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Yap1 activation by H_2O_2 or thiol-reactive chemicals elicits distinct adaptive gene responses

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ABSTRACT

The yeast *Saccharomyces cerevisiae* transcription factor Yap1 mediates an adaptive response to oxidative stress by regulating protective genes. H_2O_2 activates Yap1 through the Gpx3-mediated formation of a Yap1 Cys303–Cys598 intramolecular disulfide bond. Thiol-reactive electrophiles can activate Yap1 directly by adduction to cysteine residues in the C-terminal domain containing Cys598, Cys620, and Cys629. H_2O_2 and *N*-ethylmaleimide (NEM) showed no cross-protection against each other, whereas another thiol-reactive chemical, acrolein, elicited Yap1-dependent cross-protection against NEM, but not H_2O_2. Either Cys620 or Cys629 was sufficient for activation of Yap1 by NEM or acrolein; Cys598 was dispensable for this activation mechanism. To determine whether Yap1 activated by H_2O_2 or thiol-reactive chemicals elicits distinct adaptive gene responses, microarray analysis was performed on the wild-type strain or its isogenic single-deletion strain Δ*yap1* treated with control buffer, H_2O_2, NEM, or acrolein. Sixty-five unique H_2O_2 and 327 NEM and acrolein Yap1-dependent responsive genes were identified. Functional analysis using single-gene-deletion yeast strains demonstrated that protection was conferred by *CTA1* and *CTT1* in the H_2O_2-responsive subset and *YDR042C* in the NEM- and acrolein-responsive subset. These findings demonstrate that the distinct mechanisms of Yap1 activation by H_2O_2 or thiol-reactive chemicals result in selective expression of protective genes.

A common challenge faced by all organisms living in the presence of molecular oxygen is the generation of reactive oxygen species (ROS)[1], an inevitable consequence of aerobic metabolism. The enzymes in electron transport chains and other oxygen metabolism pathways may prematurely deliver electrons to oxygen and subsequent ROS to produce the superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$). These ROS can disturb the cellular redox balance and damage important biological molecules including proteins, lipids, and nucleic acids, thus causing oxidative stress to the cell [1]. When cells encounter sublethal concentrations of ROS or electrophiles, they exhibit resistance to a subsequent challenge at higher concentrations of these chemicals. This adaptive response is a defensive mechanism found in bacteria [2], yeast [3], and mammals [4]. Studies of these response systems have revealed a common signaling mechanism that uses cysteine residues of a variety of proteins as redox status sensors to activate transcription of genes involved in protection against oxidative damage [5].

In yeast, the basic leucine-zipper transcription factor Yap1 regulates most of the known cellular antioxidant genes and plays a major role in the adaptive response to oxidative stress [6,7]. As the first member of a family of eight yeast activator proteins (YAPs) [8], Yap1 was originally isolated by its ability to bind the SV40 AP-1 recognition element (TGACTAA) [9] and was characterized as a member of the Jun family of transcription factors with its DNA-binding bZIP domain located in the N-terminus of the protein [10]. The similarity between the cysteine thiol reactivity of Yap1 and that of the mammalian AP-1, NF-κB, and Keap1–Nrf2 systems [4] makes it a good model for investigating the general mechanisms of oxidative stress response. The normally inactive Yap1 is maintained in the cytoplasm because of its rapid nuclear export [11]. When challenged by ROS, Yap1 moves from the cytoplasm into the nucleus [12]. This is due to the inhibition of nuclear exportation [11], whereas the nuclear import of Yap1 is not affected [13]. The nuclear export sequence of Yap1, recognized by the β-karyopherin-like nuclear exporter Crm1, is located within the C-terminal cysteine-rich domain (c-CRD) of the protein. The nearby cysteine residues function as a redox sensor, which under oxidative conditions makes the nuclear export sequence unavailable to Crm1, thus inhibiting nuclear exportation of Yap1 [11].

