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GENETICS AND MOLECULAR BIOLOGY

Interactions of GMP with Human Glrx3 and with *Saccharomyces cerevisiae* Grx3 and Grx4 Converge in the Regulation of the Gcn2 Pathway

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ABSTRACT The human monothiol glutaredoxin Glrx3 (PICOT) is ubiquitously distributed in cytoplasm and nuclei in mammalian cells. Its overexpression has been associated with the development of several types of tumors, whereas its deficiency might cause retardation in embryogenesis. Its exact biological role has not been well resolved, although a function as a chaperone distributing iron/sulfur clusters is currently accepted. Yeast humanization and the use of a mouse library have allowed us to find a new partner for PICOT: the human GMP synthase (hGMPs). Both proteins carry out collaborative functions regarding the downregulation of the *Saccharomyces cerevisiae* Gcn2 pathway under conditions of nutritional stress. Glrx3/hGMPs interact through conserved residues that bridge iron/sulfur clusters and glutathione. This mechanism is also conserved in budding yeast, whose proteins Grx3/Grx4, along with *GUA1* (*S. cerevisiae* GMPs), also downregulate the integrated stress response (ISR) pathway. The heterologous expression of Glrx3/hGMPs efficiently complements Grx3/Grx4. Moreover, the heterologous expression of Glrx3 efficiently complements the novel participation in chronological life span that has been characterized for both Grx3 and Grx4. Our results underscore that the Glrx3/Grx3/Grx4 family presents an evolutionary and functional conservation in signaling events that is partly related to GMP function and contributes to cell life extension.

IMPORTANCE *Saccharomyces cerevisiae* is an optimal eukaryotic microbial model to study biological processes in higher organisms despite the divergence in evolution. The molecular function of yeast glutaredoxins Grx3 and Grx4 is enormously interesting, since both proteins are required to maintain correct iron homeostasis and an efficient response to oxidative stress. The human orthologous Glrx3 (PICOT) is involved in a number of human diseases, including cancer. Our research expanded its utility to human cells. Yeast has allowed the characterization of GMP synthase as a new interacting partner for Glrx3 and also for yeast Grx3 and Grx4, the complex monothiol glutaredoxins/GMPs that participate in the downregulation of the activity of the Gcn2 stress pathway. This mechanism is conserved in yeast and humans. Here, we also show that this family of glutaredoxins, Grx3/Grx4/Glrx3, also has a function related to life extension.

KEYWORDS GCN2, GMP synthase, Glrx3, glutaredoxin, Grx3, Grx4, PICOT, aging, chronological life span, iron

Iron is one of the most abundant metals in eukaryotic cells. It is involved in multiple essential processes, such as mitochondrial respiration and lipid, protein, and DNA synthesis, among other functions. The maintenance of correct iron homeostasis is essential for cell survival. Iron deficiency can provoke human diseases, such as anemia (1), and it also can increase the risk of cancer (2), whereas iron overload provokes severe

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diseases caused by oxidative stress and is also associated with cancer incidence (3). Monothiol glutaredoxins contain an active site, Cys-Gly-Phe-Ser (CGFS), with no catalytic activity. The active cysteine jointly coordinates a [2Fe-2S] cluster with the aid of the sulfhydryl residue of a glutathione (GSH) molecule (4–7). Cytosolic monothiol glutaredoxins contain an amino-terminal thioredoxin (Trx) domain followed by one (Grx3 and Grx4 in *Saccharomyces cerevisiae*) or two tandem glutaredoxin (Grx) domains (Glrx3 in humans). This family of cytosolic monothiol glutaredoxins is needed for iron metabolism from lower to higher eukaryotes (8, 9). Grx3/Grx4 functions in iron homeostasis require binding to the GSH-ligated iron-sulfur clusters via the CGFS domain (10). Grx3 and Grx4 use iron/sulfur clusters ligated at the interface with partner proteins. Binding occurs through the cysteines located at the active sites of the glutaredoxins, glutathione, and specific histidine residues in Grx3/Grx4 proteins (5, 11–14). Evolutionarily, the cysteine residue corresponding to the active sites and the glutathione binding residues of the Grx domains of human Glrx3 are also the residues required to independently form iron/sulfur-bridged complexes with partner proteins, such as BolA (15). In budding yeast, the iron-sensing transcriptional activator Aft1 and its paralog, Aft2, regulate the subset of genes involved in iron homeostasis (16, 17). Double deletion of the monothiol Grx3 and Grx4 glutaredoxins leads to nuclear localization and the constitutive transcriptional activation of Aft1, affecting cellular iron homeostasis (18). Consequently, an excess of biologically unavailable iron is accumulated in a *grx3 grx4* mutant, impairing a large number of iron-dependent enzymes, suggesting that both Grx3 and Grx4 are involved in iron trafficking (19). Again, binding to iron/sulfur clusters through cysteine and glutathione residues is required for iron delivery to different substrates. A similar effect occurs when the human Glrx3 (PICOT) is depleted from human cells, affecting the function of proteins requiring iron/sulfur clusters (20, 21). The Grx domains of the human Glrx3 share 40% to 50% sequence identity with the Grx domains of *S. cerevisiae* Grx3 and Grx4; however, the identity found between the Trx domains is very low (less than 27%) (6, 22). Although iron-trafficking proteins that chaperone iron through the cytoplasm have been properly identified, there is a general consensus attributing a role for the conserved family of monothiol glutaredoxins Grx3/Grx4 and Glrx3 as [2Fe-2S] cluster chaperones, thereby playing an important role in iron metabolism and trafficking (7, 9, 23, 24).

The integrated stress response (ISR) is a signal transduction pathway highly conserved in all eukaryotic cells. Uncharged tRNAs, oxidative stress, glucose or amino acid deprivation, general nutritional stress, endoplasmic reticulum (ER) stress, viral infection, and many other injuries activate this pathway (for reviews, see references 25 and 26). In yeast, all these stimuli activate the kinase Gcn2 of the ISR pathway, whereas in human cells several kinases (*GCN2*, *PKR*, *HRI*, and *PERK*) receive different stresses converging and activating eIF2α, the core of the ISR pathway (25). The factor eIF2α is highly relevant for the initiation of mRNA translation (27). Upon ISR activation, eIF2α becomes phosphorylated, blocking the exchange of GDP to GTP mediated by eIF2, which attenuates the global protein synthesis but induces the translation of specific mRNAs, such as the transcription factor *GCN4* in yeast or ATF4 in humans (28). Thus, the basic mechanism for the activation of either *GCN4* or ATF4 in response to a number of different stresses (endoplasmic reticulum stress, hypoxia, oxidative stress, TOR inactivation, glucose depletion, etc.) occurs through translational regulation in all eukaryotic cells, from yeast to humans (25, 29–31).

The purine nucleotides ATP and GTP are required for cellular life. They are basic components to build nucleic acids, but they are also involved in conserved and essential processes, such as translation, building GTP, signaling, enzymatic reactions, etc. (32). The synthesis of guanine nucleotides requires IMP conversion into XMP by the IMP dehydrogenase (33), and then the GMP synthase (GMPs) (*GUA1* in yeast) catalyzes the amination of XMP to GMP (34).

A connection between the general amino acid control system of *S. cerevisiae* and the levels of purines has been identified by Rolfes and Hinnebusch (35), who observed that purine starvation provoked a compensatory induction of the ISR pathway. In this

respect, further studies have reported the exquisite relationship between purine metabolism and amino acid biosynthesis. Some authors (32) demonstrated that threonine metabolism is important for deoxynucleoside triphosphate (dNTP) homeostasis and showed that deficiencies in guanine synthesis lead to the derepression of the transcription factor *GCN4*. However, the exact mechanism that fully explains the cross talk between these pathways is not known.

With the aim of finding novel substrates for the human Glrx3, we used an updated two-hybrid approach and a mouse library. We isolated GMP synthase as a novel Glrx3/Grx3/Grx4 interactor protein. Our studies reveal an evolutionary conservation regarding the interaction between monothiol glutaredoxins and GMPs. Yeast allowed us to find a functional connection between the Grx/GMPs complex and the integrated stress response.

