

Boron Stress Activates the General Amino Acid Control Mechanism and Inhibits Protein Synthesis

Irem Uluisik¹, Alaattin Kaya^{1,2}, Dmitri E. Fomenko³, Huseyin C. Karakaya¹, Bradley A. Carlson⁴, Vadim N. Gladyshev², Ahmet Koc^{1*}

1 Izmir Institute of Technology, Department of Molecular Biology and Genetics, Izmir, Turkey, **2** Division of Genetics, Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, **3** Department of Biochemistry, University of Nebraska, Lincoln, Nebraska, United States of America, **4** Molecular Biology of Selenium Section, Laboratory of Cancer Prevention, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

Boron is an essential micronutrient for plants, and it is beneficial for animals. However, at high concentrations boron is toxic to cells although the mechanism of this toxicity is not known. Atr1 has recently been identified as a boron efflux pump whose expression is upregulated in response to boron treatment. Here, we found that the expression of *ATR1* is associated with expression of genes involved in amino acid biosynthesis. These mechanisms are strictly controlled by the transcription factor Gcn4 in response to boron treatment. Further analyses have shown that boron impaired protein synthesis by promoting phosphorylation of eIF2 α in a Gcn2 kinase dependent manner. The uncharged tRNA binding domain (HisRS) of Gcn2 is necessary for the phosphorylation of eIF2 α in the presence of boron. We postulate that boron exerts its toxic effect through activation of the general amino acid control system and inhibition of protein synthesis. Since the general amino acid control pathway is conserved among eukaryotes, this mechanism of boron toxicity may be of general importance.

Citation: Uluisik I, Kaya A, Fomenko DE, Karakaya HC, Carlson BA, et al. (2011) Boron Stress Activates the General Amino Acid Control Mechanism and Inhibits Protein Synthesis. PLoS ONE 6(11): e27772. doi:10.1371/journal.pone.0027772

Editor: Arthur J. Lustig, Tulane University Health Sciences Center, United States of America

Received: June 10, 2011; **Accepted:** October 25, 2011; **Published:** November 17, 2011

Copyright: © 2011 Uluisik et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by TUBITAK Grant No. 104T213, 110T917 and TUBA-GEBIP grant to (A.Koc). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ahmetkoc@iyte.edu.tr

Introduction

Boron is required for plant growth and development, and adequate levels of boron are crucial for high crop yield [1,2,3]. It has been established that the primary role of boron is the cross linking of cell wall components, rhamnogalacturonan-II and pectin, in plants [4]. Boron also forms complexes with glycoproteins in membrane structures [5] and it has been hypothesized that it is required for stabilization of molecules with *cis*-diol groups [6]. Apart from these structural functions, boron can interact with transcription factors and plays roles in cell signaling [7] and quorum sensing [8,9]. This element also has beneficial effects for animal growth and development. It stimulates embryogenesis in zebrafish and trout [10,11] and reduces the risk for prostate and lung cancers in humans [12,13].

Saccharomyces cerevisiae emerged as a useful model to identify and characterize boron tolerance genes [14,15,16,17,18,19]. *S. cerevisiae* is boron tolerant and can tolerate boric acid levels of up to 80 mM [14,15]. Recently, the *ATR1* gene has been found to be a major boron tolerance gene [14]. Overexpression of *ATR1* provides high resistance to boron and reduces the intracellular boron levels. Mutants lacking the *ATR1* gene have been found to be sensitive to boron and have higher intracellular boron levels. Boron treatment has been shown to induce the *ATR1* gene and many amino acid biosynthesis genes that are normally regulated by the Gcn4 transcription factor [14].

In the case of amino acid starvation, yeast induces amino acid biosynthesis genes which are known as general amino acid control

(GAAC) [20,21,22]. The Gcn4 transcription factor is the master regulator of GAAC that governs the concerted induction of a large group of biosynthesis genes [20,22]. Interestingly, the expression of *GCN4* itself is regulated primarily at the translational level by four short open reading frames in its 5'-leader region [22,23]. Activation of GAAC also triggers phosphorylation of the eukaryotic initiation factor-2 α (eIF2 α) through the Gcn2 kinase and inhibits general translation [21,24]. Activation of the Gcn2 kinase involves direct binding of uncharged tRNAs to the histidyl-tRNA synthetase domain of Gcn2 [21,24,25]. In yeast, eIF2 α phosphorylation also leads to preferential translation of *GCN4* mRNA, while inhibiting general translation [21,24].

In this study, we demonstrate that boron is sensed via its disruption of amino acid metabolism and translation. We postulate that induction of GAAC in response to boron stress is toxic because of its inhibitory effects on translation. However, GAAC-dependent *ATR1* activation is required for resistance to boron stress.

Results

Transcriptional Regulation of *ATR1*: Roles of the Gcn4 Transcription Factor in Boron Stress Response

Recently, Atr1 has been identified as a membrane transporter with boron efflux function. Treating cells with boric acid causes a four-fold increase in *ATR1* mRNA levels [14]. However, the factors that regulate *ATR1* in response to boron stress are not

known. Previously, transcription factors Yap1 and Gcn4 were suggested to play a role in the regulation of *ATR1* [26]. Yap1 is a well known transcription factor that controls oxidative stress response genes and is likely to play a role in the activation of *ATR1* [27]. Gcn4 is the major regulator of gene expression during amino acid starvation and also under conditions of nutrient limitation in yeast [21]. Knowing that boron stress also induces amino acid biosynthetic genes [14], we reasoned that the Gcn4 transcription factor should regulate *ATR1* in response to boron treatment. To determine whether Yap1 or Gcn4 is required for *ATR1* induction by boron, global gene expression patterns of $\Delta yap1$ and $\Delta gcn4$ mutants were analyzed after boron treatment using DNA microarrays. The absence of *GCN4* prevented the activation of

many transporters including *ATR1* (Fig. 1A) and amino acid biosynthesis genes (Fig. 1B), whereas the absence of *YAP1* had no effect on the expression of these genes. Thus, *ATR1* and the genes under the control of GAAC, such as amino acid biosynthesis genes and transporters, appear to be regulated by the transcription factor Gcn4 during boron stress. Gcn4 requirement for *ATR1* expression was also confirmed by a real time-PCR approach, in which *ATR1* mRNA levels were almost similar in control and mutant untreated cells, but upon boron treatment $\Delta gcn4$ mutants failed to induce *ATR1* transcription. However, deletion of *YAP1* partially prevented *ATR1* upregulation in response to boron (Fig. 1C).

