forces to internalize iron but quickly are

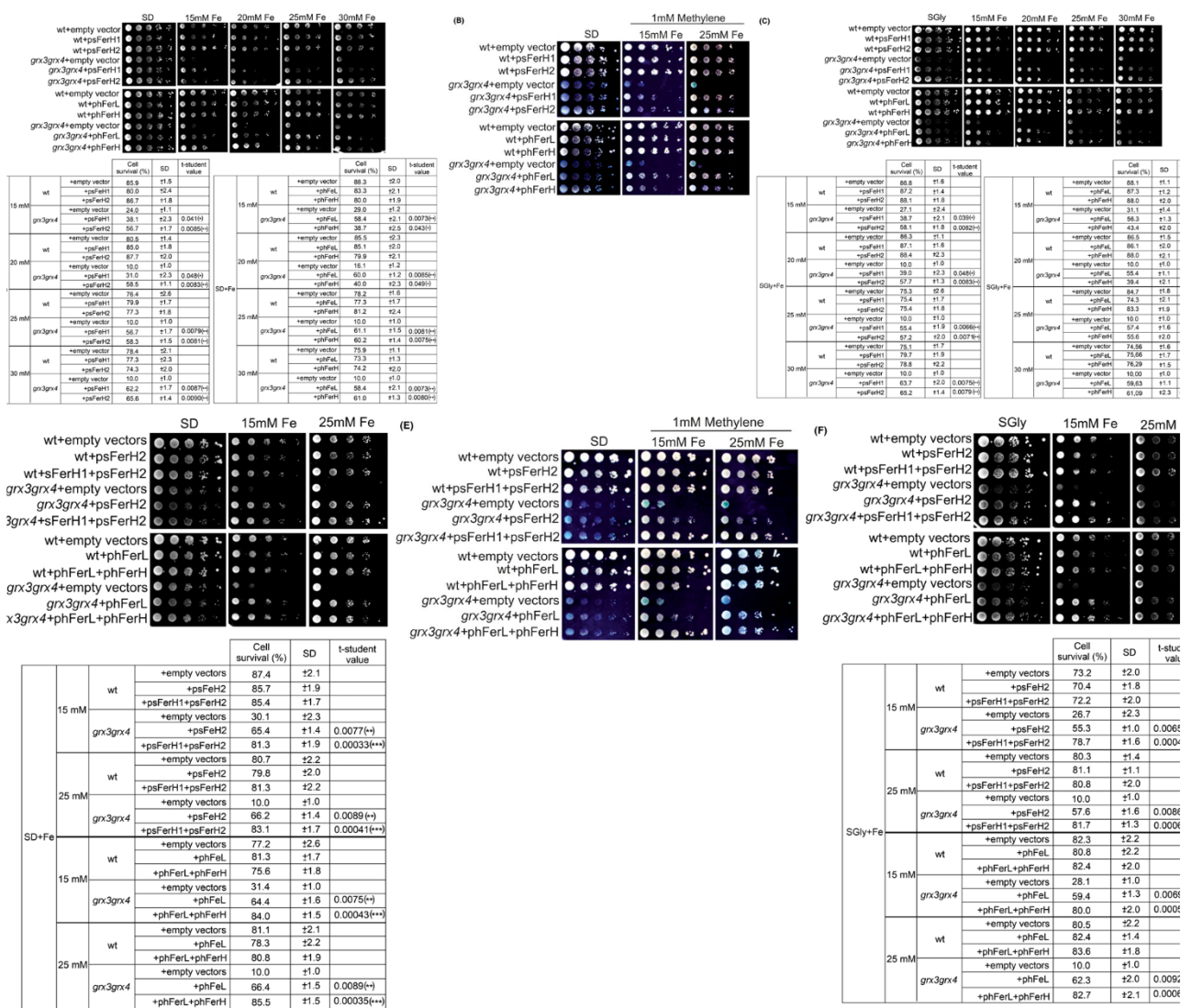


Fig. 4. Both human and soya bean ferritin chains possess antioxidant properties to detoxify for iron accumulation in the absence of Grx3 and Grx4 glutaredoxins.

A. Wt and *grx3grx4* strains were transformed with pUG35 (+empty vector), phFerL, phFerH1, psFerH1 or psFerH2 respectively. Cultures were logarithmically grown (O.D.₆₀₀:0.6) in SD medium plus amino acids at 30°C, to be subsequently serially diluted and plated in triplicate onto SD plates containing or not different iron concentrations (15, 20, 25 and 30 mM Fe). Plates were grown at 30°C for three days. Numerical data represent the average values between three independent experiments regarding cell survival (%) in all the concentrations tested for each strain. SD: standard deviations. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001).

