Proteolytic Degradation of the Yap1 Transcription Factor Is Regulated by Subcellular Localization and the E3 Ubiquitin Ligase Not4*

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Saccharomyces cerevisiae Yap1 is a transcriptional regulatory protein that serves as a central determinant of oxidative stress tolerance. Activity of this factor is regulated in large part by control of its subcellular location. In the absence of oxidants, Yap1 is primarily located in the cytoplasm. Upon oxidant challenge, Yap1 accumulates rapidly in the nucleus where it activates expression of genes required for oxidative stress tolerance such as the thioredoxin TRX2. Here, we demonstrate that Yap1 degradation is accelerated in response to oxidative stress. Yap1 is folded differently depending on the oxidant used to induce its nuclear localization but is degraded similarly, irrespective of its folded status. Mutant forms of Yap1 that are constitutively trapped in the nucleus are degraded in the absence of an oxidant signal. Degradation requires the ability of the protein to bind DNA and a domain in the amino-terminal region of the factor. Inhibition of the proteasome prevents Yap1 turnover. Screening a variety of mutants involved in ubiquitin-mediated proteolysis demonstrated an important role for the nuclear ubiquitin ligase Not4 in Yap1 degradation. Not4 was found to bind to Yap1 in an oxidant-stimulated fashion. The Candida albicans Yap1 homologue (Cap1) also was degraded after oxidant challenge. These data uncover a new, conserved pathway for regulation of the oxidative stress response that serves to temporally limit the duration of Yap1-dependent transcriptional activation.

Use of oxygen as a terminal electron acceptor allows for the efficient utilization of carbon as an energy source but also causes cells to constantly produce reactive oxygen species (ROS). ROS can directly damage macromolecules of biological importance and also trigger loss of a physiologically tolerant intracellular redox environment. Cells have developed a variety of means to sense and respond to threats caused by increases in ROS levels. One of the best studied redox-activated transcription factors is the Saccharomyces cerevisiae Yap1 protein (seeRefs. 1–3 for review).

Yap1 activates the expression of a large number of different genes encoding a variety of proteins with antioxidant function (4, 5). This Yap1-dependent transcriptional network is stimulated in response to several different ROS-generating compounds such as H2O2 and diamide. One of the major routes of Yap1-oxidant regulation occurs at the level of control of the nuclear localization of this transcription factor (6). In the absence of excessive ROS production, Yap1 is found almost exclusively in the cytoplasm. Upon oxidant challenge, Yap1 rapidly moves to and accumulates in the nucleus where it induces its characteristic transcriptional program. This regulated nuclear localization is accomplished by controlling the rate at which Yap1 is exported from the nucleus, a process catalyzed by the nuclear exportin Crm1 (7, 8).

Two clusters of cysteine residues, referred to cysteine-rich domains (CRD), are essential for the regulation of Yap1 nuclear export in response to redox challenge. Cysteine residues in the C-terminal CRD can form two pairs of disulfide bonds with cysteine residues in the N-terminal CRD (n-CRD) (9–11). This dually disulfide-bonded form of Yap1 is only formed upon challenge with the oxidant H2O2. H2O2-specific oxidative folding also requires the presence of the Yap1-binding protein Ybp1 and the glutathione peroxidase homologue Gpx3 (11–13). This H2O2-folded form of Yap1 is required to allow recruitment of a specific transcriptional mediator component called Rox3 to key Yap1 target genes such as the thioredoxin TRX2 (14, 15). Only when the properly folded form of Yap1 arrives at TRX2 can the appropriate antioxidant response be induced to detoxify H2O2.

Induction of oxidative stress by exposure to oxidants such as diamide or diethylmaleate triggers the same nuclear accumulation of Yap1 but has a number of different molecular features. First, these oxidants seem to act directly on Yap1 to oxidize cysteine residues in the C-terminal CRD that in turn block Crm1 interaction with Yap1 (7, 8). Second, no disulfide bond formation is required for diamide- or diethylmaleate-induced

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3 The abbreviations used are: ROS, reactive oxygen species; CRD, cysteine-rich domains; TAP, tandem affinity purification; UPS, ubiquitin proteasomal system; YRE, Yap1 response element; n-CRD, N-terminal CRD.
nuclear localization of Yap1. Finally, no additional protein factors are required for Yap1 nuclear accumulation in response to these oxidants. Loss of cysteines in the C-terminal CRD either constitutively trapped in the nucleus with an accompanying elevation in diamide and diethylmaleate resistance. These same oxidants are required for Yap1 nuclear accumulation in response to oxidative stress onset. In this work, we demonstrate that Yap1 is degradation of Yap1 mutants that were trapped genetically in this mutant form of Yap1 was localized to the nucleus in response to oxidative stress. A BY4742 strain con-

