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Yap1 mediates tolerance to cobalt toxicity in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

Background: Cobalt has a rare occurrence in nature, but may accumulate in cells to toxic levels. In the present study, we have investigated how the transcription factor Yap1 mediates tolerance to cobalt toxicity.
Methods: Fluorescence microscopy was used to address how cobalt activates Yap1. Using microarray analysis, we compared the transcriptional profile of a strain lacking Yap1 to that of its parental strain. To evaluate the extent of the oxidative damage caused by cobalt, GSH was quantified by HPLC and protein carbonylation levels were assessed.
Results: Cobalt activates Yap1 under aerobiosis and anaerobiosis growth conditions. This metal generates a severe oxidative damage in the absence of Yap1. However, when challenged with high concentrations of cobalt, *yap1* mutant cells accumulate lower levels of this metal. Accordingly, microarray analysis revealed that the expression of the high affinity phosphate transporter, *PHO84*, a well-known cobalt transporter, is compromised in the *yap1* mutant. Moreover, we show that Yap1 is a repressor of the low affinity iron transporter, *FET4*, which is also known to transport cobalt.
Conclusions: Cobalt activates Yap1 that alleviates the oxidative damage caused by this metal. Yap1 partially controls cobalt cellular uptake via the regulation of *PHO84*. Although *FET4* repression by Yap1 has no effect on cobalt uptake, it may be its first line of defense against other toxic metals.
General significance: Our results emphasize the important role of Yap1 in mediating cobalt-induced oxidative damages and reveal new routes for cell protection provided by this regulator.

1. Introduction

Cobalt is a biologically important metal as it is an essential enzyme cofactor in most living organisms. However, a tight homeostatic control of cobalt is a preponderant requirement, since when present in excess, this metal may be life threatening. Overexposure to cobalt often occurs in several industrial occupations and, in humans, it can cause several diseases such as contact dermatitis, asthma, pneumonia and lung cancer [1].

Cobalt toxicity arises not only from the ability of cobalt ions to generate reactive oxygen species (ROS) via Fenton reactions, but also from its competition with other essential metals such as iron, calcium and zinc, for the binding to macromolecules — possibly resulting in the inhibition of their proper function [2]. In addition, the chemical affinity of cobalt for sulfur atoms has also been invoked as a possible mechanism of cobalt toxicity, due to the likely dysfunction of proteins whose activity depends on thiol groups [3].

The exact mechanisms used by organisms to protect from cobalt toxicity remain to be elucidated. In the yeast *Saccharomyces cerevisiae*, cobalt may enter the cell through the low affinity iron transporter, Fet4, via the manganese transporter Smf2, or via the phosphate transporter Pho84 [4–6]. In line with the mechanisms of cobalt-induced toxicity, the cellular response to cobalt excess includes the activation of the major iron responsive transcription factor, Aft1 [7,8]. One target of Aft1 is the *COT1* gene that codes for a vacuolar transporter [7,9]. Cot1 presumably renders protection against the damaging effects of cobalt through cobalt sequestration in the vacuole [9,10]. Nevertheless, overexpression of *COT1* gene does not confer increased cobalt tolerance to cells deficient in high affinity iron transporter system, which met their iron requirements by increasing the expression of low affinity iron transporters [10].

In a second line of defense against cobalt surplus, yeast also increases the expression of genes involved in oxidative stress response [7]. The yeast transcription factor Yap1, is an essential regulator of the cellular response to oxidative stress, being activated upon exposure to peroxide

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or to thiol modifying agents [11]. Moreover, Yap1 also plays a prime role in cadmium and arsenic detoxification by regulating the *YCF1* gene that encodes a vacuolar ATP binding cassette (ABC) transporter which functions as a vacuolar glutathione S-conjugated pump [12,13].

Regulation of Yap1 occurs mainly through the control of its nuclear localization. Yap1 contains a C-terminal non-canonical nuclear export signal (NES), containing three cysteine residues, which are important for the interaction with the exportin protein Crm1 [14–16]. Crm1 rapidly exports Yap1 to the cytoplasm under normal growth conditions [14,15]. When exposed to oxidants Yap1 undergoes oxidation-induced conformational changes; as a consequence NES becomes masked, the Yap1-Crm1 interaction is lost and Yap1 becomes localized into the nucleus, activating its targets [17].

In this work, we have investigated the role of Yap1 in the response of yeast cells exposed to cobalt excess. We observed that in the absence of Yap1, cells exhibit a higher sensitivity towards cobalt and therefore we sought to understand how cobalt activates Yap1 and how Yap1 contributes to cobalt tolerance. Using genetic and biochemical approaches, we show that cobalt induces oxidative stress in yeast cells and that Yap1 is required to alleviate the oxidative damages. The transcriptomic analysis of a strain lacking Yap1 revealed novel targets of this regulator involved in the low affinity metal uptake (*PHO84* and *FET4*).

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The yeast strains and plasmids used in this study are listed in Table S1 and Table S2, respectively. Yeast strains were grown in synthetic media (SC: 0.67% ammonium sulfate-yeast nitrogen base without amino acids [Difco], 2% glucose, supplemented with the appropriate selective amino acids) or SC lacking specific requirements (SD). Phenotypic growth assays were carried out by spotting 5 μl of early exponential phase cultures ($A_{600} = 0.4$) sequentially diluted (approximately 5×10^3 to 50 cells) in medium containing the indicated $CoSO_4$ concentrations. Growth was recorded after 2 days at 30 °C. The bacterial *Escherichia coli* strain XL1-Blue (Stratagene) was used as a host for routine cloning purposes. Standard methods were used for genetic analysis, cloning and transformation. The anaerobiosis assay was performed with cultures transferred to a glove box, for at least 24 h, where the O_2 concentration was kept below 1 ppm. Low and high-phosphate media were prepared as described in [18].

2.2. Fluorescence microscopy

Cells transformed with *GFP-YAP1* [16] or *GFP*-cCRDYap1 [19] were grown to early exponential phase and challenged with 2 mM $CoSO_4$ for the indicated time points. DAPI (4′, 6-diamidino-2-phenylindole) was added as a DNA marker at the final concentration of 5 μg/ml before microscopy. After washing with PBS, cells were resuspended in a solution of DABCO (1,4-diazadicyclo[2.2.2]octane) in 75% (v/v) glycerol and 0,25× PBS (Sigma-Aldrich). GFP signals were analyzed in living cells with a Leica DMRXA fluorescent microscope equipped with a Roper Scientific Micro-Max cooled CCD (charge-coupled device) camera and MetaMorph software (Universal Imaging Inc.).

