Interaction between GMP synthase and either human Glrx3 or *Saccharomyces cerevisiae* Grx3/Grx4 converges in the regulation of Gcn2 pathway

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Running Head: Glrx3/Grx3/Grx4 bind GMPs to downregulate Gcn2 pathway

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Saccharomyces cerevisiae is an optimal eukaryotic microbial model to study biological processes in superior organisms, despite the divergence in evolution. The molecular function of yeast glutaredoxins Grx3 and Grx4 has an enormous interest since both proteins are required to maintain a 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 participates in the down regulation of the activity of Gcn2 stress pathway. This mechanism is conserved in yeast and humans. Here we also show that this family of glutaredoxins, Grx3/Grx4/Glrx3 also shares a function related to life extension.

ABSTRACT

The human monothiol glutaredoxin Glrx3 (PICOT) is ubiquitously distributed in cytoplasm and nuclei in mammalian cells. Its overexpression has been associated to 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 chaperone distributing iron/sulphur clusters is currently accepted. Yeast humanization and the use of a mouse library has allowed us to find a new partner for PICOT: the human GMP synthase (hGMPs), both proteins carry out collaborative functions regarding down-regulation of the S. cerevisiae Gcn2 pathway, in conditions of nutritional stress. Glrx3/hGMPs interact through conserved residues which bridge iron/sulphur clusters and glutathione. This mechanism is also conserved in budding yeast whose proteins Grx3/Grx4 along with GUA1 (S. cerevisiae GMPs) also
downregulate ISR pathway. Heterologous expression of Glrx3/hGMPs efficiently complements Grx3/Grx4. Moreover, 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 family Glrx3/Grx3/Grx4 present an evolutive and functional conservation in signaling events, partly related to GMPs function, that contribute to cell life extension.

INTRODUCTION

Iron is one of the most abundant metals in eukaryotic cells. It is involved in multiple essential processes such as mitochondrial respiration, lipid, protein and DNA synthesis among other functions. Maintenance of a correct iron homeostasis is essential for cell survival. Iron deficiency can provoke human diseases such as anaemia (1) and it can also increase the risk of cancer (2) whereas iron overload provokes severe diseases caused by oxidative stress and is also associated to cancer incidence (3). Monothiol glutaredoxins contain an active site: Cys-Gly-Phe-Ser (CGFS) with no catalytic activity. The active cysteine with the aid of the sulfhydryl residue of a glutathione molecule, jointly coordinate a [2Fe-2S] cluster (4,5,6,7). 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-sulphur clusters via the CGFS domain (10). Grx3 and Grx4 use iron/sulphur 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 (11, 12,
Evolutionary, 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/sulphur bridged complex with partner proteins such as BolA (15). In budding yeast, iron sensing transcriptional activators Aft1 and its paralogous Aft2 regulate the subset of genes involved in iron homeostasis (16, 17). Double deletion of the monothiol Grx3 and Grx4 glutaredoxins, leads to nuclear localisation and the constitutive transcriptional activation of Aft1, affecting cellular iron homeostasis (18). Consequently, an excess of bio-unavailable iron is accumulated in *grx3grx4* mutant impairing a wide number of iron-dependent enzymes, suggesting that both Grx3 and Grx4 are involved in iron trafficking (19). Again, binding to iron/sulphur 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/sulphur clusters (20, 21). The Grx domains of the human Glrx3 share 40-50% 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%) (22, 6). 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 thus playing an important role in iron metabolism and trafficking (23, 7, 9, 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 and in general nutritional stress, endoplasmic reticulum (ER) stress, viral infection and many other injuries activate this pathway (for reviews, 25, 26). In yeast, all these stimuli activate the kinase Gcn2 of the ISR pathway, whereas in human cells...
several kinases (GCN2, PKR, HRI, PERK) receive different stresses converging and activating eIF2α, the core of the ISR pathway (25). The factor eIF2α is very 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 activation of either GCN4 or ATF4 in response to a number of different stresses (ER stress, hypoxia, oxidative stress, TOR inactivation, glucose depletion, etc.) occurs through translational regulation in all the eukaryotic cells, from yeast to humans (29, 30, 31, 25).

The purine nucleotides ATP and GTP are required for cellular life. They are basic components to build nucleic acids but they both are also involved in conserved and essential processes, such as translation, building GTP, signalling, enzymatic reactions etc. (32). Synthesis of guanine nucleotides requires IMP (inosine monophosphate) conversion into XMP (xanthosine monophosphate) by the IMP dehydrogenase (33), then the GMP-synthase (GUA1 in yeast) catalyses 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 (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 dNTP homeostasis and showed that deficiencies in Guanine synthesis leaded to 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 Grx/GMPs complex and the integrated stress response.