The detailed mechanism of H_2O_2-induced Yap1 activation has been well studied. Oxidation of Yap1 leads to the formation of disulfide

Abbreviations: CRD, cysteine-rich domain; NEM, *N*-ethylmaleimide; ROS, reactive oxygen species; GPX, glutathione peroxidase; LB, Luria broth; YEPD, yeast extract peptone dextrose medium; ATCC, American Type Culture Collection; GFP, green fluorescence protein; GO, gene ontology; SGD, *Saccharomyces* genome database.

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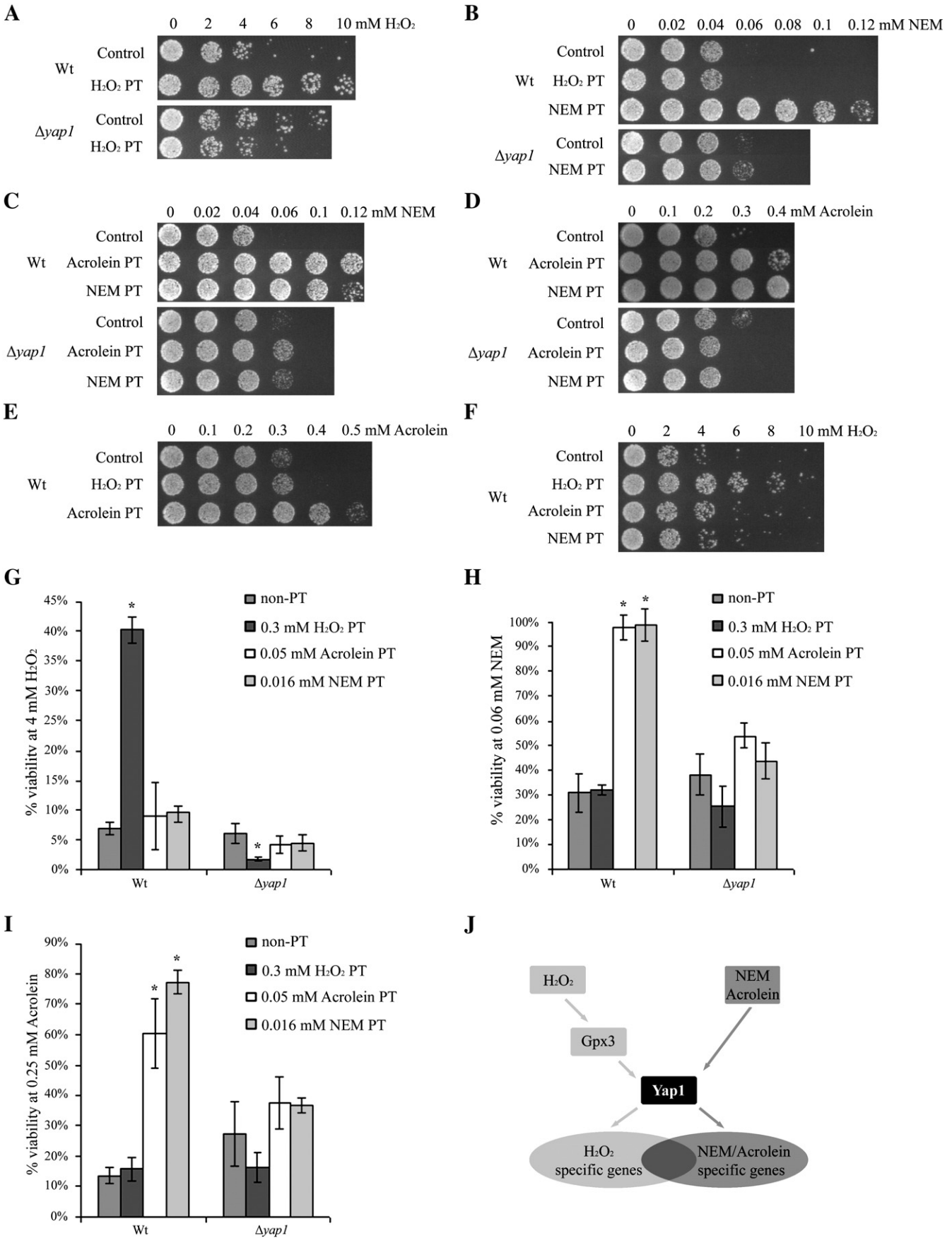
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0 2 4 6 8 10 mM H2O2
Wt
Control
H2O2 PT
Δyap1
Control
H2O2 PT
B
0 0.02 0.04 0.06 0.08 0.1 0.12 mM NEM
Wt
Control
H2O2 PT
NEM PT
Δyap1
Control
NEM PT
C
0 0.02 0.04 0.06 0.1 0.12 mM NEM
Wt
Control
Acrolein PT
NEM PT
Δyap1
Control
Acrolein PT
NEM PT
D
0 0.1 0.2 0.3 0.4 mM Acrolein
Wt
Control
Acrolein PT
NEM PT
Δyap1
Control
Acrolein PT
NEM PT
E
0 0.1 0.2 0.3 0.4 0.5 mM Acrolein
Wt
Control
H2O2 PT
Acrolein PT
F
0 2 4 6 8 10 mM H2O2
Wt
Control
H2O2 PT
Acrolein PT
NEM PT
G
% viability at 4 mM H2O2
non-PT
0.3 mM H2O2 PT
0.05 mM Acrolein PT
0.016 mM NEM PT
Wt
Δyap1
H
% viability at 0.06 mM NEM
non-PT
0.3 mM H2O2 PT
0.05 mM Acrolein PT
0.016 mM NEM PT
Wt
Δyap1
I
% viability at 0.25 mM Acrolein
non-PT
0.3 mM H2O2 PT
0.05 mM Acrolein PT
0.016 mM NEM PT
Wt
Δyap1
J
H2O2
NEM
Acrolein
Gpx3
Yap1
H2O2 specific genes
NEM/Acrolein specific genes

