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Anti-diabetic and anti-obesity agent sodium tungstate enhances GCN pathway activation through Glc7p inhibition

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ABSTRACT

Tungstate counteracts diabetes and obesity in animal models, but its molecular mechanisms remain elusive. Our *Saccharomyces cerevisiae*-based approach has found that tungstate alleviated the growth defect induced by nutrient stress and enhanced the activation of the GCN pathway. Tungstate relieved the sensitivity to starvation of a gcn2-507 yeast hypomorphic mutant, indicating that tungstate modulated the GCN pathway downstream of Gcn2p. Interestingly, tungstate inhibited Glc7p and PP1 phosphatase activity, both negative regulators of the GCN pathway in yeast and humans, respectively. Accordingly, overexpression of a dominant-negative Glc7p mutant in yeast mimicked tungstate effects. Therefore tungstate alleviates nutrient stress in yeast by in vivo inhibition of Glc7p. These data uncover a potential role for tungstate in the treatment of PP1 and GCN related diseases.

1. Introduction

The phosphorylation of the eukaryotic initiation factor 2-α subunit (eIF2α) through the General Control of Nutrients (GCN) pathway occurs in response to amino acid deprivation in yeast and mammals [1]. In yeast, Gcn2p protein kinase is the responsible of phosphorylating eIF2α at serine 51 [2]. This phosphorylation diminishes general protein synthesis and enhances the specific transcription of genes involved in amino acid biosynthesis. Translational regulation of *GCN4* is mediated by four short open reading frames located in its 5′-untranslated region [2]. This peculiarity allows the GCN4 mRNA being translated in deprivation conditions and thus stimulating transcription of genes entitled to overcome the starvation state, like *HIS4* [3].

FK506 is an immunosuppressant used to prevent allograft rejection after organ transplantation. Through calcineurin inhibition it impedes transcription of genes involved in immune response [4]. FK506 impairs *Saccharomyces cerevisiae* cell growth by inhibiting tryptophan, histidine and leucine import in strains auxotrophic for these amino acids [5,6]. Previous evidences indicate that tryptophan starvation is mainly responsible for this growth defect [7]. Other inductor of amino acid deprivation in yeast is 3-amino-1,2,4-triazole (3AT), an inhibitor of histidine biosynthesis [8] that causes scarcity of this amino acid.

Stimuli other than amino acid have been reported to trigger the GCN pathway, such as purine starvation [9], glucose limitation, ethanol [10], DNA damage [3], high salinity [11,12], rapamycin [13–15] and volatile anesthetics [16]. In mammals, the GCN pathway is triggered by UV irradiation, proteasome inhibition, misfolded protein in the ER, double-stranded RNA, haem deprivation, as well as oxidative and heat stresses in erythroid tissues (reviewed in [17]). Many lines of evidence linking the GCN pathway and human disease have been described. Mutations in the Gcn2p mammalian orthologue, PERK, causes the Wolcott–Rallison syndrome, a rare childhood diabetic disorder [18–20]. Accordingly PERK-deficient mice develop hyperglycemia and β-cell death [21,22]. Furthermore, mutant mice with non-phosphorylatable eIF2α (Ser51Ala) mutant die because of hypoglycemia [22]. Recently, the relevance of eIF2α for insulin production in adaptation to a high-fat diet has been underscored [23]. Other disease conditions, like tumor progression [24], connective tissue diseases [25], drug resistance [26], aging and sleep disorders [27] have been recently related to translational control.

The status of eIF2α phosphorylation is negatively regulated by phosphatases. In yeast, the main eIF2α phosphatase is Glc7p [28], an orthologue of the catalytic subunit of the mammalian

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PP1 [29], although Sit4p phosphatase is partially redundant with Glc7p. This phosphatase is required for lots of cellular functions like cell cycle progression [30], glycogen synthesis [31], glucose repression [32], ionic homeostasis [33] or DNA replication [34]. Accordingly, mammalian PP1 plays also a pivotal role in cell physiology, as is reflected by its large variety of substrates and functions [35].