RESULTS

GMP synthase is a novel target of monothiol glutaredoxin Glrx3. To find new targets for Glrx3, we screened for novel interacting proteins by using the Matchmaker gold yeast two-hybrid system mate and plate library-mouse embryo-11 day. To start the screening, we cloned the open reading frame of *GLRX3* into the pGBKT7 plasmid carrying the GAL4 activating domain. The mouse library inserts were contained in plasmid pGADT7. We tested 4.7×10^7 independent clones by following the manufacturer's instructions. Upon isolation, sequencing, and identification, the sequences identified could be classified into three categories: 68% corresponded to zinc finger protein 788 of unknown function, 25% to GMP synthase, and 7% to peroxidasine. We performed *in vivo* interaction assays through the two-hybrid approach and demonstrated a clear strong interaction between Glrx3 and the mouse GMP synthase (Fig. 1A; see also Fig. S1A in the supplemental material). Since mouse and human GMP synthase present a high degree of homology (99%), we next cloned human GMP synthase into pACT2 and performed two-hybrid analyses with Glrx3. *In vivo* β-galactosidase assays revealed a strong interaction between these two proteins that was clearly detected and validated upon coimmunoprecipitation, as depicted in Fig. 1A and Fig. S1A, B, and C.

Iron-sulfur clusters are relevant for Glrx3/Grx3/Grx4 binding to GMP synthases. The family integrated by Glrx3 and the yeast Grx3/Grx4 use iron/sulfur clusters to bridge different target proteins. To perform this investigation, we mutagenized the residues of Glrx3 known to be involved in binding to other target proteins. We mutagenized the cysteine of the active center in each of the glutaredoxin domains, C159 (GrxA domain) and C261 (GrxB domain), and also the residues WP197/198 (GrxA domain) and WP299/300 (GrxB domain), which are known to be involved in the interaction with glutathione. All of them are required to bind iron/sulfur clusters and are evolutionarily conserved between human Glrx3 and Grx3/Grx4 in *S. cerevisiae*. All these mutagenized residues clearly precluded the formation of the Glrx3/GMP synthase complex (Fig. 1A and Fig. S1A), as previously described in the characterization of the complex formed between Glrx3 and the human protein BolA (4, 5). Our results indicate that Glrx3 uses iron/sulfur-bridged clusters to bind hGMP synthase. With the aim of detecting a conserved pattern in budding yeast, we checked the potential *in vivo* interaction through a two-hybrid assay between *GUA1*, the yeast GMP synthase, and each of Grx3 and Grx4 glutaredoxins. Grx3 and Grx4 bound to *GUA1*, although less strongly than the previously observed interaction between Glrx3/hGMPs (Fig. 1A and Fig. S1A). For a better characterization of the evolutionary conservation of this interaction, we decided to investigate the possible existence of *in vivo* complementation between human and yeast proteins. We observed that Glrx3 successfully interacted with yeast *GUA1* (Fig. 1A and Fig. S1A). In addition, both Grx3 and Grx4 interacted either with mouse or human GMPs (Fig. 1A and Fig. S1A), demonstrating the complementarity of these proteins. We next tested the Grx3/Grx4 mutants in the residues involved in bridging iron/sulfur clusters described in reference 36 to perform a subsequent two-hybrid experiment with hGMP synthase. In all cases, those mutants severely diminished the binding between Grx3 or Grx4 with hGMPs (Fig. 1A and Fig. S1A). These results

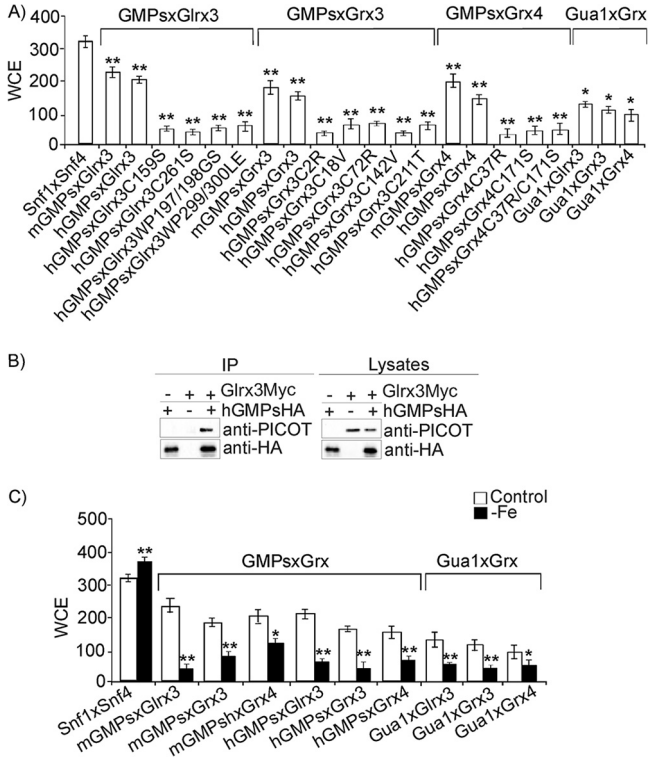


FIG 1 Formation of the complex between monothiol glutaredoxins and GMP synthases occurs through specific conserved residues, affecting binding to iron/sulfur clusters, being yeast and human proteins interchangeable for this function. (A) Two-hybrid analyses to determine the interactions between pGBKT7Glrx3 and pGADT7mGMPs or pACTIIhGMPs; pACTIIhGMPs and pGBKT7Glrx3C159S, pGBKT7Glrx3C261S, pGBKT7WP197/198GS, or pGBKT7WP299/300LE; either pGADT7mGMPs or pACTIIhGMPs and pGBT9Grx3 or pGBT9Grx4; pACTIIhGMPs and pGBT9Grx3C2R, pGBT9Grx3C18S, pGBT9Grx3C72R, pGBT9Grx3C142R, or pGBT9Grx3C211T; pACTIIhGMPs and pGBT9Grx4C37R, pGBT9Grx4C171S, or pGBT9Grx4C37R/C171; and pACTIIGlrx3/Grx3/Grx4 and pGBKT7Gua1. These assays were carried out with exponentially growing cells in SD medium plus amino acids. The β-galactosidase values obtained from the interaction between Snf1 and Snf4 were interpreted as a reference for a positive interaction. Histograms represent the average values between three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t* test (*, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$). WCE, whole-cell extract. (B) Physical interaction between Glrx3 and hGMPs. Cultures of the wild-type strain previously transformed with both pACTIIhGMPsHA and pGBKT7Glrx3Myc were grown to logarithmic phase in SD medium plus amino acids. Samples were collected for protein extraction and subsequent coimmunoprecipitation. The Glrx3 coimmunoprecipitated protein was detected in complex with the anti-PICOT monoclonal antibody. IP, immunoprecipitation. (C) Two-hybrid analysis between mGMPs/Glrx3, mGMPs/Grx3, mGMPs/Grx4, hGMPs/Glrx3, hGMPs/Grx3, hGMPs/Grx4, Gua1/Glrx3, Gua1/Grx3, and Gua1/Grx4. All cultures were exponentially grown in SD containing (control) or lacking (−Fe) iron plus the required amino acids. Histograms represent the average values of β-galactosidase activity obtained from three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t* test (*, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$).

indicate that iron/sulfur clusters also bridge the physical binding between Grx3, Grx4, and hGMP synthase. We depleted iron from the culture media and observed that low iron levels clearly precluded the *in vivo* interaction between each Glrx3/Grx3/Grx4 glutaredoxin and GMP synthases (human, mouse, or yeast), reinforcing our theory that iron bridges the complex between monothiol glutaredoxins and GMP synthases in the eukaryotic models that we have tested (Fig. 1C and Fig. S1B and C) by following a mechanism that seems to be evolutionarily conserved in this eukaryotic family of monothiol glutaredoxins.