**TABLE 1**

| Strains used in this study | BY4742 MATa his3-D1 leu2-D0 lys2-D0 ura3-D0 Open Biosystems |
|---------------------------|------------------------------------------------------|
| not4::his3-D1           | BY4742 not4::his3-D1 Open Biosystems               |
| His3::not4::his3-D1      | BY4742 not4::his3-D1 Open Biosystems               |
| rad18-1::his3-D1        | BY4742 rad18-1::his3-D1 Open Biosystems            |
| rad18-2::his3-D1        | BY4742 rad18-2::his3-D1 Open Biosystems            |
| rad6-1::his3-D1         | BY4742 rad6-1::his3-D1 Open Biosystems             |
| rad6-2::his3-D1         | BY4742 rad6-2::his3-D1 Open Biosystems             |
| rad18::his3-D1          | BY4742 rad18::his3-D1 Open Biosystems              |
| rad6::his3-D1           | BY4742 rad6::his3-D1 Open Biosystems               |
| rad5::his3-D1           | BY4742 rad5::his3-D1 Open Biosystems               |
| rad4::his3-D1           | BY4742 rad4::his3-D1 Open Biosystems               |
| rad3::his3-D1           | BY4742 rad3::his3-D1 Open Biosystems               |
| rad2::his3-D1           | BY4742 rad2::his3-D1 Open Biosystems               |
| rad1::his3-D1           | BY4742 rad1::his3-D1 Open Biosystems               |
| rad0::his3-D1           | BY4742 rad0::his3-D1 Open Biosystems               |
| rad9::his3-D1           | BY4742 rad9::his3-D1 Open Biosystems               |
| rad8::his3-D1           | BY4742 rad8::his3-D1 Open Biosystems               |
| rad7::his3-D1           | BY4742 rad7::his3-D1 Open Biosystems               |
| rad6::his3-D1           | BY4742 rad6::his3-D1 Open Biosystems               |
| rad5::his3-D1           | BY4742 rad5::his3-D1 Open Biosystems               |
| rad4::his3-D1           | BY4742 rad4::his3-D1 Open Biosystems               |
| rad3::his3-D1           | BY4742 rad3::his3-D1 Open Biosystems               |

**Proteolytic Regulation of Yap1**

Yeast Strains, Plasmids and Media—The strains used in this study are listed in Table 1. All strains used in this study were derived from the BY4741 or BY4742 backgrounds. Yeast cells grown in rich YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) or complete synthetic media lacking appropriate amino acids. These cultures (50 ml each) were treated with 1 mM H$_2$O$_2$, 1 mM diethylmaleate, 1 mM diamide, or left untreated. The cultures were incubated at room temperature for 15 min. Cells were quickly washed with water and transferred to fresh media containing 100 μg/ml cycloheximide with or without oxidant as appropriate. For the 0-h time point, 10 ml of culture was immediately taken out after the addition of cycloheximide. Cultures were further allowed to incubate at 30 °C with shaking at 250 rpm. Further samples were collected at the indicated time periods and processed by centrifugation, followed by washing the pellets with 20% TCA and frozen until ready for further steps. The pellets from different time periods were then resuspended in 200 μl of 12.5% TCA and lysed with glass beads by vigorous shaking on a Tomy shaker for 20 min. The extracts were collected and another 200 μl of 12.5% TCA was added to glass beads and vortexed for 1 min. Extracts were pooled (total volume ~ 400 μl) and spun down at full speed for 5 min. Pellets were washed with ice-cold acetone and air-dried. 100 μl of 1× Laemmli buffer (0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.002% bromphenol blue dye), and 50 μl of 1 M Tris base were added to the pellets. Samples (40 μl) were then subjected to Western blot analysis, using anti-Yap1 antibody (1:7000 dilution) (22) and anti-rabbit secondary antibody (1:12,000 dilution).

