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The *S. cerevisiae* Yap1 and Yap2 transcription factors share a common cadmium-sensing domain

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Abstract **Towards elucidating the function of Yap2, which remains unclear, we have taken advantage of the C-terminal homology between Yap1 and Yap2. Swapping domains experiments show that the Yap2 C-terminal domain functionally substitutes for the homologous Yap1 domain in the response to Cd, but not to H_2O_2. We conclude that specificity determinants of the Cd response are encoded within both Yap1 and Yap2 C-terminus, whereas those required for H_2O_2 response are only present in the Yap1 C-terminus. Furthermore, our results identify *FRM2* as Cd-responsive Yap2 target and indicate a possible role of this protein in regulating a metal stress response.**

Keywords: H_2O_2; Cadmium stress; YAP2; YAP1

1. Introduction

The *Saccharomyces cerevisiae* Yap1 and Yap2 transcription factors belong to the Yap basic-leucine zipper (bZip) family of stress response regulators [1]. Yap1 regulates the yeast peroxide detoxification pathway by being activated by H_2O_2 and activating the transcription of most cellular antioxidants. Yap1 also regulates a yeast response to several unrelated chemicals with thiol reactivity and to metals such as Cd [2]. A key step in Yap1 activation resides in the regulation of nuclear export. Under non-stress conditions, Yap1 is restricted to the cytoplasm [3] by rapid nuclear export via the nuclear export receptor Crm1. Crm1 recognizes a leucine-rich nuclear export signal (NES) located in a C-terminal cysteine rich domain (cCRD) also carrying three repeats of the cysteine motif CSE [3–5]. Upon exposure to stress signals, Yap1 accumulates into the nucleus due to loss of the Yap1–Crm1 interaction [4,5]. H_2O_2 and thiol-reactive chemicals both inhibit the Yap1–Crm1 interaction by producing distinct Yap1 post-translational modifications. H_2O_2 promotes the formation of two Yap1 intramolecular disulfide bonds, one between cCRD Cys598 and Cys303, located within a second CRD at the protein N-terminus (nCRD) [6] and the other between cCRD Cys629 and nCRD Cys310 [7]. Oxidation was shown to conceal the Yap1 NES [8]. Thiol-reactive chemicals are thought to covalently attach to cCRD cysteine residues thereby altering the NES, as shown for *N*-ethylmaleimide [2].

Yap2 was identified in a genetic screen for genes confering resistance to metal and chemical stress when overexpressed [9–11]. Although, these experiments strongly suggest a role for Yap2 in stress response regulation, this function has not yet been confirmed due to the lack of any clear tolerance phenotype in a strain lacking *YAP2* (Δyap2) under many different toxic chemicals and other adverse growth conditions (Azevedo, unpublished results). Yap2 shares extensive similarity with Yap1, especially within the bZIP and cCRD domains, retaining all three Yap1 cCRD cysteine residues and the hydrophobic residues that constitute the NES (see schematics in Fig. 2A). We have here taken advantage of the Yap1 and Yap2 C-terminal homology with the premise that it might underlie a common mechanism of regulation that would reveal some aspects of the elusive Yap2 function. We show that the Yap2 cCRD endows this transcriptional regulator with a Cd-regulated Crm1-dependent nuclear export. Furthermore, Yap2 cCRD can function in the context of Yap1 in the response to Cd but not to H_2O_2, establishing the high specificity of these responses.

2. Materials and methods

2.1. Strains, growth conditions and sensitivity analysis

Yeast cells were grown either in synthetic medium, SC (6.7 g/l yeast nitrogen without aminoacids, 6 g/l casamino acids) supplemented with 20 g/l dextrose and the appropriate amino acids or bases for maintenance of plasmids, or rich medium, YPD (20 g/l dextrose, 10 g/l yeast extract, 10 g/l bactopeptone). Standard cultures were incubated with orbital shaking (200 rpm) at 30 °C until reaching early exponential growth phase. Northern blot analysis and spot assays were performed using the wild type strains FT4 and W303a and its derivatives Δyap1, Δyap2 and Δyap1Δyap2 [12] (see Table 1). YPH98 and its isogenic

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Abbreviations: YAP, yeast AP-1 like factor; Crm1, chromosome region maintenance protein; FRM2, fatty acid repressor 2; GTT2, glutathione transferase 2; TRX2, thioredoxin 2; GFP, green fluorescent protein

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Table 1
Saccharomyces cerevisiae strains used in this study

Strain	Genotype	Source
YPH98	Mat a, ura3-52, lys2-801^{amber}, ade2-101^{ochre}, trp1Δ1, leu2Δ1	[13]
YPH98Δyap1	Mat a, ura3-52, lys2-801^{amber}, ade2-101^{ochre}, trp1Δ1, leu2 Δ1, yap1::*TRP1*	[14]
FT4	Mat a, ura3-52, trp1-Δ63, his3- Δ 200, leu2::PET56	[38]
FT4Δyap1Δyap2	FT4 Δyap1Δyap2	[26]
FT4Δyap2Δfrm2	Mat a, ura3-52, trp1-Δ63, his3- Δ 200, leu2::PET56, Δyap2, frm2:: *URA3*	This study
W303-1A	Mat a, leu2-3/112, ura3-1, trp1-1, Δ1, his 3-11/15, ade2-1, can1-100, *GAL SUC* maI0	[39]
W303-1AΔyap1	Mat a, leu2-3/112, ura3-1, trp1-1, Δ1, his 3-11/15, ade2-1, can1-100, *GAL SUC* maI0, Δyap1	[12]
W303-1AΔyap2	Mat a, leu2-3/112, ura3-1, trp1-1, Δ1, his 3-11/15, ade2-1, can1-100, *GAL SUC* maI0, Δyap2	This study
MNY7	Mat a, Δ*CRM1*::KAN^r leu2$^-$ his3$^-$ trp1$^-$ ura3$^-$⟨*CRM1*⟩	[15]
MNY8	Mat a, Δ*CRM1*::KAN^r leu2$^-$ his3$^-$ trp1$^-$ ura3$^-$⟨*CRM1*^{T539C}⟩	[15]
EGY48	Matα, ura3-52 trp1 his3 LEXA$_{op(X6)}$-*LEU2*⟨pSH18-34 *URA3*-2 μ⟩	R. Brent

strain Δyap1 were also used in proteomic analysis [13,14]. *YAP2* and *FRM2* were disrupted by one-step amplification protocol that successively replaced the entire *YAP2* and *FRM2* open reading frames (ORFs) with the Kanamycin and *URA3* genes, respectively. The Δyap1Δyap2 mutant was generated using the Δyap1 mutant. The Kanamycin gene was eliminated according to [12] and then the *YAP*2 gene was replaced by the Kanamycin one. MNY7 (carrying the wild type Crm1) and MNY8 (*CRM1T539C*, sensitive to leptomycin B) strains were also used in some assays [15]. Phenotypic growth assays were carried out on solid media containing increased concentrations of H_2O_2 and Cd, by spotting 20 μl of a serially diluted culture representing 2000 cells. Growth was recorded after 3 days at 30°C. Standard methods were used for genetic analysis [16], cloning [17] and transformation [18].

