Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor

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Running title: Ybp1 is a regulator of Yap1
Summary

We describe the characterisation of Ybp1, a novel protein, in Saccharomyces cerevisiae, that is required for the oxidative stress response to peroxides. Ybp1 is required for H$_2$O$_2$-induced expression of the antioxidant encoding gene TRX2. Our data indicates that the effects of Ybp1 are mediated through the Yap1 transcription factor. Indeed, Ybp1 forms a stress-induced complex with Yap1 in vivo and stimulates the nuclear accumulation of Yap1 in response to H$_2$O$_2$ but not in response to the thiol-oxidising agent diamide. The H$_2$O$_2$-induced nuclear accumulation of Yap1 is regulated by the oxidation of specific cysteine residues and is dependent on the thiol peroxidase Gpx3. Our data suggests that Ybp1 is required for the H$_2$O$_2$-induced oxidation of Yap1 and acts in the same pathway as Gpx3. Consequently, Ybp1 represents a novel class of stress regulator of Yap1. These data have important implications for the regulation of protein oxidation and stress responses in eukaryotes.
Introduction

The cells of all aerobic organisms are exposed to reactive oxygen species (ROS) that include the superoxide (O$_2$•−) and hydroxyl (OH•) radicals and H$_2$O$_2$. ROS are highly reactive and can damage many cellular components. Indeed, cells of the immune system make use of the damaging effects of ROS to eliminate invading pathogens such as fungi (for a review see 1). Organisms have an array of proteins that protect against ROS-induced damage. For example, antioxidant proteins scavenge and inactivate ROS. However, if the level of ROS exceeds the antioxidant defences then oxidative stress occurs, triggering a mechanism, called the oxidative stress response (OSR), that increases the production of antioxidant proteins to restore homeostasis. ROS have been linked with a wide range of diseases and hence the characterisation of the mechanisms of the OSR has important implications for understanding disease processes in higher eukaryotes.

The budding yeast *Saccharomyces cerevisiae* is an important model organism in studies of the regulation of the OSR (2). The Yap1 and Skn7 transcription factors are required for the OSR to regulate the H$_2$O$_2$-induced expression of many antioxidant encoding genes. These include the *TRX2*, *TRR1* and *TSA1* genes (3-5), encoding thioredoxin, thioredoxin reductase and thioredoxin peroxidase respectively, which together make up the thioredoxin system, a conserved pathway that reduces peroxides such as H$_2$O$_2$ to harmless products (6,7). Yap1 and Skn7 act in the same pathway to regulate *TRX2* and *TRR1* gene expression (3,4), although the mechanism of co-operation has not been elucidated.

In eukaryotes, AP-1-like transcription factors, such as mammalian c-Jun, are involved in stress responses (see 8 for a review). AP-1-like proteins contain a bZip structural motif consisting of a leucine-rich zipper region and an adjacent basic region that are important for dimerisation and DNA binding, respectively. Yap1 is a member of a sub-family of AP-1-like
transcription factors that also includes Cap1 from *Candida albicans* and Pap1 from *Schizosaccharomyces pombe* (8-10). Oxidative stress induces the accumulation of Yap1 in the nucleus (11) and this is linked to changes in the redox status of two cysteine-rich domains in Yap1, the n-CRD and c-CRD, that prevent the interaction of Yap1 with the nuclear export factor Crm1 (11-16). In contrast, the nuclear import of Yap1 has been found to be unaffected by the redox status of Yap1 (17). During non-stressed conditions Yap1 and Crm1 interact, resulting in export of Yap1 from the nucleus; whereas, oxidative stress treatment weakens this interaction resulting in the nuclear accumulation of Yap1 (12,13).

Interestingly, different oxidative stress agents appear to regulate Yap1 by different mechanisms. In particular, H$_2$O$_2$ and diamide (a free thiol oxidising agent) cause oxidation of different cysteine residues in the CRDs of Yap1 (15,16). However, both H$_2$O$_2$ and diamide result in the nuclear accumulation and activation of Yap1. Although the mechanism of regulation of Yap1 by diamide is unclear, Delaunay and colleagues (18) demonstrated that a thiol-peroxidase, Gpx3, forms a complex with Yap1 and is important for the H$_2$O$_2$-induced oxidation of the protein.

Here, we have identified an open reading frame (ORF), *YBR216c*, which we have named *YBP1* (*Yap1* binding protein), that is important for the OSR in *S. cerevisiae*. Moreover, we demonstrate that Ybp1 forms a stress-induced complex with Yap1 *in vivo* and influences the nuclear localisation of Yap1 in response to H$_2$O$_2$ but not diamide. Yap1 oxidation is induced by H$_2$O$_2$ and is important for the regulation of the protein. Our data reveals that Ybp1 is required for the H$_2$O$_2$-induced oxidation of Yap1 and acts in the same pathway as Gpx3. Thus, Ybp1 represents a new class of redox regulator protein.
Experimental Procedures

Yeast strains and growth conditions

The *S. cerevisiae* strains used were: W303-1a (*a ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3*); W303-1a diploid (*a/α ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3*) (19); *skn7Δ* (*a ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 skn7::HIS3*) (4); *yap1Δ* (*α ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 yap1::TRP1*) (4); DY (*α ade2 trp1 can1-100 leu2 his3 ura3::(3xSV40AP1-lacZ)* ) (3); DWYU (*α ade2 trp1 can1-100 leu2 his3 ura3::(3xSV40AP1-lacZ) yap1::URA3*) (3); SR1 (*a ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 yap1::HIS3*); SR2 (*a ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 yap1::HIS3*); CTY10-5d (*a ade2 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::lexA op-lacZ*) (20).