Co-immunoprecipitation—For performing co-immunoprecipitation assays to determine the identity of E3 ligase responsible for degradation of Yap1, speroplasts were prepared and subjected to oxidant exposure. In brief, 100 ml of cells was harvested at an A$_{600}$ of 1.0 and washed with speroplast solution I (1 M sorbitol, 10 mM MgCl$_2$, 30 mM diithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 50 mM K$_2$HPO$_4$), and resuspended in speroplast solution II (1 M sorbitol, 30 mM diithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 50 mM K$_2$HPO$_4$, 25 mM sodium succinate, pH 5.5) containing oxylyticase, and incubated at 30 °C for 1 h on a shaker at 100 rpm. After chilling on ice, cell suspensions were overlaid on a sucrose cushion (20 ml HEPES, 1.2 M sucrose, 0.02% sodium azide) and pelleted by centrifuging at 4000 × g for 20 min at 4 °C. A portion of the resulting speroplasts was treated with 1 mM H$_2$O$_2$ for 25 min at room temperature, suspended in ICE (lysis) buffer (100 mM potassium acetate, 50 mM KCl, 20 mM PIPES, 200 mM sorbitol, pH 6.8). Protein extracts were clarified by centrifuging lysates at 14,000 rpm for 5 min at 4 °C. The lysates were incubated with anti-TAP antibody for 4 h at 4 °C.
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a rotator. Protein A-agarose beads, washed and resuspended in the same buffer, were then added to the lysate and incubated for 2 h for immunoprecipitation. Finally, the beads were washed and collected by centrifugation, and immunoprecipitated proteins were recovered by adding 2× Laemmli buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromphenol blue dye). Protein samples were then loaded on 10% polyacrylamide gel and probed with anti-Yap1 antibodies or anti-TAP antibody.

RESULTS

Rate of Yap1 Proteolysis Increases after Activation—Activation of Yap1-dependent transcriptional regulation occurs primarily due to localization of this transcription factor to the nucleus (see Refs. 1–3 for review). Several mutants exhibiting constitutive nuclear localization also were observed to accumulate at reduced steady-state levels when evaluated by Western blotting (6, 9, 16, 22). To determine whether the decreased accumulation seen in terms of steady-state levels could be explained by enhanced proteolysis, a cycloheximide chase assay was carried out. A strain lacking the Yap1 protein (yap1Δ) was transformed with an empty vector plasmid or the same plasmid expressing wild-type YAP1 (WT) or the constitutively nuclear form of Yap1 (C629A). Transformants were grown to mid-log phase in complete synthetic (SC) medium, and whole cell protein extracts were prepared after incubation for the indicated times in the presence of 100 μg/ml cycloheximide. Proteins were resolved on SDS-PAGE, and the levels of Yap1 were detected by the use of an anti-Yap1 polyclonal antiserum (22). The pRS316 transformants are indicated as yap1Δ. B, wild-type cells were grown inYPD medium to mid-log phase. Aliquots were then incubated an additional 15 min with no treatment or with oxidative stress imposed by the addition of 1 mM H2O2, 1 mM diethylmaleate (diethylmaleate). After this incubation, cycloheximide chase was performed as described above followed by Western blot analysis of Yap1 levels.

The C629A form of Yap1 was nearly completely degraded over this time interval (Fig. 1A), whereas wild-type Yap1 did not exhibit significant degradation. A major difference between these two forms of Yap1 occurs at the level of nuclear localization. The wild-type factor resides in the cytoplasm in the absence of oxidative stress but translocates rapidly to the nucleus upon oxidative exposure (6). To determine whether the wild-type Yap1 protein exhibited oxidant-triggered instability, wild-type cells were analyzed by the cycloheximide chase protocol described above, either with no stress or with the addition of H2O2 or diethylmaleate to induce oxidative stress. The wild-type Yap1 protein also reaches the nucleus.

The addition of H2O2 caused Yap1 levels to markedly decline within 120 min after the onset of oxidative stress (Fig. 1B). diethylmaleate-induced oxidative stress induced an even more rapid decline in Yap1 levels with the majority of this transcription factor being eliminated 60 min after oxidant exposure. Together, these data suggest that localization of Yap1 to the nucleus leads to degradation of the transcription factor.

Nuclear Localization and DNA-binding Are Required for Oxidant-induced Degradation of Yap1—Previous studies have uncovered unique requirements for Yap1 nuclear translocation in the presence of H2O2. The Yap1-binding protein (Ybp1) is an integral component of Yap1 nuclear localization during H2O2 stress but is dispensable during challenge by oxidants such as diethylmaleate or diamide (12, 13). Use of a ybp1Δ strain allowed Yap1 to be selectively prevented from entering the nucleus during H2O2 stress but still accumulate in this compartment upon diamide stress. Cycloheximide chase analysis was performed to compare Yap1 degradation in wild-type or isogenic ybp1Δ cells under three different conditions: no stress or H2O2- or diamide-induced oxidative stress (Fig. 2A).

