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Repression of the Low Affinity Iron Transporter Gene *FET4*

A NOVEL MECHANISM AGAINST CADMIUM TOXICITY ORCHESTRATED BY YAP1 VIA ROX1[*]

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Background: Yap1 regulates cadmium accumulation in the vacuole and mitigates cadmium-induced ROS.
Results: Yap1 induces the gene of the hypoxic repressor Rox1 that in turn represses *FET4*, avoiding cadmium uptake.
Conclusion: Repression of *FET4*, via Rox1, is a novel line of defense mediated by Yap1 against cadmium toxicity.
Significance: Evidence of cross-talk between oxidative and hypoxic regulators that results in increased tolerance to metal toxicity.

Cadmium is a well known mutagenic metal that can enter cells via nonspecific metal transporters, causing several cellular damages and eventually leading to death. In the yeast *Saccharomyces cerevisiae*, the transcription factor Yap1 plays a key role in the regulation of several genes involved in metal stress response. We have previously shown that Yap1 represses the expression of *FET4*, a gene encoding a low affinity iron transporter able to transport metals other than iron. Here, we have studied the relevance of this repression in cell tolerance to cadmium. Our results indicate that genomic deletion of Yap1 increases *FET4* transcript and protein levels. In addition, the cadmium toxicity exhibited by this strain is completely reversed by co-deletion of *FET4* gene. These data correlate well with the increased intracellular levels of cadmium observed in the mutant *yap1*. Rox1, a well known aerobic repressor of hypoxic genes, conveys the Yap1-mediated repression of *FET4*. We further show that, in a scenario where the activity of Yap1 or Rox1 is compromised, cells activate post-transcriptional mechanisms, involving the exoribonuclease Xrn1, to compensate the derepression of *FET4*. Our data thus reveal a novel protection mechanism against cadmium toxicity mediated by Yap1 that relies on the aerobic repression of *FET4* and results in the impairment of cadmium uptake.

Cadmium contamination poses a threat to the environment and human health, because cadmium is extremely toxic and carcinogenic even at low concentrations (1). Environmental contamination with this metal arises from both natural and anthropogenic sources. Human exposure to cadmium mainly occurs through contaminated dietary sources or by inhalation of tobacco smoke or polluted air, leading to its accumulation in the liver, kidney, and lungs (2).

The precise molecular mechanism of cadmium toxicity is not fully understood, but it is thought to cause injury primarily via oxidative-induced cellular damages. Cadmium is, however, unable to directly generate free radicals, but it is assumed to induce the formation of reactive oxygen and nitrogen species by two distinct mechanisms. One of those mechanisms involves the displacement of metals from proteins that, once unbound, may generate oxidative stress via Fenton reactions; the other relies on cadmium ability to inhibit the activity of antioxidant enzymes, such as superoxide dismutase and catalase (3, 4). Other forms of cadmium cellular toxicity include the inhibition of the DNA mismatch repair system (5), the induction of iron deficiency (6–8), and the disturbance of the homeostasis of other metals (9–11).

Cadmium enters cells through transporters evolved for the uptake of essential metals, such as iron, zinc, manganese, and calcium (7, 12–16). The budding yeast *Saccharomyces cerevisiae* has long been used as model organism to study the molecular mechanisms of cadmium toxicity and tolerance. The most relevant mechanism of cadmium detoxification in yeast relies on its accumulation in the vacuole, which is strongly dependent on Ycf1, a vacuolar membrane transporter of the ABC family. Ycf1 transports cadmium conjugated with glutathione into the vacuole (17). Two Ycf1 paralogues, Bpt1 and Vmr1, and the zinc transporter Zrc1 also play a minor role in vacuolar cadmium sequestration (18, 19). Another cadmium detoxification system in yeast relies on Pca1, a plasma membrane P-type ATPase involved in cadmium efflux (20). In addition, two other plasma membrane transporters, Alr1 and Yor1, have been implicated in cadmium detoxification; however, little is known regarding their mode of action (21, 22). Also the yeast internal Ca^{2+} transporters, Pmr1 and Pmc1 can act as ancillary pathways to cope with cadmium toxicity, particularly when Ycf1 activity is compromised (23, 24).

In yeast, the transcriptional regulator Yap1 plays a central role in cadmium stress response, by activating antioxidant genes and by inducing the expression of *YCF1* (25). Yap1 senses cadmium by means of the direct interaction of its C-terminal

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TABLE 1
***Saccharomyces cerevisiae* strains used in this work**

Strain	Description	Source
BY4742	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0*	Euroscarf
BY4742 *yap1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 yap1Δ::kanMX4*	Euroscarf
BY4742 *rox1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 rox1Δ:: kanMX4*	Euroscarf
BY4742 *fet4*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 fet4Δ:: kanMX4*	Euroscarf
BY4742 *xrn1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 xrn1Δ:: kanMX4*	This study
By4742 *rnt1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 rnt1Δ:: kanMX4*	This study
BY4742 *yap1fet4*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 fet4Δ:: kanMX4 yap1Δ:: his3Δ1*	Ref. 28
BY4742 *yap1xrn1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 xrn1Δ:: kanMX4 yap1Δ:: his3Δ1*	This study
BY4742 *yap1rnt1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 rnt1Δ:: kanMX4 yap1Δ:: his3Δ1*	This study
BY4742 *yap1rox1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 yap1Δ::* kanMX4 *rox1Δ:: his3Δ1*	This study
BY4742 *yap1xrn1fet4*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 xrn1Δ:: kanMX4 yap1Δ:: his3Δ1 fet4Δ:: ura*	This study

cysteine rich domain with the drug (26). As a consequence, Yap1 nuclear export signal becomes masked, and the factor accumulates in the nucleus, activating its target genes (27).

We have recently shown that under normal growth conditions Yap1 is a negative regulator of *FET4* (28). Fet4 is a cell surface low affinity iron transporter able to transport other divalent metals, including toxic cadmium ions (14). In this context, we put forward the hypothesis that Yap1-mediated negative regulation of *FET4* may be a novel line of protection conferred by this regulator against cadmium insult. We show herewith that Yap1 represses *FET4* gene expression via Rox1, which results in a reduction of cadmium uptake. We also show that, when challenged with cadmium, *yap1* and *rox1* mutant strains trigger post-transcriptional mechanisms, dependent on the 5′-3′ exoribonuclease Xrn1 that compensates the derepression of *FET4* gene expression.

Experimental Procedures

Yeast Strains, Plasmids, and Growth Conditions—The yeast strains used in this study are listed in Table 1. All mutants constructed in this work were generated using the microhomology PCR method (30).

To construct the p*ROX1-lacZ* plasmid, a fragment comprising a 1-kb sequence upstream of *ROX1* ATG codon was amplified by PCR using the primers listed in Table 2. PCR product was first digested with BamHI, treated with Klenow, and next digested with SphI. The resulting fragment was cloned into the YEp356R vector previously digested with EcoRI, treated with Klenow, and cut with SphI.

To generate the C-terminal HA-tagged version of *FET4* (*FET4-HA*), one fragment comprising 1 kb upstream from the ATG plus the *FET4* coding region and another including 0.5 kb downstream from the stop codon were amplified by PCR using the primers listed in Table 2. The HA sequence was inserted in frame with *FET4* coding region just before the TAG stop codon. Both fragments were inserted into the pRS416 vector, previously linearized with SmaI, by homologous recombination using the In-Fusion Advantage PCR cloning kit (Clontech).

To construct the *p-ROX1* plasmid, *ROX1* gene was amplified by PCR with specific primers (Table 2). The resulting fragment was inserted into the SmaI site of pRS416. The *MUTp-ROX1* plasmid was generated using as template *p-ROX1*, and the primers depicted in Table 2 were used in a PCR-directed mutagenesis reaction to mutate the YRE[2] site located at −414 bp, as detailed in Ref. 31. A similar and sequential strategy was used to construct *MUTp-FET4-HA* using the primers listed in Table 2.

Yeast strains were grown in synthetic medium (SC) or medium lacking specific requirements (SD), as previously described (31). Phenotypic growth assays were carried out by spotting 5 μl of cultures in early exponential phase (A_{600} = 0.4–0.5) sequentially diluted (~5×10^3 to 50 cells) in medium containing the indicated concentrations of $CdCl_2$ and supplemented or not with $FeSO_4$. These assays were repeated at least twice. Cultures were grown for 2 days at 30 °C. The bacteria *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.