The two independent *ybp1Δ* mutants, SR1 and SR2, were isolated by sporulation of a *ybp1-1/ybp1Δ* heterozygous W303-1a diploid where one copy of the *ybp1-1* allele had been replaced with the *HIS3* gene from YDp-H (21). The *YBP1* strain (SR6) was created by replacing the chromosomal *ybp1::HIS3* in SR1 with the wild type *YBP1* gene contained on a PCR fragment. The *yap1Δybp1Δ* haploids SR4 (*yap1::URA3 ybp1::HIS3*) and SR8 (*yap1::TRP1 ybp1::HIS3*) were from a cross between SR2 with either DWYU or *yap1Δ*, respectively. The *yap1ΔYBP1* strain (SR5) was created by replacing the chromosomal *ybp1::HIS3* in SR4 with the wild type *YBP1* gene as described above. The *yap1Δgpx3Δ* strain SR7 (*yap1::TRP1 gpx3::HIS3*) was constructed by replacement of the *GPX3* gene in the *yap1Δ* strain with the *HIS3* gene from YDp-H (21). The *yap1Δybp1Δgpx3Δ* strain (SR9) *yap1::TRP1 ybp1::HIS3 gpx3::HIS3* was from a cross between SR7 and SR8. The *YBP1 gpx3Δ* (SR10) *YBP1 gpx3::HIS3* and *ybp1::HIS3 gpx3::HIS3* (SR14) strains were from a cross between SR6 and SR9.
Yeast media has been described previously (22). Growth was at 30°C unless stated. H₂O₂ (Sigma) and tetra-butyl hydroperoxide (tBOOH, Sigma) were used at concentrations stated. For sensitivity tests 5µl of serial 10-fold dilutions of mid-log cultures were spotted on to media containing various concentrations of H₂O₂ , tBOOH and diamide (Sigma) and plated at 30°C for 2-3 days.

**Yeast techniques**

Yeast cells were transformed using a lithium acetate method (23). Plasmids were isolated from yeast cells by the method of Robzyk and Kassir (24). Yeast genomic DNA was isolated as described by Hoffman and Winston (25).

**Plasmid constructs**

A 3.7 kb BamHI fragment, containing the YBP1 gene and promoter, was introduced into Yep24 (26), YCplac111 and YCplac22 (27) creating Yep24-YBP1, YCplac111-YBP1 and YCplac22-YBP1, respectively. To create YEplac181-YBP1 a 3.7 kb SacI/XbaI fragment from YCplac22-YBP1 was ligated with SacI/XbaI-digested YEplac181 (27). YEplac181-YBP1-PK was constructed by firstly ligating a 240 bp PstI/BamHI PCR fragment containing the C-terminus of Ybp1 into the pRep42PkC plasmid (28) digested with the same enzymes to create pRep42-YBP1-PkC. YEplac181-YBP1-PK was then obtained by ligating a 1.2 kb BglII/SacI fragment from pRep42-YBP1-PkC with BglII/SacI-digested YEplac181-YBP1.

pBTM-YBP1 was constructed by ligating YBP1 into BamHI-digested pBTM116 (20). The pGAD-Yap1(1-650) plasmid was constructed by ligating YAPI into EcoRI-digested pGAD-C1 (29). To construct pGAD-Yap1(157-650), pGAD-Yap1(379-650) and pGAD-Yap1(460-650) appropriate PCR products were ligated into EcoRI-digested pGAD-C2 (29).

To obtain the YCplac111-YBP1-6xHIS plasmid a 1.3 kb SalI/XhoI fragment of YBP1 was first ligated with SalI/XhoI-digested pET-Hnef-PFH (30). Digestion of this plasmid with
XhoI/HindIII released a fragment of YBP1 with in-frame FLAG(His)₆ which was then ligated with XhoI/HindIII-digested YCplac22-YBP1 to form YCplac22-YBP1-6xHISFlag. Finally, a fragment of YCplac22-YBP1-6xHISFlag, containing the full-length YBP1 gene with in-frame FLAG(His)₆, was ligated with KpnI/HindIII-digested YCplac111 (27) to generate YCplac111-YBP1-6xHISFlag.

The pRS–cp-GFP-YAP1 (11) and pRS316-Yap1-Myc plasmids (15) were kindly provided by S. Kuge and M. Toledano, respectively.

The sequences of oligonucleotides used for PCR can be supplied upon request.

RNA analysis

RNA was extracted (31) from mid-log cells growing in SD media. RNA analyses were performed as described previously (32) using ³²P-labelled cDNA probes. The TRX2 cDNA was obtained by PCR using gene specific oligonucleotides (4). An ACT1 cDNA probe was used as a loading control (4). Autoradiography of probed membranes was carried out using Fuji Medical X-ray film (Super RX) and membranes were also analysed using a phosphorimager (Bio-imaging analyser Fuji film Bas-1500) and quantitated using Tina 2.0 software (Raytest).

β-galactosidase assays

β-galactosidase assays were performed (33) on cells grown to mid-log in SD media.

Protein interaction studies

Crude protein extracts were prepared from ~2x10⁹ cells by lysing cells in lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 10mM Imidazole, 0.5g/100 ml Nonidet P-40, with 2 µg/ml pepstatin A, 2µg/ml leupeptin, 100mg/ml phenylmethylsulfonyl fluoride, 1 ml/100 ml aprotinin). For purification of 6xHisFlag-tagged Ybp1, crude extract was added to 30µl of Ni²⁺-NTA-agarose pre-equilibrated in the lysis buffer. After incubation at 4°C for 60 min, the
beads and any coupled proteins were pelleted, washed with lysis buffer, then resuspended in SDS PAGE loading buffer. The ability of Myc-tagged Yap1 protein to copurify with 6xHisFlag-tagged Ybp1 was analysed by SDS PAGE and Western blotting using monoclonal anti-Myc antibodies (9E10, Sigma).