2.2. Plasmid constructs

GFP-Yap1-cCRDYap2 was constructed by fusing amino acid 572 of Yap1 protein with amino acid residues 329–409 with the stop codon of Yap2 protein as indicated in scheme of Fig. 2A. The cCRDYap2 was amplified from Yap2 in pRS315 [13] with the primers 1 and 2 (Table 2). Yeast was then transformed with the purified PCR product and GFP-Yap1 both digested with *Bsm*I and *Bst*EII. GFP-Yap2 was constructed using a three-step PCR strategy. Three amplifications were performed separately. Cup1 promoter was amplified from Cup1-GFP-Yap1 molecule [3] with primers 3 and 4 (PCR 1). The GFP full-length coding sequence was amplified from pYGFP3 [19] with primers 5 and 6 (PCR 2). Yap2 coding sequence was amplified from Yap2 in pRS315 with the primers 7 and 8 (PCR 3). PCR 1 and 2 and PCR 2 and 3 were then combined using external primers (3 + 6 and 5 + 8, respectively). Amplification of the whole molecule Cup1-GFP-Yap2 was performed using primers 3 and 8. The purified PCR product was transformed in yeast with Yap2 in pRS315 hydrolyzed with *Sal*I (present in the primer) and *Bam*HI (present in Yap2 three prime). In order to transform this molecule into *CRM1* (MNY7) and *CRM1*^{T539C} (MNY8) strains, the Cup1-GFP-Yap2 was then subcloned into pRS314 [13]. A GFP-cCRDYap2 fusion was constructed using a two-step PCR method. The GFP full length coding sequence was separately amplified from pYGFP3 [19] with primer 9 (*Eco*RI site) and primer 10 (PCR 1). cCRD-Yap2 gene fragment encoding Yap2 aminoacids 325–409 was PCR-amplified from Yap2 cloned in pRS315 using primers 11 and 12 (*Bam*HI site) (PCR 2). The resulting PCR fragments overlapped about 30 bp in their 3′ GFP and 5′ cCRD-Yap2 coding regions. The two PCR products were purified and subsequently combined and subjected to a PCR with the two external primers (primers 9 and 12) used in the previous reactions that contained appropriate restriction sites to clone in pGBT9. The resulting PCR product was purified and subsequently hydrolyzed with *Eco*RI and *Bam*HI for cloning in pGBT9 [20].

Table 2
Oligonucleotide primers used in this study

No.	Sequence 5′–3′	Product
1	GGAAATGAAAGCGAAATCTCACAAAAAAAATGGCAGTAGTTTACAGAATGCTGCTTCTCATACTAAAACAATTCGAAC	
2	GGTAAGTTAAAAAAGTTTAATTGTAACATTATAGAAAAAGTTCTTTCGGTTACCCGATCAATATTACATGCTCTCATCC	cCRD Yap2
3	TTAACCCTCACTAAAGGGAACAAAAGCTGGGTACCGGGCCCCCCCTCGAG**GTCGAC**TCTTTTGCTGGCATTTCTTC	
4	TTCACCTTTAGACATGACTTCTATATGATATTGCAC	Cup1
5	TATCATATAGAAGCTATGTCTAAAGGTGAAGAATTATTC	
6	TCCGAAGGATATTGCCTTTGTACAATTCATCCATACC	yEGFP3
7	TGGATGAATTGTACAAAGGCAATATCCTTCGGAAAGGTC	
8	TCACATTGACATGCTGACGTATC	Yap2
9	**GGAATTC**ATGTCTAAAGGTGAAGAATTATTC	
10	AGCAGCCACCGGAAGTTTGTACAATTCATCCATACC	yEGFP3
11	TGGATGAATTGTACAAACTTCCGGTGGCTGCTTC	
12	CG**GGATCC**CGCATTATGTATACTCAAGATATG	cCRD Yap2
13	AAGACGC**GTCGAC**CTCTTCCGGTGGCTGCTTCTC	
14	TTCTCTTTTCCATGGATCCTCCCAGGAGCTGTCTAACC	cCRD Yap2
15	TCTGGTTAGACAGCTCCTGGGAGGATCCATGGAAAAG	
16	AAAA**CTGCAG**TCAGGTTGACTTCCCCGC	TAPtag
17	AATGGAAAAGCGTCTGCCTACCACATTCTCGAA	cCRD
18	TTCGAGAATGTGGTAGGCAGACGCTTTTCCATT	Yap2-C356A
19	GACATAGATGATTTAGCCAGCGAATTAATAATC	cCRD
20	ATTATTAAATTCGCTGGCTAAATCATCTATGTC	Yap2-C378A
21	ATCAAGGCAAAAGCTACAGATGACTGC	cCRD
22	TGCAGTCATCTGTAGCTTTTGCCTTG	Yap2-C387A
23	TGTACAGATGACGCCAAAATAGTAGTC	cCRD
24	ACTACTATTTTGGCGTCATCTGTAC	Yap2-C391A

Bold letters: restriction sites (3 and 13: *Sal*I; 9: *Eco*rI; 12: *Bam*HI; 16: *Pst*I); underlined: sequence for the amplification of the product indicated and the rest of the sequence represent: (no. 1 and 2: Yap1; 3 and 4: pRS315 and yGFF3; 5 and 6: CUP1 and YAP2; 7 and 8: yGFF3; 14: TAPTag; 15: cCRDYap2). In the case of the mutants, the sequence underlined represents the cysteine residues that were changed to alanine (15–16; 17–18; 19–20 and 21–22).