Loss of Ybp1 completely prevented Yap1 degradation following H2O2 stress but had no effect on the turnover of Yap1 elicited by diamide shock. This finding argues that the presence of H2O2 is not sufficient to cause Yap1 degradation unless the protein also reaches the nucleus.

To further explore the basis of oxidant-triggered degradation of Yap1, low-copy number plasmids expressing either wild-type (WT) or a DNA-binding mutant form (K67A) of Yap1 were introduced into yap1Δ cells. We previously characterized K67A Yap1 as being completely defective in DNA-binding as assessed by an in vitro assay (23). Degradation of wild-type and K67A Yap1 under stressed and nonstressed conditions was evaluated as above.

K67A Yap1 was not degraded in response to either H2O2 or diamide stress, whereas the wild-type protein was destabilized by both treatments (Fig. 2B). The loss of oxidant-induced instability in the K67A form of Yap1 is consistent with the notion that Yap1 must be able to engage its target promoters to exhibit the characteristic degradation during stress This conclusion was supported by analysis of the regulated nuclear localization of the K67A form of Yap1. This mutant protein was normally imported into the nucleus in response to H2O2 treatment (Fig. 2C), eliminating any concern that defective nuclear import could explain the observed stability of this mutant Yap1 derivative.
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The primary determinant of the subcellular localization of Yap1 occurs at the level of nuclear export of this protein. The steady-state distribution of Yap1 is cytoplasmic owing to its relatively slow nuclear import (24), coupled with the rapid nuclear export via the action of the nuclear exportin Crm1 (7, 8). A strain containing a temperature-sensitive allele of Crm1 (crm1ts) was used to determine whether Yap1 was destabilized by trapping the protein in the nucleus. The cmy1ts strain was transformed with a low-copy number plasmid expressing GFP-labeled Yap1 to confirm that Yap1 accumulates in the nucleus at the restrictive temperature. In parallel, the cmy1ts strain was transformed with a vector plasmid containing or lacking the wild-type CRM1 gene to verify that any effect due to temperature shift in this strain was due to the cmy1ts allele. Appropriate transformants were grown to mid-log phase, and then equal fractions were maintained at 30 °C or shifted to the restrictive temperature of 37 °C. After incubation at these temperatures, transformants were analyzed for Yap1 stability by cycloheximide chase to detect GFP-Yap1 (GFP), DNA (DAPI), or Nomarski optics (DIC).

Inactivation of Crm1 by growth at the restrictive temperature strongly destabilized Yap1 (Fig. 3A). Introduction of wild-type CRM1 into the temperature-sensitive background restored the stability of Yap1. No oxidants were added to these cultures, which supports the idea that loss of Yap1 nuclear export is sufficient to cause the protein to be susceptible to proteolysis. GFP-Yap1 was seen to accumulate in the nucleus at the restrictive temperature as expected (Fig. 3B). Together, these data suggest that accumulation of a DNA-binding competent form of Yap1 in the nucleus caused by oxidative stress, mutational inactivation of nuclear export (C629A mutation), or by inactivation of Crm1 is sufficient to lead to destabilization of the protein.

Evidence for Proteasomal Degradation of Yap1 and Positive Site for Oxidant-induced Degradation—Analyses of other transcription factors, including the basic region-leucine zipper-containing Gcn4 protein, have demonstrated that the proteasome is the proteolytic system responsible for their degradation (25–27) and the importance of this proteolytic system in control of nuclear gene expression (reviewed in Ref. 28). To determine whether the proteasome was similarly required for Yap1 degradation, a cycloheximide chase experiment was carried out in the presence or absence of the proteasome inhibitor MG132. These experiments were performed in a strain lacking the Pdr5 ABC transporter protein as this genetic background has been found to facilitate use of MG132, likely through increasing the uptake of this drug (29). MG132 was added to one culture prior to beginning the cycloheximide chase assay. Oxidative stress degradation.
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A.

B.