**Preparation of cell extracts for monitoring Yap1 oxidation**

Cell extracts were prepared as described by Delaunay *et al.* (15). Iodoacetimide-treated proteins were treated with alkaline phosphatase then separated by non-reducing SDS PAGE. Gels were analysed by Western blotting using anti-Myc monoclonal antibodies (9E10, Sigma) to detect the oxidised and reduced forms of Myc-tagged Yap1.

**Localisation of GFP-tagged Yap1**

Cells containing the GFP-Yap1 expression plasmid were concentrated into approximately 25µl medium and 5µl spotted onto a glass slide. This spot was mixed on the slide with 5µl of 40°C 1% (w/v) low melting temperature agarose (GibcoBRL) containing an appropriate concentration of H\(_2\)O\(_2\) or diamide to give the desired level of stress. A coverslip was placed on top and GFP-tagged Yap1 was detected by exciting cells with 450-490nm using a Zeiss Axioscope fluorescence microscope, with a 40x oil immersion objective, and Axiovision digital imaging system.

**Localisation of Pk-tagged Ybp1**

Cells were prepared essentially as described by Kilmartin and Adams (34) except that 5mg/ml lyticase in 50% glycerol for 30 min at 30°C was used. Anti-Pktag primary antibodies (Serotec), diluted 1 in 1000, and Alexa 488-conjugated anti-mouse secondary antibodies (Molecular Probes) were used. DAPI-stained nuclei and anti-Pk immunofluorescence were visualised by excitation at 365nm (DAPI) and 450-490nm (Alexa 488) using a Zeiss
Axioscope fluorescence microscope, with a 63x oil immersion lens, and Axiovision digital imaging system.
Results

Identification of a novel protein involved in peroxide resistance

The deletion of the *TSA1* gene, which encodes thioredoxin peroxidase, in the W3031a strain reduces the H₂O₂-induced expression of the *TRX2* promoter, and increases peroxide sensitivity (19). A high copy suppressor screen revealed a previously uncharacterised ORF, *YBR216c* (named *YBP1* from here), that increased the peroxide resistance of the *tsa1Δ* mutant and stimulated the H₂O₂-induced expression of the *TRX2* gene in *tsa1Δ* cells (data not shown). These results suggest that Ybp1 functions downstream of Tsa1, or within a separate pathway, to protect cells against peroxide stress. Analysis of the predicted amino acid sequence of Ybp1 did not reveal homology to identified domains or provide clues to the biochemical activity of the protein.

Since the Yap1 and Skn7 transcription factors regulate peroxide-induced *TRX2* gene expression (3,4) the effect of expression of *YBP1* from the Yep24 vector on H₂O₂ and tBOOH resistance was examined in the W303-1a (*YAP1 SKN7*), *yap1Δ* and *skn7Δ* strains. Yep24-YBP1 greatly increased the resistance of W303-1a cells to these peroxides (Fig. 1A) but was unable to restore tBOOH resistance to the *yap1Δ* and *skn7Δ* mutants (Fig. 1B). This suggests that the increased resistance associated with the presence of Yep24-YBP1 in W303-1a is dependent on Yap1 and Skn7 (Fig. 1B). Intriguingly, Yep24-YBP1 did not confer any increased resistance to the thiol-oxidising agent diamide and, in fact, actually slightly increased the sensitivity of W303-1a (Fig. 1A). These data suggest that Ybp1 is a new protein involved in the peroxide-induced OSR but not the general response to oxidative stress.

Ybp1 regulates H₂O₂-induced gene expression

Several genes, including those encoding proteins of the thioredoxin system, are induced by H₂O₂ in a Yap1- and Skn7-dependent manner (3-5). RNA analysis of the W303-
1a strain, either untreated or treated with H$_2$O$_2$, revealed that both the basal and H$_2$O$_2$-induced levels of TRX2 mRNA were increased in cells containing the high copy number plasmid, Yep24-YBP1 (Fig. 2A). Moreover, Yep24-YBP1 had no significant effect on the basal or H$_2$O$_2$-induced levels of TRX2 mRNA in the yap1Δ mutant and barely increased levels in the skn7Δ mutant (Fig. 2A). The expression of YBP1 from a high copy number plasmid also increased the expression of a reporter, where the TRX2 promoter was fused to the E. coli lacZ gene, indicating that Ybp1 stimulates transcription from the TRX2 promoter (data not shown).

To examine the role of Ybp1 in H$_2$O$_2$-induced expression of TRX2 RNA analysis was performed using a derivative of W303-1a where the YBP1 gene has been deleted. Less induction of TRX2 mRNA occurred after 20 and 40 min H$_2$O$_2$ treatment in the ybp1Δ mutants (SR1 and SR2) compared with the isogenic W303-1a strain (Fig. 2B) revealing that Ybp1 is normally required for the response to H$_2$O$_2$. However, the induction of expression by H$_2$O$_2$ was greater than that seen in the yap1Δ or skn7Δ mutants (Fig. 2B). As expected, a low copy number (CEN) plasmid carrying the library-encoded YBP1 gene, expressed from its own promoter, rescued the H$_2$O$_2$-induced expression of TRX2 in the ybp1Δ strain (data not shown), confirming that Ybp1 is required for H$_2$O$_2$-induced TRX2 expression.