The cCRD-Yap2-TAP tag fusion was similarly constructed using primers 13 and 14 to generate the cCRD-Yap2 fragment, primers 15 and 16 to generate the TAP tag fragment [21], and primers 13 and 16 to generate the fusion fragment that was cloned in pGBT9 at the *Sal*I and *Pst*I sites. Mutations of the cysteine residues in Yap2 were generated by PCR amplification of the entire GFP-cCRDYap2 or YcpLac33-Yap2 (Yap2 cloned as *Pst*I–*Bam*HI in YcpLac33, [22]) using complementary primers containing the desired mutation as described in Table 2 (C356A, primers 17 and 18; C378A, primers 19 and 20; C387A, primers 21 and 22 and C391A, primers 23 and 24). These amplifications were treated with *Dpn*I prior to *E. coli* transformation. Yap2^{C356AC387A} was generated after Yap2^{C356A}. The purified plasmids were then transformed in a Δyap2 strain for gene expression and GFP staining analysis or in Δyap2Δfrm2 strain for sensitivity analysis. All constructs used in this study have been sequenced. PCR primers sequences used in this work are shown in Table 2.

2.3. Northern-blot analysis

Cells were grown overnight until saturation and then diluted to $OD_{600\,nm}$ of 0.100 and allowed to grow until early exponential phase $OD_{600\,nm}$ of 0.300–0.400. Aliquots of these cells were then left untreated or were treated with either 0.4 mM H_2O_2 for 30 min or 1 mM of Cd for 60 min. RNA procedures were performed according to [12]. *U3*, a small nuclear RNA (*SNR17A*) or *ACT1* (Actin 1), was used as internal loading controls. mRNA levels were quantified using Image J software and normalized against those of the internal control U3 or Act1.

2.4. Cell extracts and Western blotting

For probing the cCRD-Yap2-TAP tag cysteine thiol state, yeast cells were grown until early exponential phase, after which they were either treated in vivo for 60 min with 1 mM Cd or left untreated. Protein was extracted using the TCA method as described previously [6] and then the TCA-precipitated pellet was washed in cold acetone, air dried, resuspended in 200 mM Tris/HCl, pH 8.8, 1 mM EDTA, 1% (w/v) SDS, complete protease inhibitors (Roche Diagnostics, Meylan, France) and then 25 min at 25 °C and 5 min at 37 °C in the presence of 15 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid, disodium salt (AMS, Molecular Probes, Eugene OR, USA). Controls were treated similarly but in the absence of the metal and/or in the absence of AMS.

Protein extracts were separated on reducing 15% SDS–PAGE and immunoblotted with a horseradish peroxidase-bound anti-peroxidase rabbit IgG (PAP, Sigma, St. Louis, MO, USA).

2.5. ^{35}S labeling, analysis of protein expression and identification of Frm2 on 2D gels

YPH98 yeast cells from a saturated culture were diluted in 2 ml of fresh SD medium containing the respective auxotrophic markers, grown overnight until $OD_{600\,nm}$ 0.300–0.400 and then treated with 1 mM cadmium for 60 min. In the last 30 min, cells were labeled with ^{35}S-Met. Protein extraction and two-dimensional PAGE were performed as previously described [23] using a Millipore apparatus. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250, dried, and processed for autoradiography by standard procedures. Identification of Frm2 was performed as described previously [23].

2.6. Cellular localization studies-GFPs

Cells were grown overnight until saturation, diluted to an $OD_{600\,nm}$ of 0.100 and allowed to grow until early exponential phase $OD_{600\,nm}$ of 0.300–0.400. Aliquots of these cells were then treated with 0.4 mM H_2O_2 for 15 min or 1 mM of Cd for 60 min. DAPI (4,6-diamino-2-phenylindole) was added as a DNA marker at a final concentration of 20 μg/ml, 5 min before analysis. After washing with phosphate-buffered saline (PBS), cells were resuspended in DABCO solution (75% glycerol, 0.25 × PBS and 200 mM diazabicyclooctane (Sigma–Aldrich)) and GFP signals were analyzed in living cells with a LEICA DMRA2 microscope coupled with a CoolSNAP™ HQ Photometrics Camera (Roper Scientific) camera and processed with Adobe Photoshop.

2.7. Two-hybrid assay

The strain EGY48 carrying pSH18-34 (2 μm plasmid carrying a *LacZ*) reporter gene under the control of eight *lexA* operators [24] was co-transformed with pKW442 (CRM1-LexA^{BD}) or pEG202 [lexA$_{(1–202)}$] [25] and one of the following pJG4-5 [B42AD haemagglutinin (HA) epitope tag TRP1] [5] or YAP2-B42. To create the YAP2-B42 chimera, *Xho*I restriction sites were introduced by PCR and the product was cloned into the vector pJG4-5 [24]. The interaction patterns were tested on solid medium. Cells pre-grown in 2% S raffinose SD medium were patched at a density of 4.5×10^7 on solid 1% S raffinose/2% galactose SD medium supplemented or not with 1 mM of Cd and incubated 2.5 h at 30 °C. The assay was revealed by overlaying the cells with a 0.5 M sodium phosphate buffer (pH 7.0) containing 0.2% (w/v) SDS, 2% (v/v) dimethyl formamide containing 100 mg X-gal/ml and 0.5% (w/v) agarose at 70 °C. Plates were analyzed after 6 h incubation at 30 °C [26].

3. Results

3.1. A Crm1-dependent nuclear export of Yap2 regulated by Cd

A strain lacking *YAP1* (Δyap1) is highly sensitive to Cd [9,27], whereas Δ*yap2* has a wild type growth in the presence of this metal (Fig. 3C). However, Cd stimulates Yap2 transcriptional activity [26] and its nuclear accumulation [28] suggesting a possible role of Yap2 in the *S. cerevisiae* Cd response. We thus sought to explore the effect of Cd on the regulation of Yap2.

Yap2 carries a C-terminal domain of extensive homology with the Yap1 cCRD that regulates its nuclear export in response to peroxide, to Cd and to others thiol-reactive chemicals. We thus investigated whether Cd could similarly regulate Yap2 at the level of nuclear export. Using a GFP-Yap2 fusion we confirmed that Cd indeed alter the subcellular distribution of the protein. In unstressed exponentially growing cells, Yap2 appeared predominantly cytoplasmic. However, in Cd-treated cells Yap2 accumulated in the nucleus (Fig. 1A).

We next investigated whether Yap2 nuclear accumulation was mediated by a Crm1-dependent mechanism by monitoring the cellular localization of Yap2 following Crm1 inhibition using the Crm1-specific inhibitor leptomycin B. We used for this experiment the MNY7 strain carrying the leptomycin-sensitive *CRM1T594C* allele [15]. Leptomycin B led to the exclusive nuclear localization of GFP-Yap2 in MNY7 cells but did not have any effect in wild type cells (Fig. 1B). To provide further support for the involvement of Crm1 in Cd-induced Yap2 nuclear accumulation, we performed a two-hybrid assay between Crm1-LexA and full-length Yap2-B42^{AD} and assessed the effect of Cd in this interaction (Fig. 1C). The data presented here show that indeed, Yap2 interacts with the exportin Crm1 and this interaction is sensitive to Cd.