2.3. Immunoblot assays

Wild type and *yap1* strains containing *cCRD-Yap1-TAP-tag* plasmid [19] were grown until early exponential phase ($A_{600} = 0.4–0.5$) and untreated or exposed to 10 mM $CoSO_4$ for 30 min. Cells were harvested and protein extracts were generated from cell cultures using cell lysis buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM KCl, 10% glycerol, 0.1% NP40) supplemented with protease inhibitors (Roche). Protein extracts were incubated 30 min at room temperature and 5 min at 37 °C with 20 mM of methoxypolyethylene glycol maleimide (MAL-PEG, Sigma-Aldrich). Samples were resolved by reducing 10% SDS-PAGE and immunoblotted with a horseradish peroxidase-bound anti-peroxidase rabbit IgG (Life Technologies). Regarding the protein carbonylation assay, wild type and *yap1* strains were grown until early exponential phase cells, either untreated or exposed to 2 mM $CoSO_4$ and collected after 60 and 90 min of treatment. Cell cultures were resuspended in cell lysis buffer [50 mM Tris buffer pH7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5% β-mercaptoethanol and protease inhibitors (Roche)]. To evaluate the presence of carbonyl groups, an OxyBlot™ Protein Oxidation Detection Kit (Intergen) was used. The samples were analyzed by immunoblotting and processed as described in [13], using rabbit ant-dinitrophenol antibody. Pgk1 was used as a loading control [20].

2.4. Microarray and Quantitative Real-Time PCR analyses

Total RNA from early log-phase cultures untreated or exposed to 2 mM of $CoSO_4$ for 1 h, was purified and used for DNA microarray analysis, as previously described [20]. SAM analyses were performed using the algorithm implemented in MeV from TM4 software [21]. The array design, spotting protocol, raw data and pre-processed data from all hybridizations were submitted to the ArrayExpress Database (E-MEXP-3874). Searches for putative Yap binding sites were carried out using the YEASTRACT database [22]. Gene clustering was performed according to the Munich Information Center for Protein Sequences database (MIPS) functional catalog (http://mips.helmholtz-muenchen.de/proj/funcatDB/).

For qRT-PCR experiments, RNA was isolated from early log-phase cultures that were either untreated or exposed to 2 mM $CoSO_4$ and harvested at the indicated time points. RNA samples were treated with DNase (TURBO™ DNase-free; Ambion) according to the manufacturer's instructions and purified by on-column DNAse I digestion (RNase-Free DNase Set; Qiagen). Total RNA (1 μg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). qRT-PCR reactions were performed in the Light Cycler 1.5 Real-Time PCR System (Roche), using Light Cycler Fast Start DNA Master SYBR Green I (Roche). Relative standard curves were constructed for each gene, using triplicate serial dilutions of cDNA. The relative expression of the genes was calculated by the relative quantification method with efficiency correction, using the LightCycler Software 4.1. Actin gene was used as a reference gene. All assays were made at least in duplicate. The primers used in this assay are listed in Table S3.

2.5. Measurement of cobalt and phosphorous

Strains were grown in SC media with 2mM of $CoSO_4$ for 20 h, collected by centrifugation and washed with 10 mM EDTA and metal-free water. The total Co and P were measured by inductively coupled plasma (ICP) atomic emission spectroscopy. Data were normalized against OD_{600}.

2.6. Glutathione (GSH) and glutathione disulphide (GSSG) quantification

The wild-type and mutant strains were grown to early log-phase, exposed to 2 mM of $CoSO_4$ and harvested after 1 h. GSH and GSSG were quantified by HPLC after sample derivatization, as described in [23]. GSH and GSSG concentrations in mM/cell were calculated on the basis of an average yeast cell volume of 4.5×10^{-14} l [24].

3. Results

3.1. Yap1 is required for resistance to cobalt

We have started to investigate the role of Yap1 in response to cobalt excess by monitoring the growth of a *S. cerevisiae* strain lacking Yap1 (*yap1*) and of its isogenic wild-type strain, in the presence of cobalt (Fig. 1A). The *yap1* mutant strain was more sensitive than the wild-

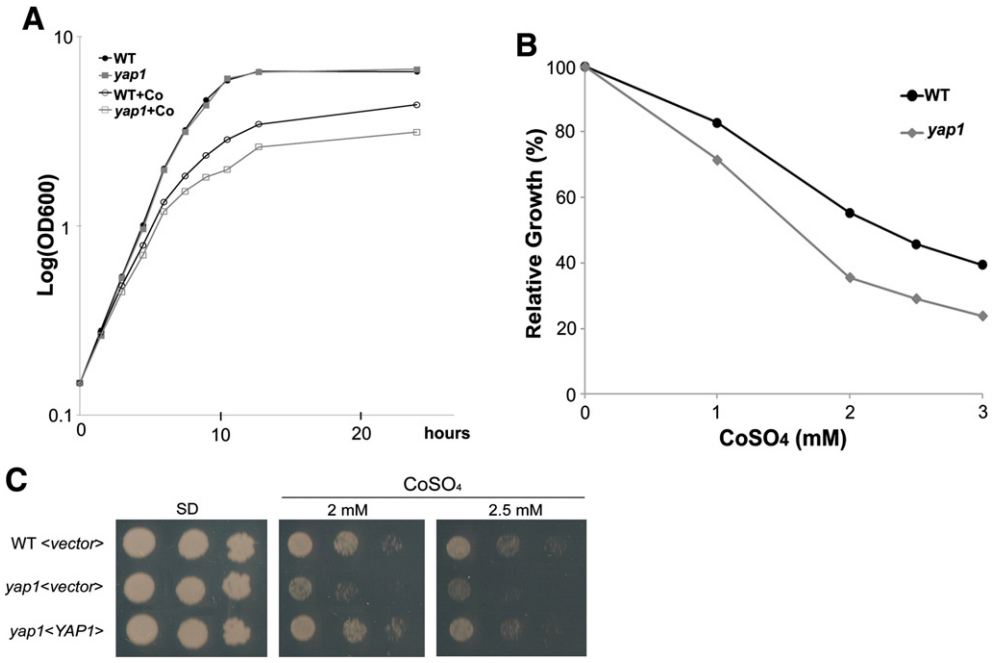

Fig. 1. Yap1 confers tolerance to cobalt excess. (A) The growth of wild-type (WT) and *yap1* mutant strains in SC medium containing 2mM of $CoSO_4$ was monitored by means of turbidity measurements. (B) WT and *yap1* cells were incubated in SC medium containing the indicated amount of $CoSO_4$ for 12 h and spread onto SC plates. Colony forming units were recorded (% survival relative to untreated cells). (C) *yap1* strain was transformed with a centromeric plasmid containing the *YAP1* gene (*yap1*<*YAP1*>), or the plasmid alone (*yap1*<*vector*>). Wild-type strain was transformed with the empty plasmid (WT). Exponentially growing cells were harvested, serially diluted and spotted onto control SD plates or SD plates containing the indicated $CoSO_4$ concentrations.

type to a wide range of cobalt concentrations (Fig. 1A and B). Reintroduction of *YAP1* into the *yap1* mutant strain rescued its cobalt sensitivity, confirming that cobalt high-toxicity was in fact due to the loss of Yap1 function (Fig. 1C).

3.2. Cobalt activates Yap1

Because Yap1 activation requires its accumulation in the nucleus, we followed the effect of cobalt in the cellular localization of a GFP-Yap1 fusion protein. Yap1 appeared predominately localized in the cytoplasm under non-stressed conditions. Ten minutes after cobalt treatment (2 mM, $CoSO_4$), a strong GFP nuclear staining was observed that persisted for up to 1 h (Fig. 2A). After 90 min, nuclear staining was still detected, although with a lower intensity. After this time period, Yap1 is still able to sense cobalt as a second challenge with this stressor led to a novel induction of its targets, which suggests that the activation of Yap1 by cobalt is a reversible process (Fig. S1). Overall, the kinetics of Yap1 localization in the cell is in according to the rapid and transient expression profile of Yap1 target genes, upon exposure to cobalt (Fig. 5A–E).