Further processing of the data obtained by microarray analyses showed that boron treatment caused upregulation (≥ 2 -fold) of 280

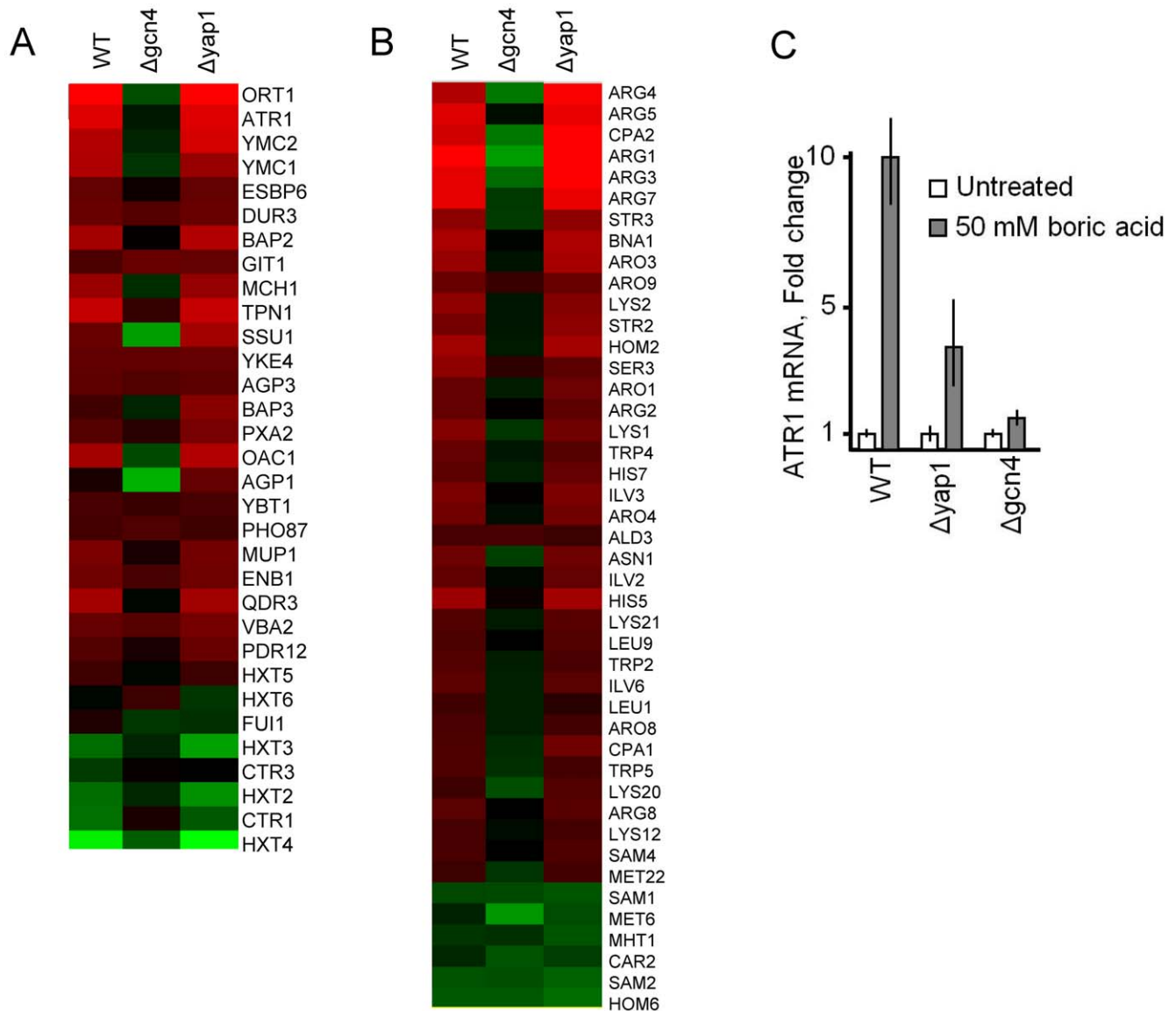


Figure 1. Gene expression analyses. To carry out microarray analyses, logarithmically growing wild type, $\Delta gcn4$ and $\Delta yap1$ mutant cells were split into two samples; one was treated with 20 mM boric acid for one hour while the other remained untreated. (A) Transporter genes that were upregulated or downregulated in response to boric acid treatment. (B) Amino acid biosynthesis genes that were upregulated and downregulated in response to boric acid treatment. Yeast *Saccharomyces cerevisiae* proteins annotation information from the yeast genome database (<http://www.yeastgenome.org/>) was used to identify transporters and other proteins. (C) Real time PCR analyses of *ATR1* in wild type, $\Delta gcn4$ and $\Delta yap1$ cells in response to boron. Cells were treated or not with 50 mM boric acid for one hour and *ATR1* expression was analyzed. Actin was used as the internal control. Bars represent the mean and SEM of triplicate assays. doi:10.1371/journal.pone.0027772.g001

genes, and down regulation of 118 genes in wild type cells (Table S1, Table S2). However, many of the boron induced genes in wild type cells were not activated in *Δgcn4* mutants (Table S3, Figure 1A and 1B). Thus Gcn4 transcription factor has a central role in mediating boron stress response in yeast cells.

Additionally, the microarray results showed that transcription of *GCN4* was 1.5-fold increased (p-value 2.8x10⁻³) in wild type cells treated with 20 mM boric acid for one hour, but none of the activators (*GCN1*, *GCN2*, *GCN3*, *GCN20*) of *GCN4* was upregulated (Table S1). The induction of internal *GCN4* expression in response to boric acid treatment was further analyzed by real time PCR (Fig. 2A). This assay confirmed that *GCN4* transcription is upregulated by boron and revealed that higher concentration of boron treatment (50 mM) causes a higher level of induction (5-fold). Since *GCN4* is mainly controlled at the translational level [21], we also tested whether boron stress increases the amount of Gcn4 protein in cells. As seen in Fig. 2B, boron treatment leads to almost two-fold induction of a chromosomal *GCN4*-dependent LacZ reporter gene activity in wild type cells. Increased protein level of *GCN4* was also shown by a western blotting approach using native *GCN4* gene tagged by MYC epitope (Fig. 2C).