3.2. Differential activation of Yap1 and Yap2 by Cd and H_2O_2 resides at the cCRD of each protein

We sought to establish whether Cd-induced Yap2 nuclear accumulation was operated by the cCRD as in the case of Yap1. To this goal, we replaced the Yap1 cCRD with the corresponding Yap2 domain, using a GFP-Yap1 fusion as template (see Fig. 2A) (GFP-Yap1-cCRDYap2). We first assayed the ability of GFP-Yap1-cCRDYap2 to rescue the Cd and H_2O_2 hypersensitive phenotype of Δyap1Δyap2. GFP-Yap1-cCRDYap2 restored a Cd wild-type tolerance to Δyap1Δyap2 but did not affect its sensitivity to H_2O_2, whereas GFP-Yap1 restored tolerance to both stress agents (Fig. 2B). We next examined the ability of GFP-Yap1-cCRDYap2 to activate gene expression in response to Cd and to H_2O_2 by

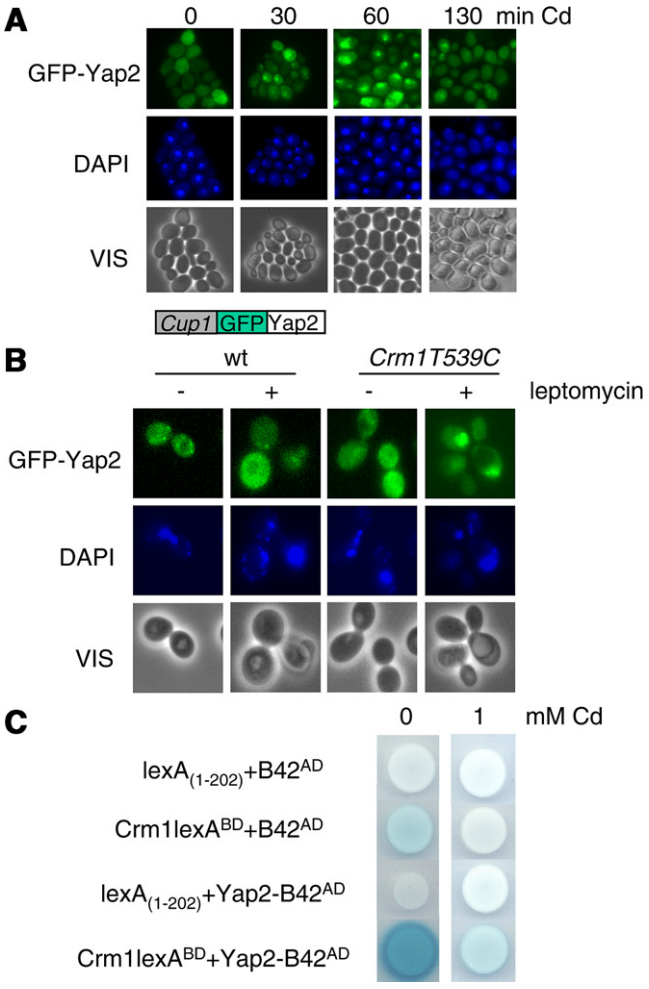

Fig. 1. The transcription factor Yap2 is activated under Cd treatment through nuclear localization in a Crm1 dependent way. (A) Cd-induced Yap2 nuclear redistribution. Cells transformed with GFP-Yap2 were analyzed either for GFP or DAPI staining after 0/30/60 and 130 min treatment with 1 mM Cd. (B) Analysis of the cellular distribution of GFP-tagged Yap2 was assayed in wt and Crm1-Thr^{539}Cys mutant strain after the addition of leptomycin B that inactivates Crm1. (C) β-galactosidase activity from a LacZ reporter gene was assayed in strain EGY48 carrying pKW442 (*Crm1*-lex^{BD}) or pEG202 (empty vector) and one of the following *Yap2*-B42^{AD} or pJG4-5 (empty vector). The assay was performed in solid media in the absence or presence of 1 mM Cd after 6 h incubation at 30 °C.

monitoring the expression of *TRX2* and *GTT2*, two known Yap1-target genes [29,30]. In keeping with the sensitivity assay, GFP-Yap1-cCRDYap2 induced the expression of both genes in response to Cd but not to H_2O_2, whereas, as expected GFP-Yap1 activated these genes in response to both stressors (Fig. 2C). Considering the above results, GFP-Yap1-cCRD-Yap2 accumulated into the nucleus in the presence of Cd but not of H_2O_2 (Fig. 2D). In addition, Western blot analysis revealed that Yap1 and Yap1-cCRDYap2 protein levels do not change in the presence of H_2O_2 or Cd treatments, demonstrating that the result above is not dependent on protein instability (results not shown). As another test of the Yap2 cCRD Cd responsiveness, we fused this domain to a Gal4 DBD-GFP protein fusion carrying a nuclear localization signal (NLS) (present in the vector pGBT9). This hybrid protein also accumulated in the nucleus upon Cd treatment but did not respond to H_2O_2 (Fig. 2E).

These experiments clearly establish that the Yap2 cCRD by its own is endowed with the ability to sense Cd, both when integrated in the context of Yap1, and when fused to GFP. However, the Yap2 cCRD cannot substitute for the homologous Yap1 domain in H_2O_2 responsiveness, confirming that two distinct mechanisms operate for Cd and H_2O_2 sensing by Yap1 [2].

3.3. Yap2 is involved in Frm2-dependent transcriptional activation upon Cd treatment

It has long been assumed that Yap1 and Yap2 have overlapping functions, this was not, however, yet fully studied. To further understand the role of Yap2, we decided to perform proteomic analysis to search Yap2-specific targets. In order to eliminate Yap1 target genes, we used a yap1 null strain (Δyap1), overexpressing either Yap2 or Yap1 in the presence/absence of Cd. Under these conditions, we found one protein that is clearly induced by Cd treatment in Δyap1 mutant strain when overexpressing Yap2 but not Yap1 (Fig. 3A). The spot corresponding to this protein was at the time unknown but after being sequenced, it was shown to be Frm2. To validate this gene as a target gene of Yap2, Northern blot analysis was performed. *FRM2* gene was indeed shown to be induced upon Cd treatment in a *YAP2* and not *YAP1* dependent way (Fig. 3B). Furthermore, we performed sensitivity analysis in the presence of increasing concentrations of Cd. We found that Δfrm2 mutant strain is slightly more sensitive than both wild type and Δyap2 strains. In contrast, Δyap2Δfrm2 double mutant is more sensitive than the single Δfrm2 mutant (Fig. 3C).