To determine whether Ybp1 affects Yap1 activity the effect of expression of YBP1 from a high copy number plasmid (YEplac181-YBP1) on the expression of a Yap1-specific lacZ reporter (regulated by SV40-derived AP-1 binding sites) was examined (3). Importantly, the presence of YEplac181-YBP1 stimulated H$_2$O$_2$-induced β-galactosidase activity (Fig. 2C) and had no detectable effect on the expression of the SV40AP1-lacZ reporter in a yap1Δ mutant treated with H$_2$O$_2$ (data not shown).
Collectively, these data suggest that Ybp1 is important for H$_2$O$_2$-induced gene expression and are consistent with the hypothesis that the increased peroxide resistance of W303-1a cells containing Yep24-YBP1 is attributed to increased Yap1-dependent antioxidant gene expression.

**Ybp1 is required for resistance to peroxide**

The role of Ybp1 in peroxide resistance was examined. The growth rates of the *ybp1Δ* strain (SR1) containing either the low copy number (*CEN*) plasmid YCplac22 or YCplac22-YBP1 (carrying the library-encoded *YBP1* gene expressed from its own promoter) were the same under non-stressed conditions (data not shown). However, in contrast, *ybp1Δ* cells containing vector took 4-5 times longer to start growing again following treatment with a non-lethal concentration of H$_2$O$_2$ than those containing YCplac22-YBP1 (Fig. 3A), although they recovered much more quickly than *yap1Δ* cells (data not shown). Surprisingly, the W303-1a strain containing vector recovered from H$_2$O$_2$ treatment much more slowly than the isogenic *ybp1Δ* strain containing YCplac22-YBP1 (Fig. 3A). Furthermore, W303-1a was much more sensitive to peroxide in plate assays than the *ybp1Δ* mutant containing YCplac22-YBP1 (Fig. 3B) or a *ybp1Δ* mutant where the *YBP1* gene from the library plasmid was integrated at the normal locus (Fig. 3C). Expression of *YBP1* from a plasmid (Fig. 1A and Fig. 3B) or from the normal genomic locus (Fig. 3C) did not increase the resistance to diamide but in fact resulted in increased sensitivity. This data suggests that the *YBP1* gene in W303-1a and the Yep24 library are different. The sequence of the *YBP1* gene from the Yep24 plasmid was found to be identical to the predicted amino acid sequence encoded by the *YBR216c* ORF in the genome sequence. However, in contrast, the sequence of the *YBP1* gene in W303-1a contains four amino acid substitutions; isoleucine at position 7 to leucine,
phenylalanine at position 328 to valine, lysine at position 343 to glutamate and asparagine at position 571 to aspartate (see Fig. 9).

This analysis reveals that Ybp1 plays an important role in the normal cellular resistance to peroxide and that W303-1a contains an allele of the *YBPI* gene, which we have named *ybp1-1*, that results in increased sensitivity to peroxide. In agreement with our data, a recent investigation of ~600 gene deletion mutants, demonstrated that a deletion of the *YBR216c* ORF inhibited growth in response to H$_2$O$_2$, cumene hydroperoxide and linoleic acid hydroperoxide (35).

**Ybp1 forms a complex with Yap1**

The relationship between Ybp1 and the Yap1/Skn7 pathway suggested that Ybp1 may form a complex with one or both of these proteins. Moreover, large scale studies of the yeast proteome have identified potential interactions between Yap1 and Ybp1 by two hybrid (36) and affinity precipitation (37) approaches. Hence, to further investigate the relationship between Ybp1 and Yap1/Skn7, the wild type *YBPI* gene was fused in-frame with the DNA binding domain of the *E. coli* LexA protein (LexA:Ybp1) in plasmid pBTM-YBP1. This plasmid was introduced into the two hybrid reporter strain CTY10-5d in conjunction with derivatives of the pGAD-C2 vector (29) expressing either full-length Yap1 (pGAD-Yap1(1-650)) or Skn7 (pGAD-Skn7) fused in-frame with the Gal4 acid activation domain. β-galactosidase assays of these strains revealed that Ybp1 can form a complex with Yap1 but not with Skn7 (Fig. 4; data not shown). Several functional domains of Yap1 have been defined; a bZip domain that is important for DNA binding, two separate transcriptional activation domains (I and II) and two cysteine-rich regions, located towards the middle of the protein (n-CRD) and at the C-terminus (c-CRD), that are important for the stress-induced accumulation of active Yap1 in the nucleus (see 8 for a review; Fig. 4A). To determine the
region(s) of Yap1 that are required for the interaction with Ybp1, the Gal4 acid activation domain was fused in-frame with several deletion derivatives of Yap1 and the ability of the LexA:Ybp1 fusion protein to interact with these analysed (Fig. 4A). This data revealed that a region near the C-terminus (amino acids 379-650), containing part of transcriptional activation domain II and the c-CRD, is sufficient to form a complex with Ybp1 (Fig. 4A).

To further investigate the role of this Yap1- and Ybp1-containing complex in the regulation of Yap1, we examined the effect of H$_2$O$_2$ on the interaction of Ybp1 and Yap1 in vivo. Myc-tagged Yap1 (pRS316-Yap1-Myc) (15) and Ybp1, tagged with six histidine residues and a Flag epitope (YCplac111-YBP1-6xHISFlag), were co-expressed in a yap1Δybp1Δ mutant (SR8) and cell extracts prepared before and after treatment with H$_2$O$_2$. When 6xHisFlag-tagged Ybp1 was partially purified from these cell extracts using Ni$^{2+}$ NTA-agarose, analysis of the precipitates revealed that, although a small amount of Yap1-Myc was co-purified with Ybp1-6xHisFlag from unstressed cells (data not shown), H$_2$O$_2$ treatment greatly increased the amount of Yap1-Myc (Fig. 4B). This suggests that the Yap1- and Ybp1-containing complex is stabilised following H$_2$O$_2$ treatment and raises the possibility that the formation of a complex between Ybp1 and Yap1 is important for the regulation of Yap1 by oxidative stress.