Characterization of a role for Glrx3/GMPs complex in the regulation of ISR pathway under conditions of nutritional stress. To ascertain the biological significance of the above-mentioned interaction, we searched for a possible connection between GMP synthases and specific signaling pathways. Some authors (37) described a functional connection between *GUA1* and the integrated stress response (ISR) in the

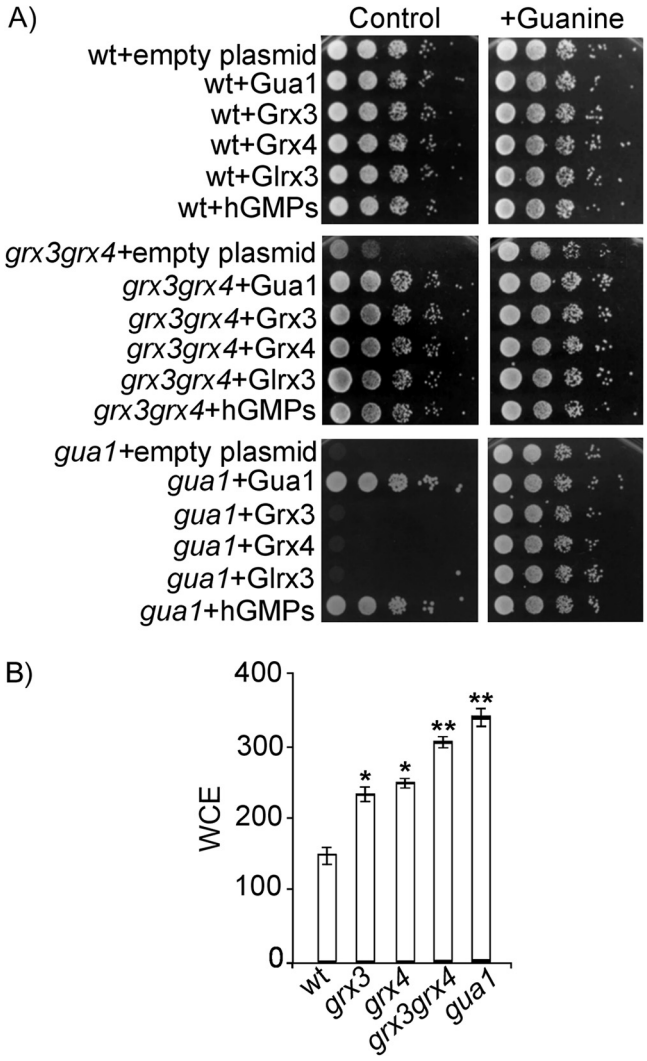


FIG 2 Lack of function of both monothiol glutaredoxins Grx3 and Grx4 provokes a constitutive induction of the ISR pathway and a defect in the synthesis of guanine. (A) Overexpression of Gua1 suppresses guanine requirements of the *grx3 grx4* double mutant. Wild-type, *grx3 grx4*, and *gua1* strains were transformed with pGua1, pGrx3, pGrx4, pGlrx3, phGMPs, or the corresponding empty plasmids (described in Materials and Methods). Cultures were logarithmically grown in SD medium with or without 130 μM guanine at 30°C and subsequently plated in triplicate on SD plates. (B) ISR pathway is constitutively activated in *grx3*, *grx4*, and *grx3 grx4* mutants. Samples from exponentially growing cultures of the wt and *grx3*, *grx4*, *grx3 grx4*, and *gua1* mutants previously transformed with plasmid p180 were exponentially grown to perform a β-galactosidase assay to determine the translational derepression of *GCN4*. Values shown in the histogram are averages from three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t* test (*, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$).

model *S. cerevisiae*. In *S. cerevisiae*, this pathway becomes activated upon Gcn2 phosphorylation in response to several stimuli: uncharged tRNAs, oxidative stress, nutritional starvation (glucose, nitrogen, and amino acids), and TORC1 inactivation, among others. Gcn2 phosphorylation then regulates eIF2α phosphorylation, which subsequently derepresses the expression of the transcriptional factor *GCN4*. In a first step, we decided to investigate the potential relationship between Grx3/Grx4 and the ISR pathway. The mutant *gua1* presents a defect in cell growth that is suppressed upon the addition of guanine to the culture media or, alternatively, upon the overexpression of hGMPs or *GUA1* (Fig. 2A). We observed that the *grx3 grx4* double mutant presents growth defects compared to wild-type (wt) cells that were also efficiently rescued upon the addition of guanine (Fig. 2A). Moreover, the overexpression of either Glrx3, Gua1, or GMP synthase

also improved the growth rate and survival of the *grx3 grx4* mutant, as depicted in Fig. 2A. As expected, the overexpression of Glrx3, Grx3, or Grx4 did not suppress the lack of growth of the *gua1* mutant (Fig. 2A), discarding an alternative function for these glutaredoxins in purine synthesis. Our results suggested a functional connection between cytosolic monothiol glutaredoxins and guanine synthesis. Some authors have previously demonstrated a link between purine synthesis and amino acid biosynthesis through the ISR pathway (35, 37). We observed that the *grx3 grx4* double mutant also presented a remarkable induction of the ISR pathway, as evidenced through the derepression of *GCN4* translation under nonstressed conditions (Fig. 2B), accompanied by significant phosphorylation of eIF2α compared to that of wt cultures (Fig. 3A and Fig. S2A). *GCN4* expression and eIF2α phosphorylation also were higher in *grx3* and *grx4* mutants than in wt cells growing exponentially in the absence of nutritional stress (Fig. 2B and 3A and Fig. S2A). In accordance with these results, the *gua1* mutant presented a higher derepression of *GCN4* translation (Fig. 2B), indicative of constitutive activation of the ISR pathway, in agreement with results reported previously (37). These results undoubtedly point to a role for Grx3/Grx4 in the activity of the ISR pathway. We speculated whether ISR constitutive activation detected in the *grx3 grx4* double mutant occurred as a consequence of alterations in iron homeostasis or because both glutaredoxins were playing a direct role in ISR regulation. The simultaneous absence of Grx3 and Grx4 provokes the constitutive hyperactivation of Aft1 due to its constant nuclear localization (10, 18). As a consequence of this hyperactivation, iron is accumulated in the cytoplasm (19). We observed that the deletion of *aft1* highly reduced eIF2α phosphorylation levels in the *grx3 grx4 aft1* triple mutant growing exponentially (Fig. 3A and Fig. S2A), which demonstrates that in the absence of exogenous stress, the effect of the simultaneous deletion of both *GRX3* and, in response to several stimuli, *GRX4* on the ISR pathway is mediated by Aft1. This observation expands to iron homeostasis the numerous stimuli that activate *GCN2* in yeast. When cells are limited for nutrients (upon amino acid deprivation or during the diauxic shift), eIF2α becomes highly phosphorylated and, consequently, ISR activated. In this context, the *grx3 grx4* mutant presented a higher eIF2α phosphorylation than wt cells. Under these nutritional conditions, we observed that *aft1* deletion restores to wt levels the activity of *GCN2* in the absence of Grx3 and Grx4 (Fig. 3A and Fig. S2A). Consequently, in the *grx3 grx4* double mutant, the total eIF2α phosphorylation detected under both conditions, amino acid deprivation or upon 1 day of growth, was the result of the addition of the response to two stimuli, one due to nutritional stress caused by nutrient exhaustion during the diauxic shift or to amino acid depletion plus a second response to iron homeostasis deregulation. Thus, eIF2α phosphorylation in a *grx3 grx4 aft1* triple mutant was equivalent to the levels detected in wt cultures (Fig. 3A and Fig. S2A). These results suggest that *GCN2* receives the signal of iron homeostasis independently of other nutritional statuses, and that these signals additively converge on *GCN2* activation. Interestingly, Aft1 deletion did not suppress the requirements of the *grx3 grx4* double mutant for guanine regarding cell viability (Fig. 3B), suggesting that the possible relationship between both monothiol glutaredoxins and the purine biosynthesis pathway is independent of Aft1 and iron homeostasis. For more in-depth details, we decided to analyze both eIF2α and *GCN4* translational induction in *grx3 grx4* and wt cells in response to several stimuli: iron deprivation, iron or guanine supplementation, and *N*-acetylcysteine (NAC) treatments. The latter agent contributes to the reduction of reactive oxygen species (ROS) levels in cases where oxidative stress also could be contributing to ISR activation in the *grx3 grx4* double mutant (38). Both guanine and NAC addition to the culture media, along with iron limitation, reduced both *GCN4* translation (Fig. 3C) and eIF2α phosphorylation (Fig. 3D and Fig. S2B). With respect to untreated control values, this reduction was more severe in *grx3 grx4* double mutant than in wt cells, supporting the model that specific stimuli activate the ISR pathway in the *grx3 grx4* double mutant, such as iron accumulation, limiting purines, or increasing ROS levels. The relevance of this preliminary finding is that guanine metabolism and the