FIGURE 4. Requirements for Yap1 proteolysis. A, a pdr5Δ strain was grown to early log phase. After dividing the culture into two equal aliquots, the proteasomal inhibitor MG132 was added (+) at 100 μM to the indicated sample. Incubation was continued for 3 h. Then each aliquot was further divided into two and either left untreated or challenged with 1 mM diethylmaleate for 15 min. After this treatment, the cultures were analyzed for Yap1 turnover by cycloheximide chase assay. B, transformants expressing the indicated forms of Yap1, along with the empty vector plasmid (denoted as yap1Δ), were grown in complete synthetic medium to mid-log phase and then assayed as above by cycloheximide chase analysis for levels of Yap1 remaining after oxidative stress. NS, no stress.

was induced with the addition of diethylmaleate and levels of Yap1 assessed as described previously (Fig. 4A).

The addition of MG132 produced a profound block in Yap1 degradation after imposition of oxidative stress. This analysis supports the view that the proteasome carries out the proteolytic degradation of Yap1 following oxidative challenge.

Previous studies on Yap1 determined that the steady-state level of deletion derivatives of the protein lacking sequences around the n-CRD were elevated compared with the wild-type factor (22). The observations above indicate that Yap1 is subject to proteolysis after oxidative stress suggesting that changes in steady-state level of particular mutants might be influenced by changes in the ability of these variants to be degraded. We selected two n-CRD deletion mutants to examine: Δ220–243 and Δ220–335 Yap1. These two n-CRD mutants have very different abilities to function in vivo. The Δ220–243 Yap1 derivative exhibited greater than wild-type resistance to H2O2 and expression of a TRX2-lacZ reporter gene, whereas Δ220–335 Yap1 was unable to correct the H2O2 hypersensitivity or drive normal TRX2 expression in a yap1Δ strain as we documented previously (22). Interestingly, both of these mutant Yap1 proteins confer greater-than-normal tolerance to diamide or diethylmaleate (22).

We focused our attention on the Δ220–243 form of Yap1 as this protein elevated resistance to all tested oxidants. To determine whether the increased oxidant resistance conferred by the presence of Δ220–243 Yap1 could be due to a change in its degradation leading to increased accumulation of the protein, we carried out a cycloheximide chase analysis of turnover of this mutant transcription factor. It is important to note that Δ220–243 Yap1 confers both hyper-resistance to H2O2 and also drives very high levels of expression of bona fide Yap1 target genes such as TRX2 (9, 14).

Δ220–243 Yap1 was very stable following oxidative stress imposed by either H2O2 or diethylmaleate (Fig. 4B). This increased stability likely contributes to the observed increment in H2O2 tolerance seen in strains expressing this form of Yap1 (22). Experiments with the Δ220–335 Yap1 also indicated that this form of the transcription factor exhibited enhanced stability after oxidative stress (data not shown). These results suggest that the region around the n-CRD element in Yap1 is an important determinant in regulating the proteolytic turnover of this protein.

Identification of Ubiquitin Proteasomal System Components Influencing Yap1 Activity—Based on the results described above, we hypothesized that Yap1 degradation would be regulated by ubiquitin-dependent targeting of the protein to the proteasome. To identify proteins that might fulfill this regulatory role, we screened a subset of the collection of haploid disruptant S. cerevisiae mutants selected on the basis of their known role in the ubiquitin proteasomal system (UPS). These UPS mutants were grown to mid-log phase and then tested for the ability to confer resistance to the oxidant H2O2. Because we already had observed that the Δ220–243 Yap1 form of Yap1 exhibited increased stability upon H2O2 challenge (Fig. 4), we hypothesized that defects in the machinery responsible for Yap1 degradation would cause a similar phenotype. Mutants were selected based on their previously determined role in the UPS, grown to mid-log phase and then tested for the ability to stabilize Yap1 after cells were treated with H2O2. Although a large number of different mutant strains were screened, a summary of these assays will be presented here.

Loss of the E3 ubiquitin ligase-encoding gene NOT4 (30) caused a readily detectable increase in stability of Yap1 target gene, whereas loss of other E3 ligases such as Ubr1 (31) or Cul3 (32) did not exhibit any significant change in Yap1 turnover (Fig. 5A). A not3Δ mutant strain was not observed to stabilize Yap1. Work from another group has provided evidence that loss of the ubr1+ gene from Schizosaccharomyces pombe caused increased stability of the Yap1 homologue (Pap1) from this yeast with an associated increase in oxidant resistance (33), but this role does not appear conserved in S. cerevisiae.