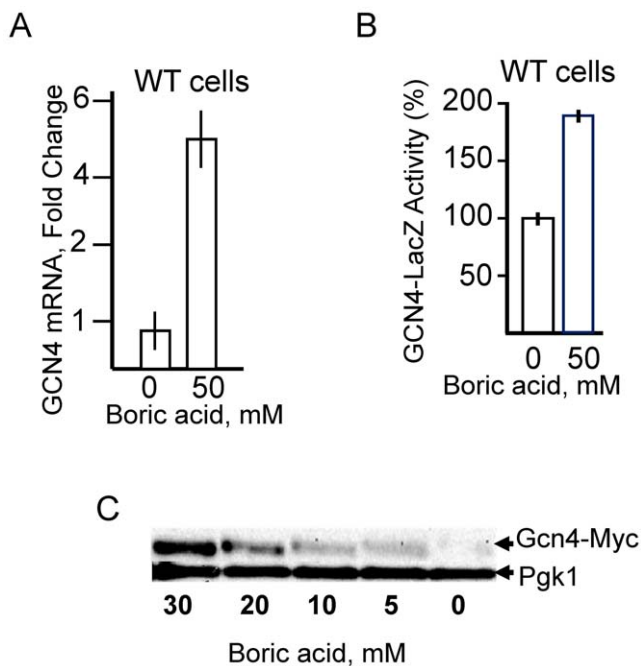


Figure 2. Induction of *GCN4* mRNA and protein expression by boric acid treatment. (A) Real time PCR analysis of *GCN4* mRNA in wild type cells treated with 50 mM boric acid for one hour. Actin was used as the internal control. (B) Wild type cells were transformed with the p180 plasmid which had the *GCN4*-lacZ reporter gene with all four uORFs of *GCN4*. Transformants were grown to logarithmic phase in YNB-URA medium. The cultures were split into two; one half was treated with 50 mM boric acid for one hour, and the other half remained untreated. β -galactosidase activities were measured as explained in Materials and Methods. (C) Effect of boric acid treatment on Gcn4 protein levels determined by western blotting. Chromosomally tagged *GCN4*-MYC cells were grown to logarithmic phase, treated with indicated amounts of boric acid and harvested (900 μ l, 0.5 OD₆₀₀). Primary (anti-c-myc) and secondary antibodies (anti-rabbit IgG) were used in a dilution of 1:3000 and 1:10000 respectively. The blot was stripped and reprobbed yeast phosphoglycerate kinase monoclonal antibody as an internal control (1:1500 dilutions) and its 1:10000 diluted secondary antibody was goat anti mouse IgG conjugated with horseradish peroxidase.
doi:10.1371/journal.pone.0027772.g002

To determine whether deletion of *GCN4* or *YAP1* renders cells boron sensitive, both mutants were inoculated to boron containing media and cell growth was followed. As seen in Fig. 3A, deletion of both of these genes sensitized cells to boron, suggesting that both Yap1 and Gcn4 might play roles in boron stress response.

It was previously shown that Gcn4 overexpression has toxic effects on yeast cell growth [28,29]. We next overexpressed *GCN4* in wild type cells from a high copy plasmid, to test whether or not its expression activates transcription of *ATR1*, and in this manner provides boron resistance or not. As seen in Fig. 3B, *GCN4* overexpression in wild type cells neither increased the transcript level of *ATR1* nor led to boron resistance (Fig. 3C), although the overall *GCN4* level was notably elevated in wild type cells overexpressing *GCN4* (Fig. 3D).

Even though deletion of *YAP1* did not inhibit *ATR1* expression in response to boron stress, overexpression of *YAP1* from a plasmid stimulated the expression of *ATR1* and provided increased boron resistance (Fig. 3B and 3C). Overexpression of *GCN4* in *Δgcn4* mutants showed effects similar to those in wild type cells (Fig. S1).

It was previously shown that Yap1 and Gcn4 bind to the same region of the *ATR1* promoter and regulate transcription in response to DNA-damaging agent 4-NQO [26]. To test whether Gcn4 is required for Yap1-dependent expression of *ATR1*, we overexpressed *YAP1* in *Δgcn4* cells and observed a reduction in the level of *ATR1* transcript compared to that in wild type cells, suggesting that Yap1 requires Gcn4 to induce *ATR1* (Fig. 3E).

Overall, our gene expression analyses suggested that Gcn4 is required for *ATR1* induction and that it serves as a physiologically relevant transcription factor in the response to boron treatment. However, Yap1 could also induce *ATR1* and offer boron resistance when expressed ectopically.

Boron Inhibits Protein Synthesis

The activation of GAAC in response to boron stress prompted us to investigate the status of translation because Gcn2 kinase has a role in inhibiting protein synthesis upon activation [21]. Wild type cells were treated with 20 mM boric acid and labeled with radioactively labeled methionine for one hour. As shown in Fig. 4, boron-treated cells contained approximately 30% less ³⁵S-labeled proteins than untreated cells (Fig. 4A, left panel) suggesting that boron inhibits protein synthesis. Translational status of boron-treated cells was further analyzed by the distribution of polysomes. Polysomes are clusters of ribosomes bound to an mRNA molecule that actively translates the message. They can be fractionated and quantified using sucrose density gradients. Cells that are grown in the absence of boron stress exhibit normal polysome profiles including peaks of 40S and 60S ribosomal subunits, 80S monosomes, and polysomes. After treatment with boric acid for one hour, we observed a dramatic shift from polysomal to monosomal states, suggesting that translation initiation collapses in the presence of boron (Fig. 4B). Inhibition of translation in response to certain stress conditions has been reported previously. For example, a recent study has shown that drugs generating oxidative stress as well as heavy metals stall initiation of translation in yeast [30].

Boron Stress Induces Gcn2-dependent Phosphorylation of eIF2 α

The protein kinase Gcn2 can be activated by a variety of conditions and it is the only known kinase in yeast that can phosphorylate eIF2 α , which is responsible for carrying methionyl initiator tRNA (Met-tRNA^{Met}) to the 40S ribosomal subunit during the initiation stage of translation. Phosphorylation of eIF2 α at serine 51 prevents GTP exchange by eIF2B and precludes the