B. Cultures described in (A) were plated in triplicate onto SD plates containing the redox state indicator Methylene Blue (1 mM) plus 15 mM or 25 mM of iron.

C. Strains described in (A) were grown to logarithmic phase (O.D.₆₀₀:0.6) in SD medium plus amino acids at 30°C to be subsequently plated in triplicate onto SGly plates (containing glycerol as unique carbon source) containing or not different concentrations of iron (15, 20, 25 and 30 mM Fe). Plates were grown at 30°C for three days.

D. Strains described in Fig. 3A, co-expressing either L and H human or H1 and H2 soya bean ferritin chains, were grown to logarithmic phase (O.D.₆₀₀:0.6) in SD medium plus amino acids at 30°C to be subsequently plated in triplicate on SD plates containing or not 15 mM and 25 mM of iron. Plates were grown at 30°C for three days. Numerical data represent the average values between three independent experiments regarding cell survival (%) in all the concentrations tested for each strain. SD: standard deviations. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, 0.001 > *P* > 0.0001).

E. Cultures and strains used in (D) were serially diluted and plated in triplicate onto SD plates containing the redox state indicator Methylene Blue (1 mM) plus 15 mM or 25 mM of iron as in (B).

F. The same cultures described in (D) were exponentially grown in SD medium plus amino acids at 30°C to be subsequently serially diluted and plated in triplicate on SGly plates (containing glycerol as unique carbon source) containing 15 mM and 25 mM of iron. Plates were grown at 30°C for three days. Numerical data represent the average values between three independent experiments regarding cell survival (%) in all the concentrations tested for each strain. SD: standard deviations. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001; ***, 0.001 > *P* > 0.0001).