The UPS has been implicated in degradation of oxidatively damaged proteins previously (reviewed in Ref. 34). To determine whether the increased Yap1 stability seen in a not4Δ strain was associated with an accumulation of active Yap1, several reporter gene fusion plasmids were introduced into the strains listed above. A fusion gene corresponding to the thioredoxin-encoding TRX2 promoter fused to Escherichia coli lacZ was used. TRX2 is a well established target of Yap1 transcriptional regulation and required for normal H2O2 resistance (14). A Yap1 response element (YRE) from the TRX2 promoter fused to a minimal CYC1 promoter-lacZ fusion gene was also tested to examine the specificity of the effect of these mutant backgrounds on Yap1-mediated gene activation. Finally, a TRPS-lacZ reporter gene that we have demonstrated previously to be Yap1-independent was used as a control for nonspecific effects on gene expression. These three fusion genes, carried on low-copy number plasmids, were transformed into isogenic wild-type, not4Δ, ubr1Δ, and cul3Δ strains. Transformants were grown to mid-log phase and then left untreated or oxidatively stressed with the inclusion of H2O2 or diamide.

Loss of Not4 led to an increase in β-galactosidase from both the YRE-CYC1-lacZ and TRX2-lacZ construct, in the absence and presence of oxidants (Fig. 5B). TRPS-lacZ expression was
not affected by the presence of the not4Δ allele. This secondary test supports the view that loss of the Not4 E3 enzyme led to an increase in the level of Yap1 with a concomitant elevation in Yap1-mediated transcriptional activation.

The UPS is involved in a wide range of different proteolytic events regulating the levels of many different proteins. The impressive spectrum of UPS-regulated proteins makes the possibility that these effects of Not4 on Yap1 might be indirect. To assess the possibility that Not4 directly associates with Yap1, co-immunoprecipitation experiments were carried out. We used a range of different TAP-tagged strains corresponding to various UPS and Not4-related components. These TAP-tagged strains were grown to mid-log phase, incubated for an additional 25 min in the absence or presence of H2O2, and then whole cell protein extracts were prepared. TAP-tagged proteins were recovered by immunoprecipitation with anti-TAP antibody as described (35). These immunoprecipitates were electrophoresed through SDS-PAGE and then analyzed by Western blotting with anti-TAP or anti-Yap1 antibodies.

Yap1 co-precipitated with the ubiquitin ligase Not4. Not4-Yap1 association was stimulated by treatment of the cells with H2O2 prior to carrying out the anti-TAP immunoprecipitation. Interestingly, two other proteins associated with the Ccr-Not complex (Not3 and Not5) did not bind to Yap1 in this assay. Other E3 ligases (Rad18, Cul3, Hrd1) and two different Ubc proteins (Rad6, Mms2) did not co-precipitate Yap1. These data are consistent with Not4 binding to Yap1 and regulating the proteolytic turnover of this transcription factor.

Trans-acting Factors and cis-determinants Regulating Yap1 Degradation—Yap1-mediated transcriptional activation after exposure to H2O2 has been shown previously to require the assistance of two other proteins. The sequence-specific transcription factor Skn7 is an essential contributor both to H2O2 resistance and also to induction of expression of genes similar to TRX2 that are required for normal H2O2 tolerance (36). Earlier work established that Yap1-dependent recruitment of the transcriptional Mediator component Rox3 occurred only during H2O2-induced folding of this transcription factor and also was required for wild-type resistance to this oxidant (15). The binding experiments above indicated that the ubiquitin ligase Not4 associated with Yap1 in response to H2O2 treatment. Although we have provided data above consistent the view that Yap1 must enter the nucleus prior to its degradation, we tested...
the contribution of these trans-acting factors to the proteolytic turnover of Yap1 in two different ways. First, we compared a series of isogenic strains lacking Not4, Skn7, or Rox3 for the ability to inducibly degrade Yap1 after challenge with H$_2$O$_2$. Second, a strain expressing the constitutively nuclear C629A Yap1 derivative was tested for its proteolytic degradation in wild-type, not4Δ, and skn7Δ strains. As described previously, we did not treat cells expressing the C629A Yap1 derivative with oxidants. Transformants were analyzed for Yap1 turnover by cycloheximide chase assay as before.

Loss of the Not4 ubiquitin ligase led to a profound block in H$_2$O$_2$-triggered turnover of Yap1 (Fig. 6B). Similarly, the not4Δ strain also exhibited a strong reduction in the rate of degradation of the constitutively nuclear C629A form of Yap1. Loss of either Skn7 or Rox3 had no significant effect on degradation of wild-type Yap1 after H$_2$O$_2$ stress and a skn7Δ allele did not influence the turnover of C629A Yap1.