Tungstate (WO_4^{2-}) is a phosphatase inhibitor [36–39] with anti-diabetic properties. This substance exhibits an excellent therapeutic profile, both in long- and short-term treatments [40,41]. When administered orally, sodium tungstate normalizes glycemia in many animal models of type 1 and 2 diabetes [41–44]. It also increases the total amount and translocation of GLUT4 transporter in muscle [45] and restores the hepatic metabolism of glucose in streptozotocin-induced diabetic rats [43,44]. In streptozotocin-treated neonatal rats tungstate administration stimulates insulin secretion [42,46] and regenerates β-cell population [42]. This compound also reduces significantly weight gain and adiposity by increasing energy dissipation and the fatty acid oxidation rate in an obese rats model system [47]. Unfortunately, the molecular mechanisms of tungstate action are not yet defined.

The budding yeast *S. cerevisiae* is being widely used as a model for investigating fundamental processes relevant to all living organisms. The genetic tractability of budding yeast, its ease of manipulation and the wealth of functional genomics tools available in this organism makes it an attractive model for investigating drug mechanisms of action. By conducting a phenotypic screening we found that tungstate relieved nutrient stress in *S. cerevisiae*. This effect seemed to be mediated by the inhibition of the eIF2α phosphatase Glc7p. This inhibition also occurred *in vitro* in the Glc7p mammalian orthologue, PP1. We propose that tungstate dependent regulation of the GCN pathway might be relevant for the anti-diabetic properties of tungstate thus uncovering potential new applications for this compound in chemotherapy.

2. Materials and methods

2.1. Yeast strains and plasmids

Yeast strains are described in Table 1. Yeast cells were transformed by standard procedures [48]. The plasmid p180, expressing a *GCN4-lacZ* fusion including the entire *GCN4* 5′-non-coding region with four upstream open reading frames inserted into YCp50, a low copy-number plasmid marked with *URA3*, has been described previously [10]. The *HIS4-lacZ* p377 reporter plasmid containing the *HIS4* promoter, upregulated by Gcn4p, and marked with *LEU2* has also been reported [49]. The plasmid p27-1, harboring a truncated version of *GLC7* (*glc7Δ209-312*) into the high-copy-number *URA3* plasmid YEp24 has been described previously [28].

2.2. Yeast growth assays

Standard methods for yeast culture and manipulations were used [50]. Synthetic medium (SD) contained 2% glucose, 0.67% yeast nitrogen base without amino acids (Pronadisa), and the amino acids, purine and pyrimidine bases required by the strains of interest. YPD medium contained 2% glucose, 2% peptone and 1% yeast extract. Sodium tungstate (Merck) was dissolved in water and diluted in YPD at the corresponding doses. FK506 was kindly provided by Astellas Pharma, dissolved in ethanol and diluted in YPD at the corresponding doses.

For analysis of cell growth by drop test, cells growing logarithmically in liquid YPD medium were 10-fold serially diluted, and volumes of around 3 μl were dropped with a stainless steel replicator (Sigma) on solid plates containing 2% Bacto-Agar (Pronadisa) and YPD medium with the corresponding doses of FK506 and/or tungstate as indicated. Growth was recorded after 2–5 days in all cases.

Table 1
Yeast strains.

Name	Genotype	Ref.
H1402	*MATα leu2-3112 ura3-52 ino1 HIS4-lacZ*	[28]
gcn2-507	H1402 *gcn2-507*	[28]
Δgcn2	H1402 *gcn2::LEU2*	[28]
w303.1a	*MATa ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1*	
Δcnb1	w303.1a *cnb1::LEU2*	[53]

2.3. Galactosidase assays

Yeast cells transformed with the appropriate reporter plasmids were grown selectively in SD medium and then diluted in YPD. Exponential cultures were treated with FK506 and/or tungstate as indicated. β-Galactosidase activity was determined at the indicated times as described [51] and represented as β-Galactosidase activity units. Data are the mean ± S.E. from three independent transformants, each measured in triplicate.

2.4. Immunoblotting

Yeast strains were grown in liquid YPD medium to mid-log phase and then treated with the corresponding doses of FK506 and/or tungstate as indicated. After treatment, equal numbers of cells were collected by centrifugation and resuspended in alkaline Laemmli buffer. Samples were boiled for 5 min and soluble extracts was recovered after centrifugation. 20 μg of total cellular protein was subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose (Hybond™, Amersham Biosciences) or PVDF (Immobilon-P; Millipore) filters. Uniform gel loading was confirmed by Ponceau S staining of membranes after transfer. Phosphorylated eIF2α was detected with an antiphospho-eIF2α antibody (Ser^{51}) from New England Biolabs. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham) using a HRP-conjugated goat anti-rabbit IgG (Amersham). The figures show an experiment representative of at least two independent ones with essentially identical results.