RESULTS

GMP synthase is a novel target of monothiol glutaredoxin Glrx3

In order 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 pGBK7 plasmid carrying out the GAL4 activating domain. The mouse library inserts were contained in plasmid pGADT7. We tested 4.7x10⁷ independent clones following the manufacturer instructions. Upon isolation, sequencing and identification, the sequences identified could be classified into three categories: 68% corresponded to a 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 (Figs 1A and S1A). 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 co-immunoprecipitation, as depicted in Fig 1A and S1A, S1C and 1B.
Iron sulphur clusters are relevant for Glrx3/Grx3/Grx4 binding to GMP synthases. The family integrated by Glrx3 and the yeast Grx3/Grx4 use iron/sulphur clusters to bridge different target proteins. In order to profound in 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 centre in each of both glutaredoxin domains, C159 (GrxA domain) and C261 (GrxB domain) and also the residues WP197/198 (GrxA domain) and WP299/300 (GrxB domain), both known to be involved in the interaction with glutathione, all of them are required to bind iron sulphur clusters and are evolutionary conserved between human Glrx3 and Grx3/Grx4 in S. cerevisiae. All these mutagenized residues clearly precluded the formation of the Glrx3/GMP synthase complex (Figs 1A and S1A), as previously described by (4, 5) in their characterisation of the complex formed between Glrx3 and the human protein Boul. Our results indicate that Glrx3 uses iron/sulphur bridged clusters to bind hGMP synthase. With the aim to detect 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 (Figs 1A and S1A). For a better characterisation 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 (Figs 1A and S1A). In addition, both Grx3 and Grx4 interacted either with mouse or human GMPs (Figs 1A and S1A), demonstrating the complementarity of these proteins. We next tested the Grx3/Grx4 mutants in the residues involved in bridging iron/sulphur clusters described in (36) to perform a subsequent Two Hybrid experiment with hGMP synthase. In all cases those mutants...
8 severely diminished the binding between Grx3 or Grx4 with hGMPs, (Figs 1A and S1A). These results indicate that iron/sulphur clusters also bridge the physical binding between Grx3 and 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 glutaredoxins with 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 (Figs 1C, S1B and S1C) following a mechanisms 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 in conditions of nutritional stress

In order to ascertain the biological significance of the above mentioned interaction we searched a possible connection between GMP synthases and specific signalling pathways. Some authors (37) described a functional connection between GUA1 and the integrated stress response (ISR) in the 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, amino acids), TORC1 inactivation, among others. Gcn2 phosphorylation then regulates eIF2α phosphorylation which subsequently derepress 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 supressed upon the addition of Guanine to the culture media or alternatively, upon overexpression of hGMPs or GUA1 (Fig 2A). We observed that the double mutant grx3grx4 presents growth defects, as compared to wt cells, which were also efficiently rescued upon the
addition of Guanine (Fig 2A). Moreover, overexpression of either Glrx3, Gua1 or GMP synthase also improved the growth rate and survival of grx3grx4, as depicted in Fig 2A. As expected, overexpression of Glrx3, Grx3 or Grx4 did not suppress the lack of growth of 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 double mutant grx3grx4 also presented a remarkable induction of the ISR pathway as evidenced through the derepression of GCN4 translation in non-stressed conditions (Fig 2B), accompanied of a significant phosphorylation of eIF2α as compared to wt cultures (Fig 3A and S2A). GCN4 expression and eIF2α phosphorylation were also higher in grx3 and grx4 than in wt cells growing exponentially in the absence of nutritional stress (Fig 2B and 3A and S2A). In accordance with these results, gua1 mutant presented a higher derepression of GCN4 translation (Fig 2B), indicative of a constitutive activation of the ISR pathway, in agreement with the results reported by (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 grx3grx4 double mutant occurred as a consequence of alterations in iron homeostasis or alternatively because both glutaredoxins were playing a direct role in ISR regulation. The simultaneous absence of Grx3 and Grx4 provokes constitutive hyper activation of Aft1 due to its constant nuclear localisation (18, 10), as a consequence of that, iron is accumulated in the cytoplasm (19). We observed that deletion of aft1 highly reduced eIF2α phosphorylation levels in the triple mutant grx3grx4aft1 growing exponentially (Fig 3A and S2A), what 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, grx3grx4 mutant presented a higher eIF2α phosphorylation than wt cells. In 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 S2A). Consequently, in the double mutant grx3grx4 the total eIF2α phosphorylation detected in 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 nutritional stress caused by nutrient exhaustion during the diauxic shift or either to amino acid depletion plus a second response to iron homeostasis deregulation. Thus, eIF2α phosphorylation in a grx3grx4aft1 triple mutant was equivalent to the levels detected in wt cultures (Fig 3A and S2A). These results suggest that GCN2 receives the signal of iron homeostasis independently of other nutritional status, and that these signals additively converge on GCN2 activation. Interestingly, Aft1 deletion did not suppress the requirements of the grx3grx4 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 on Aft1 and iron homeostasis. For more in-depth detail we decided to analyse both eIF2α and GCN4 translational induction in grx3grx4 and wt cells in response to several stimuli: iron deprivation, iron or guanine supplementation, and N-acetylcysteine treatments (NAC), this latter agent contributes to the reduction of ROS levels in case that oxidative stress could also be contributing to ISR activation in the grx3grx4 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 S2B). With respect to each untreated control values, this
descent was more severe in grx3grx4 double mutant than in wt cells, supporting the
model that specific stimuli activate the ISR pathway in grx3grx4 double mutant, such
as: iron accumulation, limiting purines or increasing ROS levels. The relevance of this
preliminary finding is that guanine metabolism and ISR pathway might meet a
connection through Grx3/Grx4 cytosolic monothiol glutaredoxins.