Measurements of β-Galactosidase Activity—The BY4742 (wild-type) and the *yap1* mutant strains were transformed with the p*ROX1-lacZ* plasmid. Cells were grown in liquid SD medium until the early exponential phase in the presence or absence of 25 μM $CdCl_2$ and harvested after 15 min. Relative β-galactosidase activity was monitored as described in Ref. 31. Enzymatic activity was assayed by following the degradation of the colorimetric substrate *O*-nitrophenyl-β-D-galactopyranoside at A_{420} and normalizing against A_{600}. The results are the average of at least six biological replicates.

Immunoblot Assays—Wild-type, *yap1* and *rox1* mutant strains containing the *FET4-HA* or *MUTp-FET4-HA* plasmids were grown until the early exponential phase (A_{600} = 0.4–0.5) and exposed to 25 μM $CdCl_2$. Cells were harvested at different time points after treatment with $CdCl_2$. Total proteins were extracted from cell cultures as described in Ref. 31. Proteins were resolved in a 10% SDS-PAGE and immunoblotted with horseradish peroxidase-bound anti-HA IgG (Roche). In what concerns PMSF treatment, cells were grown until the early exponential phase, exposed to 1.2 mM of PMSF for 90 min, and supplemented (or not) with $CdCl_2$ for 1 h. Pgk1 was used as loading control. Immunoblots were repeated at least twice with different protein extracts.

Quantitative Real Time RT-PCR Analyses—Cells were grown until the early exponential phase, cultures were left untreated or treated with 25 μM $CdCl_2$. Cells were harvested at the indi-

[2] The abbreviations used are: YRE, Yap-responsive element; ICP-AES, inductively coupled plasma atomic emission spectroscopy; qRT-PCR, quantitative RT-PCR.

TABLE 2
Oligonucleotides

Primer	Sequence	Use
ACT1	5′-CTATTGGTAACGAAAGATTCAG-3′	qRT-PCR
	5′-CCTTACGGACATCGACATCA-3′	
CTH2	5′-AGGTATGCTGCTGGAGCTGT-3′	qRT-PCR
	5′-GAGGGCCATGAAGGTATCAA-3′	
FET4	5′-GGAGAACTGCCTGTGGAAAA-3′	qRT-PCR
	5′-TTCTCCGGTGTAAGGTGGAG-3′	
FET3	5′-ACGGTGTGAATTACGCCTTC-3′	qRT-PCR
	5′-TTGGAAAGCGTGACCATGTA-3′	
ROX1	5′-AGGGCTTACAACCGGAAGAT-3′	qRT-PCR
	5′-GCTGTTGCTCGATTTCCTTC-3′	
CUP1	5′-TGAAGGTCATGAGTGCCAAT-3′	qRT-PCR
	5′-GCATTTGTCGTCGCTGTTAC-3′	
ARN2	5′-AGGTATGCTGCTGGAGCTGT-3′	qRT-PCR
	5′-GAGGGCCATGAAGGTATCAA-3′	
ACT1-ChIP	5′-GATCCTTTCCTTCCCAATCTCTCTTG-3′	ChIP
	5′-GCTCATGTAGTAGAAGATCCTATT-3′	
ARN2	5′-GGTATGCTGCTGGAGCTGT-3′	ChIP
	5′-AGGGCCATGAAGGTATCAA-3′	
ROX1-414bp	5′-GCAAAACAATTGGAAATCTGG-3′	ChIP
	5′-GAACAACAAAAGAGGCAGCA-3′	
ROX1-897bp	5′-TCTACATAATGCACGAAACTTGG-3′	ChIP
	5′-CGCAGTGTGTGTTCCTGTCT-3′	
ROX1p'1000	5′-CTAGCATGCAGTTGACCTACATTCAAC-3′	*pROX1*
A4-*ROX1*	5′-GGATTTCGCATCCTAGACCA-3′	
ROX1m Fw	5′-TGGCGATTGAAGACAAAGAAGAAA-3′	*MUT-pROX1*
ROX1m Rv	5′-TTTCTTCTTTGTCTTCAATCGCCA-3′	
ROX1-ATG-codon	5′-CTAGCATGCAGTTGACCTACATTCAAC-3′	*pROX1-lacZ*
	5′-CTTGGATCCGGATTCATTGTTGATTGTC-3′	
FET4-ORF	5′-GAATTCCTGCAGCCCCTGTGCTTGCTGTTC-3′	*pFET4-HA*
	5′-ATGTACCCATACGATGTTCCAGATTACGCTTAGCTTCATTGAACA-3′	
0.5Kb-*FET4*-terminator	5′-AGCGTAATCTGGAACATCGTATGGGTACATTTTTTCCAACATCAT-3′	*pFET4-HA*
	5′-ACTAGTGGATCCCCCGACATATAAGCGGAG-3′	
FET4m1 Fw	5′-GCCTTCTTAATTGAGTTTAGCATC-3′	*MUT-pFET4-HA (Aft1 site 1 deletion)*
FET4m1 Rv	5′-GATGCTAAACTCAATTAAGAAGGC-3′	
FET4m2 Fw	5′-GTTCCGAAAACCCACTTTTTGTTC-3	*MUT-pFET4-HA (Aft1 site 2 deletion)*
FET4m2 Rv	5′-GAACAAAAAGTGGGTTTTCGGAAC-3′	

cated time points, and RNA was isolated. RNA samples were next treated with DNase (TURBO™ DNase-free; Ambion) according to the manufacturer's instructions and purified. Total RNA (1 μg) was reverse transcribed with transcriptor reverse transcriptase (Roche Diagnostics). Quantitative PCRs were performed in the Light Cycler 480 II real time PCR system (Roche), using Light Cycler 480 SYBR Green I Master (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 Light Cycler 480 software 1.5 (32). Actin gene was used as a reference gene. All assays were made using biological and technical triplicates. Primers used in this assay are listed in Table 2.

ChIP Analysis—ChIP assays were carried out as previously described (31). Cells transformed with a c-Myc-tagged version of Yap1 (27) and treated with cadmium were harvested at A_{600} 0.6 and fixed with 1% formaldehyde. The cross-linking was stopped by addition of glycine, and cells were disrupted with a FastPrep®-24 instrument (MP Biomedical). Cell extracts were sonicated to yield DNA fragments with an average of 500 bp. c-Myc-tagged Yap1 was immunoprecipitated by incubating the cross-linked chromatin with a c-Myc antibody prebound to Dynabeads Pan mouse IgG (Invitrogen) for 16 h at 4 °C. Immunoprecipitated proteins were eluted from the beads by heating the samples in appropriate buffer, and fixation was reversed. Aliquots of total chromatin input (IN) and immunoprecipitated chromatin were simultaneously processed for subsequent normalization. After sample treatment with proteinase K and RNase A, DNA was purified. Quantification of specific DNA targets in the input and immunoprecipitated samples was performed by quantitative PCR. A standard curve, generated with a dilution series of the immunoprecipitated sample, was used to assess the PCR efficiency. The relative enrichment of a specific pRox-YRE in the immunoprecipitate was determined using the $\Delta\Delta C_T$ method through the calculation of log2 (immunoprecipitated/input). The primer sequences used are listed in Table 2. The primers *ROX1*-414bp and *ROX1*-897bp were used to amplify the regions of the *ROX1* promoter flanking both YRE sites located at −414 and −897 bp from the ATG, respectively. *ARN2* was used as a negative control.

Measurement of Cadmium and Iron—Strains were grown to early exponential phase (A_{600} 0.4–0.5) in SC medium and left untreated or treated with 25 μM $CdCl_2$. Cells were harvested after 6 h of stress induction, collected by centrifugation, and washed with 10 mM EDTA and metal-free water. This time point was chosen because, in all strains, the maximum of growth inhibition was observed after 6 h of treatment with cadmium (data not shown). The total cadmium and iron content was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The data were normalized against A_{600}. All assays were made using biological triplicates.