2.5. Expression of recombinant proteins in Escherichia coli

E. coli DH5α transformants harboring the GST fusion were grown in 500 ml of LB/ampicillin, supplemented with 0.5 mM $MnCl_2$. Transformants were grown at 37 °C until the absorbance at 600 nm reached a value of about 0.3. Isopropyl-1-thio-β-D-galactopyranoside was then added to a concentration of 0.1 mM, and cultures were grown overnight at 25 °C. Cells were harvested and resuspended in 20 ml of sonication buffer (50 mM Tris–HCl, pH 7.6, 0.2 mM EGTA, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM dithiothreitol, 2 mM $MnCl_2$, 2 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor mixture (Roche Applied Science)). Cells were disrupted by sonication, and the fusion proteins were purified by passing the extracts through a 1-ml bed volume of glutathione-Sepharose columns (BioRad). GST-Glc7was eluted from the column with 10 mM glutathione. Samples were stored at −80 °C.

2.6. Expression of recombinant proteins in HEK293T cells

HEK293T cells were seeded in p150 plates. When the cells reached confluence, they were transfected with 50 μg/plate of pcDNA3-FLAG-PP1. On the first day post-transfection, we replaced the medium by fresh Dulbecco's Modified Eagle's Medium (DMEM,

Lonza) cell culture medium supplemented with 10% fetal bovine serum (FBS). On the second day post-transfection, cell culture medium was removed, and 500 μl of cold lysis buffer (30 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 25 nM okadaic acid, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin) was added per dish. Cells were collected using cell scrapers and incubated in lysis buffer for 20 min in an orbital shaker at 4 °C and low speed. The extract was then centrifuged for 10 min at 5000×*g*. The supernatant was then collected. Next, 200 μl of anti-FLAG agarose resin (Sigma) was added to the extract, and it was incubated overnight at 4 °C using an overhead tumbler. The following day, cell extracts with resin were centrifuged at 1000×*g* and 4 °C for 5 min. The resin was transferred to an Eppendorf tube, in which it was washed once using 500 μl of cold wash buffer (30 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40) and five times more with TBS. The resin was then incubated for 10 min with elution buffer (30 mM Tris–HCl pH 7.4, 150 mM NaCl, 500 μg/ml FLAG peptide), and protein was eluted.

2.7. Protein phosphatase assays

Protein phosphatase activity using *p*-nitrophenylphosphate (pNPP) as substrate was determined essentially as described [52]. The reaction buffer was 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 2 mM $MnCl_2$, and 1 mM dithiothreitol. Samples were incubated for 10 min at 30 °C (Glc7p) or 37 °C (PP1), and the reaction was then stopped by adding 1% Tris (final concentration). For phosphatase inhibition assays, a range of concentrations of tungstate were incubated with the purified phosphatases during 5 min at 30 or 37 °C, prior to the addition of pNPP.

3. Results

3.1. Tungstate relieves the growth defect caused by FK506-induced amino acid deprivation

We tested the tungstate effects on the yeast cell growth defect caused by FK506-induced amino acid deprivation. We used the *WT w303.1a* strain and the Δ*cnb1* mutant. In the latter, the calcineurin regulatory subunit *CNB1*, a therapeutic FK506 target, is absent [53]. Tungstate restored cell growth in the FK506-treated yeast cells (Fig. 1A), indicating that tungstate somehow alleviated the amino acid deprivation caused by FK506 treatment.

3.2. Tungstate enhances FK506-dependent GCN pathway activation

FK506 activates the GCN pathway and stimulates *GCN4* selective translation, via Gcn2p kinase [7]. In order to identify the effects of tungstate on translational control, we studied the activation of the GCN pathway in presence/absence of tungstate. Tungstate alone did not induce expression of a *GCN4-lacZ* reporter in the *WT* strain. Interestingly, tungstate greatly enhanced FK506-dependent *GCN4-lacZ* reporter induction in a dose-dependent manner (Fig. 1B). Tungstate also stimulated a *HIS4-lacZ* p377 reporter activation by FK506 (Fig. 1C). We also monitored the phosphorylation status of the sole Gcn2p kinase substrate in yeast, the α subunit of the eukaryotic initiation factor 2 (eIF2α) by immunodetection with a phospho-eIF2α specific antibody. As expected, tungstate incremented the FK506-induced amount of phospho-eIF2α (Fig. 1D). Taken together, these data indicated that tungstate treatment enhanced the GCN pathway activation by FK506 in yeast.