Glrx3 and the 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 ISR pathway upon our experimental
conditions of nutritional starvation in both wt and grx3grx4 mutant (using gua1 as a
control). GCN4 derepression and eIF2a phosphorylation revealed a remarkable ISR
activation in both strains upon growth in either amino acid deprivation or 1 day in
minimum medium, as compared to non-starved cultures (Figs 4 A-D and S2C, D and
E). In accordance with the former observations depicted in Fig 3A, the double mutant
grx3grx4 presented a higher induction than wt cells in our experimental conditions, due
to the additive and independent effect caused by the starvation and Aft1 dysfunction. In
order to functionally characterize the novel interaction between Glrx3 and hGMPs, we
co-expressed both human proteins in wt, grx3grx4 and gua1 strains and analysed 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 non-starved exponentially growing wild type, grx3grx4 or gua1 cultures
(Figs 4 A, C and E). However, upon nutrient deprivation conditions, Glrx3+hGMPs co-
expression significantly reduced GCN4 expression both in wt, grx3grx4 and gua1
strains (as compared to wild type strains transformed with the empty plasmids). hGMPs
overexpression slightly reduced GCN4 translational expression in all the strains tested, although at a lesser extent than determined when Glrx3+hGMPs were co-expressed. However, Glrx3 overexpression did not cause any significant reduction in GCN4 levels in the experimental conditions tested (Figs 4A, C and E). These results in general correlated with those obtained upon analysis of eIF2α phosphorylation (Figs 4 B, D and F and S2 C, D and E). Altogether, the previous results support the hypothesis that the lack of monothiol glutaredoxin function is detrimental for purine metabolism. We decided to investigate the degree of complementation between yeast and human glutaredoxins and GMP synthase regarding downregulation of ISR pathway. For this we analysed a) Grx3 or Grx4 co-expression with human GMP synthase; b) Glrx3 co-expressed with GUA and c) Grx3 and Grx4 co-expressed with GUA1 (Fig 4). As previously described with Glrx3 and hGMPs, Grx3 or Grx4 did not cause a descent in ISR activity, however, Gua1 overexpression slightly reduced GCN4 expression and eIF2α phosphorylation in wt, grx3grx4 and gua1 strains. Moreover, in all cases the co-expressions described above in a), b) and c) significantly reduced both GCN4 levels and eIF2α phosphorylation upon both nutritional starvation conditions tested in this study (Figs 4, S1C and S2C, D and E). We observed a small reduction in the phosphorylation caused by each the human or yeast GMP synthases that was negligible as compared to the results obtained upon each coexpression. This observation points to a mild negative function of GMP synthases in the activation of GCN2 only detectable immunologically.

We next analysed the effect 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 eIF2α suffer upon amino acid starvation (Figs 5A and B, S1C and S2F). Our results suggest that Glrx3/GMPs are relevant in order to reduce the
activity of the IRS pathway and also suggest that this mechanism is conserved from yeast to humans.

Glrx3 and GMP synthase co-localise in the nucleus upon ISR pathway induction. We fused GFP to each of the human proteins, Glrx3 and GMP synthase to observe their cellular localisation. hGMPs showed a cytoplasmic localisation whereas Glrx3 presented partial nuclear accumulation in conditions of nutrient sufficiency. Nutritional stress however, induced a predominant nuclear accumulation for both proteins, supporting the previous hypothesis that both proteins could be playing a common and potentially collaborative function in this compartment when ISC is activated (Fig 6).