Results

Yap1 Regulation of FET4 Contributes to S. cerevisiae Cadmium Tolerance—Our previous work indicated that Yap1 is a negative regulator of *FET4* (28), a gene encoding a low affinity

iron transporter also involved in the transport of zinc, cobalt, manganese, and cadmium (14, 33). To evaluate whether the uptake of cadmium via *FET4* could contribute to the well known sensitivity exhibited by the *yap1* mutant to this metal, we first tested *FET4* dependence on Yap1 by qRT-PCR (Fig. 1*A*). As illustrated in Fig. 1*A*, *FET4* expression is induced in *yap1* unstressed cells. A sharp decrease of *FET4* levels was, however, observed after cadmium challenge. Indeed, after 20 min of cadmium exposure, the levels of *FET4* transcripts in the *yap1* mutant and WT strains were comparable. We have also assessed the protein expression of a HA-tagged version of Fet4 driven by its native promoter. In agreement with the gene expression data, we observed that protein levels were consistently higher in the *yap1* mutant compared with the WT strain, being rapidly reduced after cadmium exposure (Fig. 1, *B* and *C*).

We next examined the growth phenotype of the double mutant *yap1fet4* in the presence of cadmium (Fig. 1, *D* and *E*). If repression of *FET4* by Yap1 is required to prevent cadmium toxicity, one would expect the double mutant to be more resistant than the single *yap1* mutant to this metal. As anticipated, the double mutant *yap1fet4* grew better than the *yap1* strain in the presence of cadmium (Fig. 1, *D* and *E*). In addition, reintroduction of *FET4* into the *yap1fet4* mutant restores cadmium sensitivity (Fig. 1*F*). Accordingly, intracellular cadmium levels are increased ~30% in the *yap1* mutant when compared with the WT, whereas in the *fet4* and *yap1fet4* mutants, these values are 50–60% lower than in the *yap1* mutant (Fig. 1*G*). Moreover, supplementation of the growth medium with iron attenuates the *yap1* mutant sensitivity, suggesting the involvement of an iron transporter in this process (Fig. 1*E*). To test this, we also measured cadmium contents when cells were simultaneously treated with iron (Fig. 1*G*). Contrary to our expectations, we noticed that in the presence of iron, cadmium uptake increases. In an attempt to understand this apparently contradictory finding, we revisited our data on the genome-wide transcriptional analysis of *S. cerevisiae* exposed to high iron conditions (31) and searched for genes whose expression was induced by high iron and that may play a role in cadmium detoxification. We found that metallothionein genes *CUP1-1* and *CUP1-2* were up-regulated in response to high iron (Table S1 in Ref. 31). Cup1-1 overexpression confers resistance to cadmium ions (34), and by qRT-PCR we found that *CUP1* genes are highly expressed when cells are treated simultaneously with cadmium and iron (Fig. 1*H*). This finding strongly suggests that iron attenuates cadmium toxicity by inducing *CUP1* expression, which possibly binds and sequesters cadmium. Altogether, these data shed light on a novel Yap1-mediated mechanism of cadmium stress tolerance involving the Fet4 low affinity iron transporter.

Yap1 Is a Direct Regulator of the Repressor ROX1—The promoter region of *FET4* does not contain any canonical YREs (35), a fact that strongly suggests that the regulation of *FET4* by Yap1 involves another regulator. Strikingly, our previous microarray data indicate that Yap1 is a positive regulator of *ROX1* gene (28). Rox1 is a repressor of anaerobic genes and also represses *FET4* expression under oxygenated conditions (29, 36). Remarkably, microarray data equally revealed that all the genes up-regulated in the *yap1* mutant are also known Rox1 targets (see Ref. (28) and Table 3). Furthermore, using YEASTRACT (37), we verified that the promoter region of *ROX1* possesses two YRE sites, located at −414 and −897 bp upstream the ATG codon (Fig. 2*A*).

In line with these data, we bring forward the hypothesis that Yap1 might repress *FET4* via *ROX1* induction. As a first approach to test this, a plasmid including the promoter region of *ROX1* fused to the *lacZ* reporter gene (p*ROX1-lacZ*) was generated and used to transform the WT and *yap1* mutant strains. We observed that β-galactosidase activity was higher in the WT compared with the mutant strain, even in the absence of cadmium (Fig. 2*B*). A slight but significant increase of the β-galactosidase activity was noticed in both strains upon cadmium stress. These results were further confirmed by the analysis of the levels of *ROX1* transcripts in both strains, in either the absence or the presence of cadmium, by qRT-PCR (Fig. 3*B*).

We next examined whether Yap1 is a direct regulator of *ROX1* by carrying out ChIP analyses. A Yap1 c-Myc-tagged version was used to test whether each of the YRE sites found in the *ROX1* promoter region (Fig. 2*A*) was recognized by this factor. As depicted in Fig. 2*C*, after immunoprecipitation of the chromatin bound to Yap1 c-Myc, an enrichment of *ROX1* promoter harboring the YRE located at −414 bp was observed. No enrichment was noticed in the *ROX1* region comprising the YRE located at −897 bp or in the promoter region of the *ARN2* gene (used as a negative control). The increased enrichment in the −414-bp YRE-containing sequence after cadmium treatment correlates well with Yap1 nuclear accumulation kinetics (26). Although the above data clearly indicate that Yap1 is a direct regulator of *ROX1*, we observed that *rox1* strain is more sensitive to cadmium than *yap1* (Fig. 3*A*). This finding suggests that Yap1 cannot be the only regulator of *ROX1*. In agreement, *ROX1* transcripts are not fully dependent on Yap1 (Fig. 3*B*), and *FET4* mRNA (Fig. 3*C*), Fet4 protein levels (Fig. 3, *D* and *E*), and cadmium intracellular contents (Fig. 3*F*) are higher in the *rox1* mutant than in the *yap1* strain. The results here described clearly show that, although partially, Yap1 directly regulates *ROX1* expression.

Rox1 Mediates Yap1 Repression of FET4—To confirm whether Yap1 regulates *FET4* expression via Rox1, we cloned *ROX1* gene and mutated the functional YRE located at −414 bp (Figs. 2*C* and 4*A*). The resulting constructs (*p-ROX1* and *MUTp-ROX1*) were next used to transform the double mutant strain *yap1rox1* and the single mutant strain *rox1*. *FET4* expression was thereafter assayed by qRT-PCR (Fig. 4*B*).

Supporting our hypothesis, we noticed that the expression of *FET4* was higher in *rox1* cells transformed with *MUTp-ROX1* than with *p-ROX1* (Fig. 4*B*). The levels of *FET4* transcripts in the former were close to those exhibited by the double mutant *yap1rox1* transformed with *p-ROX1*. As expected, *ROX1* levels were decreased in *rox1* strain transformed with *MUTp-ROX1* and in the double mutant transformed with *p-ROX1* (Fig. 4*C*). These data support that Yap1 mediates the repression of *FET4* via Rox1.