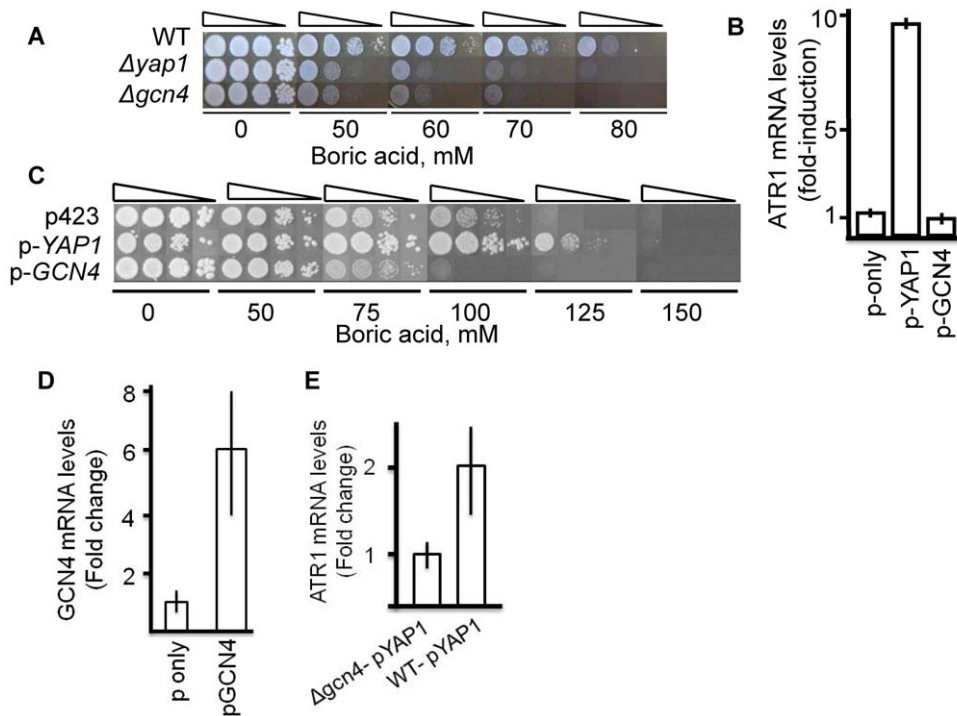


Figure 3. Effects of *GCN4* and *YAP1* overexpression on *ATR1* mRNA levels and boron tolerance. (A) Overnight cultures of wild type and isogenic $\Delta yap1$, $\Delta gcn4$ yeast cells were first diluted to an OD_{600} of 0.2, 0.02, 0.002 and 0.0002, then 5 μ l of each cell suspension were spotted onto YPD plates which contained different amounts of boric acid, as indicated. The cells were grown for 3 days at 30°C and photographed. (B) *ATR1* mRNA expression levels of wild type cells containing empty pAG423GPD vector, pAG423GPD-YAP1 and pAG423GPD-GCN4 vectors, respectively. Actin was used as an internal control. These experiments were performed three times. (C) Boric acid tolerance level of the same cells. The transformants were grown to logarithmic phase in YNB-HIS media, serially diluted to OD_{600} of 0.2, 0.02, 0.002, and 0.0002 and 5 μ l of cell suspension were spotted onto plates containing the indicated amounts of boric acid. The plates were incubated at 30°C for 5 days and photographed. (D) *GCN4* mRNA expression levels in wild type cells overexpressing pAG423GPD and pAG423GPD-GCN4 vectors. Actin was used as an internal control. (E) *ATR1* mRNA levels in wild type and $\Delta gcn4$ mutant cells containing pAG423-YAP1 vector. Actin was used as an internal control. doi:10.1371/journal.pone.0027772.g003

formation of a ternary complex for translation initiation, thus blocking protein synthesis. To determine whether the inhibition of protein synthesis in response to boron stress is dependent on the phosphorylation of eIF2 α by Gcn2 kinase, wild type and $\Delta gcn2$ cells were treated with boron and the samples were subjected to western blotting analyses using an antibody which specifically recognizes the phosphorylated form of Ser51 in eIF2 α . As seen in Fig. 5A, eIF2 α is phosphorylated in wild type cells upon boron treatment, but not in $\Delta gcn2$ mutants. Thus, boron leads to phosphorylation of eIF2 α in a Gcn2-dependent manner.

Apart from its kinase domain, Gcn2 contains a region similar to histidyl-tRNA synthetase (HisRS) which regulates Gcn2 kinase activity by monitoring uncharged tRNA levels in cells [25,31]. Gcn2 activity can also be regulated by dephosphorylation of Ser577 [21]. To investigate whether Gcn2 activation is the result of uncharged tRNA binding or dephosphorylation of Ser577 in response to boron stress, we transformed $\Delta gcn2$ mutants with plasmids containing intact and mutated forms of the *GCN2* gene (*GCN2* without m2 domain [32], *GCN2* with S577A substitution [33], or a combination of both). The ability of transformants to phosphorylate eIF2 α was investigated in boron-treated cells. As seen in Fig. 5B, eIF2 α phosphorylation was unaffected by S577A substitution. However, mutation in the HisRS domain of Gcn2 (*GCN2*-m2), which abolishes uncharged tRNA binding to Gcn2, prevents eIF2 α phosphorylation. Thus, the HisRS-like region is responsible for induction of Gcn2 kinase activity and this finding confirms that boron induces the uncharged tRNA signaling pathway.

Boron inhibits aminoacylation of tRNAs

To determine whether boron inhibits the aminoacylation of tRNAs, we aminoacylated total calf liver tRNA with either 3 H-serine or 3 H-phenylalanine in the presence of various concentrations of neutralized boric acid. We found that boric acid inhibits both tRNA^{Ser} and tRNA^{Phe} formation if there is more than 50 mM boric acid in the reaction (Fig. S2). Thus, boron interferes with aminoacylation steps of the tRNAs and causes uncharged tRNA stress.

Discussion

The essentiality of boron for plant growth is predominantly due to its presence in cell walls as a structural component of the rhamnogalacturonan II complex [4]. There has been no convincing evidence that boron toxicity is exerted through disruption of any process for which boron is required, notably via its role in cell walls. We recently suggested a potential link between translational control and boron toxicity in yeast based on the observation that mutants which show boron resistance were all lacking genes that play a role in uridine base modifications at the wobble position of tRNAs during translation [19]. Here, we found that the transcriptional activation of *ATR1* is mediated by the transcription factor Gcn4 in response to boron treatment. Further analyses have shown that boron activates the Gcn2 kinase, and leads to phosphorylation of eIF2 α and inhibition of protein synthesis. The uncharged tRNA binding site (HisRS domain) of the Gcn2 kinase is required for Gcn2 to phosphorylate eIF2 α . The