Excess or defects in monothiol glutaredoxins dosage is detrimental for life span. We also checked the influence that either Glrx3, hGMPs or co-expression of both could have in the chronological life span. We observed that Glrx3 and at lower extent GMPs over-expression caused an acceleration of yeast growth (Table1). Glrx3/GMPs sustained co-expression reduced the chronological life span (CLS) of a wt strain (Fig 7), In this study we could determine that the simultaneous absence of Grx3 and Grx4 was detrimental for chronological life span, nevertheless, Glrx3 over-expression efficiently complemented this deficiency (Fig 7). Interestingly, hGMPs overexpression in grx3grx4 also partly rescued the defects in chronological life span of the mutant, supporting the idea that both Grx3/Grx4 and possible Glrx3 might be directly or indirectly involved in purine biosynthetic pathway. We conclude that the excess 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 also a role in human life span extension and whether this role requires GMPs activity.
DISCUSSION:

Here we present evidence demonstrating that yeast system is a useful strategy for screening of new interactors with human proteins which are evolutionary conserved in the eukaryotic world. This approach also permits a faster searching for new connections with signalling 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 synthesis of Guanine and GTP. Our results suggest that the complex Glrx3/GMPs is important to regulate ISR activity in conditions of nutrient deprivation. Moreover, we show here 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 to this observation is that this complex could be required to down regulate GCN2 activity in order to avoid 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 yeast the system in which more studies have been conducted given the elevated degree of conservation (39). There are a wide number of publications demonstrating the useful tool that yeast are in order to characterise human cellular functions and processes (as examples: 40, 41). Consequently, this is another example of the contribution of yeast to decipher 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 signalling to ISR which is evolutionary conserved, from yeast to mouse and humans. We show that the absence of both monothiol glutaredoxins Grx3 and Grx4 cause a mild induction of the GCN2 signalling pathway caused by Aft1 nuclear localisation and the consequent anomalous induction of the iron regulon (18, 19, 42). However, nutrient
starvation caused by the diauxic shift or amino acid limitation are both conditions that activate the GCN2 pathway independently on Grx3/Grx4 and consequently of Aft1 proteins. This observation allowed us to conclude that in budding yeast, dysfunction of iron metabolism signals to GCN2 independently of other signalling mechanism caused by other types of nutritional stresses.

The observation that in S cerevisiae, addition of guanine to cultures of the grx3grx4 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 grx3grx4 double mutant for guanine and as a novel finding also supresses the chronological life span defect that we have characterised in this study for grx3grx4 double mutant. These findings suggest that: 1. Glrx3/Grx3/Grx4 share an evolutionary conserved function related to purine metabolism. 2. These monothiol glutaredoxins are required for the chronological life span of eukaryotic cells. Our CLS studies reveal, however, that a maintained and continuous descent of ISR expression (caused by a constant overexpression of both, Grx3/Grx4 or Glrx3 in combination with GMPs, in this study) during aging provokes a mild reduction of life span, suggesting that ISR might require a tight and punctual downregulation in order 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 complex Grx/GMPs 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). Thus, suggesting that when cells shift from proliferative to a quiescent state, a decrease in the guanylyl nucleotide pool should be required. 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 complex GMPs/Glrx3 might contribute to a negative feed-back on GCN4 function, probably in the nucleus and consequently on purine biosynthesis, since one of the outcomes of GCN4 is the translational activation of genes involved in that biochemical pathway (26, 45). The observation that NAC addition or iron starvation significantly descents activation of the GCN2 pathway in the grx3grx4 double mutant could lead to the following speculation: that iron excess provokes an increment of ROS causing activation of the GCN2 pathway. This is probably the explanation to the observed induction of ISR pathway in the grx3grx4 mutant given that deletion of Aft1 suppress the ISR activation. Moreover, it has been determined that iron excess provokes a constitutive oxidative stress in the mutant grx3grx4 (38). That monothiol glutaredoxins involved in ROS detoxification and iron metabolism interact with GMPs, an enzyme dedicated to synthetize 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 might be involved. In exponentially growing cells the lack of GUA1 function also activates GCN2 pathway in S. cerevisiae, probably due to defects in purine synthesis increasing the pool of non-chargeable tRNAs which activates the ISR pathway (35, 37). The human GMPs efficiently complemented yeast gua1 regarding signalling to ISR, this was indicative of an evolutionary conservation of this signalling in the eukaryotic world.
We present evidences suggesting that upon nutrient limitation and the concomitant GCN2 activation, both hGMPs and Glrx3 play a conjunct role in the down-regulation of the activity of ISR pathway.

Is has been recently described that Gcn2 regulates Aft1 function in order to adjust iron metabolism to amino acid biosynthesis (48). In our study we observed that in the absence of grx3grx4, 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 two different scenarios: 1. Grx3/Grx4 are required to sense iron availability and without them cells receive a constant signal of iron depletion then activating Gcn2 and Aft1. 2. Grx3/Grx4 are both negative regulators of ISR, then in their absence, GCN2 is constitutively activated, then 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 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 the common functions evolutionary conserved and regulated by cytosolic monothiol glutaredoxins and GMPs. The family of monothiol glutaredoxins: human Glrx3 and yeast Grx3 and Grx4, all interact with GMPs proteins through specific and already characterised residues which affect binding to iron/sulphur clusters.