Azevedo et al. showed that Yap1 carries two distinct molecular redox sensors, one operated by H_2O_2 and the other by thiol-reactive compounds [19]. The former relies on the formation of an intramolecular disulphide bond between N- and C-terminal cysteine residues that requires Orp1/Gpx3, the primary sensor transducing the H_2O_2 signal to Yap1 [17,25]. The latter, involves three Yap1 C-terminal cysteine residues that directly interact with the drug [19]. In order to understand which molecular device was operating in Yap1 activation by cobalt, we first examined whether a truncated GFP-Yap1 version, only bearing the C-terminal cysteine rich domain of Yap1 (GFP-cCRDYap1), was enough to drive its accumulation into the nucleus under cobalt treatment. As illustrated in Fig. 2B, the GFP-cCRDYap1 construct was found in the nucleus in the presence of cobalt. Consistent with this observation, cobalt induced the accumulation of Yap1 in the mutant *orp1* (Fig. 2B). Furthermore, we still observed Yap1 nuclear localization under anoxia after cobalt treatment (Fig. 2C), suggesting that Co-Yap1 activation may also occur via a ROS-independent mechanism.

As cobalt has high affinity for sulfhydryl groups [2], we next assessed Yap1 cCRD free sulfhydryl status by means of the high molecular mass cysteine-specific alkylating agent, MAL-PEG. MAL-PEG increases the mass of a protein by 5 kDa for each alkylated cysteine. Proteins with exposed thiols are able to react with the MAL-PEG to form covalent complexes of greater molecular mass relative to non-alkylated proteins and thus their migration is retarded in subsequent electrophoretic gel. We used a TAP tagged version of the C-terminal cysteine rich domain of Yap1 (cCRDYap1-TAP-Tag) to transform a wild-type strain. Protein extracts were then incubated with MAL-PEG in the absence or presence of cobalt, analyzed by SDS-PAGE and immunoassayed using an anti-TAP-tag antibody (Fig. 2D). Yap1 cCRD from samples untreated with cobalt and incubated with MAL-PEG (lane 2) had a slower mobility than the protein from MAL-PEG unreacted samples (lane 1), indicating modifications of the polypeptide by the high molecular mass alkylating agent. The presence, however, of three bands in the former (lane 2) indicates that under the tested conditions Yap1 cCRD was present as a mixture of modified and non-modified molecules. Yap1 cCRD from protein extracts treated with cobalt and MAL-PEG (lane 3) exhibited a third band ("b" in Fig. 2D) of intermediate size between the total alkylated ("a" in Fig. 2D) and unalkylated ("c" in Fig. 2D) polypeptides.

These data suggest that cobalt activates Yap1, driving its nuclear accumulation presumably by binding to the Yap1 C-terminal cysteines.

3.3. Yap1 targets under cobalt induced stress

Aiming at identifying Co-dependent targets of Yap1, we characterized the global changes in the transcriptome of *yap1* mutant cells grown under cobalt surplus conditions. Because all organisms encounter reactive oxygen species during the course of normal aerobic metabolism, which could lead to Yap1 activation, we also monitored transcriptomic changes of *yap1* mutant cells in the absence of cobalt. DNA microarrays analyses were conducted by comparing RNAs isolated from the *yap1* mutant vs. wild-type strains grown in the absence or

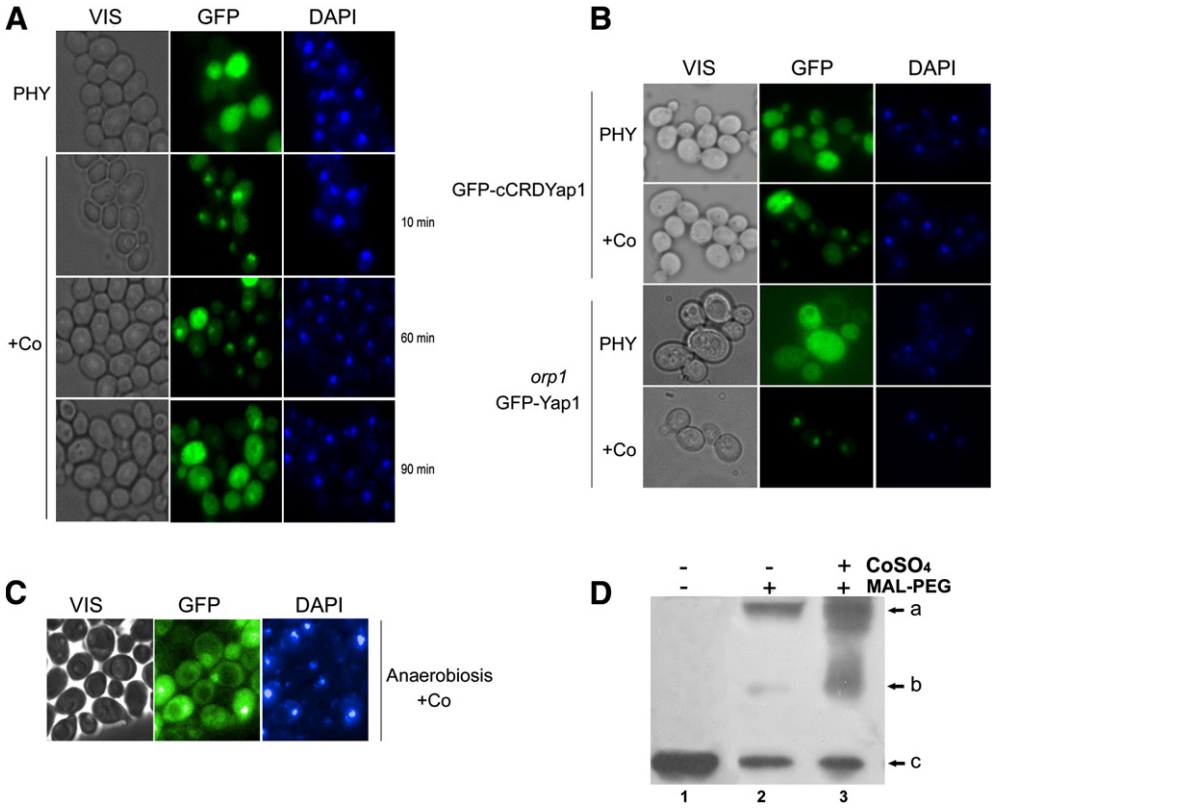

Fig. 2. Yap1 is activated by cobalt. (A) The *yap1* mutant expressing the fusion *GFP-YAP1* was induced with 2 mM of $CoSO_4$ and analyzed for GFP staining at the indicated time points, by fluorescence microscopy. (B) The construct GFP-cCRDYAP1, containing the C-terminal cysteine rich domain of Yap1, was used to transform a *yap1* strain (upper part of the panel). The *orp1* mutant was transformed with the fusion *GFP-YAP1* (lower part of the panel). Exponentially growing cells were treated with 2mM of $CoSO_4$, for 10 min and fluorescence was recorded. (C) The localization of the gfp-yap1 fusion was assayed under anaerobiosis after exposure to 2 mM of $CoSO_4$ for 30 min. PHY-physiological conditions (D) Protein extracts from cultures of a wild-type strain carrying a cCRDYap1-TAP-tag were left untreated (lanes 1 and 2) or were treated with $CoSO_4$ (lane 3). The proteins were solubilized in the absence (lane 1) or presence (lanes 2 ad 3) of 20 mM of MAL-PEG. The cCRDYap1-TAP-Tag was revealed with labeled IgG.