3.3. Tungstate overcomes 3-aminotriazole sensitivity of a hypomorphic GCN2 mutant

Glc7p is the yeast orthologue of PP1 [29] which negatively regulates *GCN4* translation through eIF2α dephosphorylation [28]. Given that tungstate is considered to be a phosphatase inhibitor, Glc7p inhibition by the compound could potentially exert its effects on the GCN pathway. To test whether tungstate inhibits in vivo the Glc7p phosphatase, we evaluated the effect of the agent on growth of the hypomorphic *gcn2-507* mutant under starvation conditions. This mutant exhibits constitutively low Gcn2p kinase activity and, consequently, reduced derepression of the Gcn4p-regulated genes in response to starvation. This set of strains is prototroph for tryptophan, so we induced histidine deprivation with 3AT, instead of FK506, who mainly causes defects in tryptophan uptake. The *gcn2-507* mutant grows slowly in the presence of 3AT concentrations that are lethal for a Δ*gcn2* strain. In the *gcn2-507* strain, eIF2α phosphorylation is defective, but the inhibition of Glc7p by overexpression of a dominant-negative Glc7p mutant can increase it sufficiently to overcome 3AT toxicity [28]. As shown on Fig. 2A, tungstate increased the tolerance of the *WT* and *gcn2-507* strains to 3AT, to the same extent as the Glc7p dominant negative expression. Furthermore, tungstate significantly enhanced the yeast 3AT tolerance induced by expression of the Glc7p dominant negative (Fig. 2A). Overall, these data are consistent with Glc7p as a mediator of tungstate effects in yeast.

3.4. A mutant with low Glc7p activity mimics tungstate-induced effects

If Glc7p inhibition contributes to the tungstate phenotype in yeast, a strain without Glc7p activity should mimic the effects of tungstate. As *GLC7* is an essential gene, in the *w303.1a* genetic background we used the plasmid p27-1, overexpressing a truncated version of the phosphatase that behaves as a negative dominant, thus yielding undetectable Glc7p activity [28]. For this purpose, we grew the strains in plates with/without FK506 (50 μg/ml) and treated or not with tungstate (10 mM). The *WT* p27-1 strain partially relieved FK506 toxicity in yeast (Fig. 2B). We also analyzed the activation of the *HIS4-lacZ* p377 reporter in the same strains (YEp24 and p27-1) under the same conditions. As expected, FK506-dependent *HIS4-lacZ* p377 reporter activation was significantly higher in the p27-1 strain than that of the control YEp24 strain (Fig. 2C), thus resembling tungstate effects on reporter activation. Interestingly, tungstate did not increase p377 induction in the p27-1 strain. Taken together, these data indicate that Glc7p inhibition mimics the effects of tungstate in yeast.

3.5. Tungstate inhibits yeast and human PP1 in vitro

To give further support to our hypothesis, we used a recombinant fusion protein Glc7p-GST as well as a fusion human PP1-FLAG and tested the effect of tungstate on phosphatase assays. Tungstate inhibited Glc7p activity with an IC_{50} around 1 mM, value that is in the pharmacological range (Fig. 2D). This compound also inhibited human PP1 activity with an IC_{50} similar to that observed with Glc7p (Fig. 2E). Therefore, Glc7p inhibition might mediate tungstate-induced effects in yeast.