**Ybp1 is located in the cytoplasm**

To understand further the role of Ybp1, its cellular localisation was examined. We were able to detect Pk epitope-tagged Ybp1 (Ybp1-Pk) by indirect immunofluorescence when expressed from a high copy but not from a low copy number vector (Fig. 5 and data not shown). Analysis of the subcellular localisation of Ybp1-Pk, expressed in ybp1Δ cells (SR1), revealed that Ybp1-Pk was distributed throughout the cytoplasm but excluded from the nucleus. This pattern was unchanged following H$_2$O$_2$ treatment (Fig. 5). Although, as
expected, the use of a high copy number plasmid gave rise to some intercellular variation in the levels of Ybp1-Pk, reflected in the varying brightness of the immunostained cells, the distribution was the same in all cells. These data indicate that, in contrast to Yap1, Ybp1 does not accumulate in the nucleus following stress.

Ybp1 is important for the H$_2$O$_2$-induced nuclear accumulation of Yap1

To examine whether Ybp1 is important for the oxidative stress-induced nuclear accumulation of Yap1, pRS-cp-GFP-YAP1, which expresses a GFP-tagged version of Yap1 (11), was introduced into the yap1Δybp1Δ strain (SR4) and the yap1ΔYBP1 strain (SR5), where the ybp1Δ locus in SR4 was replaced with the YBP1 gene. The nuclear accumulation of GFP-tagged Yap1 following H$_2$O$_2$ treatment was clearly more efficient when the YBP1 gene is present (Fig. 6). Moreover, the nuclear accumulation of GFP-tagged Yap1 was much less efficient in a ybp1-1yap1Δ strain compared with that observed in the yap1ΔYBP1 strain suggesting that the ybp1-1 allele is a loss of function mutant (data not shown).

The nuclear accumulation of Yap1 is also stimulated by diamide though in this case the c-CRD but not the n-CRD is important for regulation (11,14,16). In contrast to H$_2$O$_2$ treatment, the diamide-induced nuclear accumulation of GFP-tagged Yap1 was similar in yap1ΔYBP1 and yap1Δybp1Δ cells (Fig. 6). This data clearly demonstrates that Ybp1 affects the localisation of Yap1 in response to specific oxidative stress.

Ybp1 is important for the H$_2$O$_2$-induced oxidation of Yap1

To examine whether Ybp1 affects the redox regulation of Yap1, cells of the yap1Δybp1Δ (SR8) strain expressing Myc-epitope-tagged Yap1 (Yap1-Myc), and containing either the low copy number CEN plasmid YCplac111-YBP1 (the wild type YBP1 gene is expressed from its own promoter) or YCplac111 vector, were treated with H$_2$O$_2$. The redox changes in Yap1-Myc were preserved with iodoacetamide then examined by non-reducing
SDS PAGE and Western blotting for characteristic changes in protein mobility (15). A more mobile form of Yap1-Myc was detected following H$_2$O$_2$-treatment of cells containing the wild type $YBP1$ gene, similar to previous studies of Yap1 oxidation (Fig. 7A) (15,18). Treatment with β-mercaptoethanol confirmed that the more mobile form of Yap1-Myc is oxidised Yap1 (data not shown). Interestingly, deletion of the $YBP1$ gene results in a significant inhibition of the H$_2$O$_2$-induced oxidation of Yap1 demonstrating that Ybp1 is required for this process (Fig. 7A).

Recently, the thiol peroxidase, Gpx3, was found to be important for the oxidation of Yap1 in response to H$_2$O$_2$ (18). To investigate the relationship between Gpx3 and Ybp1 the effect(s) of the absence of Ybp1 and/or Gpx3 on peroxide-induced Yap1 oxidation were examined (Fig. 7B). As can be seen, the loss of either Ybp1 or Gpx3 results in a loss of Yap1 oxidation following peroxide treatment, although a small but reproducible level of oxidation was detected (Fig. 7B). Interestingly, a small level of Yap1 oxidation also occurred in the absence of both Ybp1 and Gpx3. This data suggests that Ybp1 and Gpx3 may act in the same pathway to regulate peroxide-induced Yap1 oxidation. Previously, Delaunay and colleagues (18) revealed that a $gpx3\Delta$ mutant is more sensitive than a $GPX3$ strain to peroxide although not as a sensitive as a $yap1\Delta$ mutant, suggesting that an alternative pathway(s) for Yap1 activation acts in the absence of Gpx3. Indeed, our studies in the W303-1a genetic background where the $ybp1$-1 allele had been replaced with the wild type $YBP1$ gene also suggested the presence of alternative pathway(s) (Fig. 7C). Hence, it was possible that Ybp1 functions in an alternative pathway to Gpx3. However, a $gpx3\Delta ybp1\Delta$ double mutant was no more sensitive to peroxide than either of the single mutants (Fig. 7C). This data is consistent with the hypothesis that Gpx3 and Ybp1 act in the same pathway to regulate the peroxide-induced oxidation of Yap1.
This data strongly suggests that Ybp1 is required for the efficient H₂O₂-induced oxidation of Yap1 which results in the nuclear accumulation of active Yap1. Furthermore, Ybp1 likely acts in the same pathway as the Gpx3 thiol peroxidase to regulate Yap1. Thus, Ybp1 is a novel regulator of the OSR.
Discussion

Here, we describe the identification of Ybp1 as a novel regulator of the OSR in *S. cerevisiae*. Ybp1 is required for resistance to peroxide (Fig. 3). Moreover, Ybp1 regulates the H$_2$O$_2$-induced expression of the TRX2 (Fig. 2) and TRR1 (data not shown) genes in a Yap1-dependent manner suggesting that Ybp1 is required for Yap1 activity. Indeed, Ybp1 specifically forms a stress-induced complex with Yap1 and stimulates the peroxide-induced oxidation, nuclear accumulation and activity of this transcription factor.