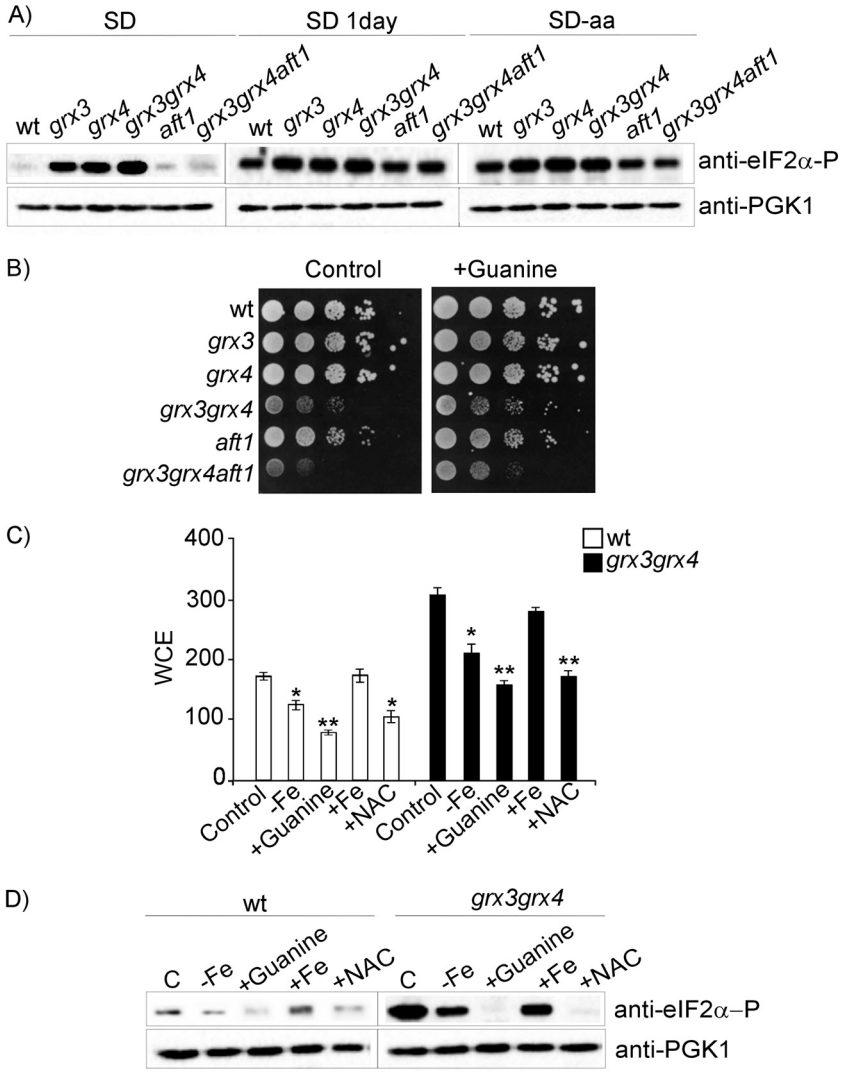


FIG 3 Iron accumulation is related to ISR induction in exponentially growing cells; however, the signal to *GCN2* activation occurs independently of and additively to nutrient starvation. (A) The phosphorylation of eIF2α was determined in WT and *grx3*, *grx4*, *grx3 grx4*, *aft1*, and *grx3 grx4 aft1* mutant cultures growing exponentially during the diauxic shift (1 day) and upon amino acid starvation. (B) Cultures shown in panel A were serially diluted and plated on YPD plates containing or lacking 130 μM guanine. Cultures from wt and *grx3 grx4* strains were subject to different treatments, i.e., iron depletion (−Fe), 130 μM guanine, 5 mM NAC, and 10 mM iron addition (+Fe) (see Materials and Methods), and subsequently grown to logarithmic phase. (C) Samples were taken to quantify *GCN4* expression as shown in Fig. 2B. Values shown in the histogram are averages 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) eIF2α phosphorylation was also determined by using the anti-phospho-eIF2α antibody and anti-PGK1 as a loading control. Western blotting was performed in duplicate, and a representative blot is shown. Quantitative and statistical values relative to the loading control are depicted in Fig. S2.

ISR pathway might form a connection through Grx3/Grx4 cytosolic monothiol glutaredoxins.

Glrx3 and yeast homologous Grx3/Grx4 downregulate the activity of the ISR pathway in conjunction with GMP synthase upon nutritional deprivation. We next sought to determine the activity of the ISR pathway under our experimental conditions of nutritional starvation in both the wt and *grx3 grx4* mutant (using *gua1* as a control). *GCN4* derepression and eIF2a phosphorylation revealed remarkable ISR activation in both strains upon growth in either amino acid deprivation or minimum medium for 1

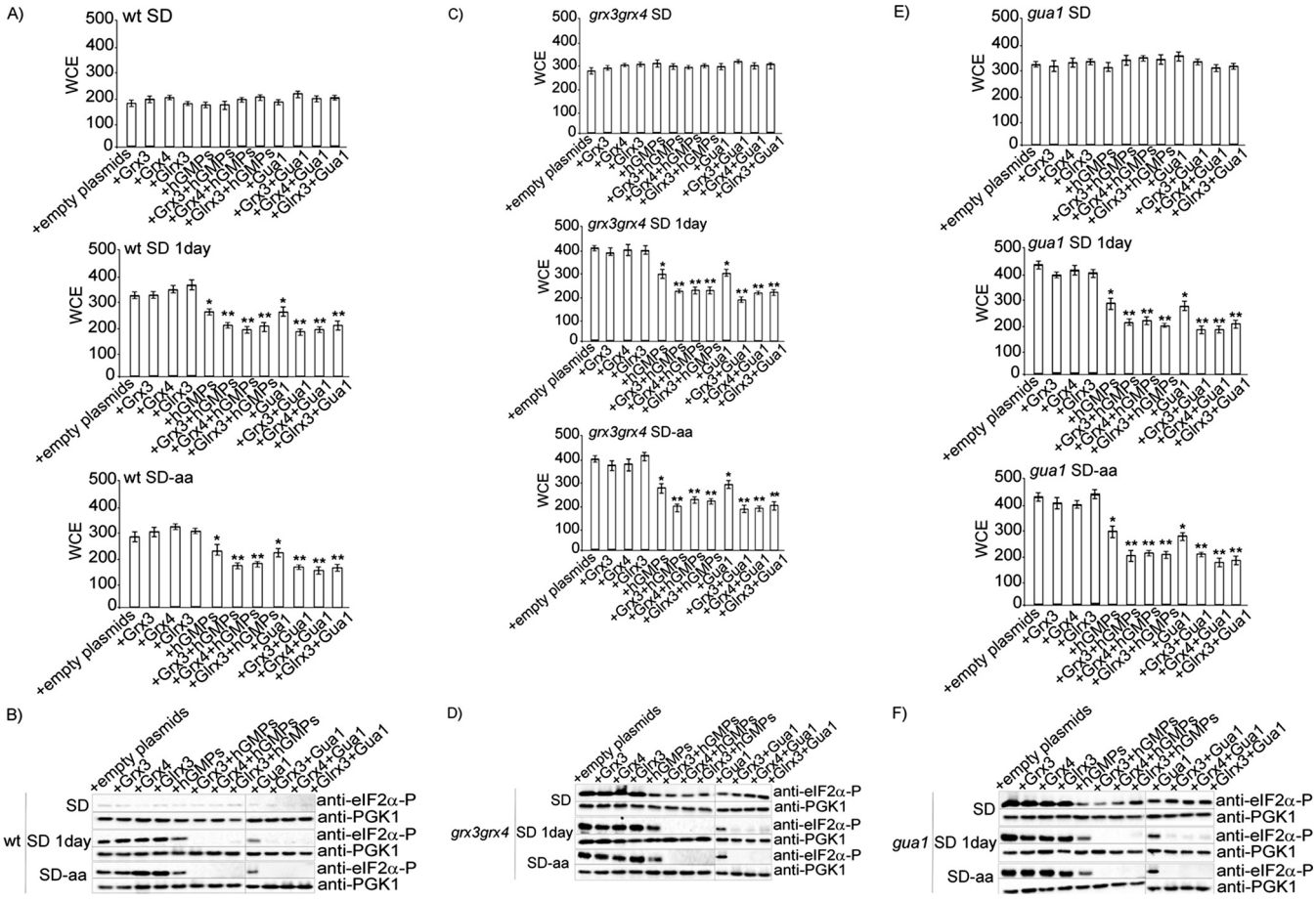


FIG 4 Coexpression of monothiol glutaredoxins Grx3/Grx4/Glrx3 and either human or yeast GMP synthase reduces the activation of ISR pathway in wt and *grx3 grx4* and *gua1* mutant cultures upon nutritional stress caused by diauxic shift or amino acid depletion. Cultures from wt (A and B) and *grx3 grx4* (C and D) or *gua1* (E and F) strains were transformed with empty plasmids, pGrx3, pGrx4, pGlrx3, phGMPs, or pGua1 or cotransformed with pGrx3+phGMPs, pGrx4+phGMPs, pGlrx3+phGMPs, pGrx3+pGua1, pGrx4+pGua1, and pGlrx3+pGua1 and grown exponentially in SD, in SD to diauxic shift, or in SD without amino acids. (A, C, and E) Values of *GCN4* expression were obtained as described for Fig. 2B. Histograms represent averages from three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t* test (*, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$). (B, D, and F) eIF2α phosphorylation was determined as described for Fig. 3A.