4. Discussion

Here we report that the anti-diabetic and anti-obesity agent sodium tungstate enhanced the activation of the GCN pathway in budding yeast. This behavior involved inhibition of Glc7p phosphatase, as a dominant negative of this enzyme mutant mim-

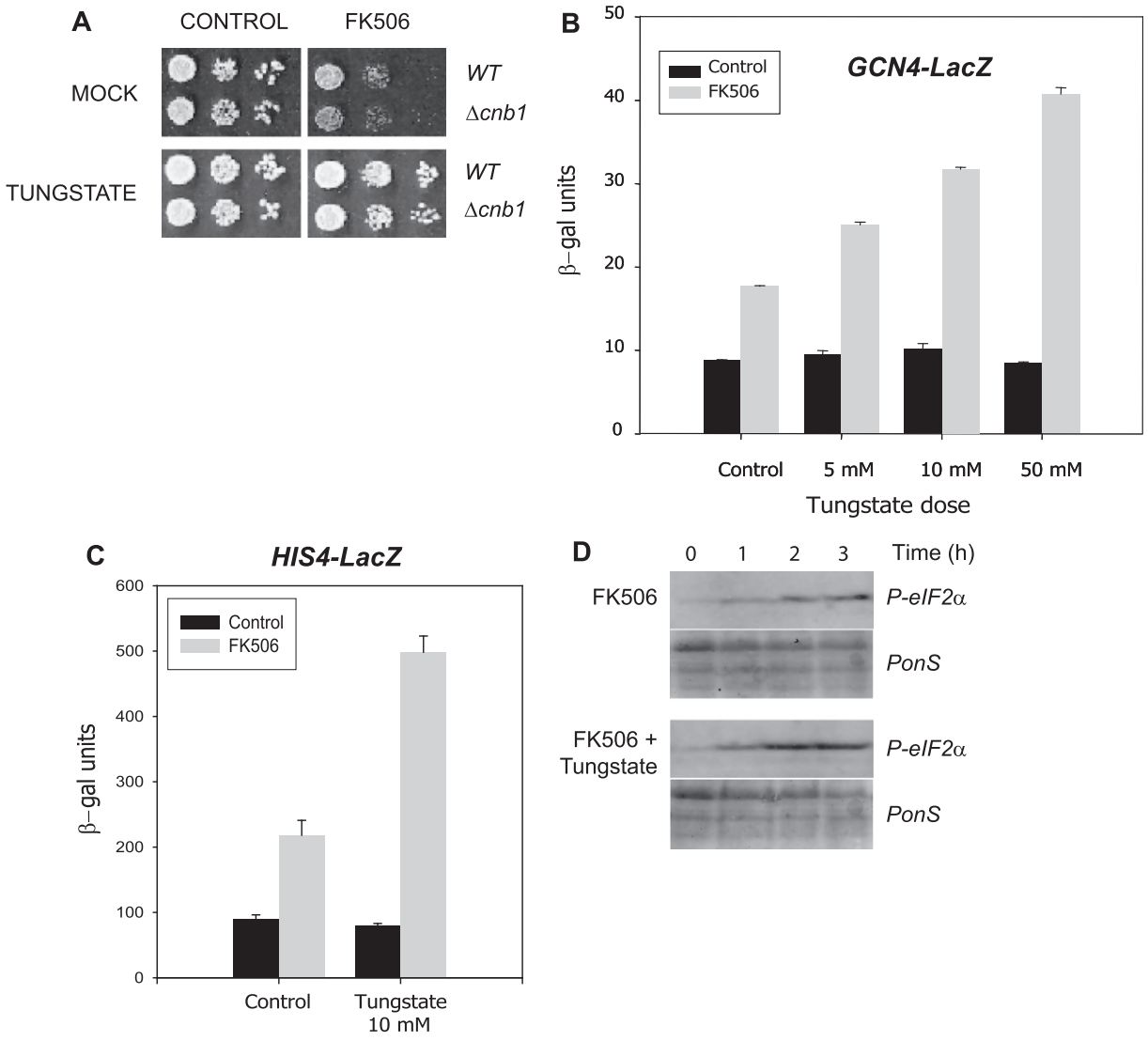

Fig. 1. Tungstate rescues the growth defect caused by FK506 and enhances FK506-dependent GCN pathway activation. (A) Growth of the *WT* (W303.1a) and Δ*cnb1* strains on YPD plates containing FK506 (50 μg/ml) and/or sodium tungstate (10 mM). (B) Dose-dependent induction of the p180 *GCN4-LacZ* reporter in a *WT* strain exposed to increasing concentrations of sodium tungstate and treated or not with the pathway activator FK506 (50 μg/ml). (C) Induction of the p377 *HIS4-LacZ* reporter in *WT* strain treated or not with FK506 (50 μg/ml) plus or minus 10 mM sodium tungstate. (D) Immunodetection of phospho-eIF2α (*P-eIF2α*) in the *WT* strain treated with FK506 (50 μg/ml) plus or minus 10 mM sodium tungstate at the indicated times. Even loading of the gels was confirmed by Ponceau S (*PonS*) staining of membranes after transfer.

icked the effects and tungstate was able to *in vitro* inhibit Glc7p/PP1 at pharmacologically relevant doses. These findings support the notion that Glc7p might be the target of tungstate effects in yeast.