bonds that are essential for its nuclear accumulation [14,15]. However, Yap1 is not oxidized directly by H_2O_2. Instead, a glutathione peroxidase (GPX)-like enzyme, Gpx3, functions as the H_2O_2 sensor and transmits the oxidative signal to Yap1 by forming an intermolecular disulfide bond. This disulfide bond is later converted into an intramolecular disulfide bond between the Yap1 N-terminal CRD Cys303 and C-terminal CRD Cys598 [16]. A second intramolecular disulfide bond between Cys310 and Cys629 stabilizes the activated form of Yap1 [17]. Together, these two disulfide bonds cause an N-terminal α-helix to mask the nuclear export sequence in the c-CRD, resulting in nuclear accumulation of Yap1 [18]. In a single study investigating gene expression, researchers demonstrated that proper disulfide bond formation between the n- and the c-CRD is essential for activating the H_2O_2-responsive gene *TRX2*, by recruiting the mediator component Rox3 to the *TRX2* promoter [19]. Whereas Trx2 has been shown to be important to resistance to H_2O_2 [20], it also promotes the export of Yap1 from the nucleus by reducing the intramolecular disulfide bonds of Yap1, thereby unmasking its nuclear export sequence [16,21].

In addition to H_2O_2 and related peroxides, Yap1 also responds to many electrophilic thiol-reactive chemicals such as *N*-ethylmaleimide (NEM) [22], and the lipid peroxidation by-products 4-hydroxynonenal [22] and malondialdehyde [7]. Unlike H_2O_2, which requires Gpx3-mediated activation of Yap1, these electrophiles activate Yap1 via a Gpx3-independent mechanism that requires its c-CRD, but not its n-CRD, cysteines [22]. This c-CRD activation mechanism may not result in any disulfide bond formation, at least in the case of NEM, because the chemical was found to form direct adducts on all three c-CRD cysteines of Yap1 [22]. Thus, by c-CRD cysteine adduction, Yap1 may be activated through a conformational change that is different from that activated by H_2O_2 [22,23]. In one study, the protective adaptive response to malondialdehyde was shown by genetics to be Yap1-dependent, yet no cross-protection was observed between H_2O_2 and malondialdehyde [7]. These results, and the current understanding of the two distinct mechanisms of Yap1 activation by H_2O_2 and thiol-reactive chemicals [22], led us to hypothesize that these distinct mechanisms of Yap1 activation result in selective Yap1 regulation of protective genes.