3.4. Yap2 cCRD Cys^{356}, Cys^{387} and Cys^{391} are required for Cd sensing

As Yap1 cCRD cysteine residues are important for the Cd response [2], we examined whether Yap2 cCRD cysteine residues, three out of the four such residues (Cys356, Cys378, Cys387) being conserved in Yap1 were also important in Cd sensing (see Fig. 2A). These residues were replaced by alanine in the context of the GFP-cCRDYap2 fusion, resulting in mutant proteins that were stably expressed at levels comparable to the unmutated cCRD Yap2 fusion (results not shown). GFP-cCRDYap2^{C391A} did not accumulate into the nucleus in response to Cd indicating the essential role of Cys391 in Cd sensing (Fig. 4A). GFP-cCRDYap2^{C356} and GFP-cCRD-Yap2^{C387A} partially accumulated in the nucleus in response to Cd, whereas GFP-cCRDYap2^{C356AC387A} did not. GFP-cCRDYap2^{C378A} accumulated in the nucleus in the presence of Cd. Cys378 appears totally dispensable for Cd response, whereas Cys391, together with either Cys356 or Cys387 are important for the Yap2 cCRD Cd treatment.

The cysteine mutations were next introduced into full-length Yap2, and the resulting mutants were tested for their ability to activate Cd-induced *FRM2* expression, when introduced into Δyap2 (Fig. 4B). Consistent with the previous results, Cys391 and either Cys356 or Cys387 appeared crucial for Cd-induced *FRM2* expression, whereas Cys378 was dispensable. Expression of the Yap1-target gene *GTT2* was not altered in any of these strains (Fig. 4B).

We found that Δyap2 became Cd-sensitive upon deletion of *FRM2* (Fig. 3C) and reintroducing *YAP2* into Δyap2Δfrm2