day compared to that of nonstarved cultures (Fig. 4A to D and Fig. S2C, D, and E). In accordance with the former observations depicted in Fig. 3A, the *grx3 grx4* double mutant presented a higher induction than wt cells under our experimental conditions due to the additive and independent effect caused by the starvation and Aft1 dysfunction. To functionally characterize the novel interaction between Glrx3 and hGMPs, we coexpressed both human proteins in wt, *grx3 grx4*, and *gua1* strains and analyzed the activity of the ISR pathway. Neither the expression of single Glrx3 and GMPs nor the simultaneous expression of Glrx3 and GMPs provoked any significant effect on *GCN4* translation in nonstarved exponentially growing wild-type, *grx3 grx4*, or *gua1* cultures (Fig. 4A, C, and E). However, under nutrient deprivation conditions, Glrx3+hGMPs coexpression significantly reduced *GCN4* expression in wt, *grx3 grx4*, and *gua1* strains (compared to wild-type strains transformed with the empty plasmids). hGMPs overexpression slightly reduced *GCN4* translational expression in all strains tested, although to a lesser extent than that determined when Glrx3 and hGMPs were coexpressed. However, Glrx3 overexpression did not cause any significant reduction in *GCN4* levels under the experimental conditions tested (Fig. 4A, C, and E). These results in general correlated with those obtained upon analysis of eIF2α phosphorylation (Fig. 4B, D, and F and Fig. S2C, D, and E). Together, the previous results support the hypothesis that the lack of monothiol glutaredoxin function is detrimental for purine metabolism. We

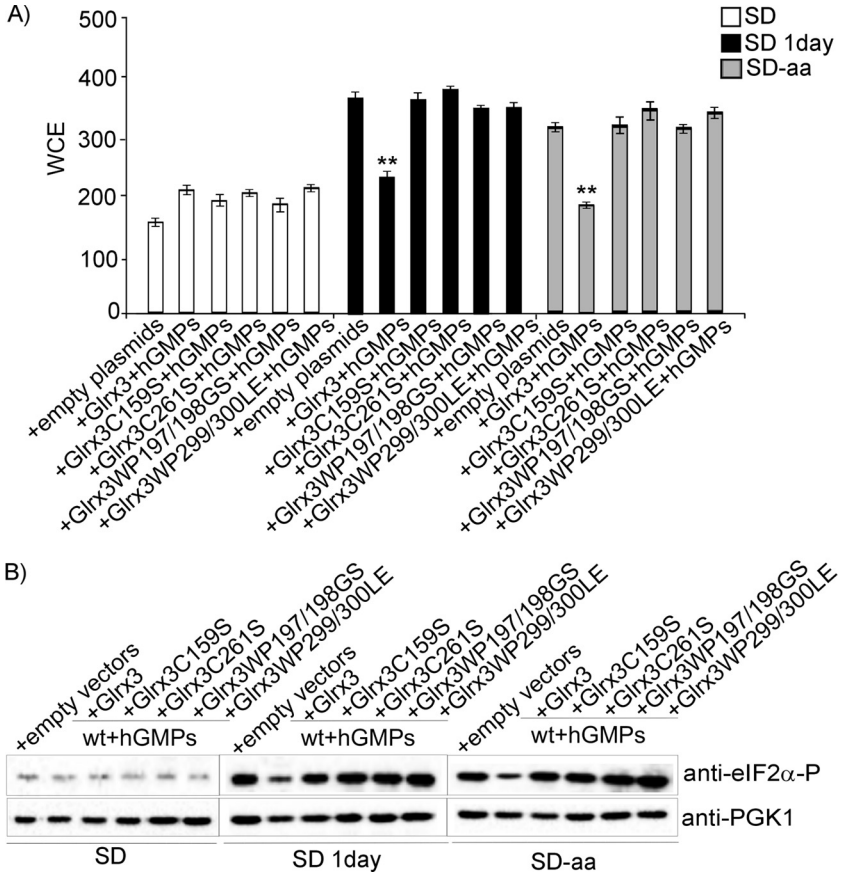


FIG 5 Residues related to iron/sulfur binding in Glrx3 are involved in ISR downregulation driven by hGMPs. Wild-type cultures were transformed with plasmids pGBKT7+pACTII, pGBKT7Glrx3, pGBKT7Glrx3C159S, pGBKT7Glrx3C261S, pGBKT7WP197/198GS, and pGBKT7WP299/300LE in combination with phGMPs. (A) Values of *GCN4* expression were obtained as described for Fig. 2B. Histograms represent averages from three independent experiments. Error bars are represented with *P* values from a Student's unpaired *t* test (*, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$). (B) eIF2α phosphorylation was determined as described for Fig. 3A.

decided to investigate the degree of complementation between yeast and human glutaredoxins and GMP synthase regarding the downregulation of the ISR pathway. For this investigation, we analyzed (i) Grx3 or Grx4 coexpression with human GMP synthase, (ii) Glrx3 coexpressed with *GUA*, and (iii) Grx3 and Grx4 coexpressed with *GUA1* (Fig. 4). As previously described for Glrx3 and hGMPs, Grx3 or Grx4 did not cause a reduction in ISR activity; however, Gua1 overexpression slightly reduced *GCN4* expression and eIF2α phosphorylation in wt, *grx3 grx4*, and *gua1* strains. Moreover, in all cases the coexpressions described above significantly reduced both *GCN4* levels and eIF2α phosphorylation under both nutritional starvation conditions tested in this study (Fig. 4 and Fig. S1C and S2C, D, and E). We observed a small reduction in the phosphorylation caused by each human or yeast GMP synthase that was negligible compared to the results obtained upon each coexpression. This observation points to a mild negative function of GMP synthases in the activation of *GCN2* that is only detectable immunologically. We next analyzed the effects that Glrx3 point mutations could have in the negative regulation of *GCN4*, since these residues are relevant for binding to hGMPs. Each of these point mutations in combination with hGMPs abrogated the negative effect that both *GCN4* derepression and eIF2a suffer upon amino acid starvation (Fig. 5A and B and Fig. S1C and S2F). Our results suggest that Glrx3/GMPs are relevant to reducing the activity of the ISR pathway and also suggest that this mechanism is conserved from yeast to humans.