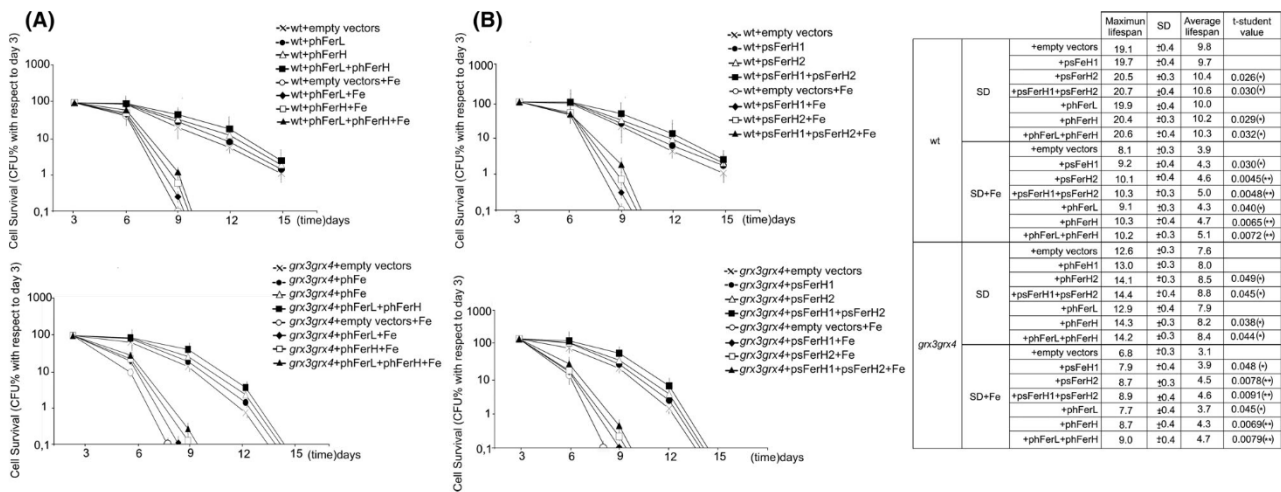


Fig. 5. Both human and soya bean ferritin chains favour life span extension in special in iron overloaded cultures. Chronological life span curves for wt+empty vectors, wt+phFerL, wt+phFerH, wt+phFerL+phFerH, *grx3grx4*+empty vectors, *grx3grx4*+phFerL, *grx3grx4*+phFerH and *grx3grx4*+phFerL+phFerH strains (A) and wt+psFerH1, wt+psFerH2, wt+psFerH1+psFerH2, *grx3grx4*+psFerH1, *grx3grx4*+psFerH2 and *grx3grx4*+psFerH1+psFerH2 strains (B). Cultures were exponentially grown OD₆₀₀:0.6 in SD medium plus amino acids at 30°C to be subsequently split into two: 8 mM Fe was added to one half, whereas the other half was taken as a control. Samples were taken at the indicated times to determine CLS, as described in Materials and Methods. Numerical data regarding maximum life span (the day when cultures reach 10% survival) and average life span (the day at which 50% survival was recorded) for each strain are depicted. SD: standard deviations. Error bars are represented with *P* values from a Student's unpaired *t*-test (*, 0.05 > *P* > 0.01; **, 0.01 > *P* > 0.001).

translocated mainly to vacuoles (Miao *et al.*, 2011), and at a lesser extent into mitochondria, in where is not available for ferritins that use to be located mainly in the cytoplasm. We believe that this is what occurs in the mutant *slt2*, recently described to accumulate high levels of iron (Pujol *et al.*, 2021), Fig. 2B, where the expression of each of the four ferritins did not render the expected levels of iron chelated and stored in the ferritins core. One interpretation is that this excess of iron might be stored inside vacuoles not available for ferritins.

Eid *et al.* (2016) determined that human ferritin H chain has a prosurvival function in yeast. Other authors (Desmyter *et al.*, 2004) demonstrated that human ferritin L chain was capable to partly suppress the oxidative stress caused by iron excess in the frataxin mutant *yfh1* of *Saccharomyces cerevisiae*. Recently, de Llanos *et al.* (2016) presented evidence demonstrating that H1 and H2 soya bean ferritins acted as prosurvival and antioxidants in the yeast mutant *ccc1*, deficient in iron vacuole storage. Our results support those results and extends those functions to the human ferritin L chain. Here, we demonstrate that each human (L and H) or soya bean (H1 and H2) ferritin chains and to a greater extended upon the simultaneous expression of both L/H and H1/H2 ferritins was capable to act as prosurvival and antioxidant molecules preventing the production of reactive oxygen species, such H₂O₂, which are related to Fenton reactions, when the oxidative stress is caused by iron overload.

L human ferritin has been reported to extend life in *yfh1* mutants, the yeast frataxin that plays a similar role

to ferritins (Shin *et al.*, 2001). Our results expand this evidence to heavy chains from human and soya bean systems with oxidoreductase function. By doing chronological life span experiments, we demonstrate that both human and soya bean ferritins efficiently extend life in the *grx3grx4* mutant and in wild-type cells. We also present evidence, demonstrating that heavy chains (from human and soya bean) are more efficient than light chains in life extension in standard and iron overload conditions of growth. Furthermore, we present additional relevant information: soya bean ferritins extend the chronological life of budding yeast at a similar level to human ferritins. Whether this function is related to the mechanisms by which iron can be released from the mineral core of ferritins and to became useful to cells or alternatively, whether ferritins play and iron independent function in life extension deserves to be more profoundly investigated and yeast are a suitable model for that.

Most neurodegenerative disorders are linked to altered homeostasis of biometals such as iron being accumulated in aged cells. There is evidence that iron accumulates in the brain during ageing (Ward *et al.*, 2014). Certain mutations in the human L ferritin chain provoke iron accumulation in neurons associated with neurological diseases and cell death (Cozzi *et al.*, 2010). Recently, it has been identified that iron not bound to ferritin causes ferroptotic cell death and promotes ageing in human neurons and fibroblasts (Cozzi *et al.*, 2019). The ability to heterologous over-express functional human or soya bean ferritins in wild type or yeast mutant opens future perspectives for

research into iron bioavailability and ferritin functions in budding yeast, such as their role in ageing. A very recent paper in which the authors engineer the high-affinity Fe transporter (PiFTR) from the fungus *Piriformospora indica* through co-cultivation with rice leading to the increase in plant Fe intake with a future application in plant biofortification (Verma *et al.*, 2021). Our findings have a projection towards biotechnology applications in human and animal health and nutrition based on iron production and regulation of iron storage.