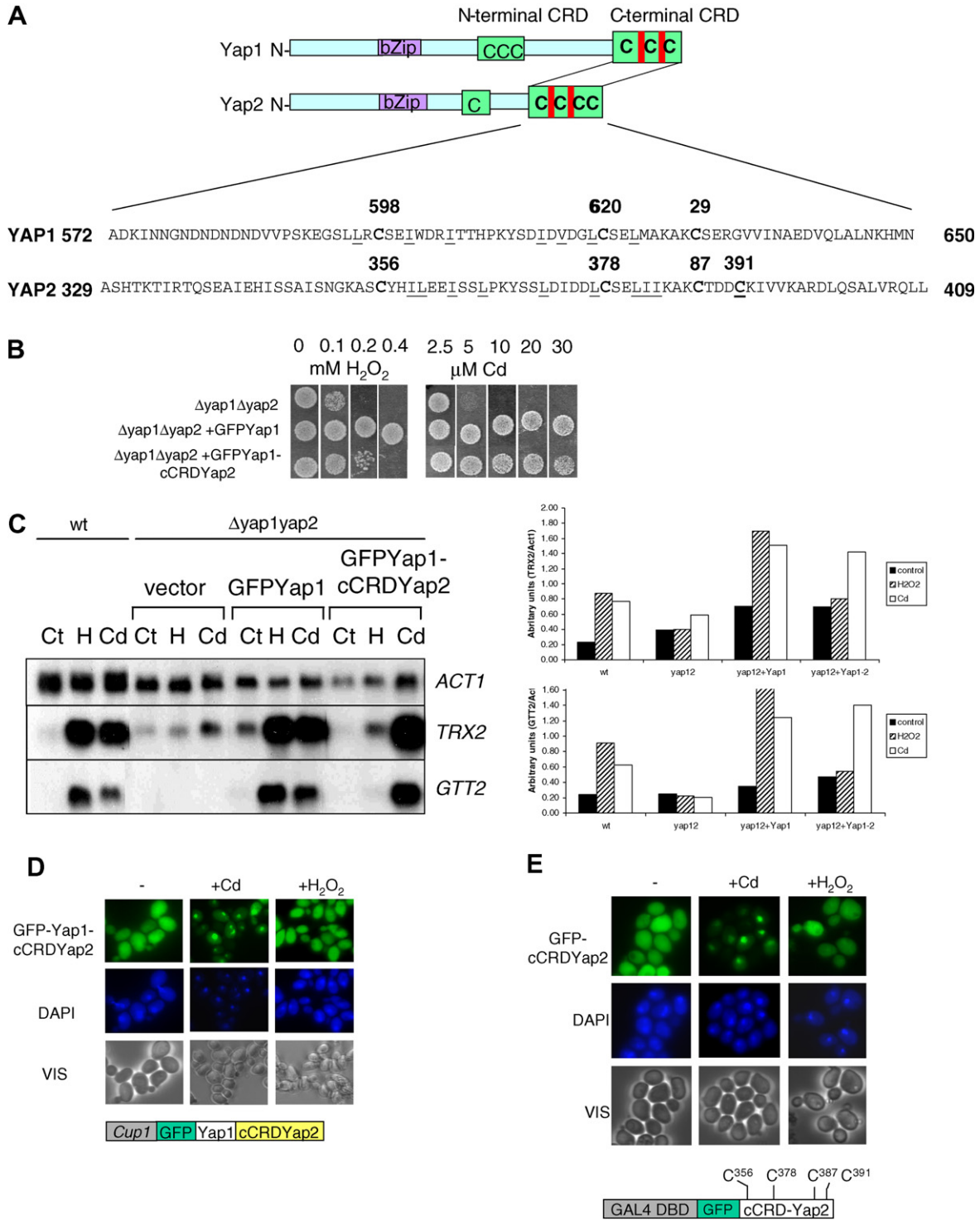

Fig. 2. Yap2 full-length protein is regulated in response to Cd but not H_2O_2. (A) Schematic overview of homology of the cCRD of Yap1 and Yap2, being represented the entire swapped regions of both Yap1 and Yap2. The hydrophobic residues characteristic from NES in other microorganisms are underlined [5]; the cysteine residues present in both cCRDs are in bold, note that Yap2 has an additional cysteine (Cys391 – in bold and underlined). (B) Sensitivity assays of Δyap1Δyap2 strain transformed with the fusions GFP-Yap1 or GFP-Yap1-cCRDYap2 (diagrammed in Fig. 2E). Strains were spotted onto SD medium containing increasing concentrations of H_2O_2 and Cd as described in experimental procedures. Growth was analyzed after 3 days at 30 °C. (C) Analysis of the mRNA expression of *TRX2* and *GTT2* in a wild type strain (wt) and in the Δyap1Δyap2 strains transformed with vector alone, with GFP-Yap1 or the fusion GFP-Yap1-cCRDYap2. Expression of target genes was followed before (Ct-control cells) and after treatment with H_2O_2(H) or cadmium (Cd). Cells were diluted from saturated cultures and grown until early exponential phase after which, they were treated with 0.4 mM H_2O_2 for 30 min or 1 mM Cd for 60 min. RNA was extracted, run on a formaldehyde gel, blotted and hybridized with *TRX2*, *GTT2* probes and *ACT1* probe as internal control. mRNA levels of *TRX2* and *GTT2* against those of Act1 are shown in the right side of the figure. (D) Nuclear redistribution of Yap1-cCDRDYap2. A wild type strain expressing the fusion GFP-Yap1-cCRDYap2 was treated with 1 mM Cd for 30 min or 0.3 mM H_2O_2 for 15 min and analyzed for GFP staining. The scheme of the fusion is also shown. (E) Cellular localization of GFP-cCRDYap2 after treatment with 1 mM Cd for 30 min and 0.3 mM H_2O_2 during 15 min.

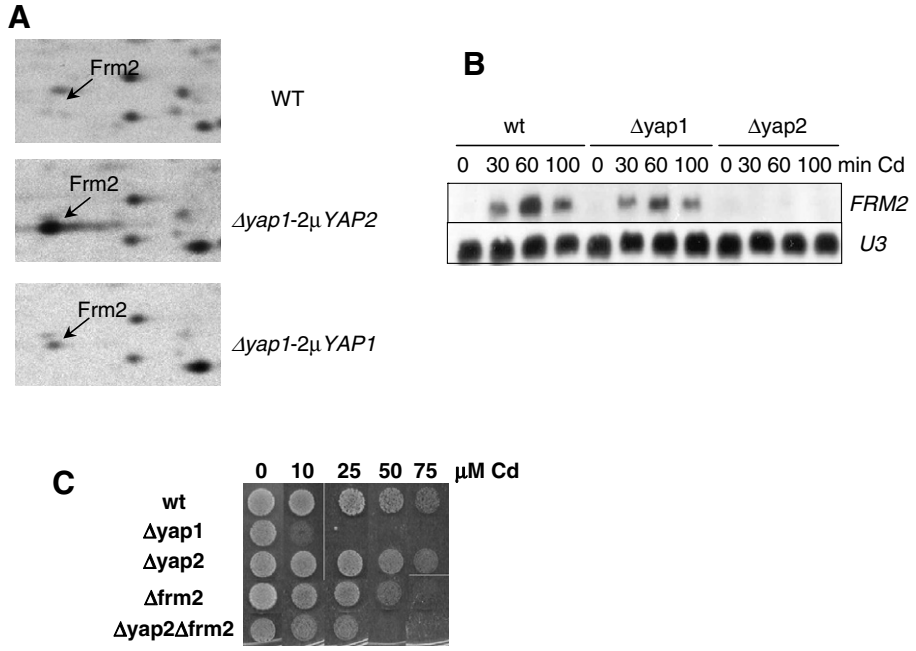

Fig. 3. Frm2 is a target gene of Yap2 under Cd conditions (A) Comparative proteomic analysis of cadmium response: effect of overexpression of Yap1 and Yap2 in a yap1 deleted strain. Autoradiograms of two-dimensional gel electrophoresis of total yeast proteins from [^{35}S]-methionine-labeled strains. Protein extracts were prepared from control untreated cells (YPH8) or cells treated with 1 mM cadmium for 1 h from its YPH8 isogenic strain Δyap1 transformed with *YAP1* or *YAP2* in multicopy plasmids as described in Section 2. (B) *FRM2* gene expression. A wild type strain and its isogenic strains, Δyap1 and Δyap2 were grown until early-exponential phase and then treated or not treated with 1 mM Cd for 0, 30 and 60 min. (C) Sensitivity assays of wt, Δyap2, Δfrm2 and Δyap2Δfrm2 strains. Diluted cells were spotted in SD plates containing increasing concentrations of Cd.

rescued this sensitivity (Fig. 4C). We used this strain background to further test the importance of the Yap2 cCRD cysteine residues in the Cd response. The Δyap2Δfrm2 strain carrying plasmids expressing either one of the four Yap2-cysteine substitution mutants were tested for Cd tolerance (Fig. 4C), also showing that Cys391 and either of Cys356 or Cys387 are important for the Cd response. These results indicate that in the absence of Frm2, Yap2 becomes important for Cd tolerance, possibly by regulating other target genes involved in Cd detoxification.

3.5. Yap2 cCRD cysteines directly sense Cd

Cd may regulate Yap2 by binding cCRD cysteine residues or promoting their oxidation, thereby altering Crm1-recognition of the Yap2 NES. We thus sought to monitor the redox state of Yap2 cysteine residues and their free sulfhydryl status before and after Cd treatment.

To assess the Yap2 free sulfhydryl status, we used the TAP-Tag-cCRD-Yap2 fusion (see diagram, Fig. 5) because of its small size amenable to analysis by cysteine derivatization, using the high molecular mass thiol alkylating reagent AMS (0.5 kDa, cf. [2]). AMS increases the mass of the protein by 0.5 kDa for each alkylated cysteine (Fig. 5, compare lanes 1 and 2). We performed a set of experiments addressing the in vivo effect of Cd. We thus treated cells with 1 mM Cd for 60 min, extracted protein using the TCA method and then the free thiol status of Yap2 cCRD cysteines was assayed by AMS derivatization (Fig. 5). Yap2 cCRD from untreated and AMS-reacted extracts (lane 2) had a slower mobility than the protein from AMS-unreacted extracts (lane 1), indicating modification of the polypeptide by the high-molecular weight alkylating agent. However, Yap2 cCRD from Cd-treated and AMS-reacted extracts migrated at a subtle but reproducible intermediate size between the fully alkylated and unalkylated polypeptides (lane 3 comparing with lanes 1 and 2) indicating incomplete AMS alkylation.