Glrx3 and GMP synthase colocalize in the nucleus upon ISR pathway induction. We fused green fluorescent protein (GFP) to each of the human proteins, Glrx3 and

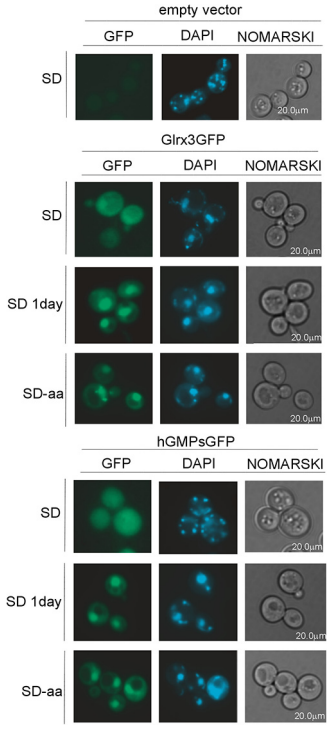


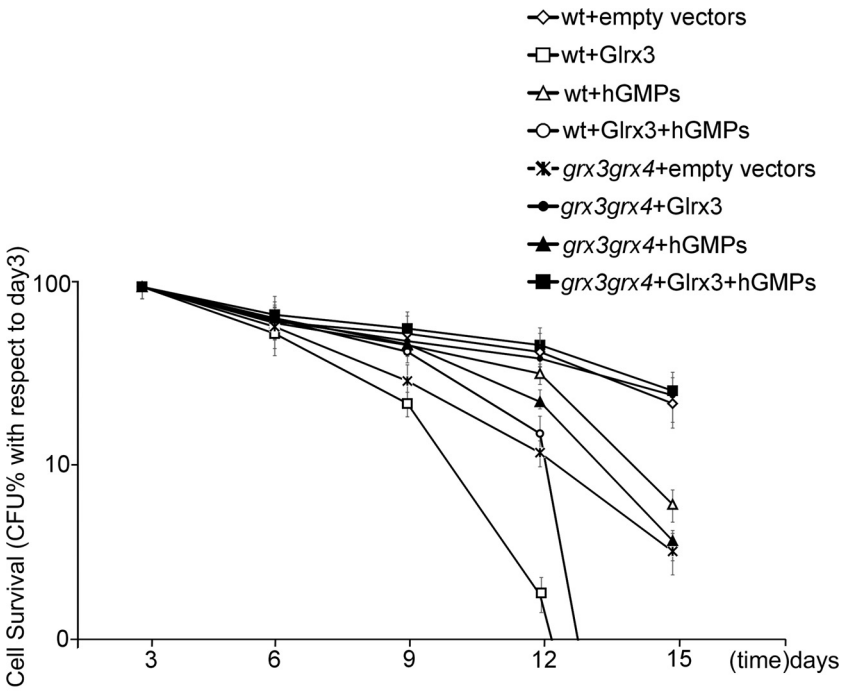
FIG 6 *In vivo* localization of the human proteins Glrx3 and hGMPs. Wild-type cells were transformed with plasmids pGlrx3GFP, phGMPsGFP, and pUG35 and subsequently grown either in SD plus amino acids to each logarithmic and diauxic phase or in SD without amino acids. Cultures were also *in vivo* stained with 4′,6-diamidino-2-phenylindole (DAPI) to identify Glrx3 and hGMPs nuclear localization. Samples were taken for observation under the fluorescence microscope.

GMP synthase, to observe their cellular localization. hGMPs showed a cytoplasmic localization, whereas Glrx3 presented partial nuclear accumulation under conditions of nutrient sufficiency. Nutritional stress, however, induced a predominant nuclear accumulation for both proteins, supporting the previous hypothesis that both proteins have a common and potentially collaborative function in this compartment when ISR is activated (Fig. 6).

Excesses or defects in monothiol glutaredoxin dosage are detrimental to life span. We also checked the influence that either Glrx3, hGMPs, or the coexpression of both could have on chronological life spans (CLS). We observed that Glrx3 and, to a lesser extent, GMPs overexpression caused an acceleration of yeast growth (Table 1). Glrx3/GMPs sustained coexpression reduced the CLS of a wt strain (Fig. 7). In this study, we determined that the simultaneous absence of Grx3 and Grx4 was detrimental for chronological life span; nevertheless, Glrx3 overexpression efficiently complemented this deficiency (Fig. 7). Interestingly, hGMPs overexpression in the *grx3 grx4* mutant also partly rescued the defects in chronological life span of the mutant, supporting the idea

TABLE 1 Generation time and standard deviations calculated for cultures of strains growing in SD plus amino acids at 30°C with continuous shaking

Strain	Generation time (h) ± SD
wt+empty vectors	2.5 ± 0.097
wt+Glrx3	1.7 ± 0.112
wt+hGMPs	2.3 ± 0.102
wt+Glrx3+hGMPs	1.9 ± 0.116
grx3 grx4+empty vectors	3.0 ± 0.132
grx3 grx4+Glrx3	2.6 ± 0.135
grx3 grx4+hGMPs	2.8 ± 0.127
grx3 grx4+Glrx3+hGMPs	2.5 ± 0.130



Strains	Maximun lifespan	SD	Average lifespan
wt+empty vectors	19.6	±0.39	10.3
wt+hGMPs	17.1	±0.41	8.6
wt+Glrx3+hGMPs	11.2	±0.45	5.6
wt+Glrx3	10.7	±0.43	5.3
grx3grx4+Glrx3+hGMPs	20.4	±0.44	10.8
grx3grx4+Glrx3	19	±0.46	10.6
grx3grx4+hGMPs	16.7	±0.38	7.9
grx3grx4+empty vectors	14.5	±0.42	7.2

FIG 7 Chronological life span curves for wt+empty plasmids, wt+pGlrx3, wt+phGMPs, wt+pGlrx3+phGMPs, *grx3 grx4*+empty plasmids, *grx3 grx4*+pGlrx3, *grx3 grx4*+phGMPs, and *grx3 grx4*+pGlrx3+phGMPs strains. Cultures were exponentially grown in SC medium plus amino acids at 30°C. 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 is depicted.

that both Grx3/Grx4 and Glrx3 are directly or indirectly involved in the purine biosynthetic pathway. We conclude that the excess amount of or defect in Glrx3/Grx3/Grx4 monothiol proteins is detrimental for life span extension. It would be interesting to test in future studies whether Glrx3 plays a role in human life span extension and whether this role requires GMPs activity.

DISCUSSION

Here, we present evidence demonstrating that the yeast system is a useful strategy for the screening of new interactors with human proteins that are evolutionarily conserved in the eukaryotic world. This approach also permits a faster searching for new connections with signaling pathways that might explain the functional relevance of the novel characterized interaction. We have found a novel interactor with the monothiol glutaredoxin Glrx3, the enzyme GMP synthase involved in the synthesis of guanine and GTP. Our results suggest that the Glrx3/GMPs complex is important to regulate ISR activity under conditions of nutrient deprivation. Moreover, we show here

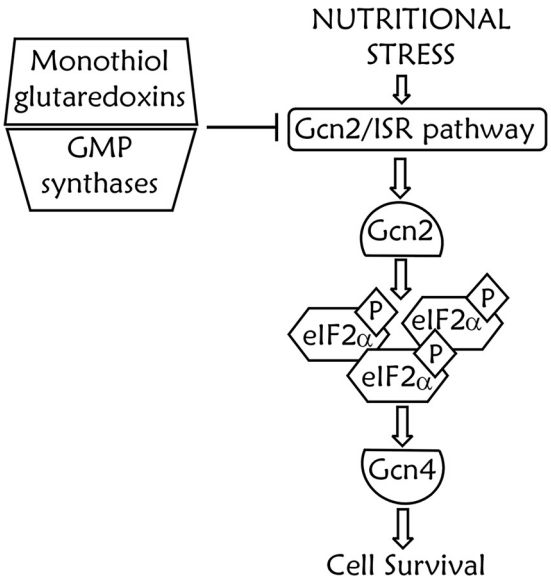


FIG 8 Schematic representation of the possible regulatory mechanisms that the complex formed between Glrx3/Grx3/Grx4 and GMPs/Gua1exerces and the regulation of the Gcn2/ISR pathway under conditions of nutritional stress.

that yeast Grx3 and Grx4 also play a role as mild repressors of the ISR pathway upon nutritional limitation, in cooperation with GMP synthase (Fig. 8). One possible interpretation of this observation is that this complex is required to downregulate *GCN2* activity to avoid the exhaustion of GTP in the cells in a context of general limitation of nutrients. Gcn2 is present in most of the known eukaryotic models, being the yeast system in which more studies have been conducted given the elevated degree of conservation (39). A wide number of publications demonstrate the usefulness of yeasts as tools to characterize human cellular functions and processes (see, for example, references 40 and 41). Consequently, this is another example of the contribution of yeast to deciphering new functions for Glrx3 and *GCN2*. In this study, *S. cerevisiae* has allowed us to identify a novel interaction between Glrx3 and GMPs in the process of signaling to ISR that is evolutionarily conserved from yeast to mice and humans.

We show that the absence of both monothiol glutaredoxins Grx3 and Grx4 causes a mild induction of the *GCN2* signaling pathway caused by Aft1 nuclear localization and the consequent anomalous induction of the iron regulon (18, 19, 42). However, nutrient starvation caused by the diauxic shift or amino acid limitation both are conditions that activate the *GCN2* pathway independently of Grx3/Grx4 and, consequently, of Aft1 proteins. This observation allowed us to conclude that in budding yeast, the dysfunction of iron metabolism signals *GCN2* independently of other signaling mechanisms triggered by other types of nutritional stress.