Glrx3 and its accompanying protein Bol2 store iron, the complex function as iron chaperones by direct binding to target proteins to which they apparently deliver clusters.
iron/sulphur (15). Glrx3/Grx3/Grx4 could be playing a role as chaperones possibly transferring iron to GMPs or alternative to other protein/s involved in guanine metabolism. Thus, one possible explanation for the formation of the complex Glrx3/Grx3/Grx4 and GMP synthase might be the requirement for iron/sulphur clusters at some point to sense or synthesize GTP, or alternatively, that iron or iron/sulphur might serve as a limiting molecule to regulate GMPs function in a context of nutritional limitation. These interesting aspects require further investigation.

MATERIALS AND METHODS

Yeast strains and plasmids

Saccharomyces cerevisiae wild type (MATa leu2-3,112, trp1, his4, can1r) and the isogenic mutant strains grx3 (MATa grx3::NatMX4), grx4 (MATa grx4::KanMX4), grx3grx4 (MATa grx3::NatMX4, grx4::KanMX4) and grx3grx4aft1 (MATa aft1::URA3, grx3::NatMX4, grx4::KanMX4) have been previously described in (18). The gua1 mutant was obtained in this study by the 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) vectors and plasmids carrying each 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 in (36).

Saccharomyces cerevisiae Y2HGold and Y187 strains were used for the screen-based two-hybrid system (Clontech, CA, USA). Y2HGold (MATa, 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 the Y187 (MATa, 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 (MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4, gal80, GAL2-ADE2, LYS2::GAL1-HIS3, met::GAL7-lacZ) and PJ69-4a (MATa, 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 cloning of the PCR amplification sequences into the multiple cloning site of: pGBKT7 plasmid (Clontech, CA, USA) (under the ADH1 promoter and GAL4 binding domain in N terminus, fused in frame with Myc epitope in C terminus), pUG35 (under the MET25 promoter and fused in frame to GFP epitope in 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 pGBKT7 vector was used as template. The mutant forms of the different proteins were: C159S (Forward primer: 5’-TCGTGGTTCTTGAGGAGTTCCTTTC-3’ and Reverse primer: 5’-
TCGGGTTCAGCAAGCAGATGGTG-3’) C261T (Forward primer: 5’-GGTTTTTGCGTCTTGGTGTTTCCCTTTC-3’ and Reverse primer: 5’-
GGTTGACTCAAAAGTCTGGAATAC-3’) WP(197/198)GS (Forward primer: 5’-TCCACTGGAATAGGCTTTGAGTCCCTG-3’ and Reverse primer: 5’-
TCCACCTATCGCTCTTAGGTCTGTTCTG-3’) and WP(299/300)LE (Forward primer: 5’-GAGATTTGAGTAAGCTTTTAATCCTTG-3’ and Reverse primer: 5’-
GAGACATACCTAGCTGATGTGAAAG-3’).

Yeast GUA1 gene was amplified from chromosomal DNA from S. cerevisiae CML128 (MATa, 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 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 GFP epitope in C terminus). Plasmid
p180 (Low copy vector expressing GCN4-lacZ, uORFs 1-4 in YCp50), was kindly
provided by Dr. Mercedes Tamame. This plasmid was transformed into wild type and in
the mutant strains grx3, grx4, gua1 and grx3grx4, to analyse GCN4 expression upon
quantification of the reporter gene β-galactosidase.

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 on SD medium whose nitrogen base
component was free of iron plus the addition of 80,µM of 4,7-diphenyl-1,10-
phenanthrolinedisulfonic acid (BPS) (Sigma,146617). When required, 5 mM of N-
acetyl glucosamine (Sigma, A9165), Guanine 130 µM (Sigma, 51030) were added to
the culture media, and iron was added as ammonium iron (III) sulfate hexahydrate
(NH₄Fe(SO₄)₂.6H₂O) (+Fe, Sigma, F1543) at a final concentration of 10mM. Cell
cultures were exponentially grown (OD₆₀₀ of 0.6) or grown to the diauxic shift (1 day in
SD plus amino acids, OD₆₀₀ of 2.5). For amino acid starvation experiments, we prepared a
cultures in SD medium plus required amino acids to log phase, OD₆₀₀:0.6. Cells were then
collected, washed three times in SD medium without amino acids and transferred to the
required volume of the same fresh medium at a starting OD₆₀₀: 0.3. Cultures were grown over
night for 10 hours, after that, cells were collected to be subsequently processed. β-
galactosidase activity was basically determined according to (52) with small variations:
we collected 5ml of cell cultures to be centrifuged and the pellets were suspended in