presence of 2 mM of $CoSO_4$ for 60 min. Genes differentially expressed were sorted into functional categories according to MIPS (Tables S4 and S5). Fig. 3 shows a schematic representation of the results of these experiments from triplicate Agilent DNA microarray studies. A total of 40 genes displayed an altered expression, by at least 1.5 fold, in the *yap1* strain, following growth shift to high-Co medium, with 30 transcripts being down-regulated. Remarkably, roughly 70% of these genes (27 out of 40) are already dependent on Yap1 under normal growth condition (Fig. 3). Among all the MIPS functional categories, the stress response and oxidative stress response categories were well represented in our microarrays, accounting for more than 40% (17 out of 40) of the total number of genes. We also found that Yap1 regulates the expression of 5 genes involved in iron homeostasis (Fig. 3 and Tables S4 and S5), namely in plasma membrane iron uptake (*FIT2*, *FIT3* and *FET4*); mitochondrial iron import (*MRS4*) and iron-sulfur protein synthesis (*ISU2*) [8]. All of them are up-regulated by Yap1, with the exception of *FET4*, a low affinity iron transporter that has a broad metal specificity [10]. Indeed, *FET4* expression in the *yap1* mutant strain was up-regulated by 2 and 4.5 fold, in the absence and presence of cobalt excess, respectively (Tables S4 and S5), suggesting that Yap1 is a negative regulator of *FET4*. Nevertheless, we could not find any canonical YRE (Yap Response Element) [11] in the promoter region of *FET4* indicating that Yap1 is not most probably directly regulating *FET4* gene. Interestingly, we observed that Yap1 is a positive regulator of two other genes involved in phosphate metabolism: *PHO84* and *SPL2* (Fig. 3, Table S4, Table S5 and Fig. S2). Pho84 is a high affinity phosphate transporter [6,26,27] and Spl2 is a negative regulator of the low affinity phosphate transporters [28]. Neither *PHO4* nor *SPL2* have YREs in the respective

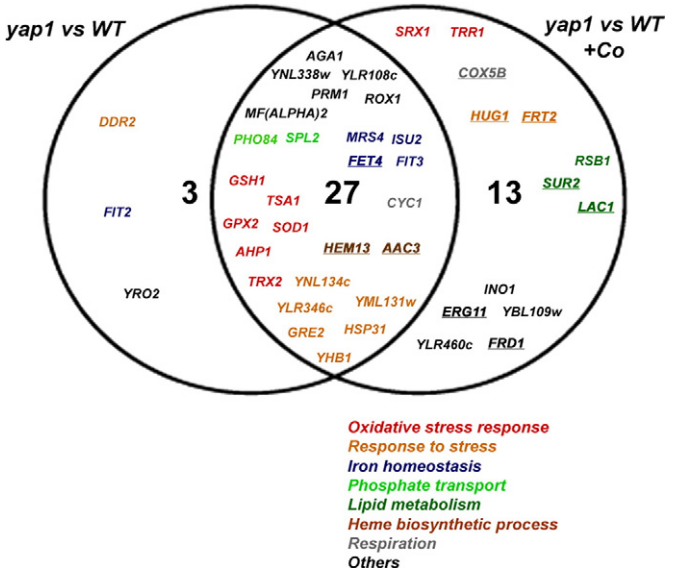

Fig. 3. Summary of genes whose expression is altered in the *yap1* mutant strain under normal or cobalt-induced stress conditions. In the *yap1* mutant, 40 genes showed altered expression levels by at least 1.5 fold, relative to the wild-type, after treatment with 2 mM of $CoSO_4$, for 1 h (*yap1 vs wt + Co*). A total of 30 genes were already dependent on Yap1 under normal growth conditions (*yap1 vs wt*). The intersect represents the number of genes regulated by Yap1 in both conditions (27 genes). Down-regulated genes are underlined. Genes were sorted into functional categories accordingly to the indicated color-code.

promoter regions, suggesting that Yap1 may not directly orchestrate their expression.

These results indicate that the majority of genes regulated by Yap1 upon stress are already being regulated by this transcription factor under normal growth conditions. The main differences observed concern the up-regulation of a larger number of genes implicated in the stress response. Furthermore, our data also unveil new targets of Yap1 involved in metal metabolism, pointing out towards new lines of cell protection given by this regulator.

3.4. Integration of Yap1-mediated gene regulation in the overall transcriptomic response of yeast cells to cobalt

In order to provide a clear picture of the relevance of Yap1 transcription factor in the overall yeast response to cobalt stress, we performed a genome-wide transcriptional analysis of *S. cerevisiae* exposed to this stressor. As such, we first compared the mRNA expression profile of wild-type cells upshifted to Co-supplemented medium (2mM $CoSO_4$, 60 min) to cells grown under unstressed conditions. Cobalt challenge led to an increase in the steady-state levels of 242 genes (Table S6). Approximately 25% of these genes (61 out of 242) are related to stress response, with 26 genes assigned to the oxidative stress response category. As described by others [7], the metal category is also well represented (17 out of 242 genes) in the yeast response to cobalt, being 14 genes involved in iron homeostasis (Table S6).

We next searched for potential Yap1 regulated genes in the above dataset. To accomplish this task we used the YEASTRACT database [22] and compared our dataset with previous studies on the Yap1 regulon or search for the presence of YREs in the promoter region of the genes (Yap Responsive Elements). We found that 152 out of 242 genes possesses YREs in the corresponding promoter regions or have been already documented to be regulated by Yap1. However comparison of those Yap1-regulated candidates with the list of genes dependent on Yap1 under cobalt surplus conditions (Table S5) just retrieved 13 genes (Fig. 4 and Table 2). Interestingly, only two of these genes (*INO1* and *SRX1*) are not dependent on Yap1 under normal growth conditions (Table 2) suggesting that Yap1 specifically regulates them under cobalt surplus. *INO1* however does not have YREs and as such Yap1 cannot be a direct regulator of this gene.