The observation that in *S. cerevisiae* the addition of guanine to cultures of the *grx3 grx4* strain clearly complemented the growth problems of the double mutant was suggestive of a functional connection between monothiol glutaredoxins and purine metabolism. Our investigation shows that Glrx3 suppresses the requirements of the *grx3 grx4* double mutant for guanine and, as a novel finding, also suppresses the chronological life span defect that we have characterized in this study for the *grx3 grx4* double mutant. These findings result in two suggestions. (i) Glrx3/Grx3/Grx4 share an evolutionarily conserved function related to purine metabolism. (ii) These monothiol glutaredoxins are required for the chronological life span of eukaryotic cells (i.e., the chronological life span without them is shorter). Our CLS studies reveal, however, that a maintained and continuous reduction of ISR expression (caused by the constant overexpression of both Grx3 and Grx4 or Glrx3 in combination with GMPs in this study) during aging provokes a mild reduction of life span, suggesting that ISR requires a tight

and punctual downregulation to delay the process of aging. This is in agreement with the current idea that *GCN2* kinases are metabolic reprogramming controllers and contributors to the process of aging (43). In this context, the potential role of the Grx/GMPs complex becomes more relevant for future analyses.

It has been demonstrated that the enzyme *GUD1*, encoding a guanine deaminase, was highly expressed during the diauxic shift (44). This suggests that when cells shift from a proliferative to a quiescent state, a decrease in the guanylyl nucleotide pool should be required. The derepression of *GCN4* is required for wild-type levels of purine biosynthesis under conditions of purine limitation, conditioned to an active *GCN2* (35). However, the nature of the purine starvation signal impinging on *GCN2* is not well characterized to date. Hence, one possible interpretation for our results is that during the diauxic shift, the GMPs/Glrx3 complex contributes to negative feedback on *GCN4* function, probably in the nucleus and consequently on purine biosynthesis, since one of the outcomes of *GCN4* expression is the translational activation of genes involved in that biochemical pathway (26, 45). The observation that NAC addition or iron starvation significantly reduces the activation of the *GCN2* pathway in the *grx3 grx4* double mutant could lead to the speculation that iron excess provokes an increment of ROS, causing the activation of the *GCN2* pathway. This is probably the explanation for the observed induction of the ISR pathway in the *grx3 grx4* mutant, given that the deletion of Aft1 suppresses ISR activation. Moreover, it has been determined that iron excess provokes constitutive oxidative stress in the *grx3 grx4* mutant (38). That monothiol glutaredoxins involved in ROS detoxification and iron metabolism interact with GMPs, an enzyme dedicated to synthesizing purines required to sustain the dNTP pool of the cell, might not be fortuitous. As an example, pathogens might develop strategies to survive the oxidative burst caused by macrophages; therefore, increasing dNTP expression and repairing possible oxidative damage to reinforce DNA repair seems to be a general survival strategy (46, 47) in which Glrx3/Grx4/Grx3 with GMPs is involved.

In exponentially growing cells, the lack of *GUA1* function also activates the *GCN2* pathway in *S. cerevisiae*, probably due to defects in purine synthesis increasing the pool of nonchargeable tRNAs, which activates the ISR pathway (35, 37). The human GMPs efficiently complemented yeast *gua1* regarding signaling to ISR, and this was indicative of the evolutionary conservation of this signaling in the eukaryotic world.

We present evidence suggesting that upon nutrient limitation and concomitant *GCN2* activation, both hGMPs and Glrx3 play a role in the downregulation of the activity of the ISR pathway.

It has been recently described that Gcn2 regulates Aft1 function to adjust iron metabolism to amino acid biosynthesis (48). In our study, we observed that in the absence of *grx3 grx4*, the ISR pathway becomes activated, drawing a new scenario in which Aft1 is anomalously located in the nucleus and iron is abnormally accumulated in the cytoplasm (19) and, consequently, cannot be used by the cell. In this context, cells receive a signal of iron depletion. We can speculate on two different scenarios. (i) Grx3/Grx4 are required to sense iron availability, and, without them, cells receive a constant signal or iron depletion and then activate Gcn2 and Aft1. (ii) Grx3/Grx4 both are negative regulators of ISR, and, in their absence, *GCN2* is constitutively activated, activating Aft1. These are speculative conclusions that will be proximally solved with the aid of more experimental results.

Iron deprivation is sensed by HRI protein to phosphorylate eIF2α in mammalian cells. In yeast, *GCN2* is the only protein that phosphorylates eIF2α; however, we have shown that the deregulation of iron homeostasis leading to abnormal iron accumulation and ROS production (18, 19, 38, 42) is a novel signal converging in yeast *GCN2*. Our studies suggest that guanine metabolism, ISR activation, and chronological life span are common functions evolutionarily conserved and regulated by cytosolic monothiol glutaredoxins and GMPs. Members of the family of monothiol glutaredoxins, human Glrx3 and yeast Grx3 and Grx4, interact with GMPs proteins through specific and already-characterized residues that affect binding to iron/sulfur clusters.

Glrx3 and its accompanying protein, Bol2, store iron, and the complexes function as

iron chaperones by direct binding to target proteins to which they apparently deliver iron/sulfur clusters (15). Glrx3/Grx3/Grx4 could be playing roles as chaperones, possibly transferring iron to GMPs or to another protein(s) involved in guanine metabolism. Thus, one possible explanation for the formation of the Glrx3/Grx3/Grx4 complex and GMP synthase is the requirement for iron/sulfur clusters at some point to sense or synthesize GTP, or, alternatively, that iron or iron/sulfur serves as a limiting molecule to regulate GMPs function in the context of nutritional limitation. These interesting aspects require further investigation.

MATERIALS AND METHODS

Yeast strains and plasmids. The *Saccharomyces cerevisiae* wild type (*MAT***a** *leu2-3,112 ura3-52 trp1 his4 can1r*) and the *grx3* (*MAT***a** *grx3*::*NatMX4*), *grx4* (*MAT***a** *grx4*::*KanMX4*), *grx3 grx4* (*MAT***a** *grx3*::*NatMX4 grx4*::*KanMX4*) and *grx3 grx4aft1* (*MAT***a** *aft1*::*URA3 grx3*::*NatMX4 grx4*::*KanMX4*) isogenic mutant strains have been previously described (18). The *gua1* mutant was obtained in this study by a one-step disruption method that uses the *kanMX4* module (49). Plasmids carrying either wild-type, Grx3, or Grx4 proteins cloned in either pGBT9 or pACTII (Clontech; https://www.takarabio.com/assets/documents/Vector%20Documents/pACT2%20AD%20Vector%20Information.pdf) vector and plasmids carrying Grx3 and Grx4 point mutations cloned into pGBT9 plasmid (Clontech; https://www.takarabio.com/assets/documents/Vector%20Documents/pGBT9%20Vector%20Map_070219.pdf) were extensively described previously (36).

Saccharomyces cerevisiae Y2HGold and Y187 strains were used for the screen-based two-hybrid system (Clontech, CA, USA). Y2HGold (*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4 gal80 LYS2*::*GAL1UAS-Gal1TATA-His3 GAL2UAS-Gal2TATA-Ade2URA3*::*MEL1UAS-Mel1TATA AUR1-C MEL1*) was used as the bait strain, and Y187 (*MAT*α *ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4 met-gal80 MEL1 URA3*::*GAL1UAS-Gal1TATA*-LacZ) was used as the prey strain.

The PJ69-4a (*MAT***a**, *trp1-901 leu2-3,112 ura3-52 his3-200 gal4 gal80 GAL2-ADE2 LYS2*::*GAL1-HIS3 met*::*GAL7-lacZ*) and PJ69-4α (MATα, *trp1-901 leu2-3,112 ura3-52 his3-200 gal4 gal80 GAL2-ADE2 LYS2*::*GAL1-HIS3 met*::*GAL7-lacZ*) strains were used for the rest of the two-hybrid experiments.

Human glutaredoxin 3 gene (Glrx3) was amplified from human cDNA. Restriction sites were incorporated to allow the cloning of the PCR amplification sequences into the multiple cloning sites of pGBKT7 (Clontech, CA, USA) (under the ADH1 promoter and GAL4 binding domain in the N terminus, fused in frame with the Myc epitope in the C terminus) and pUG35 (under the MET25 promoter and fused in frame to the GFP epitope in the C terminus).