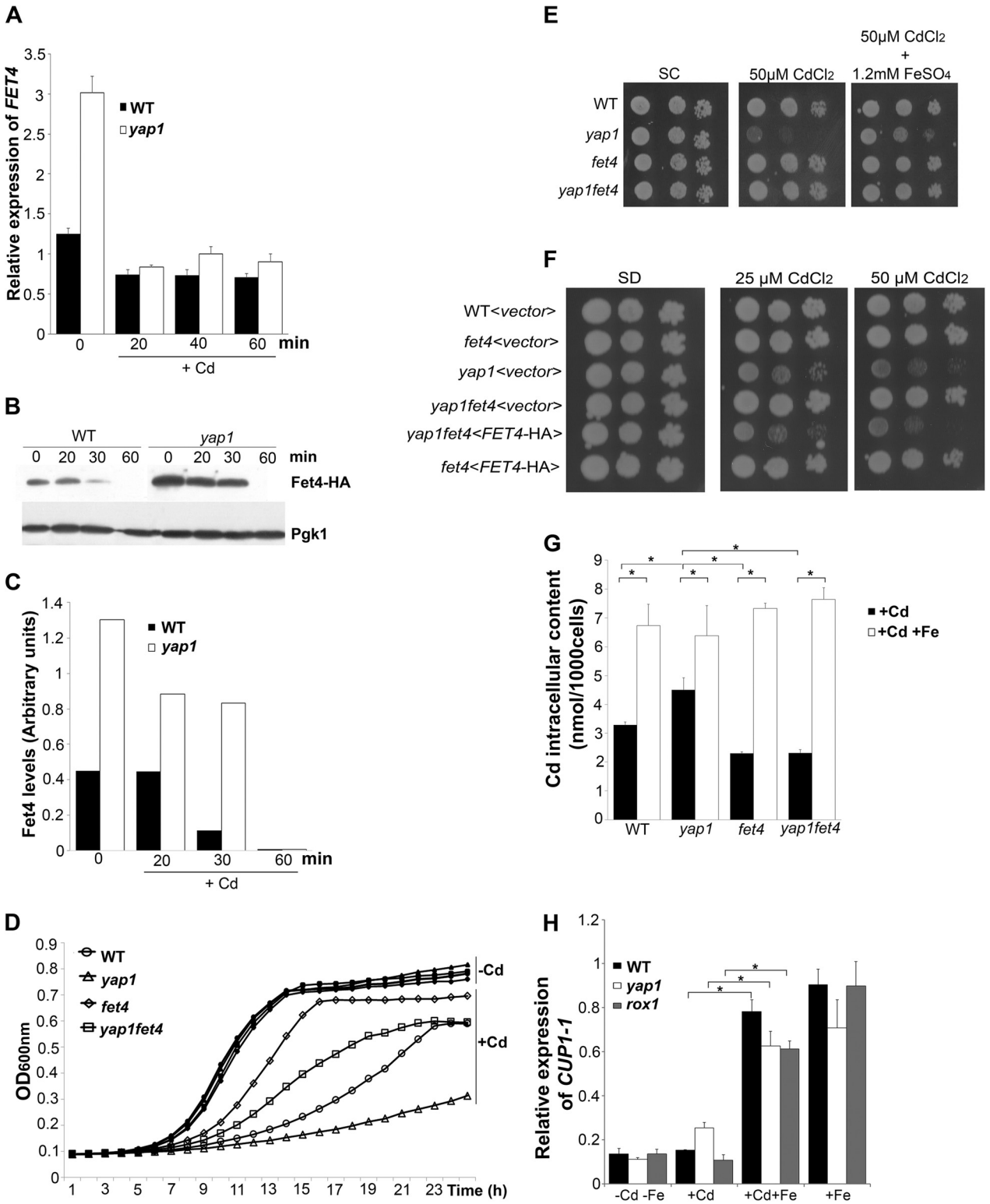

FIGURE 1. **Yap1 mediates *FET4* repression.** *A*, WT and *yap1* strains were challenged with 25 μM $CdCl_2$ and harvested at the indicated time points. The expression of *FET4* was monitored by qRT-PCR. *B*, the WT strain and the *yap1* mutant were transformed with a plasmid containing *FET4*-HA, cultures were treated with 25 μM $CdCl_2$, and Fet4 expression was analyzed by Western blot with an anti-HA antibody. *C*, Fet4 protein levels were normalized to Pgk1, from *B*. *D*, growth exhibited by the WT, *yap1*, *fet4*, and *yap1fet4* strains in SC medium was recorded over a period of 24 h. The *open* and *closed symbols* represent strains grown in media containing or not 25 μM $CdCl_2$, respectively. *E*, growth sensitivity exhibited by the WT, *yap1*, *fet4*, and *yap1fet4* strains in SC plates containing 50 μM $CdCl_2$ and supplemented with 1.2 mM $FeSO_4$. *F*, exponentially growing WT, *yap1*, *fet4*, and *yap1fet4* cells were transformed with the *FET4*-HA plasmid (<*FET4-HA*>) or with the empty vector (<*vector*>), harvested, serially diluted, and spotted onto SD plates or SD plates supplemented with 25 or 50 μM $CdCl_2$. *G*, cadmium content of WT, *yap1*, *fet4*, and *yap1fet4* strains were determined by ICP-AES, after treatment with 25 μM $CdCl_2$ (+*Cd*) or after treatment with 25 μM $CdCl_2$ and 1.2 mM of $FeSO_4$ (+*Cd* +*Fe*) for 6 h. *H*, WT, *yap1* and *rox1* strains were grown in medium left untreated (−*Cd*−*Fe*) or treated with 25 μM $CdCl_2$ (+*Cd*); with 25 μM $CdCl_2$ and 1.2 mM of $FeSO_4$ (+*Cd* +*Fe*) or 1.2 mM of $FeSO_4$ (+*Fe*). *CUP1* expression levels were assessed by qRT-PCR after 15 min of treatment. In this figure, all values are the means of at least biological triplicates ($n = 3$) ± S.D. Significance of differences was calculated with the *t* test. *, $p < 0.05$.

TABLE 3

Rox1 target genes that appear upregulated in *yap1* mutant cells

This information is adapted from Ref. 28.

Systematic name	Gene name	Fold change	Description
YLR034C	*SMF3*	1.3	Member of the Nramp family of the metal transport proteins
YMR319c	*FET4*	4.5	Low affinity Fe(II) transporter of the plasma membrane
YER014w	*HEM14*	1.5	Protoporphyrinogen oxidase
YKL008c	*LAC1*	1.6	Ceramide synthase component
YHR007c	*ERG11*	1.7	Lanosterol 14-α-demethylase
YDR297w	*SUR2*	1.7	Sphinganine C4-hydroxylase
YDR004w	*HEM13*	2.2	Coproporphyrinogen III oxidase
YIL11w	*COX5B*	1.7	Subunit Vb of cytochrome c oxidase
YBR085w	*AAC3*	2.2	Mitochondrial inner membrane ADP/ATP translocator
YEL047c	NA	1.6	Soluble fumarate reductase
YDR518w	*EUG1*	1.4	Disulfide isomerase of the endoplasmatic reticulum lumen
YHR179w	*OYE2*	1.4	Conserved NADPH oxidoreductase
YAL028w	*FRT2*	1.5	Tail-anchored endoplasmic reticulum membrane protein
YNR014w	NA	1.4	Putative protein of unknown function