In this study, we confirm that H_2O_2 and NEM are Yap1-dependent and provide no cross-protection against each other. We also show that another lipid peroxidation product, acrolein, activates Yap1 and affords cross-protection against NEM, but not H_2O_2. The mechanism of Yap1 activation by both NEM and acrolein requires either Cys620 or Cys629, whereas Cys598 is dispensable for this activation mechanism. Two subsets of Yap1-dependent genes, corresponding to H_2O_2- or NEM- and acrolein-specific adaptive responses, were identified by microarray analysis. Functional analyses using yeast strains with single-gene deletions demonstrate that selective Yap1-dependent gene expression provides a molecular basis for the protection and lack of cross-protection of the H_2O_2 and thiol-reactive chemicals.

Materials and methods

Bacterial and yeast strains

Escherichia coli strain DH5α was used to construct, propagate, and maintain all plasmids in this study. For *E. coli* cells transformed with plasmids, the antibiotic ampicillin (100 μg/ml) was added to Luria broth (LB) medium (1% w/v tryptone, 0.5% w/v yeast extract, and 0.5% w/v NaCl). The yeast *Saccharomyces cerevisiae* strain BY4741 (MAT*a his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*) [24] and the isogenic single-gene-deletion strains (created by the *Saccharomyces* Genome Deletion Project: http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html) that were used in this study were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) or Invitrogen (Carlsbad, CA, USA).