The main mechanism by which oxidative stress activates Yap1 is by stimulating its accumulation in the nucleus (11). In unstressed cells Yap1 is exported from the nucleus by Crm1. However, oxidative stress induces changes in the redox status of cysteines in the n-CRD and c-CRD of Yap1 (15,16,18) that inhibit the interaction of Yap1 with Crm1 (12,13). Ybp1 stimulates the H$_2$O$_2$-induced nuclear accumulation (Fig. 6) and activity (Fig. 2C) of Yap1 but does not affect the ability of a constitutively nuclear and active derivative of Yap1 to activate a Yap1-dependent reporter (data not shown). Furthermore, Ybp1 did not affect the nuclear localisation of GFP-tagged Yap1 where the c-CRD and NES (nuclear export sequence) were replaced with a non-redox regulated, Crm1-dependent NES (12) (data not shown). Thus, the c-CRD is required for redox regulation of Yap1 and for stimulation of nuclear accumulation by Ybp1.

Recently, large scale analyses of yeast protein complexes, by affinity precipitation and two hybrid studies, revealed that Ybp1 forms a complex with Yap1 (36, 37). We have found that Ybp1/Yap1 complex formation is further stimulated by prior treatment of cells with H$_2$O$_2$. Different domains of Yap1 have specific regulatory roles; the c-CRD is required for diamide-induced regulation, whereas both the n-CRD and the c-CRD are required for H$_2$O$_2$-induced regulation (14, 38). Our two hybrid interaction data suggests that the...
of Yap1 containing the c-CRD is sufficient to support the interaction of Yap1 and Ybp1. However, Ybp1 is required for the H$_2$O$_2$-induced but not the diamide-induced, nuclear accumulation of Yap1. Moreover, ybp1Δ and ybp1-1 strains were more resistant to diamide than strains containing the YBP1 gene. The basis of this diamide resistance is unclear but together these results suggest that Ybp1 is required for peroxide-specific aspects of Yap1 regulation.

Several aspects of the mechanism of H$_2$O$_2$-dependent regulation of Yap1 have been characterised. In particular, the thiol peroxidase Gpx3 is important for the H$_2$O$_2$-induced oxidation of Yap1 (18) while reduced thioredoxin can reverse H$_2$O$_2$-induced oxidation (15). The effects of loss of Ybp1 (this study) or Gpx3 (18) function on the H$_2$O$_2$-induced oxidation and nuclear accumulation of Yap1 are very similar. Furthermore, a gpx3Δybp1Δ double mutant is no more sensitive to peroxide than either of the single mutants. These data suggest that Ybp1 and Gpx3 act in the same pathway to regulate Yap1. The precise nature of the relationship between Gpx3 and Ybp1 is under investigation. However, Delaunay et al. (18) demonstrated that a transient intermolecular disulphide bond forms between Gpx3 and Yap1 following H$_2$O$_2$ treatment and it is possible that Ybp1 may have a role in this process. The H$_2$O$_2$-induced stimulation of Yap1/Ybp1 complex formation, together with the observation that Ybp1 is located predominantly in the cytoplasm, both before and after peroxide stress, support a model where stress-induced binding of Ybp1 is important for the H$_2$O$_2$-induced oxidation of Yap1 by Gpx3 in the cytoplasm whereupon Yap1 accumulates in the nucleus (Fig. 8).

In this study W303-1a was found to contain a mutant allele of YBP1, ybp1-1, containing four amino acid substitutions, that result in increased peroxide sensitivity. This increased sensitivity correlated with reduced H$_2$O$_2$-induced oxidation and nuclear
accumulation of Yap1, compared with cells containing the \textit{YBPI} gene and previous studies in other strains of \textit{S. cerevisiae} (eg. 15,18). However, \textit{ybp1}-1 is not a complete loss of function allele as it is more resistant to peroxides than the \textit{ybp1}\textsuperscript{Δ} strain (Fig. 3). Many labs have investigated diverse biological processes, including the OSR, in the W303 genetic background. Furthermore, the identification of an allele of \textit{YBPI} in a commonly used lab strain has important implications for studies of Yap1 regulation. Indeed, the very fact that this strain had a mutant allele that affected the OSR to peroxide allowed us to identify \textit{YBPI}. We previously utilised this strain to identify a mutation in the \textit{TSA1} gene, encoding a thioredoxin peroxidase, that reduced \textit{TRX2} expression in response to H\textsubscript{2}O\textsubscript{2} (19). The basis of regulation of gene expression by Tsal in W303-1a is not understood. However, the introduction of the \textit{YBPI} gene into the \textit{ybp1}-1\textit{tsa1}\textsuperscript{Δ} strain rescued the peroxide-induced expression of the \textit{TRX2} gene suggesting that Ybp1 and Tsal act in separate pathways (data not shown). Interestingly, a small level of residual peroxide-induced oxidation of Yap1 can be detected in the \textit{ybp1}\textsuperscript{Δ}\textit{gpx3}\textsuperscript{Δ} double mutant (Fig. 7B) which raises the possibility that Tsal may affect Yap1 oxidation in the absence of Gpx3 and/or Ybp1.