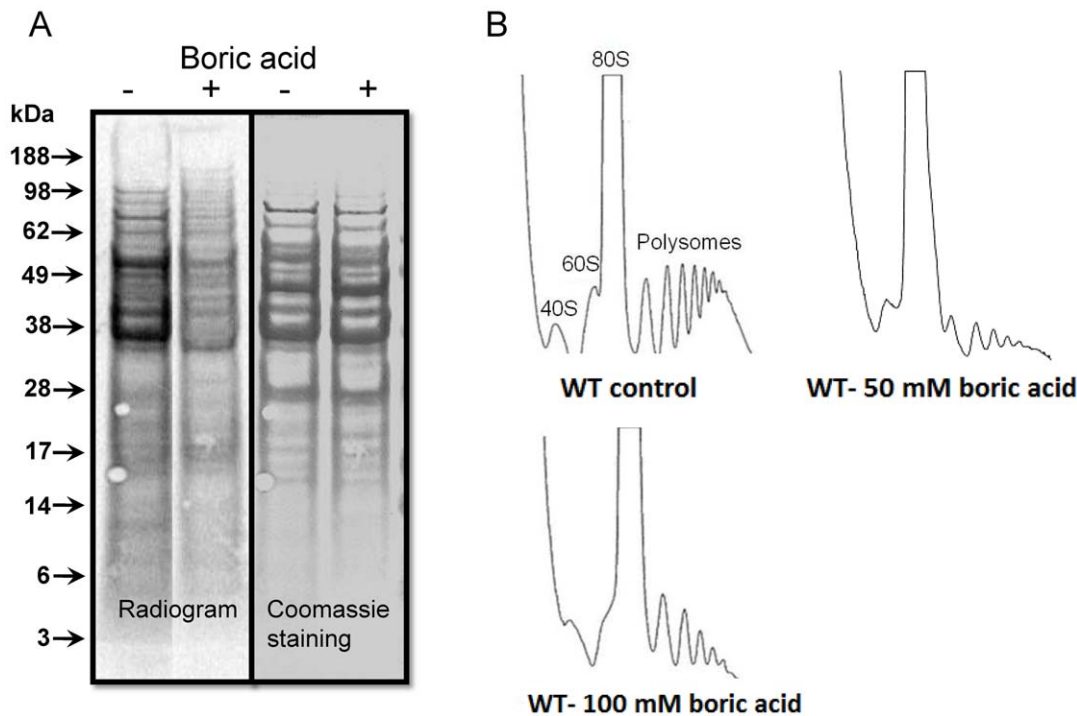


Figure 4. Protein synthesis is inhibited by boron. (A) Radiogram (left panel) showing incorporation of radioactive methionine into newly synthesized proteins. Coomassie staining of the same gel is shown in the right panel as a loading control. (B) Boron treatment decreases the abundance of polysomes. Wild type cells were grown to logarithmic phase and then treated with 50 and 100 mM of boric acid or left untreated. The cell lysates were analyzed by sucrose density gradient centrifugation. The absorbance profiles at 254 nm show 40S, 60S, 80S and polysome fractions, as indicated.

doi:10.1371/journal.pone.0027772.g004

requirement for HisRS-like domain (uncharged tRNA binding region) to phosphorylate eIF2 α suggests that the Gcn2 kinase activity is stimulated by uncharged tRNAs. Eventhough, boron impairs the acylation of tRNAs, the mechanism by which boric acid interferes with tRNA acylation should be further investigated. Boric acid is a relatively unreactive compound, however, it can form ester bonds with several metabolites with multiple hydroxyl groups in the cis-configuration [6,34]. Among such metabolites, one of the most important moieties is the ribose sugar found in ribonucleotides. It is likely that boric acid can bind to RNA species and interfere with the acylation reaction of tRNAs. In opposition to this, a recent study has shown that oxidative stress leads to degradation of tRNAs, mediated by Rny1 nuclease and induction of the Gcn2 kinase [35,36]. We argue against this possibility since yeast cells lacking the *RNY1* gene still show a wild type level of eIF2 α phosphorylation in response to boron treatment (data not shown).

It is also unclear how boron induces the transcription of Gcn4. Many stress and signaling pathways converge on the Gcn4 transcription factor and there are more than a dozen transcription factor binding sites in the *GCN4* promoter region [37]. Thus, further studies are required to identify upstream players in the boron stress response pathway.

Our results suggest a role for GAAC in boron stress response. It is known that several other stress conditions also activate GAAC. Among these stressors, transcriptional response to hydrogen peroxide [30], 3-AT and rapamycin [38] have also been analyzed in Δ *gcn4* cells and their datasets are publicly available. In order to understand whether the cellular response to boron stress is mediated via a unique or a common mechanism, we compared our microarray data to those of hydrogen peroxide, 3-AT and

rapamycin treated cells. We also compared expression profiles of Δ *gcn4* mutants exposed to boron and other stress conditions to determine how Gcn4-dependent boron regulon differs from other regulons. We observed that the transcriptional response of wild type cells to boron and other stress conditions is quite different (Fig. S3). The major gene clusters, up- or down-regulated in response to boron treatment, did not show similarity to the patterns observed for 3-AT, rapamycin or hydrogen peroxide treatments. However, it is clear that the expression profiles of 3-AT and rapamycin treated cells share some common patterns. The expression profiles of Δ *gcn4* mutants exposed to boron and other stressors are also different (Fig. S3). This data clearly shows that the Gcn4-dependent boron regulon is different from those of other stress conditions and that boron stress has specific effects on the transcriptome.

Recently, it was shown that amino acid starvation in response to 3-AT treatment leads to repression of genes encoding ribosomal proteins via the transcription factor Gcn4 [39]. Our results showed that boron treatment does not change the expression pattern of most of the ribosomal protein genes, and that Gcn4 has no roles in regulating either *RPL* or *RPS* genes in response to boron stress (Table S1).

Overall, our results suggest that the general amino acid control pathway contributes to boron toxicity and transcriptional regulation of *ATR1* in response to boron stress. In this respect, we propose a model, wherein Gcn2 is activated by boron in an uncharged tRNA binding domain-dependent manner, activates Gcn4 and inhibits protein synthesis. Upon activation, Gcn4 induces the transcription of its boron specific target genes, *ATR1*, and many genes under the control of GAAC. Since the Gcn4-specific boron regulon shows differences with the activation of

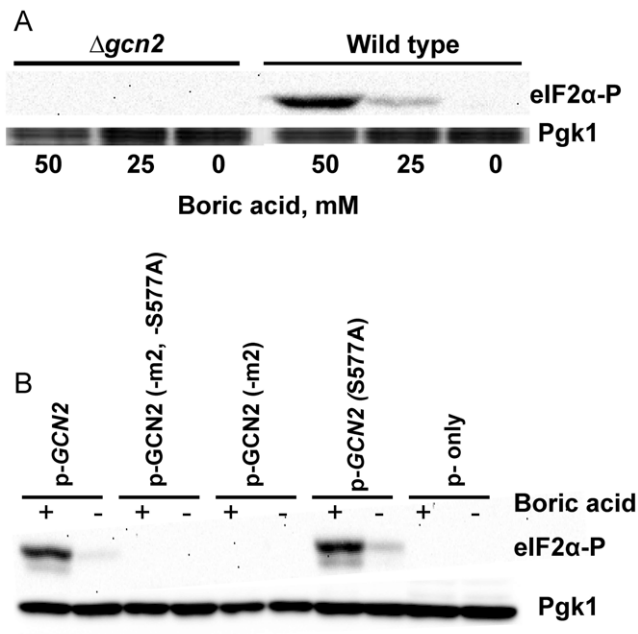


Figure 5. eIF2 α is phosphorylated by boron treatment. (A) Wild type and Δ *gcn2* cells were grown to logarithmic phase in YPD medium; the cultures were split and treated with 25 mM or 50 mM boric acid for one hour or left untreated. The levels of eIF2 α phosphorylation at Ser51 were measured by western blot analysis using an antibody against phospho Ser51 on eIF2 α . The membrane was stripped and reprobed with antibodies against yeast phosphoglycerate kinase (Pgk) antibody as an internal control. (B) Effect of boric acid on eIF2 α phosphorylation depends on uncharged tRNA binding to an intact HisRS-like domain in Gcn2. The Δ *gcn2* transformants carrying plasmids for an empty vector (p-only), GCN2 with S577A substitution (p-GCN2-S577A), GCN2 without HisRS domain (p-GCN2-m2), GCN2 without HisRS domain and with S577A substitution (p-GCN2-S577A-m2), and intact GCN2 (p-GCN2) were grown to logarithmic phase in YNB-URA medium and split in half; one of which was treated with 50 mM boric acid for one hour while the other was left untreated. Western blot analyses were performed with cell extracts using an antibody against eIF2 α phosphorylated on Ser51. The membrane was stripped and reprobed with antibodies against yeast phosphoglycerate kinase (Pgk) as an internal control. doi:10.1371/journal.pone.0027772.g005

Gcn4 by other stress conditions, we speculate that there are other transcription factors that are involved in boron stress specific gene induction.