4. Discussion

Yap2 and Yap1 are two stress responsive bZIP regulators in *S. cerevisiae*. Although sharing extensive similarity, especially in the cCRD, Yap1 and Yap2 are physiologically distinct. Yap1 responds to several stress signals such as H_2O_2, thiol-reactive chemicals and Cd [2,31,32], which Yap2 does not respond to [9,26]. Yet, a role of Yap2 in Cd detoxification has been previously suggested, based on the effect of this agent to stimulate its transcriptional activity [26], and to promote its nuclear accumulation [28].

Our data reveal that Yap2 shares with Yap1 a very similar mechanism of activation by Cd. We provide evidence that under non-stress conditions, as Yap1, Yap2 shuttles between the cytoplasm and nucleus by virtue of permanent export by the nuclear export receptor Crm1 (Fig. 1C). Cd activates Yap2 by inhibiting the Crm1-dependent nuclear export, thus leading to Yap2 protein nuclear accumulation (Fig. 1B). As in the case of Yap1, the effect of Cd on Yap2 requires the cCRD, which by itself confers Cd responsiveness to a heterologous protein when fused to it. The Yap2 cCRD thus constitutes a *bona fide* Cd-sensing domain, as the homologous Yap1 domain. We also show that, as anticipated in the case of Yap1 [2], cCRD cysteine residues are crucial for sensing Cd, probably serving as binding site for the metal. The binding of Cd to cCRD cysteine residues probably modify the

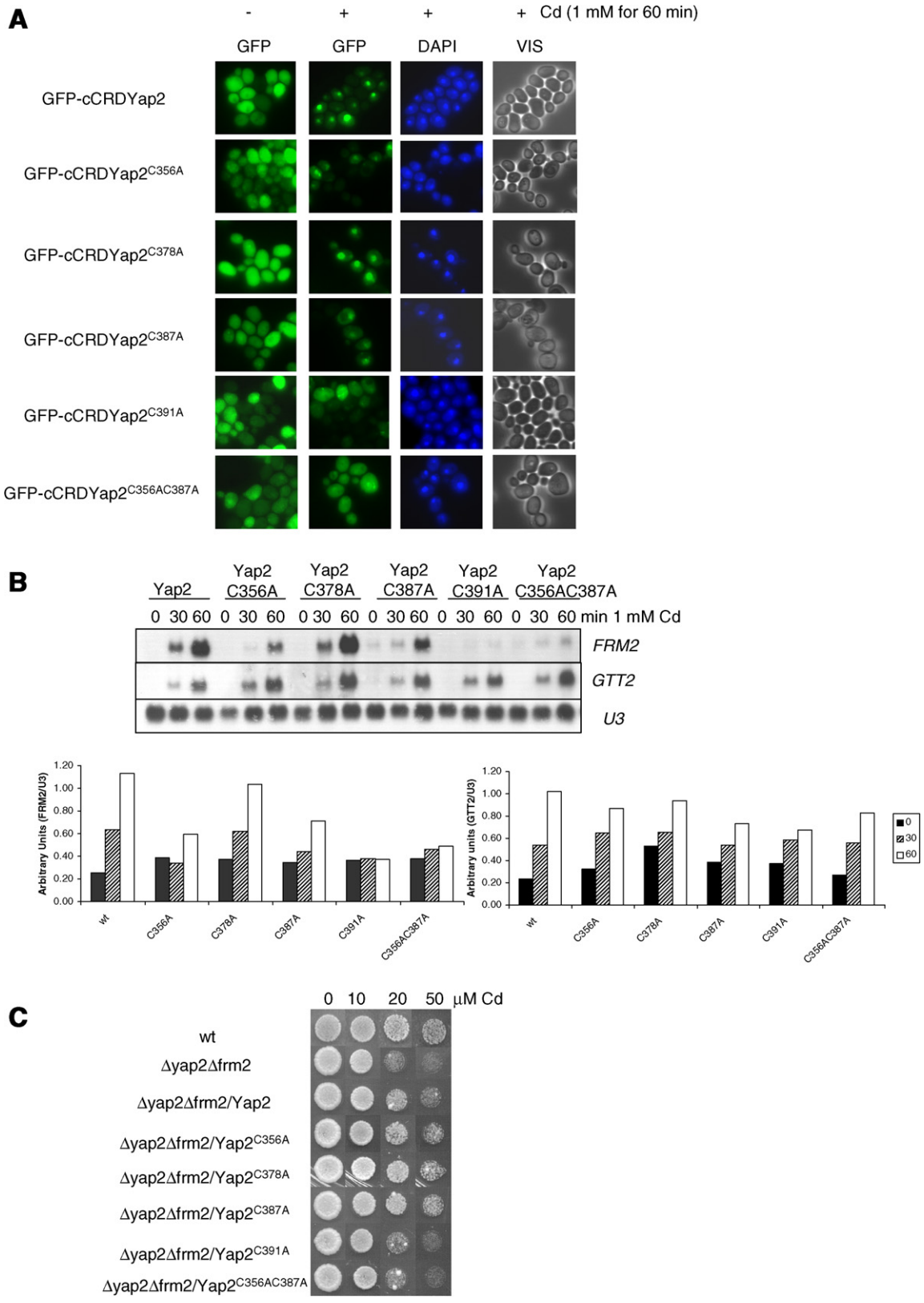

Fig. 4. Cys356, Cys387 and Cys391 are required for Yap2 activation by Cd. (A) Nuclear redistribution of cCRD-Yap2. A wild type strain transformed with GFP-cCRDYap2, GFP-cCRDYap2^{C356A} GFP-cCRDYap2^{C378A} GFP-cCRDYap2^{C387A} GFP-cCRDYap2^{C391A} and GFP-cCRDYap2^{C356AC387A} were assessed for its GFP staining before and after treatment with 1 mM Cd for 1 h. (B) Expression of *FRM2* and *GTT2*. Total RNA was isolated from an early exponential growth phase of a Δyap2 transformed with yCplac33-Yap2, yCplac33-Yap2^{C356A}, yCplac33-Yap2^{C378A} yCplac33-Yap2^{C387A}, yCplac33-Yap2^{C391A} and yCplac33-Yap2^{C356AC391A} before and after treatment with Cd for the indicated period of time. mRNA levels of *FRM2* and *GTT2* were normalized against the internal control, U3, and are shown below the Northern blot. (C) A Δyap2Δfrm2 strain transformed with only vector, YcpLac33, with YcpLac33-Yap2 or the corresponding mutants as indicated in Fig. 4B were assessed for Cd tolerance under plate assays.