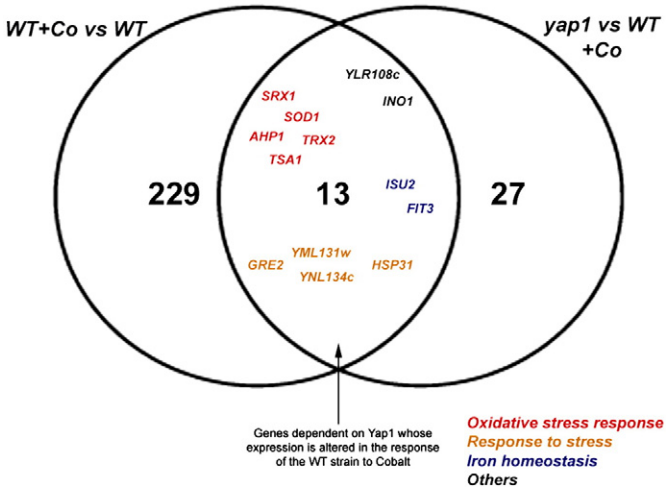

Fig. 4. Involvement of Yap1 in the overall transcriptomic changes observed in yeast cells exposed to cobalt. In the wild-type strain 242 genes were induced after treatment with 2 mM of $CoSO_4$, for 1 h (*wt+Co vs wt*). In the *yap1* mutant, 40 genes showed altered expression levels by at least 1.5 fold, relative to the wild-type, after treatment with 2 mM of $CoSO_4$, for 1 h (*yap1 vs wt + Co*). The intersection represents the number of genes dependent on Yap1 whose expression is altered in the wild-type response to cobalt (13 genes). Genes were sorted into functional categories accordingly to the indicated color-code.

3.5. Cobalt generates severe oxidative damage in the absence of Yap1

To evaluate whether Yap1 was important to deal with the oxidative damage presumably generated by exposure to cobalt, we first monitored by qRT-PCR, the expression of genes coding for several antioxidant defenses (*TRR1*, *TRX2*, *GSH1*, *SOD1* and *GPX2*) and confirmed their dependence on Yap1 (Fig. 5A–E). All tested genes were up-regulated after treatment with cobalt and were shown to be dependent on Yap1. Some level of Yap1-independent gene expression, however, was observed suggesting that other factors might be regulating their expression. The exception is the *GPX2* gene that encodes a 2-Cys peroxiredoxin [29] whose expression is completely abrogated in the *yap1* mutant (Fig. 5E).

In response to an oxidant challenge, yeast cells elevate GSH levels both by means of increased synthesis and by recycling the oxidized form (GSSG) to the reduced form (GSH) [30]. The compromised levels of *GSH1* gene expression in the *yap1* mutant, prompted us to further analyze, by HPLC, the cellular levels of reduced and oxidized glutathione in the wild-type and mutant strains subjected to cobalt treatment (Table 1). As expected, GSH levels were significantly compromised in the *yap1* mutant strain, being reduced from approximately 40% and 26% in unstressed and stressed samples, respectively, in comparison to the wild-type strain. Notably, GSSG/GSH ratios were decreased in both strains, after treatment with cobalt. This finding was rather unexpected as the utilization of GSH usually results in its conversion to the oxidized form (GSSG) and in the consequent increase of the GSSG/GSH ratio [13].

To examine the possible oxidative effects of cobalt treatment at the protein level, we next monitored the changes in the protein carbonyl status of yeast cells exposed to 2 mM of $CoSO_4$ for 60 and 90 min (Fig. 5F). Protein carbonylation is an early and largely used indicator of protein oxidation, and results from oxidative modifications on amino acid residues and by oxidative cleavage of the peptide chain [31]. Our results clearly indicate that the amount of carbonylated proteins increase after cobalt treatment, being more pronounced in the *yap1* mutant.

Overall our data indicate the existence a redox imbalance and oxidative damage triggered by cobalt excess which are more accentuated in cells lacking Yap1, highlighting the relevance of this regulator in cobalt induced stress response.

3.6. Yap1 is a negative regulator of FET4

Li et al., have demonstrated that yeast cells compensate for the absence of the high iron affinity transporter, Fet3, by up-regulating *FET4* gene. The overexpression of *FET4* observed in the *fet3* mutant strain, however, led to an increase of transient metal accumulation that renders this strain high sensitive towards metals [10].

Microarray analyses showed Yap1 as a negative regulator of *FET4* expression (Fig. 3, Tables S4 and S5). Aiming to understand whether the uptake of cobalt via *FET4* could be contributing to the sensitivity exhibited by the *yap1* mutant, we first tested *FET4* dependence on Yap1 by qRT-PCR (Fig. 6A). Confirming our microarray data, *FET4* expression was induced in the *yap1* mutant, being however its induction no longer observed after 90 min of cobalt exposure.

We next monitored the total cellular concentrations of cobalt in cells exposed to 2 mM of $CoSO_4$. Surprisingly, we observed a 25% decrease of the cobalt levels in the *yap1* mutant strain relative to the wild type, as measured by ICP-MS (Fig. 6B). In addition, the double mutant *yap1fet4* did not exhibit an increased resistance to cobalt, comparatively to the single mutant *yap1* (Fig. 6C).

Overall, these results indicate that overexpression of *FET4* observed in the *yap1* strain is not responsible by the toxicity displayed by this mutant, and suggest that another Yap1 target is involved in cobalt uptake.