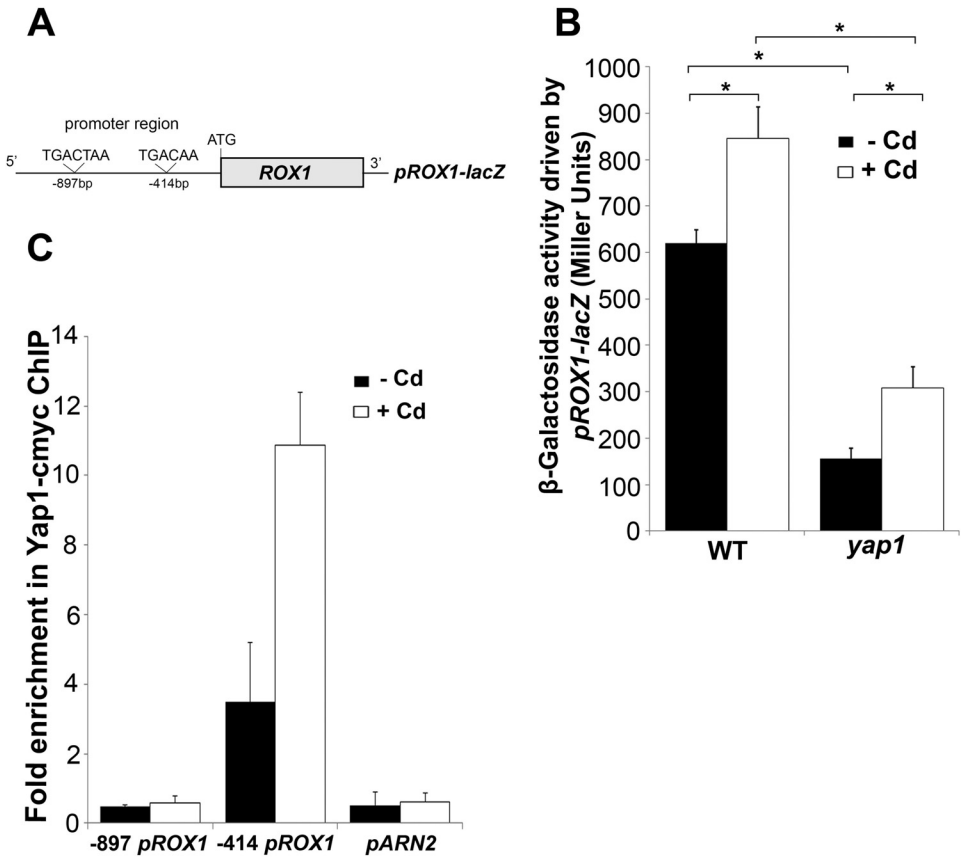

FIGURE 2. **Yap1 is a direct regulator of *ROX1*.** *A*, schematic representation of the *ROX1* promoter region containing the two YREs sites located at −897 and −414 bp upstream the ATG. *B*, WT and *yap1* mutant strains were transformed with the p*ROX1-lacZ* plasmid. Cells were grown exponentially in SD medium and challenged with 25 μM $CdCl_2$ for 15 min. β-Galactosidase activity was assayed as detailed under "Experimental Procedures." The values are the means of biological decaplicates ± S.D. Significance of differences was calculated with the *t* test. *, $p < 0.05$. *C*, *yap1* mutant cells transformed with a plasmid containing a c-Myc-tagged version of Yap1 were grown exponentially in SD medium and treated with 25 μM $CdCl_2$ for 10 min. ChIP analyses combined with quantitative PCR were used to determine the fold enrichment of each YRE. The promoter region of *ARN2* was used as a negative control, because it does not possess YREs. The values are the means of at least biological triplicates ± S.D.

The Exoribonuclease 5′-3′ Xrn1 Alleviates the FET4 Derepression Observed in the yap1 Mutant—Xrn1 is an exoribonuclease responsible for the degradation of mRNAs from the 5′ to the 3′ end. This protein is conserved in all eukaryotes and is involved in the normal mRNA decay (38). We have recently shown that arsenate stress triggers Xrn1-mediated degradation of *FET3* transcripts (39).

In an attempt to determine whether Xrn1 is also involved in the decrease of *FET4* mRNAs levels observed in the *yap1* mutant after cadmium treatment (see Figs. 3*C* and 1*A*), we first constructed the double mutant *yap1xrn1* and assayed its cadmium sensitivity. We observed that the double mutant is more sensitive to cadmium than the *yap1* or *xrn1* single mutants (Fig. 5*A*). We next monitored *FET4* mRNA levels in these strains and observed that in both *xrn1* and *yap1xrn1* mutants the accentuated drop of *FET4* levels after stress was no longer evident (Fig. 5*B*). To assess whether the higher sensitivity of the *yap1xrn1* mutant to cadmium was due to higher levels of *FET4* expres-

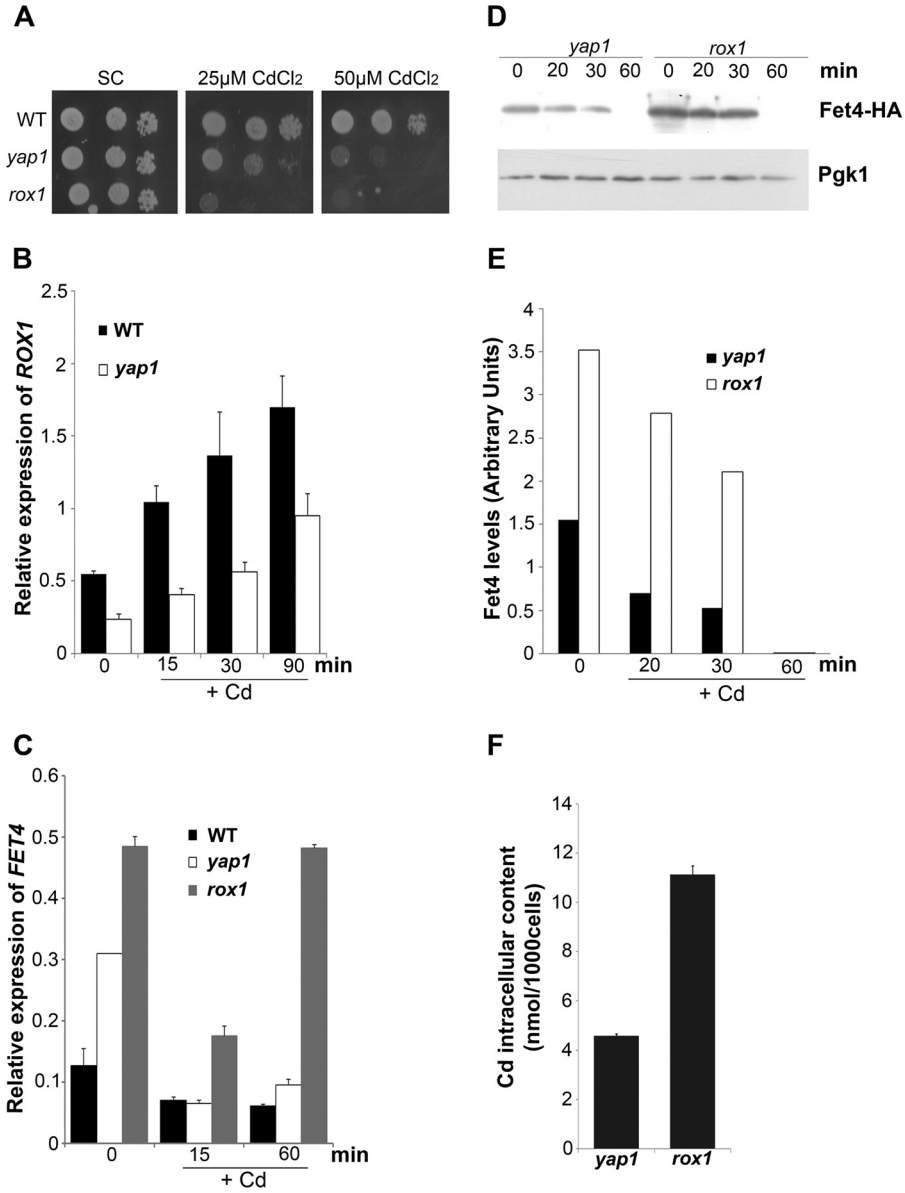

FIGURE 3. **Yap1 partially regulates *ROX1*.** *A*, growth sensitivity exhibited by WT, *yap1*, and *rox1* strains in SC plates containing 25 or 50 μM $CdCl_2$. *B*, *ROX1* expression in the WT and *yap1* mutant was analyzed by qRT-PCR at the indicated time points, after treatment with 25 μM $CdCl_2$. *C*, *FET4* expression in the WT and *yap1* and *rox1* mutants was analyzed by qRT-PCR, at the indicated time points after treatment with 25 μM $CdCl_2$. *D*, *yap1* and *rox1* mutants were transformed with *FET4-HA* plasmid and treated with 25 μM $CdCl_2$. Fet4 protein levels were analyzed by Western blot, at the indicated time points. *E*, Fet4 protein levels were normalized to Pgk1, from *D*. *F*, cadmium intracellular content in *yap1* and *rox1* mutant strains was determined by ICP-AES, after treatment with 25 μM $CdCl_2$ for 6 h. The values are the means of at least biological triplicates ± S.D.

sion, we constructed the triple mutant *yap1xrn1fet4* and observed that it is more tolerant to cadmium compared with the double mutant (Fig. 5, *C* and *D*). Overall, these data strongly suggests that a post-transcriptional mechanism involving Xrn1 may counteract the derepression of *FET4* observed in the *yap1* mutant after cadmium insult.

Degradation of FET4 by Xrn1 Appears to Be Stress-specific—To understand whether *FET4* degradation mediated by Xrn1 was stress-specific, we examined and compared the levels of *FET4* and *FET3* in the mutant *xrn1* after treatment with cadmium or arsenate. In the absence of stress, *FET3* and *FET4* mRNA levels are dependent on Xrn1 (Fig. 6, *A* and *B*, time point 0). As we have previously described (39) and herein confirmed by qRT-PCR, the drop of *FET3* mRNA levels in response to arsenate is dependent on Xrn1 (Fig. 6*A*). Contrary to *FET3*, *FET4* mRNA levels are no longer dependent on Xrn1 after arsenate treatment (Fig. 6*B*). In response to cadmium, however, *FET4* mRNA levels continue to be strongly dependent on Xrn1 (Fig. 6*C*), whereas *FET3* transcripts become insensitive to this exoribonuclease (Fig. 6*D*).