β-galactosidase activity was basically determined according to (52) with small variations:
400 ml buffer Z (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 01 mM MgSO4, 50mM βME, pH 7.0) plus 20µl de sarcosyl (10%) and 4µl toluene. After that, samples were vortexed and split into two eppendorfs to which 600µl buffer were added. Samples were incubated at 28ºC for 5 min, then added 50µl ONPG (4mg/ml) to be subsequently incubated at 28ºC for 5 min. Finally, the addition of 500µl Na2CO3 (1M) stopped the reaction. Absorbance was measured at λ=420nm

Two-hybrid system

To start the screen, we cloned the open reading frame of GLRX3 into pGBK7 plasmid carrying out the GAL4 activating domain. A mouse cDNA library (Matchmaker® Gold Yeast Two-Hybrid System Mate and Plate Library-Mouse embrio-11 day) was used as prey to identify interactions with human GLRX3. The mouse library is constructed in pGADT7 plasmid, which contains a GAL4 activation domain (DNA-AD) and a constitutively active ADH1 promoter (PADH1). The mouse library was transformed into Y187 strains and incubated in SD/-Leu broth. Y2HGold strains containing the pGBK7-GLRX3 constructs were cultured in SD/-Trp both. Matings were performed in 50 ml YDPA (2x) medium containing 12.5 µg/ml kanamycin. At least 2 × 10⁷ Y187 cells and 2 × 10⁸ Y2HGold cells we incubated at 30 ºC and 70 rpm for 24 h. After observation of zygotes, cultures were washed in 0.5x YDPA, submitted to serial dilutions 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 3-5 days incubation at 30 ºC. Positive mating controls between Y2HGold yeast transformed with pGBK7-53 and Y187 yeast cells transformed with pGADT7-T were employed, as well as negative mating controls with Y2HGold yeast transformed with pGBK7-Lam and
Y187 yeast transformed with pGADT7-T. Colonies obtained were isolated and kept frozen for further analysis.

Positive interactions analysis: We tested $4.7 \times 10^7$ independent clones following the manufacturer instructions. Plasmids from the diploids obtained from the matings were recovered by using 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 the different profiles were sent for sequencing analysis. Positive interactions were confirmed by transforming isolated prey plasmids into Y187 strains and repeating matting with Y2HGold strains containing pGBK7-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 O.D. $600: 0.6$ in SC medium supplemented with the required amino acids. Viability was registered through serial dilutions and plated by triplicate onto YPD plates.

We measured the chronological life span (CLS) in the different strains based on the survival of populations of non-dividing yeast cells according to (53). The viability was scored by counting the number of cells able to form colonies, CFU (colony-forming units). Cultures were started at an O.D600: 0.6. The same number of cells collected from each culture were plated in triplicated into YPD plates and allowed to grow at 30°C for 3-4 days. Viability at day 3 is considered to be the 100% survival (time 0 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 (36). The antibodies for western blotting were as follows: anti-HA 3F10 (Roche Applied Science, Cat. No.12158167001) was used at a dilution of 1:2000 in 0.25% non-fat milk, anti-GFP (Living Colors, Cat. No. 632381) at a dilution 1:2000, anti-PICOT (D-10) (Santa Cruz Biotechnology, Cat. No. sc-390068) at a dilution 1:1000, anti-MYC (Roche) at a dilution 1:2000, antiphospho eIF2α Ser–51 (Cell Signaling, Cat. No. 3597S) at a dilution 1:1000, anti-Grx3/4 (a gift from Rolland Lill) at a dilution 1:4000, and anti-PGK1 (Invitrogen, Cat. No. 459250) at a dilution 1:10000 were used as indicated by the manufacturers. The protein-antibody complexes were visualised by enhanced chemiluminescence, using the Supersignal substrate (Pierce) in a Chemidoc (Roche Applied Science). Secondary antibodies at a dilution 1:10000: Anti-mouse IgG horseradish peroxidase (from sheep) (GE Healthcare, Cat. No. Na931) and Anti-rabbit IgG horseradish peroxidase (from donkey) (GE Healthcare, Cat. No. Na934), and Goat anti-rat IgG HRP conjugate (Millipore, Cat. No. AP136P).