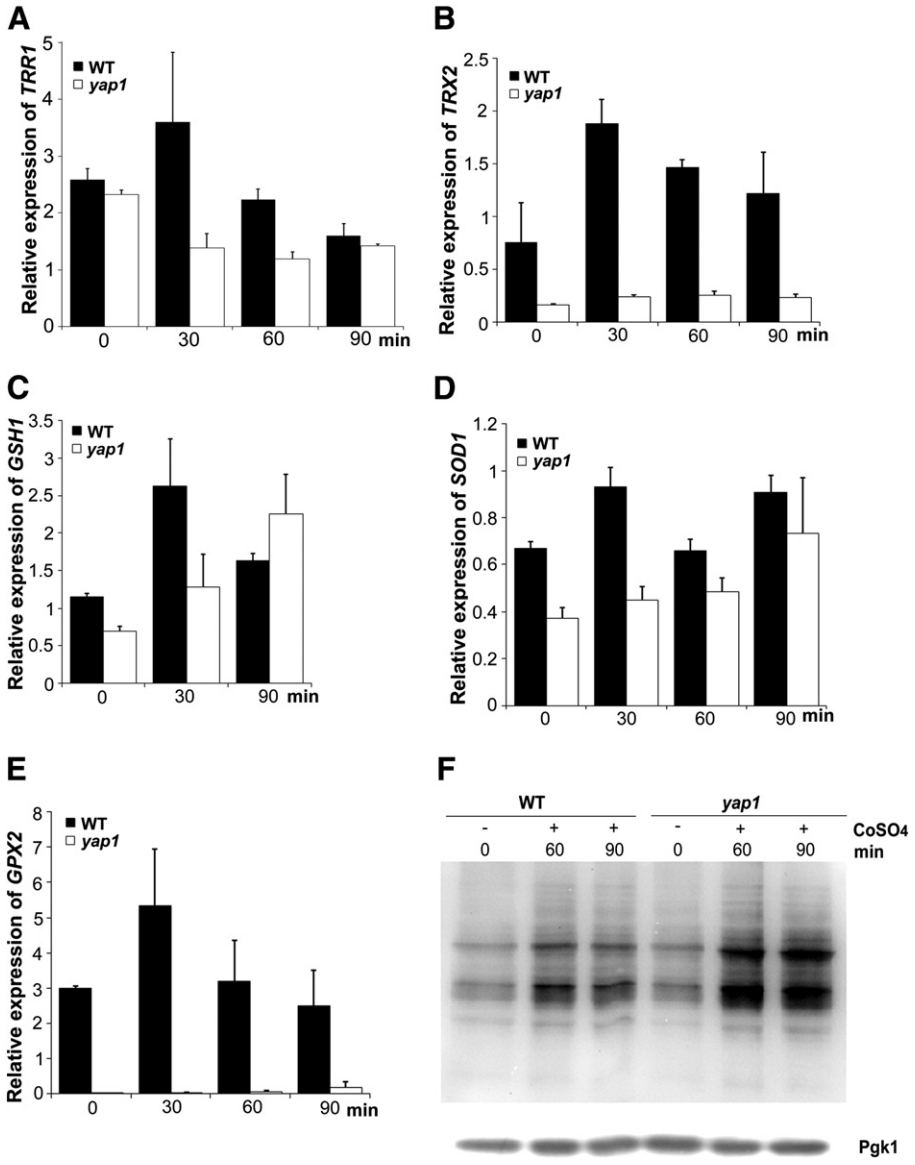

Fig. 5. Yap1 provides protection against cobalt-induced oxidative stress. (A–E) WT (wild-type) and *yap1* strains were upshifted to high-cobalt medium, by supplementation of SC medium with 2 mM of $CoSO_4$, and harvested at the indicated time-points. The expression of the indicated genes was assessed by qRT-PCR. Values are the mean of biological triplicates ± s.d. *TRR1*— thioredoxin reductase gene; *TRX2*— thioredoxin isoenzyme gene; *GSH1*— gamma glutamylcysteine synthetase gene; *SOD1*— copper-zinc superoxide dismutase gene; *GPX2*— glutathione peroxidase gene. (F) The content of carbonyl groups in proteins after cobalt treatment were evaluated by derivatization with 2,4-dinitrophenylhydrazone (DNP), followed by immunoblot with an anti-DNP antibody. Pgk1 protein levels were used as loading control. A representative experiment is shown.

3.7. The lack of Yap1 partially affects cobalt and phosphorus uptake

In an attempt to understand why the levels of cobalt were diminished in the *yap1* mutant, the microarray data were inspected as to find other Yap1-dependent genes known to be involved in metal homeostasis. In this search we have found *PHO84* gene (Fig. 3 and Tables S4 and S5) that codes for a high affinity inorganic phosphate transporter located on the yeast cell surface [32,33]. In the absence of *PHO84* yeast cells become more resistant to several metals, including cobalt, as a consequence of a reduced metal accumulation [6,27]. Accordingly, in this work, under the tested stress conditions, approximately 46% of cellular cobalt enters the cell via Pho84 (Fig. 6B). It is known that *PHO84* is regulated by the Pho4 transcription factor [34] and the microarray data (Fig. 3) confirmed by qRT-PCR analysis (Fig. 7A), provide evidence that Yap1 is also regulating this gene even under physiological growth conditions. Indeed, in the double mutant *yap1pho4* the expression of *PHO84* is lower than in *pho4* and *yap1* single mutants (Fig. 7A). This finding may justify the lower cobalt uptake, observed in the *yap1* mutant (Fig. 6B). Further corroborating this assumption, we noted that the levels of total phosphorus were slightly but significantly compromised in the *yap1* strain, as measured by ICP-MS (Fig. 7B). Notably, whereas the deletion of *PHO84* from the *yap1* background completely rescues the growth sensitivity displayed by this strain, deletion of *PHO4* does not restore the normal growth (Fig. 7C). In our view, these findings point towards one of the following possibilities: or the basal levels of expression of *PHO84*, observed in the *yap1pho4* mutant, still

Table 1
GSH measurements in WT and yap1 strains upon cobalt induced stress.

Strain	GSH (mM/cell)	GSSG (mM/cell)	GSSG/GSH
WT	12.23 ± 0.03	3.35 ± 0.14	0.274 ± 0.011
WT+Co	8.11 ± 1.50	0.61 ± 0.09	0.077 ± 0.025
yap1	7.3 ± 1.10	1.87 ± 0.30	0.256 ± 0.003
yap1+Co	6.0 ± 0.07	0.22 ± 0.07	0.037 ± 0.010

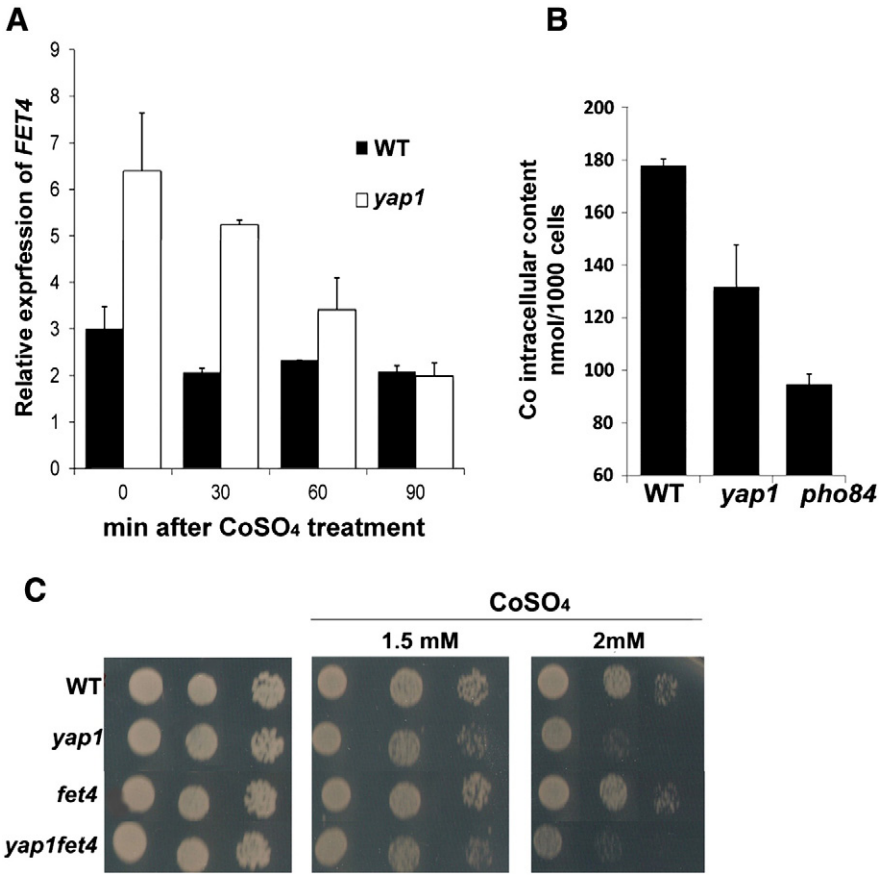

Fig. 6. The negative regulation of *FET4* by Yap1 does not contribute to *yap1* cobalt-sensitivity. (A) Wild-Type (WT) and *yap1* strains were treated with 2 mM of $CoSO_4$, and harvested at the indicated time-points. The expression of *FET4* gene was monitored by qRT-PCR. Values are the mean of biological triplicates ± s.d. (B) Cobalt accumulation in the WT (By4741), *yap1* and *pho84* mutants, after treatment with 2 mM of $CoSO_4$, was determined by inductively coupled plasma atomic emission spectroscopy. The data (± s.d.) are from at least two independent experiments (C) Exponentially growing cells from WT, single mutants *yap1* and *fet4*, and double mutant *yap1fet4* were harvested, serially diluted and spotted onto control SC plates or SC plates containing the designated $CoSO_4$ concentrations.

upshift cobalt intracellular levels to toxic concentrations (Fig. 7A), or Pho4 is regulating other genes relevant for cobalt tolerance.