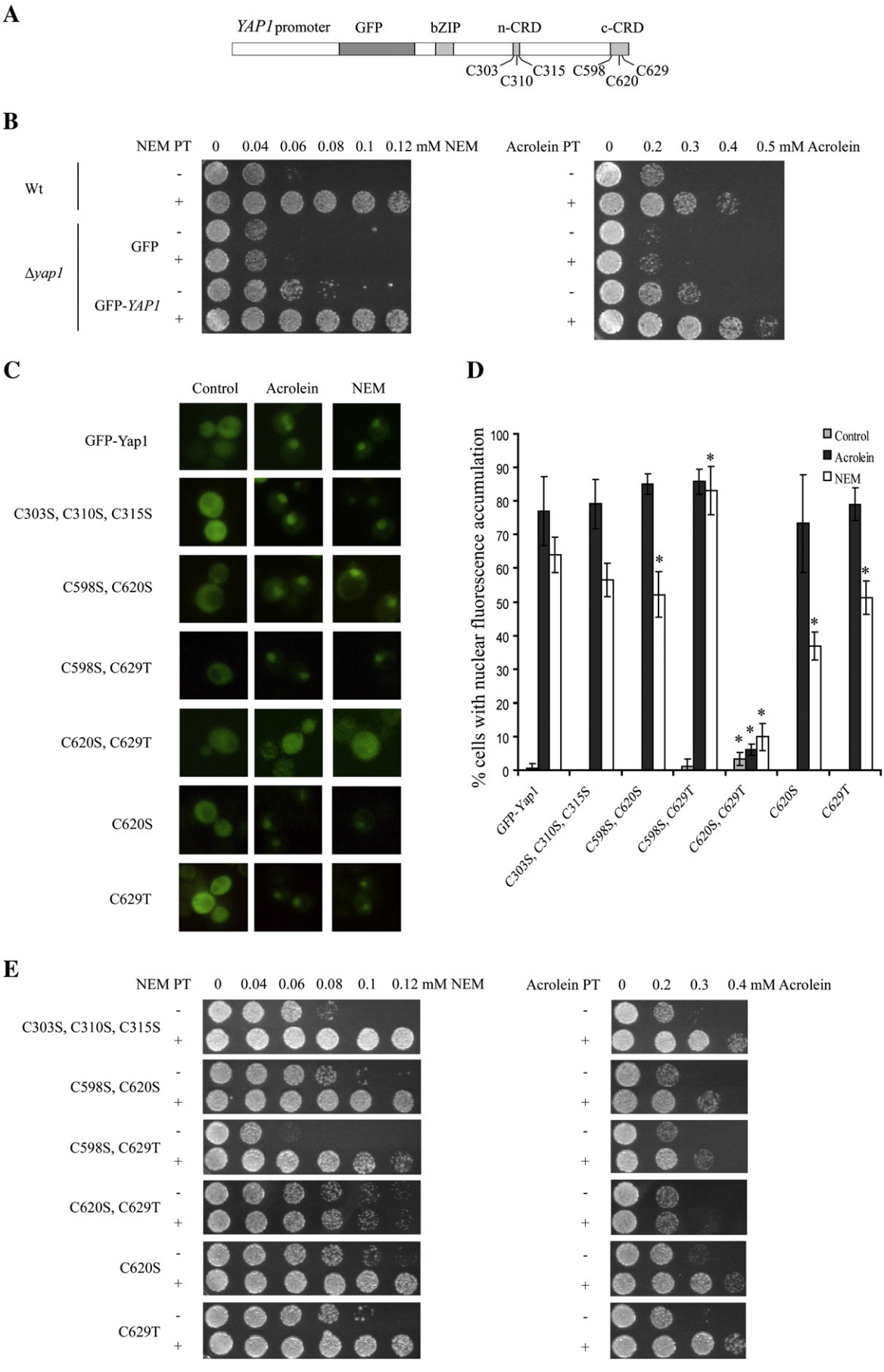
Media and growth conditions

E. coli strains were grown in LB medium at 37 °C with shaking at 250 rpm. The yeast strain BY4741 was grown in yeast extract peptone dextrose (YEPD) medium (1% w/v yeast extract, 2% w/v glucose, and 2% w/v peptone), and deletion strains were grown in YEPD medium with 0.02% w/v Geneticin sulfate (G418). Yeast cells transformed with plasmids were grown in modified synthetic complete $G418^+$ medium (0.17% w/v yeast nitrogen base, 2% w/v glucose, 0.1% w/v glutamic acid, 0.19% w/v amino acid supplement mixture without uracil (ATCC), 0.02% w/v G418) in which the ammonium sulfate was replaced by glutamic acid sodium to maintain the G418 sensitivity of the yeast in this medium [25]. All yeast cultures were grown at 30 °C and shaken at 250 rpm.

Plasmid constructs

The *YAP1* promoter region (1 kb sequence upstream of the *YAP1* gene in the yeast genome, on chromosome XIII between chromosomal coordinates 252848 and 253847) and the *YAP1* gene (chromosome XIII, chromosomal coordinates 253848 to 255800) were amplified separately from yeast BY4741 genomic DNA by PCR with primers 1 and 2 and primers 3 and 4, respectively (see Supplementary Table S1 for the sequences of the primers used to make constructs). The green fluorescent protein (GFP) gene encoding the GFP variety "3-cycle" was amplified from plasmid pGLO (Bio-Rad, Hercules, CA, USA) with primer 5 (containing AgeI and SacII sites) and primer 6 (containing SacI and EcoRI sites). The restriction endonuclease sites were introduced into the *YAP1* promoter PCR product with primer 1 (AgeI site) and primer 7 (SacII site), and into the *YAP1* gene with primer 8 (SacI site) and primer 9 (EcoRI site). The PCR product of the GFP gene was digested by AgeI and SacI and cloned into plasmid pYES2. The resulting plasmid was designated pYES2-GFP. The *YAP1* promoter was cloned into pYES2-GFP between the AgeI and the SacII sites, and the *YAP1* gene was then inserted between the SacI and the EcoRI sites.