We have identified several homologues of Ybp1 (Fig. 9). For example, the \textit{S. cerevisiae} ORF \textit{YGL060w} (\textit{YBP2}) encodes a protein with 35\% identity. Interestingly, the expression of \textit{YGL060w} is induced by the DNA damaging agent MMS (39) and transposon mutagenesis has revealed a role for \textit{YGL060w} in the response to osmotic and temperature stresses (40). We have also identified homologues of Ybp1 in other fungi, including the human pathogen \textit{Candida albicans} (Fig. 9). In \textit{C. albicans}, Cap1, a homologue of Yap1, is important for oxidative stress-induced gene expression (41, 42) and regulated nuclear localisation of Cap1 is crucial for function (42). Yap1 and Cap1 represent a sub-family of AP-1-like proteins that are found exclusively in fungi (8). Thus, Ybp1 and its homologues may
represent a novel class of regulators of these fungal stress-induced transcription factors possibly providing new targets for the development of effective anti-fungal treatments.
Acknowledgements

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References


Figure legends

Figure 1  Ybp1 protects cells against peroxides. Cultures of the (A) W303-1a, (B) skn7Δ and yap1Δ strains containing Yep24 (vector) or Yep24-YBP1 (+YBP1) were grown to mid-log and 10-fold serial dilutions spotted on to SD media containing the indicated concentrations of tBOOH, H₂O₂ and diamide.

Figure 2  Ybp1 stimulates H₂O₂-induced gene expression. Northern blot analysis of RNA isolated from mid-log growing cultures of (A) W303-1a, skn7Δ and yap1Δ strains, containing either Yep24 (vector) or Yep24-YBP1 (+YBP1), either untreated or treated with 0.1 mM H₂O₂ for 20 min, and (B) W303-1a, yap1Δ, skn7Δ and two independent ybp1Δ strains (SR1 and SR2), either untreated or treated with 0.8 mM H₂O₂ for 20 or 40 min. Probes used were specific for the TRX2 and ACT1 genes. In (A) quantitation of TRX2 mRNA levels relative to the amount of ACT1 mRNA was performed using a phosphorimager. (C) Ybp1 affects Yap1-dependent gene expression. β-galactosidase assays were performed on mid-log cultures of the wild type YAPI strain (DY), containing the integrated SV40AP1-lacZ reporter and either YEplac181 (vector) or YEplac181-YBP1 (+YBP1), either untreated or treated with 0.8 mM H₂O₂. LacZ activities are shown relative to the activity of the untreated vector control.

Figure 3  W303-1a contains a mutant allele of YBP1. (A) Growth analysis of mid-log cultures of the W303-1a and ybp1Δ (SR1) strains, containing either the low copy number CEN plasmid YCplac22 (+vector) or YCplac22-YBP1 (+YBP1), after the addition of 0.8 mM H₂O₂. (B) Cultures of the W303-1a (ybp1-1) and ybp1Δ (SR1) strains, containing the low copy number CEN plasmid YCplac22 (vector) or YCplac22-YBP1 (+YBP1), were grown to mid-log and 10-fold serial dilutions spotted on to SD media containing the indicated concentrations of tBOOH and diamide. (C) Cultures of the W303-1a (ybp1-1), ybp1Δ (SR1)
and YBP1 (SR6) strains were grown to mid-log and 10-fold serial dilutions spotted on to YPD media containing the indicated concentrations of tBOOH and diamide.

**Figure 4** Ybp1 forms a complex with Yap1. (A) Ybp1 interacts with Yap1 in two hybrid assays. β-galactosidase assays were performed on cultures of the two hybrid strain (CTY10-5d) containing pBTM-YBP1 and either the pGAD-C2 vector (pGAD), pGAD-Yap1(1-650), pGAD-Yap1(157-650), pGAD-Yap1(379-650) or pGAD-Yap1(460-650). LacZ activities were calculated as (OD_{420} x 1000)/(OD_{600} x time of incubation [min] x cells [ml]). The positions of the Gal4 acid activation domain (GAD) in the fusion proteins are shown. The previously identified domains of Yap1, including the bZip domain, the n-CRD, the c-CRD, and transcription activation domains I and II, are also indicated. (B) Ybp1 forms a stress-induced complex with Yap1 in vivo. Cell lysates were prepared from yap1Δybp1Δ (SR8) cells containing pRS316-Yap1-Myc, expressing Myc epitope-tagged Yap1 (Yap1-Myc) (15), and either YCplac111-YBP1-6xHISFlag, expressing Ybp1 tagged with six histidine residues and a Flag epitope (Ybp1-6xHisFlag), or vector control, following treatment with 0.8 mM H_{2}O_{2} for 0, 5 or 10 min. Ybp1 was partially purified from these cell lysates using Ni^{2+} NTA-agarose and precipitated proteins (Ni^{2+} pulldown) were analysed by SDS PAGE and Western blotting using anti-Myc and anti-Flag antibodies. The amount of Yap1-Myc co-purified with Ybp1 is shown relative to the amount of Yap1-Myc present in 2% of the cell lysate used in the pulldown.

**Figure 5** Ybp1 is localised in the cytoplasm. Ybp1 tagged with the Pk epitope (Ybp1-Pk) was expressed in ybp1Δ (SR1) cells from the YEplac181-YBP1-PK plasmid. Cells were fixed before (unstressed) or after treatment with 0.3 mM H_{2}O_{2} for 5 min. Ybp1-Pk was visualised by immunofluorescence using mouse monoclonal anti-Pk antibodies and Alexa 488-conjugated anti-mouse antibodies. Nuclei were stained by DAPI treatment.
**Figure 6** Ybp1 is required for efficient H$_2$O$_2$-specific nuclear accumulation of Yap1. GFP-tagged Yap1 was visualised by fluorescence microscopy of cells of the *yap1ΔybplΔ* (SR4) strain and the *yap1ΔYBP1* derivative of SR4 (SR5), containing pRS-cp-YAP-GFP, before (unstressed) and after treatment with 0.8 mM H$_2$O$_2$ or 1.5 mM diamide for 5 min.