The simplest explanation for the observed tungstate-induced phenotypes is that the drug modulates the status of eIF2α phosphorylation in the cell. This modulation seems to be independent of Gcn2p, as the 3AT sensitivity of the hypomorphic *gcn2-507* mutant was alleviated by tungstate. Therefore, eIF2α phosphatases are good candidates for mediating tungstate effects. Accordingly, a mutant with low Glc7p activity was partially resistant to FK506 (Fig. 2B) and to GCN pathway regulation by tungstate (Fig. 2C). Tungstate-dependent inhibition of Glc7p and its mammalian orthologue PP1 *in vitro* indicates that suppression might occur directly on the catalytic activity of the enzyme. However, we cannot exclude the involvement of Glc7p regulatory subunits, as tungstate has been described to inhibit the interaction between Cdc14p and its regulator Net1p [54].

Tungstate exerts anti-diabetic effects in many experimental models. It potentiates insulin effects in rat adipocytes [55,56], stimulates insulin release in rat pancreas [57], favors β-cell regeneration in rat pancreas [58] and is an efficient oral anti-diabetic agent in Zucker fatty [44] and streptozotocin-induced [41,42] diabetic rats. In the latter model this compound also improves other parameters, such as cardiac [59] and reproductive [60] functions, and alleviates oxidative stress in brain [61]. However, most of the mechanisms underlying these effects are unknown. Our data obtained with yeast suggest that PP1 phosphatase could be a potential candidate target for tungstate action. Several pharmacological studies have revealed the relevance of PP1 in insulin-dependent regulation of metabolic enzymes such as glycogen synthase, hormone-sensitive lipase and acetyl-CoA carboxylase [62,63]. Furthermore, many studies have reported the importance of eIF2α kinases in diabetic syndromes [18–20], hyperglycemia, and β-cell death in mice [21,22] and also in the translational control of insulin mRNA [23]. Nevertheless, taken into account the high range of potential phosphatase inhibition by tungstate in vivo [36–39], we cannot rule out the possibility of phosphatases other than Glc7p/PP1 contributing to the cellular effects of this compound.