Fig. 1. Yap1 mediates selective adaptive responses to H_2O_2 versus NEM or acrolein. (A) Patch analysis of the H_2O_2 adaptive response. Wild-type (Wt) and Δ*yap1* cells were pretreated (PT) with 0.3 mM H_2O_2 for 1 h, recovered in YEPD medium for 1 h, and then challenged with H_2O_2 at the indicated concentrations for 1 h. The cell suspensions were diluted and 5 μl (1×10^3 cells) was spotted onto a YEPD plate. (B) Patch analysis of the NEM adaptive response. Wt cells were pretreated with 0.3 mM H_2O_2 or 0.016 mM NEM, whereas Δ*yap1* cells were pretreated with NEM. Untreated and pretreated cells were challenged with NEM at the indicated concentrations. (C) Patch analysis of NEM or acrolein adaptive response against NEM. Wt and Δ*yap1* cells were pretreated with 0.05 mM acrolein or 0.016 mM NEM before challenge with NEM at the indicated concentrations. (D) Patch analysis of acrolein or NEM adaptive response against acrolein. Wt and Δ*yap1* cells were pretreated with 0.05 mM acrolein or 0.016 mM NEM before challenge with acrolein at the indicated concentrations. (E) Patch analysis of acrolein or H_2O_2 adaptive response against acrolein. Wt cells were pretreated with 0.3 mM H_2O_2 or 0.05 mM acrolein before challenge with acrolein at the indicated concentrations. (F) Patch analysis of H_2O_2, acrolein, or NEM adaptive response against H_2O_2. Wt cells were pretreated with 0.3 mM H_2O_2, 0.05 mM acrolein, or 0.016 mM NEM before challenge with H_2O_2 at the indicated concentrations. (G–I) Shown are the means and standard deviations of three independent measurements. *Significant difference in survival between the nonpretreated and the pretreated samples within a yeast strain. (G) Quantitative analysis of the H_2O_2 adaptive response after pretreatment with each of the three oxidants. Wt (left) or Δ*yap1* (right) cells were pretreated with 0.3 mM H_2O_2, 0.05 mM acrolein, or 0.016 mM NEM before challenge with 4 mM H_2O_2. (H) Quantitative analysis of the acrolein adaptive response after pretreatment with each of the three oxidants. Wt (left) or Δ*yap1* (right) cells were pretreated with H_2O_2, acrolein, or NEM before challenge with 0.25 mM acrolein. (I) Quantitative analysis of the NEM adaptive response after pretreatment with each of the three oxidants. Wt (left) or Δ*yap1* (right) cells were pretreated with H_2O_2, acrolein, or NEM before challenge with 0.6 mM NEM. (J) The current understanding of the Yap1-mediated adaptive responses to H_2O_2, acrolein, and NEM. H_2O_2 activates Yap1 through a Gpx3-dependent mechanism [16], whereas the activation of Yap1 by NEM [22] and acrolein does not require Gpx3. The hypothesis tested in this study is that these two mechanisms of Yap1 activation lead to the expression of distinct sets of Yap1-dependent genes that protect against H_2O_2 or acrolein and NEM.


A
YAP1 promoter
GFP
bZIP
n-CRD
c-CRD
C303
C310
C315
C598
C620
C629
B
NEM PT 0 0.04 0.06 0.08 0.1 0.12 mM NEM
Acrolein PT 0 0.2 0.3 0.4 0.5 mM Acrolein
Wt
Δyap1
GFP
GFP-YAP1
C
Control
Acrolein
NEM
GFP-Yap1
C303S, C310S, C315S
C598S, C620S
C598S, C629T
C620S, C629T
C620S
C629T
D
% cells with nuclear fluorescence accumulation
Control
Acrolein
NEM
GFP-Yap1
C303S, C310S, C315S
C598S, C620S
C598S, C629T
C620S, C629T
C620S
C629T
E
NEM PT 0 0.04 0.06 0.08 0.1 0.12 mM NEM
Acrolein PT 0 0.2 0.3 0.4 mM Acrolein
C303S, C310S, C315S
C598S, C620S
C598S, C629T
C620S, C629T
C620S
C629T