**Figure 7** Ybp1 is required for efficient H$_2$O$_2$-induced oxidation of Yap1 by the Gpx3 dependent pathway. (A) Cells of the *yap1ΔybplΔ* strain (SR8) containing pRS316-Yap1-Myc, expressing Myc epitope-tagged Yap1 (Yap1-Myc), and either YCplac111 (vector) or YCplac111-YBP1 (+YBP1), were treated with 0.8 mM H$_2$O$_2$ for the indicated times before isolation of proteins using iodoacetimide to preserve reduced cysteines. Proteins were treated with alkaline phosphatase prior to analysis by non-reducing SDS PAGE and Western blotting using anti-Myc antibodies. Oxidised (ox) and reduced (red) forms of Yap1-Myc are indicated. (B) Cells of the *yap1ΔybplΔGPX3* (SR8) and the *yap1ΔybplΔgpx3Δ* (SR9) strains, containing pRS316-Yap1-Myc, expressing Myc epitope-tagged Yap1 (Yap1-Myc), and either YCplac111-YBP1 (+YBP1) or YCplac111 (vector), were treated with 0.8 mM H$_2$O$_2$ for the indicated times before analyses as described above. (C) Cultures of the *GPX3YAP1YBP1* (SR6), *GPX3YAP1ybplΔ* (SR1), *gpx3ΔYAP1YBP1* (SR10), *gpx3ΔYAP1ybplΔ* (SR14) and *GPX3yap1ΔYBP1* (SR5) strains were grown to mid-log and 10-fold serial dilutions spotted on to YPD media containing the indicated concentration of tBOOH.

**Figure 8** Model for the regulation of Yap1 by Ybp1. Under non-oxidising conditions Yap1 is in a reduced form (Yap1$^{\text{red}}$) which is exported from the nucleus by Crm1. Upon treatment with H$_2$O$_2$ Yap1 becomes oxidised through the action of the thiol peroxidase Gpx3. Oxidised Yap1 (Yap1$^{\text{ox}}$) no longer interacts with Crm1 and therefore accumulates in the nucleus where it activates transcription of H$_2$O$_2$-induced genes such as *TRX2*. Ybp1 is
important for the oxidation of Yap1 in response to H₂O₂ in a Gpx3-dependent manner and hence stimulates the nuclear accumulation of Yap1 and the transcription of TRX2.

**Figure 9** Alignment of Ybp1 family members. The primary amino acid sequences of Ybp1 (encoded by *YBR216c*), Ybp2 (encoded by *YGL060w*) and CaYbp1 (encoded by CA5230) were aligned using ClustalW (43). Dashes indicate single-residue gaps introduced to maximise the alignment. Amino acids conserved between Ybp1 and either Ybp2 or CaYbp1 are indicated; black boldface=identical, black type=highly similar, grey boldface=similar. Non-conserved amino acids are shown in grey type. * represents the amino substitutions I7L, F328V, K343E, N571D encoded by the *ybp1-1* allele of W303-1a.
Figure 1
Figure 2
Figure 3

A

![Graph showing fold growth over time after \( \text{H}_2\text{O}_2 \) (hr)]

- W303-1a (\( ybp1-1 \)) + vector
- \( ybp1\Delta \) + vector
- \( ybp1\Delta + \text{YBP1} \)

B

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Figure 4
Figure 5

Ybp1+Pk

Nuclei

unstressed

H₂O₂
Figure 7

A

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Yap1^red^-Myc
Yap1^ox^-Myc

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Yap1^red^-Myc
Yap1^ox^-Myc

C

GPX3 YAP1 YBP1
GPX3 YAP1 ybp1Δ
gpx3Δ YAP1 YBP1
gpx3Δ YAP1 ybp1Δ

control
0.2mM tBOOH
Figure 8
Additions and Corrections


Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor.

Elizabeth A. Veal, Sarah J. Ross, Panagiota Malakasi, Emma Peacock, and Brian A. Morgan

Page 30903: In the original paper, Fig. 9 indicated that the protein encoded by the ybp1-1 allele was predicted to contain four amino acid changes. We have subsequently discovered another change in the ybp1-1 allele; a “C” is inserted in the coding strand (see below). This insertion alters the third base of the codon for Pro-243, and the codon for Asp-244 is changed to a Stop codon (TGA). Importantly, this additional change does not affect the conclusions of the paper with regard to the role of Ybp1 in the regulation of Yap1 or that W303-1a contains a mutant allele of the YBP1 gene. Interestingly, the phenotypes associated with the ybp1-1 allele (see paper) suggest that the mutant protein retains some activity. Thus, the insertion of a premature stop codon suggests that this residual activity is located in amino acids 1-243. We thank Dr. S. Kuge for bringing this error to our attention, and we apologize to the readers of the Journal for any confusion or inconvenience this error may have caused.

The predicted amino acid sequence (original Fig. 9) of the ybp1-1 allele between amino acids 241 and 245 was:

....(aa241) I P P D P(aa245)....
....ATT CCC CCT GAT CCA....

The corrected predicted sequence of the protein encoded by the ybp1-1 allele is:

....(aa241) I P P Term
....ATT CCC CCC TGA....
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