Some of the mRNAs degraded by Xrn1 are first cleaved by Rnt1, a double-stranded RNA endonuclease (40). Rnt1 specifically recognizes particular RNA hairpins and therefore recognizes its targets (40). Although *FET4* transcripts do not contain such hairpin structures and are not affected by Rnt1 under normal growth conditions (41), we could not certainly rule out the possibility that a different scenario occurs in response to cadmium. Indeed, if Rnt1 specifically recognizes *FET4* transcripts

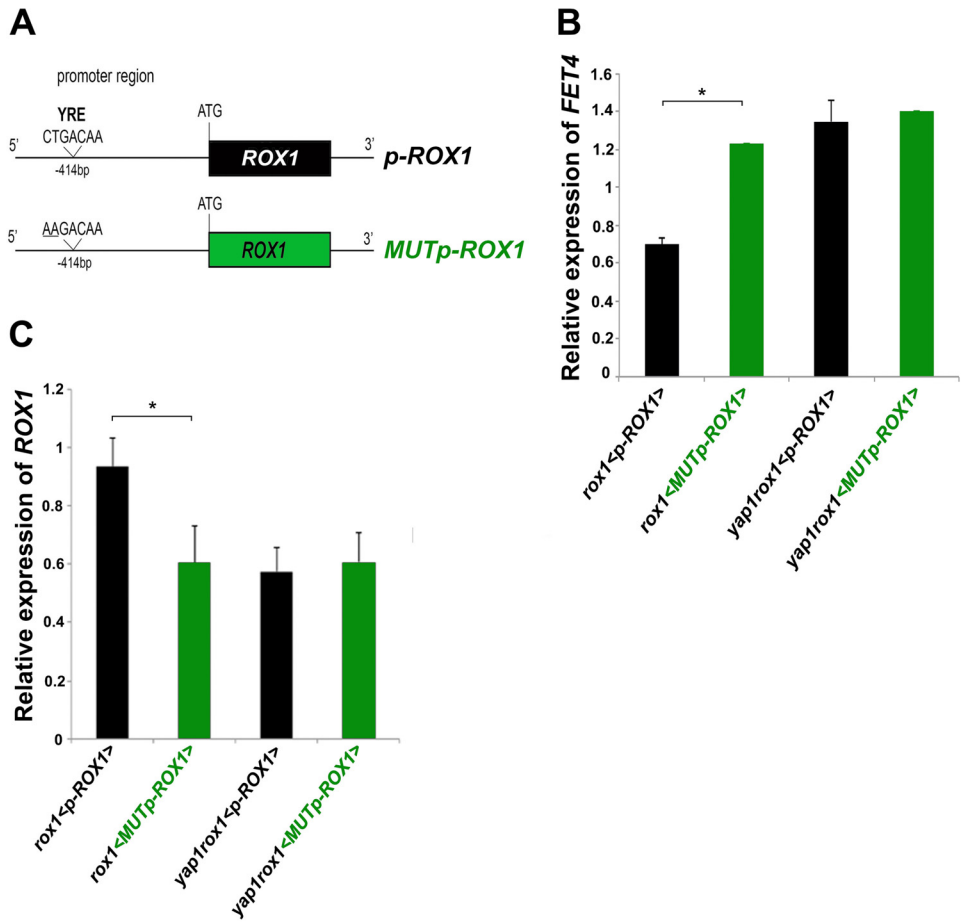

FIGURE 4. **Rox1 mediates Yap1 repression of *FET4*.** *A*, schematic representation of the constructs *p-ROX1* and *MUTp-ROX1*. *MUTp-ROX1* possesses two mutations in the functional YRE (CTGACAA to AAGACAA). *B* and *C*, *FET4* (*B*) and *ROX1* (*C*) expression in *rox1* and *yap1rox1* mutant strains transformed with *p-ROX1* (*black*; <*p-ROX1*>) or *MUTp-ROX1* (*green*; < *MUTp-ROX1*>) was analyzed by qRT-PCR. The values are the means of at least biological triplicates ± S.D. Significance of differences was calculated with the *t* test. *, $p < 0.05$.

in the presence of cadmium, one would expect *FET4* mRNA levels to be highly dependent on Xrn1. To test this, we constructed the single and the double mutants *rnt1* and *yap1rnt1* and examined their growth phenotype in the presence of cadmium, as well as *FET4* gene expression by qRT-PCR. We observed that cells lacking Rnt1 are not sensitive to cadmium toxicity and that deletion of *RNT1* gene from the *yap1* background does not aggravate the sensitive growth phenotype exhibited by the *yap1* mutant to cadmium (Fig. 6*E*). In addition, our results indicate that the absence of Rnt1 does not affect *FET4* mRNA levels either under normal (as previously shown in Ref. 41) or under cadmium stress conditions (Fig. 6*F*). Overall, our results show that the mechanism by which Xrn1 controls *FET4* transcripts is stress-dependent and does not rely on the endoribonuclease Rnt1.

Iron Homeostasis Is Perturbed by Cadmium Treatment in the Mutant Strains yap1 and rox1—Remarkably, we found that *FET4* transcript levels in the *rox1* mutant tend to increase after prolonged cadmium treatment (Fig. 3*C*). The fact that cadmium induces iron deficiency (36), together with the knowledge that the iron sensing transcription factor, Aft1, controls *FET4* expression in response to depletion of cellular iron levels (42), led us to examine whether iron homeostasis could be over affected in *rox1* strain after extended cadmium treatment. To this end, we have first evaluated iron contents in WT, *yap1*, *rox1*, *fet4*, and *yap1fet4* strains, after and before cadmium stress (Fig. 7*A*). In the absence of treatment, iron levels did not differ among the strains. In the presence of cadmium, however, iron levels were significantly increased in the *yap1* and *rox1* mutants, as compared with the WT and control conditions (no cadmium). Among the tested strains, *yap1* was the one that exhibited the highest iron content after cadmium addition (Fig. 7*A*). Because deletion of *FET4* from the *yap1* background restored iron contents to control levels, we conclude that the derepression of *FET4* observed in this strain (Fig. 1, *A*–*C*) was mediating iron increase.

We next monitored Aft1 activity in WT, *yap1* and *rox1* strains by evaluating the expression of *CTH2* and *ARN2*, two target genes of Aft1 (43, 44). After prolonged cadmium exposure (60 min), the expression of these genes was increased in all the strains, but in a more pronounced way in the *rox1* mutant (Fig. 7, *B* and *C*), suggesting that Aft1 was more active in this strain. If this was the case, then the increase of *FET4* levels observed in *rox1* cells after prolonged cadmium treatment (Fig. 3*C*) could be ascribed to Aft1 activity. To test this hypothesis, we have deleted the two Aft1 *consensus* sites (36) from the promoter region of the construct *pFET4-HA.* The resulting plasmid (*MUTp-FET4-HA*; Fig. 7*D*) was used to transform WT and