Point mutations in Glrx3 protein that yielded the desired amino acids were obtained by site-directed mutagenesis by using the ExSite method (50). The Glrx3 wild-type protein, previously cloned into the pGBKT7 vector, was used as the template. The mutant forms of the different proteins were the following: C159S (forward primer, 5′-TCGTGGTTCTTGAGGAGTTCCTTTC-3′; reverse primer, 5′-TCGGGTTTCAGCAAG CAGATGGTGG-3′), C261T (forward primer, 5′-GGTTTTTGCTTCCTGTTTGTTTCCTTTC-3′; reverse primer, 5′-GGTTTCAGCAAACAAATTCTGGAAATAC-3′), WP(197/198)GS (forward primer, 5′-TCCACTGGAATAGGCT TTGAGTCCCTG-3′; reverse primer, 5′-TCCACCTATCCTCAGCTCTATGTTTCTG-3′), and WP(299/300)LE (forward primer, 5′-GAGATTTGAGTAAGCTTTTAATCCTTGC-3′; reverse primer, 5′-GAGACATACCCTCAGCTGTA TGTGAAAG-3′).

The yeast *GUA1* gene was amplified from chromosomal DNA from *S. cerevisiae* CML128 (*MAT***a** *leu2-3,112 ura3-52 trp1 his4*) to be subsequently inserted into pGBKT7 as described above.

Human GMP was amplified from human cDNA cloned into the pDONR221 vector (purchased from The PlasmID Repository, Harvard Medical School, Boston, MA, USA). Human GMP was inserted into pACTII (which carries a GAL4 activating domain, a hemagglutinin [HA] epitope, and a constitutive ADH1 promoter) and pUG35 (under the MET25 promoter and fused in frame to the GFP epitope in the C terminus). Plasmid p180 (low-copy-number vector expressing *GCN4-lacZ*, upstream open reading frames 1 to 4 in YCp50) was kindly provided by Mercedes Tamame. This plasmid was transformed into the wild-type and *grx3*, *grx4*, *gua1*, and *grx3 grx4* mutant strains to analyze *GCN4* expression upon quantification of the β-galactosidase reporter gene.

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) plus amino acids (51). Iron depletion conditions (−Fe) consisted of SD medium with a nitrogen base component free of iron plus the addition of 80 μM 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (BPS) (146617; Sigma). When required, 5 mM *N*-acetylglucosamine (A9165; Sigma) and 130 μM guanine (51030; Sigma) were added to the culture media, and iron was added as ammonium iron(III) sulfate hexacahydrate [$NH_4Fe(SO_4)_2{\cdot}6H_2O$] (+Fe; F1543; Sigma) at a final concentration of 10 mM. Cell cultures were exponentially grown (optical density at 600 nm [OD_{600}] of 0.6) or grown to the diauxic shift (1 day in SD plus amino acids, OD_{600} of 2.5). For amino acid starvation experiments, we prepared a culture in SD medium plus required amino acids to log phase (OD_{600} of 0.6). Cells then were collected, washed three times in SD medium without amino acids, and transferred to the required volume of the same fresh medium at a starting OD_{600} of 0.3. Cultures were grown overnight for 10 h, and then cells were collected to be subsequently processed. β-Galactosidase activity was determined basically according to reference 52, with small variations. We collected 5 ml of cell culture to be centrifuged, and the pellets were suspended in 400 ml buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) plus 20 μl sarcosyl (10%) and 4 μl toluene. After that, samples were vortexed

and split into two Eppendorf tubes, to which 600 μl buffer was added. Samples were incubated at 28°C for 5 min, and then 50 μl *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml) was added and subsequently incubated at 28°C for 5 min. Finally, the addition of 500 μl Na_2CO_3 (1 M) stopped the reaction. Absorbance was measured at 420 nm.

Two-hybrid system. To start the screening, we cloned the open reading frame of GLRX3 into the pGBKT7 plasmid carrying the GAL4 activating domain. A mouse cDNA library (Matchmaker gold yeast two-hybrid system mate and plate library-mouse embryo-11 day) was used as the prey to identify interactions with human GLRX3. The mouse library is constructed in the pGADT7 plasmid, which contains a GAL4 activation domain (DNA-AD) and a constitutively active ADH1 promoter (PADH1). The mouse library was transformed into the Y187 strain and incubated in SD/−Leu broth. Y2HGold strains containing the pGBKT7-GLRX3 constructs were cultured in SD/−Trp broth. Matings were performed in 50 ml yeast extract-peptone-dextrose-adenine (YPDA) (2×) medium containing 12.5 μg/ml kanamycin. At least 2×10^7 Y187 cells and 2×10^8 Y2HGold cells were incubated at 30°C and 70 rpm for 24 h. After the observation of zygotes, cultures were washed in 0.5× YPDA, serially diluted, and plated onto SD/−Trp, SD/−Leu, SD/−Trp/−Leu (DDO), and SD/−Trp/−Leu/X-α-Gal/AbA (DDO/X/A) agar plates. Colony observation was performed after 3 to 5 days of incubation at 30°C. Positive mating controls between Y2HGold yeast transformed with pGBKT7-53 and Y187 yeast cells transformed with pGADT7-T were employed, as well as negative mating controls with Y2HGold yeast transformed with pGBKT7-Lam and Y187 yeast transformed with pGADT7-T. Colonies obtained were isolated and kept frozen for further analysis.

Positive interaction analysis. We tested 4.7×10^7 independent clones by following the manufacturer's instructions. Plasmids from the diploids obtained from the matings were recovered by using a Zymoprep yeast plasmid miniprep II kit (Zymo Research, CA, USA) and then transformed into *Escherichia coli* DH5α competent cells. Prey plasmids were recovered by plating transformant cells in LB containing 50 μg/ml ampicillin. Restriction enzyme digestions were performed to select the digestion profiles, and all of the different profiles were sent for sequencing analysis. Positive interactions were confirmed by transforming isolated prey plasmids into Y187 strains and repeated mating with Y2HGold strains containing pGBKT7-GLRX3 and plating onto SD/−Trp, SD/−Leu, DDO, and DDO/X/A agar plates.

Cell survival and chronological life span. To assay cell viability, cells were grown to mid-log phase at an OD_{600} of 0.6 in SC medium supplemented with the required amino acids. Viability was registered through serial dilutions and plated in triplicate onto YPD plates.

We measured the chronological life span (CLS) in the different strains based on the survival of populations of nondividing yeast cells according to reference 53. Viability was scored by counting the number of cells able to form colonies (CFU). Cultures were started at an OD_{600} 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 (36). The antibodies for Western blotting were anti-HA 3F10 (no. 12158167001; Roche Applied Science), used at a dilution of 1:2,000 in 0.25% nonfat milk, anti-GFP (no. 632381; Living Colors) at a dilution of 1:2,000, anti-PICOT (D-10) (no. sc-390068; Santa Cruz Biotechnology) at a dilution of 1:1,000, anti-MYC (Roche) at a dilution of 1:2,000, anti-phospho-eIF2α Ser-51 (no. 3597S; Cell Signaling) at a dilution of 1:1,000, anti-Grx3/4 (a gift from Rolland Lill) at a dilution of 1:4,000, and anti-PGK1 (no. 459250; Invitrogen) at a dilution of 1:10,000. They were used as indicated by the manufacturers. The protein-antibody complexes were visualized by enhanced chemiluminescence using SuperSignal substrate (Pierce) in a Chemidoc (Roche Applied Science). Secondary antibodies at a dilution 1:10,000 were anti-mouse IgG horseradish peroxidase (from sheep) (no. Na931; GE Healthcare), anti-rabbit IgG horseradish peroxidase (from donkey) (no. Na934; GE Healthcare), and goat anti-rat IgG horseradish peroxidase conjugate (no. AP136P; Millipore).

Coimmunoprecipitation of proteins. Protein extracts were prepared from exponentially growing cells to be subsequently bound to anti-HA affinity matrix (Roche) as described previously (53). For immunoblotting, we used monoclonal anti-HA 3F10 to bind the proteins to the affinity matrix and monoclonal anti-PICOT (Santa Cruz) to detect the coimmunoprecipitated protein by Western blotting.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

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