The interaction of Not4 and Yap1 also was assessed in these mutant backgrounds. A NOT4-TAP allele was integrated into the genome of wild-type, rox3Δ, and skn7Δ strains. These strains were then analyzed for H$_2$O$_2$-dependent Not4-Yap1 association as described previously.

Yap1 association with Not4 was unaffected by the absence of these key co-factors required for H$_2$O$_2$-inducible transcription (Fig. 7A). This is consistent with the lack of an effect caused by loss of Rox3 or Skn7 on Yap1 turnover seen earlier (Fig. 6B). Because we had found previously that deletion derivatives of Yap1 lacking sequence elements around the n-CRD also exhibited increased steady-state protein levels (9, 22), the interaction of Not4 with a mutant lacking the entire n-CRD was evaluated using the Not4-TAP association assay.

A yap1Δ strain carrying the NOT4-TAP allele was transformed with low-copy number plasmids expressing either wild-type or Δ220–335 Yap1. The Δ220–335 Yap1 derivative was found to accumulate to higher steady-state levels than wild-type in an earlier study (22). Transformants were grown and then left untreated or challenged with H$_2$O$_2$. The level of Yap1 associated with Not4-TAP was analyzed by co-immunoprecipitation as described above.

Loss of the Yap1 region from Δ220–335 reduced the ability of the resulting mutant to associate with Not4 (Fig. 7B) by ~3-fold. This analysis suggests that the region around the n-CRD is critical but not essential for recruitment of Not4 to Yap1 to induce the degradation of this transcription factor.

Oxidant-induced Degradation of Candida albicans Homologue of Yap1—The pathogenic yeast C. albicans contains a Yap1 homologue called Cap1 (37, 38). This transcription factor is recruited inducibly to the nucleus in response to oxidative stress as is Yap1 in S. cerevisiae (39). We produced a polyclonal antibody directed against Cap1 in this earlier study and used this reagent to determine whether oxidant exposure also induced Cap1 degradation in C. albicans. Isogenic wild-type and cap1Δ/cap1Δ homozygous strains were grown to mid-log phase, either left untrated or treated with diethylmaleate or H$_2$O$_2$, followed by incubation in the presence of 100 μg/ml cycloheximide. Equal samples of cells were withdrawn, and whole cell protein extracts were prepared at the indicated time points. The polyclonal anti-Cap1 antibody was used to assess the levels of Cap1 by Western blotting.

Treatment of C. albicans with either H$_2$O$_2$ or diethylmaleate induced the degradation of Cap1 (Fig. 7C). Cap1 was fairly stable in the absence of oxidative stress as very little degradation was seen over the 60-min time period of this experiment. Cap1
is cleared from promoters after oxidative stress in *C. albicans* in a manner directly analogous to that of Yap1 in *S. cerevisiae*.

**DISCUSSION**

Yap1 is regulated primarily by control of its nuclear localization, which was thought previously to represent a reversible process (40, 41). Here, we provide the first evidence that the movement of Yap1 into the nucleus, coupled with engagement of a target promoter, represents a one-way trip for this transcription factor. The proteolysis of Yap1 explains previous observations of the diminished expression of this protein when expressed in a constitutively nuclear form (6, 9, 22). Yap1 derivatives that escape nuclear export by Crm1 bind to YREs and are then subject to proteolysis. Under normal circumstances, ongoing protein synthesis would maintain this pool of Yap1 but when this is blocked (here by the addition of cycloheximide), the rapid degradation of DNA-bound Yap1 can be visualized.