Experimental procedures

Yeast strains and plasmids

Saccharomyces cerevisiae strains, wild type (*MATa leu2-3,112 ura3-52 trp1 his4 can1r*), the single mutant *slt2::KanMX4* and *wtAft1C291F.HA* have been previously described in Pujol *et al.* (2021), whereas the double-mutant *grx3::NatMX4grx4::KanMX4* was previously reported in (Pujol *et al.*, 2006). Strain *grx3grx4Aft1C291F.HA* was obtained in this study upon integration of plasmid pMM392 (Pujol *et al.*, 2021) previously digested with EcoRV in the *LEU2* locus. The strains *wtFerL.HA* and *grx3grx4hFerL.HA* were obtained upon integration of the plasmid pTP581, whereas the strains *wtFerH2.HA* and *grx3grx4sFerH2.HA* were obtained upon integration of the plasmid pTP579 respectively. Both plasmids were previously digested with EcoRV to be integrated in the *LEU2* locus. Both pTP581 and pTP579 plasmids were obtained upon cloning L human and H2 soya bean chains, respectively, into PmeI and NotI sites of the integrative vector pMM351 (Pujol *et al.*, 2006).

Human L and H ferritin chains were amplified from human cDNA contained in two plasmids purchased from Addgene: ID 22653 and ID122652, respectively, to be cloned into the BamHI and Sall sites of pUG35 vector (this vector is under the MET25 promoter and fused in frame to the GFP epitope in the C terminus (Pujol *et al.*, 2006). Both soya bean H1 and H2 ferritin chains were amplified from plasmids p416GPD-sFerH1 and p416-sFerH2 kindly provided by Dr. Sergi Puig (de Llanos, *et al.*, 2016) to be subsequently cloned into the BamHI and Sall sites of pUG35 vector.

Media and growth conditions

Yeasts were grown at 30°C in SD medium (2% glucose, 0.67% yeast nitrogen base that lacked the corresponding amino acids for plasmid maintenance) or glycerol medium (3% Glycerol, 0.67% yeast nitrogen base that lacked the corresponding amino acids for plasmid maintenance) plus amino acids (Kaiser *et al.*, 1994). Cell cultures were exponentially grown (optical density at 600 nm [OD₆₀₀] of 0.6) or at longer times as indicated.

Iron was added as ammonium iron (III) sulfate hexadecahydrate [NH₄Fe(SO₄)₂·6H₂O] (+Fe; F1543; Sigma). The final concentrations and the incubation times were indicated in each experiment. For oxidation stress experiments, the redox state indicator Methylene Blue (M9140; Sigma) was added to plates containing iron to a final concentration of 1 mM from a 1% stock solution (de Llanos *et al.*, 2016).

Endogenous iron measurements

Endogenous iron measurements were performed according to the colorimetric assay described in Tamarit *et al.* (2006).

Cell survival and chronological life span

To assay cell viability, cells were grown to mid-log phase at an OD₆₀₀ of 0.6 in SD medium supplemented with the required amino acids. Viability was registered through serial dilutions and plated in triplicate onto YPD plates (2% glucose, 2% peptone and 1% yeast extract).

Chronological life span (CLS) values calculated in the different strains were based on the survival of populations of non-dividing yeast cells according to the directions described in (Mechoud *et al.*, 2020). Viability was scored by counting the number of cells capable of forming colonies CFU (colony-forming units). Cultures were started at an OD₆₀₀ of 0.6. The same number of cells collected from each culture was plated in triplicate onto YPD plates and allowed to grow at 30°C for 3 to 4 days. Viability at day 3 is considered 100% survival (time zero in the CLS curves), since it is the moment when the great majority of the cells stop dividing. CLS curves were plotted with the corresponding averages and standard deviations from three independent experiments.

Protein extraction and immunoblot analyses

Total yeast protein extracts were prepared as previously described in Pujol *et al.* (2017). The antibodies for Western blotting were anti-HA 3F10 (no. 12158167001; Roche Applied Science, Penzberg, Upper Bavaria, Germany) used at a dilution of 1:2000 in 0.25% non-fat milk, anti-GFP (no. 632381; Living Colours, Takara Bio Europe, Saint Germain en Laye, France) at a dilution of 1:2000 and anti-Phospho-Glycerate Kinase (anti-PGK1) (no. 459250; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) at a dilution 1:10 000. The corresponding secondary antibodies were goat anti-rat IgG horseradish peroxidase conjugate (no. AP136P; Millipore, Merck, Burlington, MA, USA) for the primary anti-HA 3F10 and anti-mouse IgG horseradish peroxidase (from sheep) (no. NA931; GE Healthcare, Chicago, IL,

USA) for anti-GFP and anti-PGK1. All these antibodies were used as indicated by the manufacturers. The protein–antibody complexes were visualized by enhanced chemiluminescence using the Supersignal substrate (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) in a Chemidoc (Roche Applied Science).

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Conflict of interest

None declared.

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