Materials and Methods

Yeast Growth, Media and Transformation

WT strain BY4741 (*MATa* his3 leu2 met15 ura3) and its isogenic deletion mutants were obtained from the yeast deletion library (Invitrogen). Cells were grown in either YEPD (2% glucose, 1% yeast extract, 2% peptone and 2% agar for solid media) or YNB (yeast nitrogen base) media supplemented with required amino acids and bases. Yeast transformations were performed using the LiAc method [40].

DNA Microarray Analysis

BY4741 wild type, Δ *yap1*, Δ *gcn4* mutant cells were grown to an optical density of 0.2–0.3 at 600 nm in 200 ml YPD medium, with or without 20 mM boric acid treatment for 1 hour. Cells were then harvested by centrifugation. Total RNA was isolated using the Ambion RiboPureTM-Yeast Kit. The Agilent Two-Color Low RNA Input Linear Amplification Kit was used to generate

fluorescently labeled cRNA for two-color microarray hybridizations. Fluorescently labeled cRNA molecules were purified from the reaction mixture using the QiaGen RNeasy mini kit. The resulting cRNA samples (825 ng each) were mixed with Agilent Hi-RPM hybridization buffer. Microarray hybridizations were performed using Agilent SureHyb hybridization chambers. The hybridization chambers were loaded onto a rotisserie in an Agilent Hybridization oven and were incubated at 65°C for 17 hours with a rotational speed of 10 rpm. Following incubation, the microarray slides were washed for 1 min each in Gene Expression wash buffer 1 (6 \times SSPE, 0.005% N-lauroylsarcosine, at room temperature) and Gene Expression Wash Buffer 2 (0.06 \times SSPE, 0.005% N-lauroylsarcosine, at 31°C) for 1 min each. The microarray slides were briefly dipped in a solution of acetonitrile, dried and scanned in an Agilent Technologies G2505B microarray scanner at 5 μ m resolution. Microarray data is MIAME compliant; the raw data has been deposited in the GEO database (Accession number GSE29887).

Western Blot Analyses

Wild type, and the isogenic *Agcn2* and *GCN4-MYC* strains were grown to mid-log phase in either YPD or selective YNB-Ura media, with or without boron treatment and equal numbers of cells were harvested by centrifugation for each strain. Cell extracts were resuspended in sample buffer (0.06 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.0025% (w/v) bromophenol blue) and heated at 95°C for 5 min. Samples were separated in 5–15% SDS-PAGE and electrotransferred to a PVDF (Sigma) membrane. Primary antibodies against phospho (Ser51) specific eIF2 α were acquired from USBiological and anti-c-myc antibody used for detection of *GCN4-MYC* was from Sigma. The blots were stripped and reprobed with monoclonal antibodies specific for yeast phosphoglycerate kinase (Invitrogen) as an internal control. As secondary antibodies, anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) or goat anti mouse IgG conjugated to horseradish peroxidase (Sigma) were used. The membranes were treated with enhanced chemiluminescence system (Bio-Rad) and visualized using the VersaDoc Imaging System (Bio-Rad).

Metabolic Labeling

BY4741 yeast cells were grown in 200 ml of YNB medium to OD₆₀₀ = 0.5, split to 100 ml aliquots, and one aliquot was treated with 20 mM of boric acid. After 1 hour of incubation, both aliquots were metabolically labeled with 0.5 mCi of EX-PRE35S35S Protein Labeling Mix (Perkin-Elmer) for 30 min. Cells were harvested by centrifugation, washed three times with PBS buffer and disrupted with glass beads for 10 min in PBS buffer. The resulting cellular lysates were normalized according to protein concentration and 10 μ g of each lysate was subjected to SDS-PAGE. Resolved proteins were transferred onto PVDF membrane. ³⁵S-labeled proteins were visualized with a Storm PhosphorImager system (Molecular Dynamics). ImageQuant package was used to quantify the autoradiogram data.

Polysome Profiling

Density gradient centrifugation system was used in order to separate total RNA based on the molecular weight. In brief, 5% and 70% sucrose solutions [RiboLockTM RNase inhibitor (Fermentas), 100 mM NaCl, 10 mM MgCl₂, 20 mM HEPES] were prepared and thereafter sucrose gradients were prepared by using ISCO systems. BY4741 cells were grown to an optical density of 0.7–0.9 at 600 nm in 30 ml YPD medium. These cells were either exposed to 50 mM or 100 mM boric acid for 1 hour

or not exposed. They were harvested by centrifugation, washed twice with distilled water, and frozen at -80°C . Then, yeast cells were homogenized in 5 ml lysis buffer [100 mM NaCl, 10 mM MgCl_2 , 20 mM HEPES, 40 $\mu\text{l}/\mu\text{l}$ RiboLockTM RNase inhibitor (Fermentas), 1% Triton-X 100, 1% sodium deoxycolate (NADOC), 10 mg/ml cycloheximide (Sigma)] by vortexing with glass beads for 10 min. After ten minutes of incubation on ice, homogenates were centrifuged for 10 min at 12,000 g and 4°C . The supernatants were transferred to 5%–70% (w/v) sucrose gradients and sedimented for 2.5 h, at 27,000 rpm, 4°C , in an SW28 rotor using a Beckman Coulter OptimaTM L-XP Ultracentrifuge System. Forty fractions were collected from the top of the gradient by using an ISCO Tris pump while monitoring absorbance at 254 nm with an ISCO UA-6 monitor. The RNP populations were grouped according to their sedimentation rates: mRNP, 40S, monosomes and polyribosome fractions.

Boron Tolerance Assays

YPD (2% glucose, 2% peptone, 1% yeast extract and 2% agar) or YNB (2% glucose, 0.67% yeast nitrogen base without amino acids and 2% agar) agar plates with different concentrations of boric acid were prepared. Overnight cultures were diluted to an optical density of 0.2 at 600 nm and then serially diluted to 0.02, 0.002, and 0.002. A 5 μl aliquot of each dilution was spotted on plates and incubated for 3 days at 30°C .