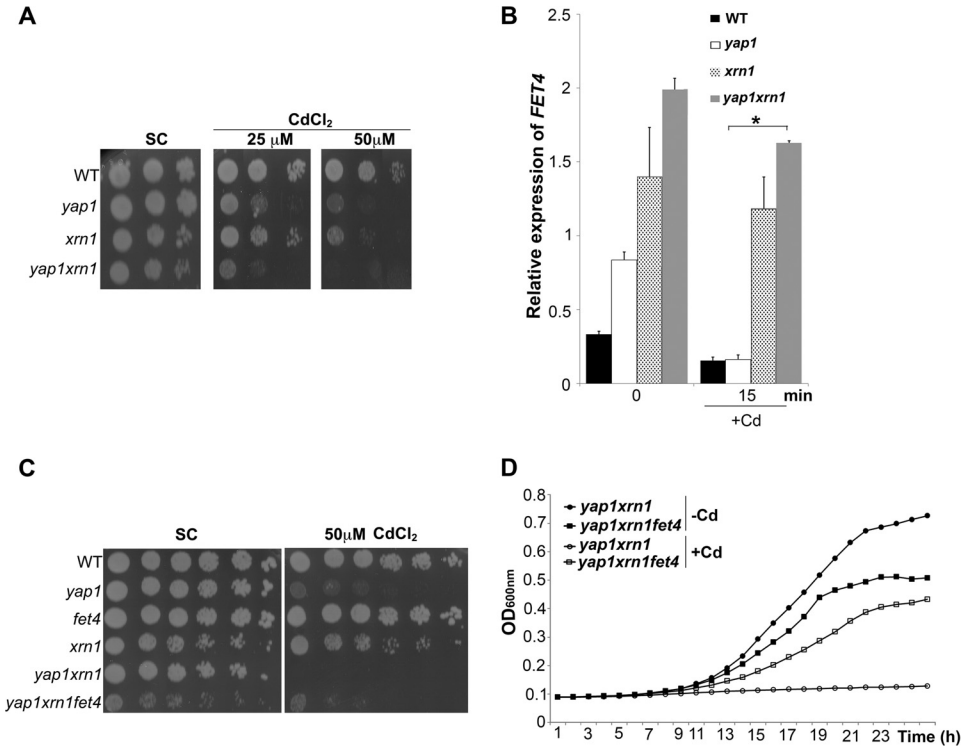

FIGURE 5. **The exoribonuclease Xrn1 controls *FET4* transcript levels and has a role in cadmium tolerance.** *A*, growth sensitivity exhibited by WT, *yap1*, *xrn1*, and *yap1xrn1* strains in SC plates containing 25 and 50 μM $CdCl_2$. *B*, *FET4* expression in WT, *yap1*, *xrn1*, and *yap1xrn1* strains was assessed by qRT-PCR at the indicated time points, after treatment with 25 μM $CdCl_2$. The values are the means of at least biological triplicates ± S.D. *, $p < 0.05$. *C*, deletion of *FET4* from the *yap1xrn1* background partially renders cells more tolerant to cadmium. The indicated strains were spotted onto SC plates supplemented or not with 50 μM $CdCl_2$. *D*, this effect is also observed when cultures are grown in liquid SC medium with a lower cadmium concentration (25 μM $CdCl_2$).

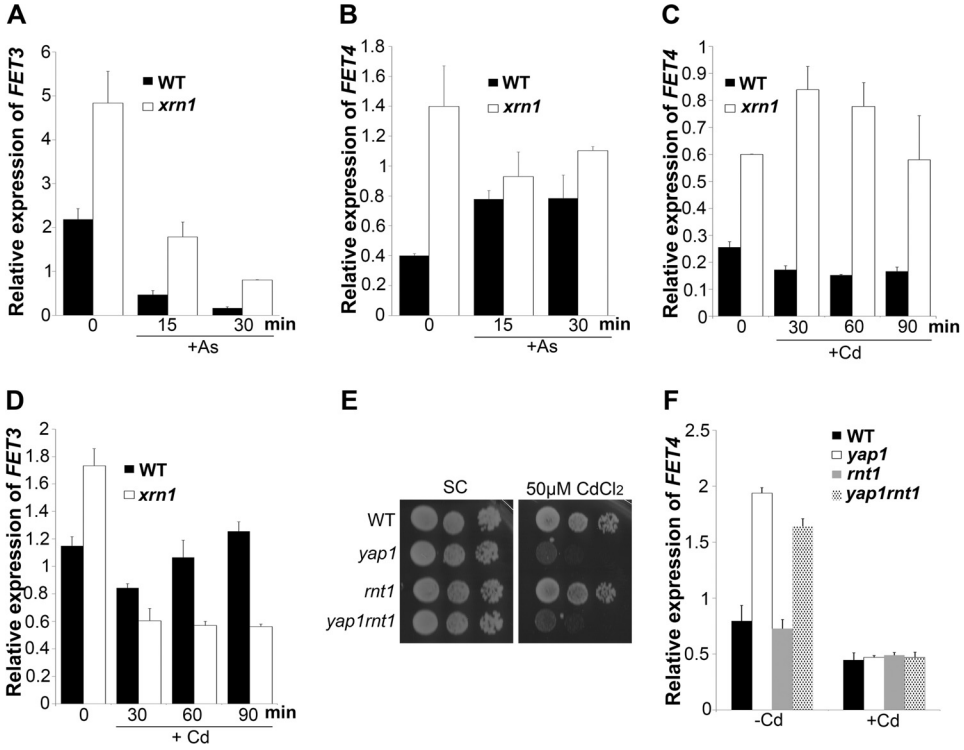

FIGURE 6. **FET4 transcripts dependence on Xrn1 is stress-specific.** *A–D*, *FET3* (*A*) and *FET4* (*B*) expression in WT and *xrn1* strains were assessed by qRT-PCR at the indicated time points, after treatment with 1 mM AsV (+*As*) or and after treatment (*C* and *D*) with 25 μM $CdCl_2$ (+*Cd*). The values are the means of at least biological triplicates ± S.D. *E*, growth sensitivity exhibited by WT, *yap1*, *rnt1*, and *yap1rnt1* strains spotted on SC plates supplemented or not with 50 μM $CdCl_2$. *F*, *FET4* expression levels in WT, *yap1*, *rnt1*, and *yap1rnt1* strains were examined by qRT-PCR after treatment with 25 μM $CdCl_2$ (+*Cd*) for 15 min. The values are the means of at least biological triplicates ± S.D.