Co-immunoprecipitation of proteins

Protein extracts were prepared from exponentially growing cells to be subsequently bound to Anti-HA Affinity Matrix (Roche) as described in (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 co-immuno precipitated protein by western blot.
FIG 1. The formation of the complex between monothiol glutaredoxins and GMP synthases occurs through specific conserved residues, affecting binding to iron/sulphur clusters, being yeast and human proteins interchangeable for this function. A) Two Hybrid analyses to determine the interactions between: pGBK7Glrx3 and each of both pGADT7mGMPs or pACTIIhGMPs; pACTIIhGMPs and pGBK7Glrx3C159S, pGBK7Glrx3C261S, pGBK7WP197/198GS, pGBK7WP299/300LE; either pGADT7mGMPs or pACTIIhGMPs and pGBT9Grx3 or pGBT9Grx4; pACTIIhGMPs and pGBT9Grx3C2R, pGBT9Grx3C18S, pGBT9Grx3C72R, pGBT9Grx3C142R, pGBT9Grx3C211T; pACTIIhGMPs and pGBT9Grx4C37R, pGBT9Grx4C171S, pGBT9Grx4C37R/C171 and pACTIIGlrx3/Grx3/Grx4 and pGBK7Gua1. These assays were carried out from 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 denoted as follows (∗=0,05>P>0,01/ ∗∗=0,01>P>0,001/ ∗∗∗=P<0,001). B) Physical interaction between Glrx3 and hGMPs. Cultures of wild type strain previously transformed with both pACTIIhGMPsHA and pGBK7Glrx3Myc were grown to logarithmic phase in SD medium plus amino acids. Samples were collected for protein extraction and subsequent co-immunoprecipitation. The Glrx3 co-immunoprecipitated protein was detected in the complex with the anti-PICOT monoclonal antibody. C) Two Hybrid analysis between the couples mGMPs/Glrx3, mGMPs/Grx3, mGMPs/Grx4, hGMPs/Glrx3, hGMPs/Grx3, hGMPs/Grx4, Gua1/Glrx3, Gua1/Grx3 and Gua1/Grx4. All cultures were exponentially grown.
grown in SD containing (Control) or not iron (-Fe) 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 denoted as follows (*=*0.05>P>0.01/ **=*0.01>P>0.001/ ***=P<0.001).

FIG 2. The 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) Over-expression of Gua1 suppresses Guanine requirements of the double mutant grx3grx4. Wild type, grx3grx4 and gual strains were transformed with each pGua1, pGrx3, pGrx4, pGlrx3, pGMPs or the corresponding empty plasmids (described in Materials and Methods). Cultures were logarithmically grown in SD medium plus or minus 130µM Guanine at 30º C and subsequently plated in triplicate in SD plates. B) ISR pathway is constitutively activated in grx3, grx4 and grx3grx4 mutants. Samples from exponentially growing cultures of wt, grx3, grx4, grx3grx4 and gual 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 the average of three independent experiments. Error bars are represented with P-values from a Student’s unpaired t-test denoted as follows (*=*0.05>P>0.01/ **=*0.01>P>0.001/ ***=P<0.001).

Fig 3. Iron accumulation is related to ISR induction in exponentially growing cells, however, the signal to GCN2 activation confluents independently and additively to nutrient starvation. A) The phosphorylation of eIF2α was determined in wt, grx3, grx4, grx3grx4, aif1 and grx3grx4aif1 cultures growing exponentially, during the diauxic shift.
(1 day) and upon amino acid starvation. B) Cultures from A were serial diluted and plated in YPD plates containing or not 130µM guanine. Cultures from wt and grx3grx4 strains were subject to different treatments: iron depletion (-Fe), 130µM guanine, 5mM NAC and 10mM iron addition (+Fe) (see Materials and Methods) to be subsequently grown to logarithmic phase. Samples were taken to: C) quantify GCN4 expression as in Fig. 2B. Values shown in the histogram are the average of three independent experiments. Error bars are represented with P-values from a Student’s unpaired t-test denoted as follows (**=0,05>P>0,01/ ***=0,01>P>0,001/ ****=P<0,001). D) eIF2α phosphorylation was also determined by using the anti-phospho-eIF2α antibody and anti-PGK1 as a loading control. Western blot were performed in duplicated, this is a representative blot. Quantitative and statistical values relative to the loading control are depicted in Fig.S2.

FIG 4. Co-expression of monothiol glutaredoxins Grx3/Grx4/Glrx3 and either human or yeast GMP synthase reduces the activation of ISR pathway in wt, grx3grx4 and gua1 cultures upon nutritional stress caused by diauxic shift or amino acids depletion. Cultures from wt (A and B), grx3grx4 (C and D) or gua1(E and F) strains were transformed with empty plasmids, pGrx3, pGrx4, pGlrx3, phGMPs, pGua1 or co-transformed with pGrx3+phGMPs, pGrx4+phGMPs, pGlrx3+phGMPs, pGrx3+pGua1, pGrx4+pGua1 and pGlrx3+pGua1 were grown exponentially in SD, in SD to diauxic shift or exponentially in SD without amino acids. A), C) and E) Values of GCN4 expression were obtained as in Fig. 2B. In the histograms are represented the average of three independent experiments. Error bars are represented with P-values from a Student’s unpaired t-test denoted as follows (**=0,05>P>0,01/ ***=0,01>P>0,001/ ****=P<0,001). B), D) and F) eIF2α phosphorylation was determined as in Fig 3A.
FIG 5. Residues related to iron/sulfur binding in Glrx3 are involved in ISR down-regulation driven with hGMPs. Wild type cultures were transformed with plasmids empty plasmids pGBKT7+pACTII, pGBKT7Glrx3, pGBKT7Glrx3C159S, pGBKT7Glrx3C261S, pGBKT7WP197/198GS, pGBKT7WP299/300LE in combination with phGMPs to analyse: A) Values of GCN4 expression were obtained as in Fig. 2B. In the histograms are represented the average of three independent experiments. Error bars are represented with P-values from a Student’s unpaired t-test denoted as follows (∗=0.05>P>0.01/∗∗=0.01>P>0.001/∗∗∗=P<0.001). B) eIF2α phosphorylation was determined as in Fig 3A.