Experiments aimed at uncovering the substrates of the ubiquitin ligase Ubr1 in *S. pombe* have provided evidence that the Pap1 transcription factors is a substrate of this ligase in this fission yeast (33). Given the striking sequence and regulatory conservation between Yap1 and Pap1 (42), we anticipated that *S. cerevisiae* Ubr1 (ScUbr1) would be involved in controlling Yap1 stability. Surprisingly, we found that *S. cerevisiae* Not4 rather than *S. cerevisiae* Ubr1 was the ubiquitin ligase linked to control of Yap1 stability in *S. cerevisiae*. Our determination that *S. cerevisiae* Not4 can be co-immunoprecipitated with Yap1 (Fig. 7) provides data in support of a direct role of *S. cerevisiae* Not4 in Yap1 degradation. Association of *S. pombe* Ubr1 with Pap1 could not be demonstrated (33), leaving open the possibility that the action of *S. pombe* Ubr1 on Pap1 stability may be indirect. Coupled with these recent data demonstrating the proteolysis of Pap1, our findings that both Yap1 and *C. albicans* Cap1 are also degraded after oxidative stress suggest that this regulatory feature is likely to be conserved in fungi that express an oxidant-responsive Yap1 homologue. We hypothesize that the regulation of Cap1 will be more similar to that of Yap1 than Pap1 as both *S. cerevisiae* (12, 13) and *C. albicans* express a homologue of the oxidative folding co-factor Ybp1, whereas *S. pombe* does not (data not shown). Experiments are currently underway to determine whether the *C. albicans* Not4 homologue is required for oxidant-induced degradation of Cap1 as is the cognate *S. cerevisiae* protein.

The finding that Yap1 is degraded by proteolysis after it binds to the YRE is consistent with a requirement to ensure that Yap1-mediated gene induction is regulated carefully and inactivated rapidly when no longer necessary. Only oxidatively activated or mutationally altered Yap1 is capable of evading Crm1-dependent export (7, 8). Removal of YRE-bound Yap1 by proteolysis requires that appropriately oxidized Yap1 continuously arrives to maintain high level target gene transcription. Once redox conditions have returned to a range in which Yap1 is no longer oxidized, Crm1 export will predominate and promoter-bound Yap1 will be degraded and no longer replenished. The combination of these regulatory mechanisms will ensure tight and rapid control of Yap1-mediated transcriptional activation.

The specificity of Yap1 degradation induced by Not4 is linked tightly to the nuclear accumulation and DNA binding of this transcription factor. Not4 can function in the cytoplasm as the nascent polypeptide complex protein Egd2 gets ubiquitinated by Not4 in cytoplasm, and translation arrest products are subject to Not4-dependent turnover (44). The exclusion of Yap1 from the nucleus is unlikely to present an ability to evade Not4. We hypothesize that Yap1 binding to promoter DNA renders the factor sensitive to Not4-dependent degradation. One interesting possibility would be the involvement of the transcriptional Mediator component Cdk8 (Srbi10). This cyclin-dependent kinase phosphorylates and triggers the degradation of a number of transcription factors, including Gcn4 (27), Ste12 (45), Msn2 (46, 47), and Hac1 (48). However, direct examination of Yap1 turnover in cdk8Δ cells failed to detect any alteration in proteolysis (data not shown).

Not4 has previously been implicated in regulation of nuclear functions. The DNA polymerase α encoded by *CDC17* is degraded in a Not4-dependent manner (49). Strikingly, degradation of the histone demethylase Jhd2 is regulated by Not4 and required to support normal levels of methylation of histone H3 (50). Although Skn7 is tightly linked to Yap1-dependent oxidative stress transcription and this winged helix transcription factor associates with Not4, we determined that Skn7 degradation was unaffected by loss of Not4 (data not shown).

Previous work from others indicated that Yap1 was the target of proteolysis in a manner regulated by the presence of the calcium-regulated phosphatase calcineurin (51). We tested a *cnb1Δ* cell and found that loss of calcineurin activity had no influence on Yap1 turnover upon oxidant challenge (data not shown). The *cnb1Δ* strain also exhibited no increase in oxidant resistance, which might be expected if the absence of calcineurin activity caused an increase in Yap1 level. The lack of an effect of calcineurin on Yap1 degradation may be due to the different strain background used here or a selective role for this phosphatase in calcium-triggered degradation.

An important theme illustrated by these findings is the mechanism by which transcription factors are inactivated when their regulatory influences are no longer required. The *S. cerevisiae* MATα2 repressor protein is removed from its DNA target site via the Cdc48 AAA ATPase (52). Some transcription factors are thought to cycle on and off their DNA binding sites in a fairly rapid manner that would facilitate their inactivation by the proteasome (53–55). However, given the wide range of transcription factors and differences in their modes of regulation, multiple mechanisms undoubtedly are invoked to modulate their transcriptional influences. Our findings support the view that proteolytic turnover of site-specific transcription factors may be a common avenue used by cells to regulate the function of these important regulatory molecules. Because some transcription factors may exhibit prolonged association with promoter elements beyond the time their respective transcriptional influences are required, active degradation will limit inappropriate gene regulation.

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4 J. Quinn, personal communication.
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