RNA Isolation and Real Time PCR Analyses

Total RNA was isolated from boron-treated and untreated samples using an RNA isolation kit (Invitrogen). Genomic DNA traces were removed by DNase treatment (Fermentas). Complementary DNA (cDNA) from total mRNA was obtained using the First Strand cDNA Synthesis Kit (Fermentas). The cDNA was used as a template for amplifying a 186 bp fragment for the *ATRI* gene, a 155 bp fragment for the *GCN4* gene and a 103 bp fragment for the internal control *ACT1* gene. Triplicate real-time PCR analyses were performed with the IQ5 real-time PCR system (Bio-Rad) using Fermentas MaximaTM SYBR Green qPCR Master Mix (2X). The primers used for the amplification of *ATRI* were *ATRI*F (5'-ACGCGTATAGCATAGCCGCTT-TCA-3') and *ATRI*B (5'-TGTAAGCCTGGTTCCAACCC-GATA-3'); the primers used for the amplification of *GCN4* were *GCN4*F (5'-ATCCAATGGGTTTCTCACCA-3') and *GCN4*B (5'-CGAAGGGTATCCTGTTTGA-3'); and those used for the amplification of *ACT1* were *ACT1*F (5'-ACGTTCCAG-CCTTCTACGTTTCCA-3') and *ACT1*B (5'-ACGTGAGTAA-CACCATCACCGGAA-3'). The conditions of PCR amplification for *ATRI* were as follows: 40 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, and for *GCN4*: 40 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s.

Cloning of YAP1 and GCN4 Genes

Wild type alleles of *YAP1* and *GCN4* were amplified using High Fidelity Enzyme (Fermentas) from yeast genomic DNA using the following primers: *YAP1*F (5'GGGGACAAGTTTGTACAAA-AAAAGCAGGCTCTTAAACCATTGAGTGTGTCT-3') and *YAP1*B (5'GGGGACCAACCTTTGTACAAGAAAGCTGGG-TGTTCCCGCTTTAGTTCATAT-3') for *YAP1*, and *GCN4*F (5'GGGGACAAGTTTGTACAAAAAAGCAGGCTAATGTC-CGAATATCAGCCAA-3') and *GCN4*B (5'GGGGACCACT-TTGTACAAGAAAGCTGGGTATGAAATCAGCGTTCCG-CAA-3') for *GCN4*. The amplified fragments were first cloned into the pDONR vector using 2 μl BP ClonaseTM II enzyme (Invitrogen) with an overnight incubation at 25°C and then moved to LR clonase reaction. This reaction was performed

with 2 μl of isolated entry clones, 1.5 μl pAG423GPD expression vector, 3 μl TE buffer and 2 μl LR ClonaseTM II enzyme mixture (Invitrogen). Isolated plasmids were verified by sequence analyses, and then yeast cells were transformed with the empty vector or with a gene expression construct, and selected for histidine prototrophy. Plasmids with *Gcn2* (p4384, flag epitope-tagged form of *GCN2*; p4055, nonphosphorylatable S577-Alanine mutation; p4056, substitutions in the m2 motif of the HisRS-like domain; p4057, with substitutions both in the m2 motif and the S577A motif) were obtained from Alan Hinnebusch (NIH).

β -galactosidase activity assays

Wild type cells were transformed with the p180 plasmid which carries the *GCN4-lacZ* gene [41]. Overnight cultures of wild type cells in 25 ml of YNB-URA were diluted to 0.1 at OD_{600} . After 2 hours incubation, cultures were split into two flasks with or without 50 mM boric acid. After 1 hour growth in the presence of boron, 100 μl of cell solution, at an optical density between 0.2 and 0.6 at 600 nm, was taken and homogenized in 400 μl of Z-buffer [60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 50 mM β -mercaptoethanol and 0.2% N-lauryl sarcosine]. After 30 min preincubation at 30°C , 150 μl of Z-buffer with 4 mg/ml, ONPG (o-nitrophenyl- β -D-galactopyranoside) (FlukaBioChemika) was added to the suspensions and the resulting solutions were incubated at 30°C for one hour. The reaction was stopped by adding 400 μl 1.5 M Na_2CO_3 . After centrifugation for 1 min, supernatants were taken and their absorbance assayed at 420 nm [42]. The obtained absorbance values were normalized according to the OD values of cells.

Aminoacylation of tRNAs in boric acid

Total calf liver tRNA (Sigma) was aminoacylated under limiting tRNA conditions with either ^3H -serine (Moravak Biochemicals, specific activity 20 Ci/mmol) or ^3H -phenylalanine (GE Healthcare, specific activity 123 Ci/mmol) and 19 unlabelled amino acids in the presence of rabbit reticulocyte synthetases as described [43] and various concentrations of boric acid (Sigma).

Supporting Information

Figure S1 Growth rates of cells transformed with *GCN4* or *YAP1* plasmids.

(TIFF)

Figure S2 Inhibition of aminoacylation of tRNAs by boric acid.

(TIF)

Figure S3 *Gcn4* regulates different sets of genes in response to various stress conditions.

Comparison of genomic expression profiles in response to boric acid, rapamycin, 3-AT and H_2O_2 in wild type and *gcn4 Δ* cells using publicly available datasets [38].

(TIF)

Table S1 Gene expression profiles of WT, *gcn4-* and *yap1-* cells in response to 20 mM boric (1 h treatment).

(XLS)

Table S2 Genes whose expression changed more than 2-fold in response to boron stress in wild type cells.

(XLS)

Table S3 Genes whose expression changed more than 2-fold in *gcn4 Δ* cells in response to boron.

(XLS)

Acknowledgments

We thank Alan Hinnebusch (NIH) for providing GCN2 plasmids, Bunyamin Akgul and Ozge Tuncel (Izmir Institute of Technology, Turkey) for their help with polysome analyses and Biotechnology Center (Izmir Institute of Technology) for their help in instruments.