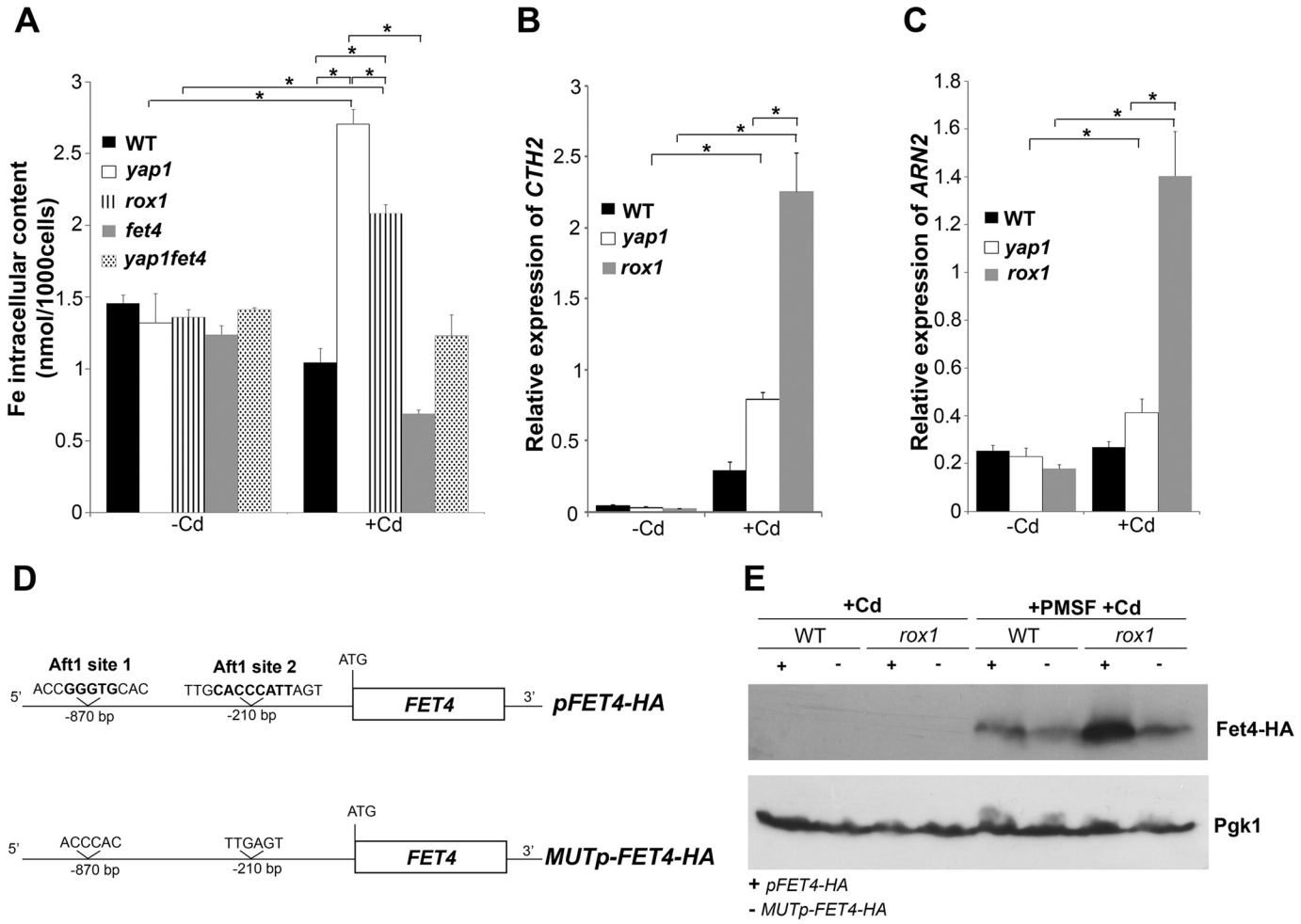

FIGURE 7. **Iron homeostasis is perturbed in the *yap1* and *rox1* mutants.** *A*, iron intracellular contents in WT, *yap1*, *rox1*, *fet4*, and *yap1fet4* strains were determined by ICP-AES in unstressed cultures (−*Cd*) and after culture treatment with 25 μM $CdCl_2$ (+*Cd*) for 6 h. *B* and *C*, *CTH2* (*B*) and *ARN2* (*C*) expression in WT, *yap1*, and *rox1* strains was analyzed by qRT-PCR after treatment with 25 μM $CdCl_2$ for 60 min (+*Cd*). The values are the means of at least biological triplicates ± S.D. Significance of differences was calculated with the *t* test. *, $p < 0.05$. *D*, schematic representation of the constructs *pFET4-HA* and *MUTp-FET4-HA*. In the *MUTp-FET4-HA* construct, both Aft1 sites (1 and 2) were deleted. *E*, WT and *rox1* mutants transformed with *pFET4-HA* or *MUTp-FET4-HA* constructs were treated with Cd for 1 h (+Cd) or treated with 1.2 mM of PMSF for 90 min and then induced with 25 μM $CdCl_2$ for 1 h (+*PMSF*+*Cd*). Fet4 protein levels were analyzed by Western blot. Pgk1 was used as a loading control.

rox1 cells, and Fet4 protein levels were assessed after extended cadmium treatment. Because Fet4-HA protein levels were rapidly reduced after cadmium exposure (Fig. 3, *D* and *E*), we performed these experiments in the presence of PMSF to block the activity of vacuolar proteases (and as such, the vacuolar degradation pathway) and in the presence of MG132, a drug that blocks the proteolytic activity of the proteasome complex. Our results indicate that Fet4 degradation induced by cadmium is not dependent on the proteasome (data not shown) but rather on the vacuolar degradation pathway (Fig. 7*E*). Moreover our data clearly show that, in the presence of both PMSF and cadmium, deletion of Aft1 *consensus* sites from *FET4* gene compromises Fet4 protein levels in the *rox1* but not in the WT strain, indicating that in the *rox1* strain *FET4* gene is up-regulated by Aft1 when cells are exposed to prolonged cadmium treatment.

Discussion

In *S. cerevisiae*, eight stress-responsive transcription factors, Yap1 to Yap8, orchestrate the regulation of gene expression in response to a plethora of environmental cues (reviewed in Ref. 45). Yap1, the master regulator of oxidative stress, plays a pivotal role in cell tolerance against metal toxicity, mainly by inducing genes coding for proteins involved in (i) vacuolar metal sequestration, (ii) metal reduction and extrusion, or (iii) detoxification of reactive oxygen species generated by metal-catalyzed Fenton chemistry.

In this work, we have identified a new line of action of Yap1 toward cadmium toxicity. We showed that the negative regulation of the low affinity iron transporter gene, *FET4*, mediated by Yap1 (Fig. 1), is important for yeast resistance to cadmium. Indeed, the *yap1* mutant accumulates higher cadmium levels compared with the WT strain, whereas the deletion of *FET4* gene from the *yap1* background restores cadmium tolerance (Fig. 1). Other authors have also reported an increase of cadmium levels in *yap1* cells, but didn't clarify the underlying mechanism (15).

Another set of data clearly indicates that repression of *FET4* by Yap1 is exerted via Rox1, an aerobic repressor of hypoxic genes, previously implicated in cadmium toxicity through a

mechanism involving the repression of *FET4* (36). We have in fact shown that Yap1 directly regulates *ROX1* expression through the recognition of an YRE located 414 bp upstream of its ATG codon (Fig. 2) and that this *consensus* is relevant for Rox1-mediated *FET4* repression (Fig. 4). The mutant *yap1*, however, is more tolerant to cadmium than the *rox1* strain (Fig. 3). Accordingly, *ROX1* expression is not fully dependent on Yap1 and *FET4* mRNA, and protein levels are therefore consistently higher in the *rox1* mutant than in the *yap1* strain (Fig. 3). This observation is in agreement with the fact that Rox1 is also regulated by Hap1 (46), a heme-dependent transcription factor (47). Moreover, *ROX1* transcript levels and the expression of Rox1 target genes were reported to be only moderately decreased in the *hap1* mutant, suggesting the presence of another regulator (46, 48).

The observed drastic decrease of *FET4* transcripts after cadmium addition to the medium, suggests that *yap1* and *rox1* mutant cells tend to counteract *FET4* derepression (Fig. 3*C*). We further showed that the 5′-3′ exoribonuclease Xrn1 is mediating this reduction (Fig. 5). Although *in vitro* Xrn1 shows little specificity to particular mRNAs, *in vivo* this is not the case. Jones *et al.* (49) have proposed that binding of a specific RNA sequence by ncRNAs and/or RNA-binding proteins may recruit the 5′-3′ degradation complex. As such, it is tempting to speculate that cadmium can promote the binding of such an element to *FET4* transcripts leading to their specific degradation. Moreover, it now seems likely that a translational/post-translational regulation of Fet4 activity occurs in response to cadmium, because in the WT strain, the protein levels decrease after cadmium insult (Fig. 1*B*), whereas the mRNA levels do not vary (Fig. 1*A*), and the blocking of the vacuolar degradation pathway restores protein levels (Fig. 7*E*).

The intriguing finding that *FET4* mRNA levels in the *rox1* mutant tend to increase over time after the initial cadmium-induced depression (Fig. 3*C*) may result from the combinatorial control of this gene by several transcription factors (36, 50). Aft1, the major regulator of the iron depletion response, also controls *FET4* expression (42). Cadmium stress induces iron starvation (36), implying that differences in the cadmium status of both mutants (*rox1* and *yap1*) may differently activate Aft1. After cadmium treatment, the *yap1* strain accumulates lower cadmium levels compared with the *rox1* strain (Fig. 3*F*), and accordingly, *rox1* cells appear to be more iron-starved because their iron content is lower (Fig. 7*A*), and Aft1 is more active (Fig. 7, *B*, *C*, and *E*).

The fact that *ROX1* regulation mediated by Yap1 occurs under normal growth conditions (Fig. 2) raises the question whether this regulation could serve a broader purpose, in addition to hindering cellular cadmium uptake *via* the repression of *FET4*. In line with this possibility, Liu and Barrientos (51) have recently reported that reactive oxygen species induce the expression of hypoxic genes in a Rox1-independent manner, although the levels of *ROX1* transcripts are strongly increased upon oxidative stress. Induction of *ROX1* expression was, however, shown to be Yap1-independent. The discrepancy between the data of Liu and Barrientos (51), and our own data are likely due to differences in the quantitative analysis of *ROX1* transcripts (relative expression *versus* fold change). Here, we clearly show that Yap1 up-regulates *ROX1* gene (Fig. 2), and this may act as a compensatory mechanism of Rox1-defective repression of hypoxic genes, under oxidative environments. Interestingly, controlled hypoxia is often used as a treatment to overcome the catastrophic effects observed after intoxication by ingestion with paraquat, a potent superoxide generator that accumulates in lungs (52, 53). In this case, hypoxia can be used to mitigate reactive oxygen species. As such, it is reasonable to hypothesize that Yap1 regulation of hypoxic genes through Rox1 may be relevant to overcome oxidative stress in a scenario where Yap1 activity is impaired and intracellular reactive oxygen species accumulate.

Author Contributions—C. P. and C. R.-P. conceived and designed the experiments; S. M. C., R. M., C. A., and C. P. performed the experiments; C. P. and S. M. C. analyzed the data; and C. P. and C. R.-P. wrote the paper.

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