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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).