References

- Nielsen FH (1997) Boron in human and animal nutrition. *Plant and Soil* 193: 199–208.
- Tanaka M, Fujiwara T (2008) Physiological roles and transport mechanisms of boron: perspectives from plants. *Plügers Archiv-European Journal of Physiology* 456: 671–677.
- Warington K (1923) *The Effect of Boric Acid and Borax on the Broad Bean and certain other Plants*. Oxford University Press.
- Kobayashi M, Matoh T, Azuma J (1996) Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiology* 110: 1017–1020.
- Goldbach HE, Wimmer MA (2007) Boron in plants and animals: Is there a role beyond cell-wall structure? *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 170: 39–48.
- Bolanos L, Lukaszewski K, Bonilla I, Blevins D (2004) Why boron? *Plant Physiology and Biochemistry* 42: 907–912.
- Gonzalez-Fontes A, Rexach J, Navarro-Gochicoa MT, Herrera-Rodriguez MB, Beato VM, et al. (2008) Is boron involved solely in structural roles in vascular plants? *Plant Signal Behav* 3: 24–26.
- Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelzer I, et al. (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* 415: 545–549.
- Miller ST, Xavier KB, Campagna SR, Taga ME, Semmelhack MF, et al. (2004) Salmonella typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Molecular Cell* 15: 677–687.
- Eckhart CD (1998) Boron stimulates embryonic trout Growth. *Journal of Nutrition* 128: 2488–2493.
- Rowe RI, Eckhart CD (1998) Boron is essential for zebrafish embryogenesis. *Faseb Journal* 12: 1193.
- Cui Y, Winton MI, Zhang ZF, Rainey C, Marshall J, et al. (2004) Dietary boron intake and prostate cancer risk. *Oncology Reports* 11: 887–892.
- Mahabir S, Spitz MR, Barrera SL, Dong YQ, Eastham C, et al. (2008) Dietary boron and hormone replacement therapy as risk factors for lung cancer in women. *American Journal of Epidemiology* 167: 1070–1080.
- Kaya A, Karakaya HC, Fomenko DE, Gladyshev VN, Koc A (2009) Identification of a Novel System for Boron Transport: Atr1 Is a Main Boron Exporter in Yeast. *Molecular and Cellular Biology* 29: 3665–3674.
- Nozawa A, Takano J, Kobayashi M, von Wiren N, Fujiwara T (2006) Roles of BOR1, DUR3, and FPS1 in boron transport and tolerance in *Saccharomyces cerevisiae*. *Fems Microbiology Letters* 262: 216–222.
- Sutton T, Baumann U, Hayes J, Collins NC, Shi BJ, et al. (2007) Boron-toxicity tolerance in barley arising from efflux transporter amplification. *Science* 318: 1446–1449.
- Takano J, Miwa K, Yuan LX, von Wiren N, Fujiwara T (2005) Endocytosis and degradation of BOR1, a boron transporter of *Arabidopsis thaliana*, regulated by boron availability. *Proceedings of the National Academy of Sciences of the United States of America* 102: 12276–12281.
- Takano J, Wada M, Ludewig U, Schaaf G, von Wiren N, et al. (2006) The *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *Plant Cell* 18: 1498–1509.
- Ulusik I, Kaya A, Unlu ES, Avsar K, Karakaya HC, et al. (2011) Genome-wide identification of genes that play a role in boron stress response in yeast. *Genomics* 97: 106–111.
- Hinnebusch AG (1997) Translational regulation of yeast GCN4 - A window on factors that control initiator-tRNA binding to the ribosome. *Journal of Biological Chemistry* 272: 21661–21664.
- Hinnebusch AG (2005) Translational regulation of GCN4 and the general amino acid control of yeast. *Annual Review of Microbiology* 59: 407–450.
- Hinnebusch AG, Natarajan K (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryotic Cell* 1: 22–32.
- Dever TE (2002) Gene-specific regulation by general translation factors. *Cell* 108: 545–556.
- Vattem KM, Wek RC (2004) Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* 101: 11269–11274.

Author Contributions

Conceived and designed the experiments: A.Koc DF HK. Performed the experiments: IU A.Kaya DF BC A.Koc. Analyzed the data: A.Koc DF VG. Wrote the paper: A.Koc IU DF VG HK.

- Wek RC, Jackson BM, Hinnebusch AG (1989) Juxtaposition of domains homologous to protein-kinases and histidyl-transfer RNA-synthetases in Gcn2 protein suggests a mechanism for coupling GCN4 expression to amino-acid availability. *Proceedings of the National Academy of Sciences of the United States of America* 86: 4579–4583.
- Coleman ST, Tseng E, MoyeRowley WS (1997) *Saccharomyces cerevisiae* basic region-leucine zipper protein regulatory networks converge at the ATR1 structural gene. *Journal of Biological Chemistry* 272: 23224–23230.
- Lee J, Godon C, Lagniel G, Spector D, Garin J, et al. (1999) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *Journal of Biological Chemistry* 274: 16040–16046.
- Herzog B, Streckfuss-Bomeke K, Braus GH (2011) A Feedback Circuit between Transcriptional Activation and Self-Destruction of Gcn4 Separates Its Metabolic and Morphogenic Response in Diploid Yeasts. *Journal of Molecular Biology* 405: 909–925.
- Tavernarakis N, Thireos G (1995) Transcriptional interference caused by GCN4 overexpression reveals multiple interactions mediating transcriptional activation. *Molecular & General Genetics* 247: 571–578.
- Mascarenhas C, Edwards-Ingram LC, Zeef L, Shenton D, Ashe MP, et al. (2008) Gcn4 is required for the response to peroxide stress in the yeast *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* 19: 2995–3007.
- Dong JS, Qiu HF, Garcia-Barrio M, Anderson J, Hinnebusch AG (2000) Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-Binding domain. *Molecular Cell* 6: 269–279.
- Garcia-Barrio M, Dong JS, Cherkasova VA, Zhang XL, Zhang F, et al. (2002) Serine 577 is phosphorylated and negatively affects the tRNA binding and eIF2 alpha kinase activities of GCN2. *Journal of Biological Chemistry* 277: 30675–30683.
- Cherkasova VA, Hinnebusch AG (2003) Translational control by TOR and TAP42 through dephosphorylation of eIF2 alpha kinase GCN2. *Genes & Development* 17: 859–872.
- Reid RJ, Hayes JE, Post A, Stangoulis JCR, Graham RD (2004) A critical analysis of the causes of boron toxicity in plants. *Plant Cell and Environment* 27: 1405–1414.
- Thompson DM, Lu C, Green PJ, Parker R (2008) tRNA cleavage is a conserved response to oxidative stress in eukaryotes. *Rna-a Publication of the Rna Society* 14: 2095–2103.
- Yamasaki S, Ivanov P, Hu GF, Anderson P (2009) Angiogenin cleaves tRNA and promotes stress-induced translational repression. *Journal of Cell Biology* 185: 35–42.
- Monteiro PT, Mendes ND, Teixeira MC, d'Orey S, Tenreiro S, et al. (2008) YEASTRACT-DISCOVERER: new tools to improve the analysis of transcriptional regulatory associations in *Saccharomyces cerevisiae*. *Nucleic Acids Research* 36: D132–D136.
- Staschke KA, Dey S, Zaborse JM, Palam LR, McClintick JN, et al. (2010) Integration of General Amino Acid Control and Target of Rapamycin (TOR) Regulatory Pathways in Nitrogen Assimilation in Yeast. *Journal of Biological Chemistry* 285: 16893–16911.
- Joo YJ, Kim JH, Kang UB, Yu MH, Kim J (2011) Gcn4p-mediated transcriptional repression of ribosomal protein genes under amino-acid starvation. *Embo Journal* 30: 859–872.
- Gietz RD, Schiestl RH (1995) Transforming yeast with DNA. *Methods in Molecular and Cellular Biology* 5: 255–269.
- Hinnebusch AG (1985) A hierarchy of trans-acting factors modulates translation of an activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* 5: 2349–2360.
- Kippert F (1995) A rapid permeabilization procedure for accurate quantitative-determination of beta-galactosidase activity in yeast-cells. *Fems Microbiology Letters* 128: 201–206.
- Hatfield D, Matthews CR, Rice M (1979) Aminoacyl transfer RNA populations in mammalian-cells chromatographic profiles and patterns of codon recognition. *Biochimica Et Biophysica Acta* 564: 414–423.