Together these data suggest that Yap1 partially controls cobalt and phosphorus uptake via the regulation of *PHO84*.

3.8. Phosphate uptake alleviates superoxide stress

The role of Yap1 as a cobalt resistance determinant appears however to be contradictory to its role as *PHO84* activator. Why would Yap1, the major oxidative surveyor of yeast cells, participate in the activation of a gene that contributes to the uptake of cobalt, a known oxidative damage inducer? One possibility is that phosphate uptake, somehow, counteracts the oxidative injury. Remarkably, growing evidence suggests that manganese-phosphate complexes act as potent scavengers of superoxide [35,36]. On the other hand, Pho84 functions as a manganese-phosphate transporter [6]. To test this hypothesis we growth the aforementioned strains in high (7.3 mM) or low (0.1 mM) phosphate medium supplemented with paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride), a superoxide generator. We observed that low phosphate concentrations render all the tested strains more sensitive to paraquat (Fig. 8). In addition, deletion of *PHO4* or *PHO84* from the *yap1* background makes cells less resistant to this stressor (Fig. 8). This data indicates that phosphate uptake should be relevant to cope with superoxide-induced stress.

4. Discussion

Our data revealed that the stress responsive bZIP regulator Yap1 is important for *Sacharomyces cerevisaie* tolerance to cobalt (Fig. 1). We showed that Yap1 cCRD cysteine residues are crucial for sensing cobalt, probably serving as a binding site for this metal. As proposed for Cd-mediated Yap1 activation [19], the putative binding of cobalt to Yap1 C-terminal cysteine residues may modify Yap1 NES, thereby inhibiting its interaction with the exportin Crm1 (Fig. 2). As a result, Yap1 accumulates in the nucleus and drives the expression of its target genes.

As previously reported by other authors [37] we found that the regulatory role of this transcription factor is not restricted to stress, but that Yap1 also controls gene expression under normal growth conditions (Fig. 3). Consistently, we had shown that Yap1 possesses a high transactivation potential even in standard growth conditions [38]. ROS are by-products of aerobic metabolism, and therefore the activation of Yap1 under such conditions is not an unexpected finding. When cells are exposed to external stressors, however, the basal activation of Yap1 is likely insufficient to re-establish the redox-homeostasis and yeast cells have therefore evolved a mechanism to keep Yap1 in the nucleus, assuring an increased and/or stress-specific expression of its targets. Our data further indicates that Yap1 is not the sole regulator of many of those genes, as basal expression was observed in the *yap1* mutant under normal and stress conditions (Fig. 5A–D). Indeed, Skn7 is a transcription factor that cooperates with Yap1 in the activation of H_2O_2-inducible target genes such as *TRR1* (thioredoxin reductase), *TRX2* (thioredoxin 2) and *SOD1* (cooper/zinc superoxide dismutase), among others [39,40]. Together with Yap1, Met4, another transcription factor, was shown to regulate *GSH1*, the gene coding for the enzyme that catalyzes the limiting step of glutathione biosynthesis, under glutathione depletion and cadmium injury [41,42]. As such, it is possible that Yap1 functions in cooperation with these transcription factors in the activation of antioxidant genes under normal and cobalt stress conditions.

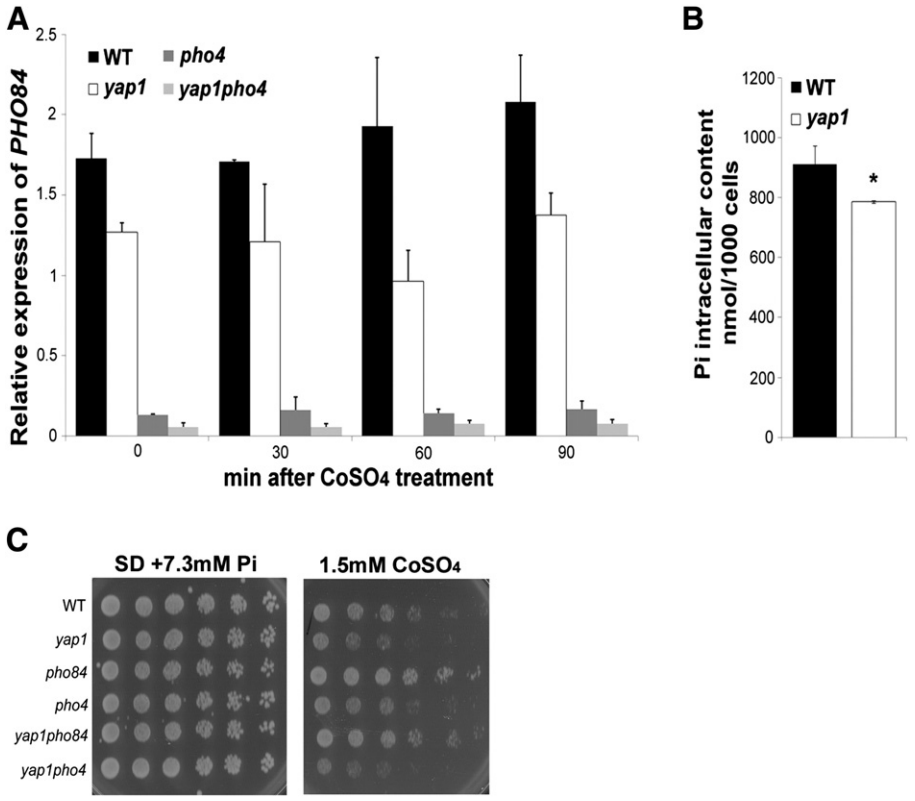

Fig. 7. Yap1 interferes with cobalt and phosphorus intracellular levels. (A) Wild-Type (WT), *yap1*, *pho4 and yap1pho4* strains were challenged with 2 mM of $CoSO_4$ and harvested at the indicated time-points. The expression of *PHO84* gene was evaluated by qRT-PCR. Values are the mean of biological triplicates ± s.d. (B) WT and *yap1* strains were grown to mid-log phase and then upshifted to high-cobalt (2 mM $CoSO_4$), for 16 h. Phosphorus contents were determined by inductively coupled plasma atomic emission spectroscopy. The values are expressed in nmol/1000 cells and correspond to the mean of at least three independent measurements ± standard deviation. Differences between WT and *yap1* strains are denoted as *$p<0.05$. (C) Exponentially growing cells from WT, single mutants *yap1*, *pho4* and *pho84* and double mutant *yap1pho4 and yap1pho84* were harvested, serially diluted and spotted onto SC medium supplemented with cobalt.