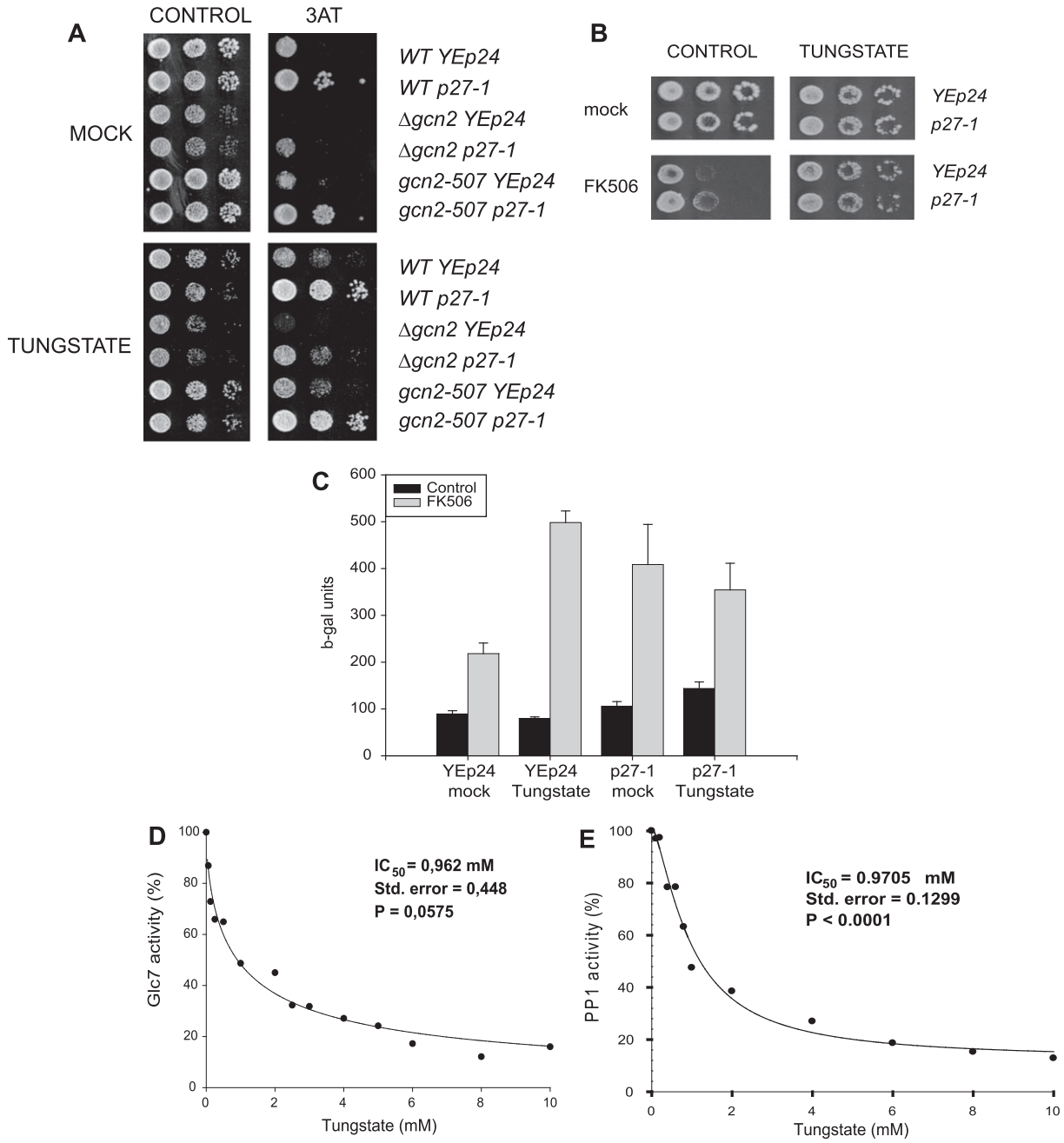

Fig. 2. Inhibition of Glc7p phosphatase is consistent with tungstate-induced effects. (A) Growth of *H1402* strains (*WT*, *Δgcn2* and *gcn2-507*) transformed with empty vector (YEp24) or a plasmid carrying a Glc7p dominant negative (p27-1) on SD plates containing 12,5 mM 3-amino-1,2,4-triazole and/or sodium tungstate (10 mM). (B) Growth of a *w303.1a WT* strain expressing either a Glc7p dominant negative (p27-1) or empty vector (YEp24) on YPD plates containing FK506 (50 μg/ml) and/or sodium tungstate (10 mM). (C) Induction of the p377 *HIS4-LacZ* reporter in *w303.1a* YEp24 and p27-1 strains treated with FK506 (50 μg/ml) in the presence or absence of 10 mM sodium tungstate. Protein phosphatase activity using *p*-nitrophenylphosphate as substrate in the presence of increasing concentrations of sodium tungstate: (D) yeast Glc7p expressed in *E. coli* and purified as explained in Section 2; and (E) human PP1, expressed in and purified from HEK293T cells as explained in Section 2. IC_{50} data were calculated with Sigma-Plot software (Systat Software, Inc.).

Tungstate also counteracted FK506 toxicity in yeast, suggesting that the interaction between FK506 and tungstate could be of therapeutic significance. One of the most important side effects of FK506 immunosuppression is post-transplantation diabetes mellitus (PTDM). Therefore, tungstate could be used to alleviate the adverse side effects of FK506. This hypothesis is currently being tested in a rat model of FK506-induced diabetes.

Furthermore, given the strong link between eIF2α phoshorylation and human disease, our findings suggest that the therapeutic use of tungstate could be relevant for treating other pathologies involving eIF2α phosphorylation such as (1) tumoral processes [64,65], (2) viral infections (reviewed in [66]), (3) organ preservation [67] and (4) sleep disorders [68]. Testing the effects of tungstate in cellular/animal models of these diseases would undoubtfully expand the applications of sodium tungstate in chemotherapy.

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