FIG 6. In vivo localisation of the human proteins Glrx3 and hGMPs. Wild type cells were transformed with plasmids pGlr3GFP and phGMPsGFP and pUG35 and subsequently grown either in SD plus amino acids to each logarithmic and diauxic phases or alternatively grown in SD without amino acids. Cultures were also in vivo stained with DAPI to identify Glrx3 and hGMPs nuclear localization. Samples were taken for observation in the fluorescence microscope.

FIG 7. Chronological life span curves for wt+empty plasmids, wt+pGlrx3, wt+phGMPs, wt+pGlrx3+phGMPs, grx3grx4+empty plasmids, grx3grx4+pGlrx3, grx3grx4+phGMPs, grx3grx4+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 Material and Methods. Numerical data regarding maximum life span (the day when cultures reach 10 % of survival) and
average life span (as the day at which 50% survival was recorded) for each strain is depicted in the Figure.

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

Table 1. Generation time and standard deviation calculated in cultures of the strains indicated growing in SD plus amino acids at 30°C in continuous shaking.

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We acknowledge Dr Concepcion Mora (IRBLleida) for providing us the mouse library to perform the screening as a starting point in this study and Dr M. Tamame and Dr A.G. Hinnemus for strains and plasmids. We are very grateful to Dr R. Lill for anti-Grx antibodies. We acknowledge the invaluable technical assistance of Inmaculada Montoliu. The research described in this publication was partly supported by the Plan Nacional de I+D+I of the Spanish Ministry of Economy, Industry and Competitiveness (BIO2017-87828-C2-2-P) and the University of Lleida (TR265). Monica A. Mecheoud was supported by the Marie Curie Actions Program of the European Union COFUND 2014-51501. Sandra Montella is funded by a fellowship from the Catalan Government (Spain).

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Fig. 1

A)  

B)  

C)
Fig. 2

A) Control + Guanine

- wt+empty plasmid
- wt+Gua1
- wt+Grx3
- wt+Grx4
- wt+Glrx3
- wt+hGMPs
- grx3grx4+empty plasmid
- grx3grx4+Gua1
- grx3grx4+Grx3
- grx3grx4+Grx4
- grx3grx4+Glrx3
- grx3grx4+hGMPs
- gua1+empty plasmid
- gua1+Gua1
- gua1+Grx3
- gua1+Grx4
- gua1+Glrx3
- gua1+hGMPs

B) Graph showing WCE
Fig. 3

A) 

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<th>SD-aa</th>
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B) Control +Guanine

C) 

D) 

wt grx3 grx4

anti-eIF2α-P

anti-PGK1

wt grx3 grx4

anti-eIF2α-P

anti-PGK1
Fig. 5

A) SD 1day

SD

SD-aa

100
200
300
400
0

WCE

anti-PGK1

B) WCE

+Glrx3C159S
+Glrx3C261S
+Glrx3WP197/198GS
+Glrx3WP299/300LE

+empty vectors

+Glrx3

+Glrx3WP299/300LE

+Glrx3WP299/300LE+hGMPs

+empty plasmids

+Glrx3+hGMPs

+Glrx3C159S+hGMPs

+Glrx3C261S+hGMPs

+Glrx3 WP299/300LE+hGMPs

+empty plasmids

+Glrx3WP197/198GS+hGMPs

+Glrx3WP197/198GS+hGMPs

+empty plasmids

+Glrx3WP299/300LE+hGMPs

+empty vectors

+empty plasmids

+Glrx3+hGMPs

+empty plasmids

+empty plasmids
Fig. 7

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<tr>
<td>wt + Glrx3 + hGMPs</td>
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<td>20.4 ± 0.45</td>
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Cell Survival (CFU% with respect to day 3)
Monothiol glutaredoxins
GMP synthases

Gcn2/ISR pathway

Gcn2

eIF2α

Cell Survival

NUTRITIONAL STRESS

Fig. 8
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Table 1