Yap1 only drives the expression of a very small subset of its known targets, under cobalt stress, (Fig. 3). Remarkably only 13 of these genes were detected in the wild-type response to cobalt (Table 2 and Fig. 4) and just two of them (*INO1* and *SRX1*) are not dependent on Yap1 under normal growth conditions (Table 2). The exiguity of the data suggests that cobalt tolerance mediated by this transcription factor is cobalt-specific, but rather should relies on the role of Yap1 as a surveyor of cell injury under normal growth conditions. Alternatively, the

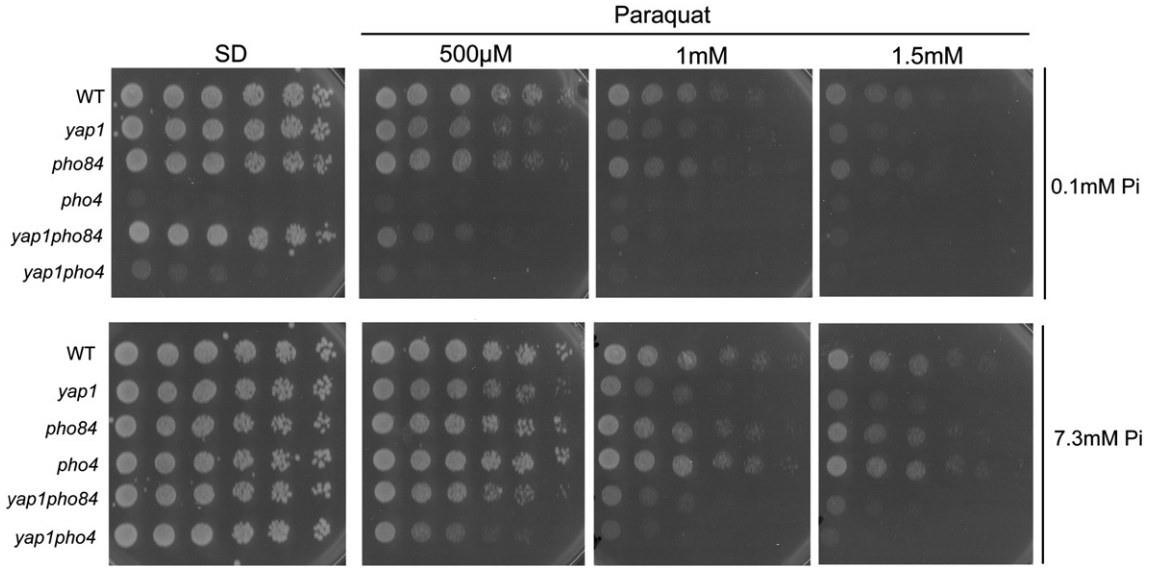

Fig. 8. Phosphate uptake alleviates superoxide stress. Exponentially growing cells from WT, single mutants *yap1*, *pho4* and *pho84* and double mutant *yap1pho4 and yap1pho84* were harvested, serially diluted and spotted onto high phosphate (7.3 mM) or low phosphate (0.1 mM) plates supplemented with the designated paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride) concentrations.

Table 2
Genes dependent on Yap1 whose expression is altered in the WT response to cobalt.

Systematic name	Gene	YREs	Description	Previously documented as dependent on Yap1?[a]	Dependent on Yap1 under normal growth conditions?[b]
YLR109W	AHP1	−313	Thiol-specific peroxiredoxin	Y	Y
YOR383C	FIT3	−126	Mannoprotein	N	Y
YOL151W	GRE2	−777	3-methylbutanal reductase and NADPH-dependent	Y	Y
YDR533C	HSP31	−261	Possible chaperone and cysteine protease	Y	Y
YJL153C	INO1	–	Inositol-3-phosphate synthase	N	N
YOR226C	ISU2	−438	Required for synthesis of mitochondrial and cytosolic iron-	Y	Y
YJR104C	SOD1	−142	Cytosolic copper-zinc superoxide dismutase	Y	Y
YKL086W	SRX1	−144; −255; −294	Sulfiredoxin, contributes to oxidative stress resistance	Y	N
YGR209C	TRX2	−181; −210	Cytoplasmic thioredoxin isoenzyme	Y	Y
YML028W	TSA1	−180	Thioredoxin peroxidase	Y	Y
YLR108C		−343; −169	Protein of unknown function	Y	Y
YML131W		−500	Protein of unknown function	Y	Y
YNL134C		−289; −860; −748	Protein of unknown function	Y	Y

Y — Yes ; N — No.
[a] Based on Yeastract information (http://www.yeastract.com/).
[b] See Table S4.

relative low growth inhibition exerted by the used cobalt concentration (Fig. 1) may as well explain the low number of genes dependent on Yap1 under such conditions.

Notwithstanding with the fact that the *yap1* mutant exhibits lower levels of intracellular cobalt (Fig. 6B), we noticed that disruption of Yap1 increases protein carbonyl content of cells challenged with cobalt (Fig. 5F), highlighting its important role in the protection against cobalt-mediated oxidative stress.

Our data point out a significant redox imbalance of cells treated with cobalt that was more pronounced in the absence of Yap1 (Table 1). Notably, the decrease of GSH levels observed after cobalt treatment is not reflected on GSSG levels increase. This finding suggests that when challenged with cobalt, GSH recycling might be impaired which may be itself a mechanism of cobalt toxicity.

Mitochondrial Fe–S proteins were identified as being major targets of cobalt ions within cells [43,44]. Because Aft1 activation responds to the production of mitochondrial Fe-S clusters [45,46], cobalt attack of the mitochondrial Fe–S enzymes has been proposed to be the mechanism underlying cobalt-induced Aft1 activation [44]. On the other hand, yeast cells depleted of GSH activate Aft1 and exhibit an intense iron starvation response [24,47]. It is therefore possible that cobalt-depleted GSH levels may as well contribute to Aft1 activation.

Accumulated evidences suggest that the role of Yap1 as a mediator of stress response is not confined to the activation of the cellular antioxidant defenses. Indeed, Yap1 also controls the expression of *YCF1* that encodes a vacuolar pump important for arsenic and cadmium sequester into the vacuole [13,48]. We here show that Yap1 also regulates several genes involved in iron homeostasis (Fig. 3). Five of these genes (*FIT2*, *FIT3*, *MRS4*, *ISU2*) are targets of Aft2 [49,50], a transcription factor that together with Aft1 coordinates the cellular response to iron deprivation in yeast [51]. Aft2 has been shown to be dependent on Yap1 [51], which may explain the down-regulation of the aforementioned genes observed in the *yap1* mutant.

Noteworthy, we showed that Yap1 is a negative regulator of the low affinity iron transporter gene, *FET4* (Fig. 6). Although in the particular case of cobalt, this negative regulation had no effect on the intracellular cobalt content (Fig. 6B), we cannot rule out the possibility that Yap1-mediated negative regulation of *FET4* may be the first line of protection conferred by this regulator regarding other metals.

One of the most intriguing findings of this work was the identification of Yap1 as a regulator of the expression of *PHO84*, the gene that encodes the high affinity phosphate transporter (Fig. 7). We have shown that Yap1-dependent regulation of *PHO84* may be an additional mean used by yeast cells to overcome oxidative stress induced by superoxide (Fig. 8). This regulation is observed under normal growth conditions possibly as a defense mechanism against superoxide generated during the aerobic metabolism (Fig. 7A). As such, the paradoxical increase of cobalt uptake mediated by Yap1 (Fig. 6B) may be a side effect of Yap1-dependent *PHO84* regulation under non-stressed conditions.

In conclusion these studies reiterate the relevant role played by Yap1 in providing protection against metal-induced oxidative stress and bring to light new possible Yap1-mediated mechanisms of stress tolerance.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.01.032.

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