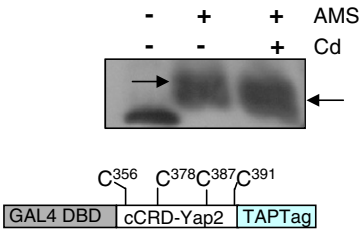

Fig. 5. Cd coordinates to the cCRD cysteines of Yap2. Protein extracts from cultures of a wild type strain carrying a cCRD-Yap2-TAP tag fusion were left untreated (lanes 1 and 2) or were treated during 1 mM Cd for 60 min in vivo (lane 3). The proteins were solubilized in the absence (lane 1) or presence of AMS (15 mM) (lanes 2 and 3). The cCRD-Yap1-TAP tag fusion was revealed with labeled IgG. A schematic representation of the cCRD-Yap2-TAP tag fusion is also shown.

Crm1-cognate nuclear export signal, thereby inhibiting the interaction between Crm1 and the Yap proteins. Still, cysteine residues requirements appear distinct between Yap1 and Yap2, as shown by the effect of their substitution on the Cd response. While only any two out of the three Yap1 cCRD cysteine residues are required for full Cd response (not shown), three out of the four Yap2 cCRD cysteine residues are required, with one being crucial by its own (Cys391, not conserved in Yap1) and two others (Cys356, Cys387) having each an intermediary requirement (Fig. 4A–C; see also suggested model in Fig. 6).

Yap1 responds to H_2O_2 through a mechanism distinct from that of Cd, involving the upstream Orp1 sensor and not only cCRD but also nCRD cysteines residues. Orp1 Cys36 is oxidized to a sulfenic acid, Cys36-SOH, which reacts with Yap1 Cys598, forming a Yap1-Orp1 disulfide linkage triggering an intermolecular Cys303–Cys598 disulfide bond of Yap1 [33]. The absence of an intramolecular disulfide bond formation in the fusion protein GFPYap1-CRDYap2 might be related with different reactivity of the cysteines present in Yap2 cCRD. The level of its nucleophilicity and pKa values might prevent reaction with Orp1-Cys36-SOH, suggesting that Cys598 in Yap1 is crucial for intramolecular disulfide bond formation. Furthermore, another fusion protein GFPYap2-cCRDYap1 does not respond to H_2O_2 (not shown), demonstrating that both Cys598 and Cys303 must have a reactivity that no other cysteine can substitute. In summary, replacement of the Yap1 cCRD by the homologous Yap2 domain preserved the response to Cd but not to H_2O_2, confirming the equivalency of cCRDs domains in Cd sensing functions and demonstrating that the built-in specificity of the Yap1 cCRD towards H_2O_2 is not conserved in the homologous Yap2 domain. Another hypothesis by which neither GFPYap1-cCRDYap2 nor GFP-Yap2-cCRDYap1 can respond to peroxides could be related to the fact that the fusion proteins could have such a conformation that hinders Orp1 from getting access to reactive cysteines present in both chimeras. Altogether these data provide strong evidence that Yap2 is specifically endowed with a Cd-sensing function operated by specific cysteine residues and regulating its function of a transcription factor through subcellular localization control. What could be the role of this Yap2 Cd-sensing and regulatory mechanism in view of the lack of a Cd phenotype of Yap2 deleted strains? The nature of Yap2 target genes should help decipher this question, but up to now the identification of such targets has been lacking. By proteomic analysis after Cd treatment, we have found a protein that is highly induced upon Cd treatment in cells overexpressing Yap2 and not Yap1 (Fig. 3A). Indeed Northern blot analysis (Fig. 3B) shows that *FRM2* is as a *bona fide* Yap2 target gene regulated by Cd in a strictly Yap2-dependent way, thus pointing to a Yap2-specific function independent of Yap1. *FRM2* was initially identified in a screen for mutants defective in *OLE1* repression by unsaturated fatty acids [34], and was proposed to function in lipid metabolism, based on the sensitivity of Δfrm2 to arachidonic acid [35]. Whether Yap2 serves to regulate lipid metabolism in response to Cd, known to exert its toxicity by promoting lipid peroxidation cascades [36], remains to be further explored. However, the fact that Δfrm2 is sensitive to increasing concentrations of Cd and Δfrm2 Δyap2 is slightly more sensitive that Δfrm2 (Fig. 3C) can suggest that indeed Yap2 and its target gene, Frm2 could play a role in Cd response, probably related to lipid metabolism.

Other putative targets of Yap2 that encode activities involved in protein folding have been suggested by microarray analyses, but these have not yet been validated [37]. It has also

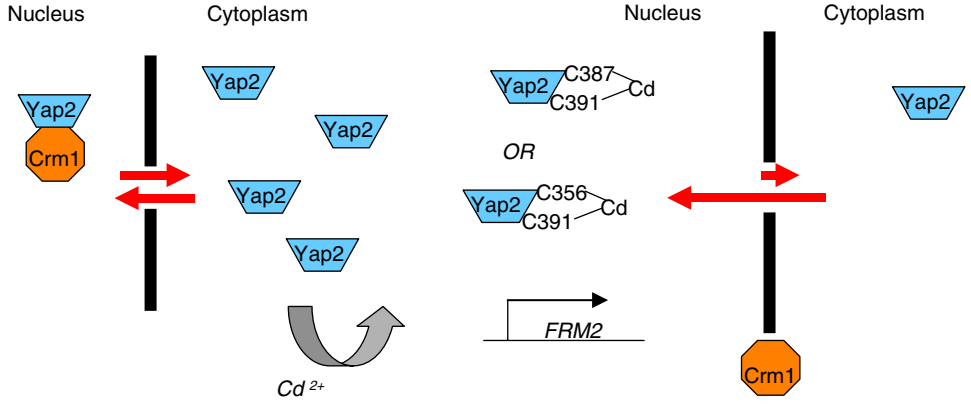

Fig. 6. Proposed model for the mechanism of Yap2 activation by Cd. Under physiological conditions, Yap2 shuttles between the cytoplasm and the nucleus, since we can observe protein both in the nucleus and cytoplasm. The export of Yap2 is more efficient than the import resulting in the majority of the protein being in the cytoplasm. After treatment with the metal Cd, Yap2 protein export no longer occurs due to an inhibition with the exportin Crm1. This inhibition results possibly from the coordination of Cd with two of the four cCRD cysteines of Yap2. Cys391 has an important role in this coordination, whereas Cys356 and Cys387 can play alternative roles.

been shown that Yap2 interacts with the kinase Rck1 under conditions of oxidative stress [28], although the nature and physiological relevance of this interaction remains elusive.

In conclusion, despite more work is required to clarify Yap2 function, this work opens new perspectives towards the identification of its function.

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