The final plasmid, Spro_yap1_2, contains a full-length GFP gene (without a stop codon) fused to the 5′ end of a full-length *YAP1* gene under the control of the *YAP1* promoter. The amplified GFP gene was also excised by AgeI and EcoRI, which retained its stop codon, and cloned into pYES2. The *YAP1* promoter sequence was later inserted, as described above, to construct plasmid Epro_2, which contains the complete GFP gene under control of the *YAP1* promoter. Mutations were introduced into the *YAP1* gene in plasmid Spro_yap1_2 to substitute its cysteine residues with serine or threonine by single- or multiple-site-directed mutagenesis reactions using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the primer pairs described in Supplementary Table S1 (primers 10 and 11, C303S; primers 12 and 13, C310S; primers 14 and 15, C315S; primers 16 and 17, C598S; primers 18 and 19, C620S; and primers 20 and 21, C629T). All constructs and mutations were verified by DNA sequence analysis.

Yeast adaptive-response patch assay

The adaptive-response assay was conducted as described [7], with modifications. Yeast from a saturated culture were diluted to OD_{600} ~0.1 in YEPD medium and grown to early exponential phase (OD_{600} ~0.3, approximately 2×10^7 cells/ml). Cells were harvested by centrifugation and pretreated with H_2O_2 (Sigma–Aldrich, St. Louis, MO, USA), acrolein (Sigma–Aldrich), or NEM (Fluka, Milwaukee, WI, USA) in 0.1 M sodium phosphate buffer, pH 7.0, for 1 h at 30 °C with shaking at 250 rpm. Both pretreated and control cell samples were harvested, suspended in YEPD medium, and allowed to recover for 1 h at 30 °C with shaking at 250 rpm. Aliquots of each sample were then challenged with H_2O_2, acrolein, or NEM at the indicated concentrations in 0.1 M sodium phosphate buffer, pH 7.0, for 1 h at 30 °C. The treatment was terminated by centrifugation and the cell pellet was washed and resuspended in fresh 0.1 M sodium phosphate buffer, pH 7.0. These cell suspensions were diluted, and the cell survival rates were estimated by a patch assay as described [26]. Five microliters of diluted sample containing approximately 1×10^3 cells was dropped onto a YEPD plate and the plate was incubated at 30 °C for 2 days before the growth was analyzed.

Quantitative survival rate measurements

For determining quantitative survival, the nonpretreated or pretreated (1 h with 0.3 mM H_2O_2, 0.016 mM NEM, or 0.05 mM acrolein) yeast cells were challenged with each of the three chemicals at one concentration (H_2O_2 at 4 mM, NEM at 0.6 mM, or acrolein at 0.25 mM). After challenge, the cell suspensions were diluted 10,000-fold in 0.1 M sodium phosphate buffer, pH 7.0, and 100 μl of the diluted sample was spread on a YEPD plate (two replicate plates for each sample). All plates were incubated at 30 °C for 2 days before the colonies on the plates were counted. Each survival rate measurement was repeated three times. Survival rates were evaluated using two-way ANOVA to determine whether chemical treatments and genetic factors had significant effects on colony survival across any groups under study. This test was performed using SAS 9.2 (SAS Institute, Cary, NC, USA). As these tests were significant ($p<0.05$), post hoc Student's t tests ($p<0.05$) were performed using a Bonferroni multiple-significance-test correction [27].

Microarray analysis

The wild-type strain BY4741 and its isogenic single-deletion strain BY4741Δ*yap1* were used in this study. Four independent cultures of each strain grown to early exponential phase were harvested by centrifugation (12,000 *g* for 10 min at room temperature) and divided into four aliquots in 0.1 M sodium phosphate buffer, pH 7.0. One aliquot was the control, and the other three aliquots were treated with 0.3 mM H_2O_2, 0.05 mM acrolein, or 0.016 mM NEM, respectively, for 1 h at 30 °C. Total RNA was isolated using RNA STAT-60 (Tel-Test, Friendswood, TX, USA) and glass beads to disrupt the cell wall. All 32 RNA samples ($n=4$ per group) were analyzed using the Yeast 2.0 GeneChip from Affymetrix (Santa Clara, CA, USA). The microarray data have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus repository, http://www.ncbi.nlm.nih.gov/projects/geo/ (Series Accession No. GSE19213).

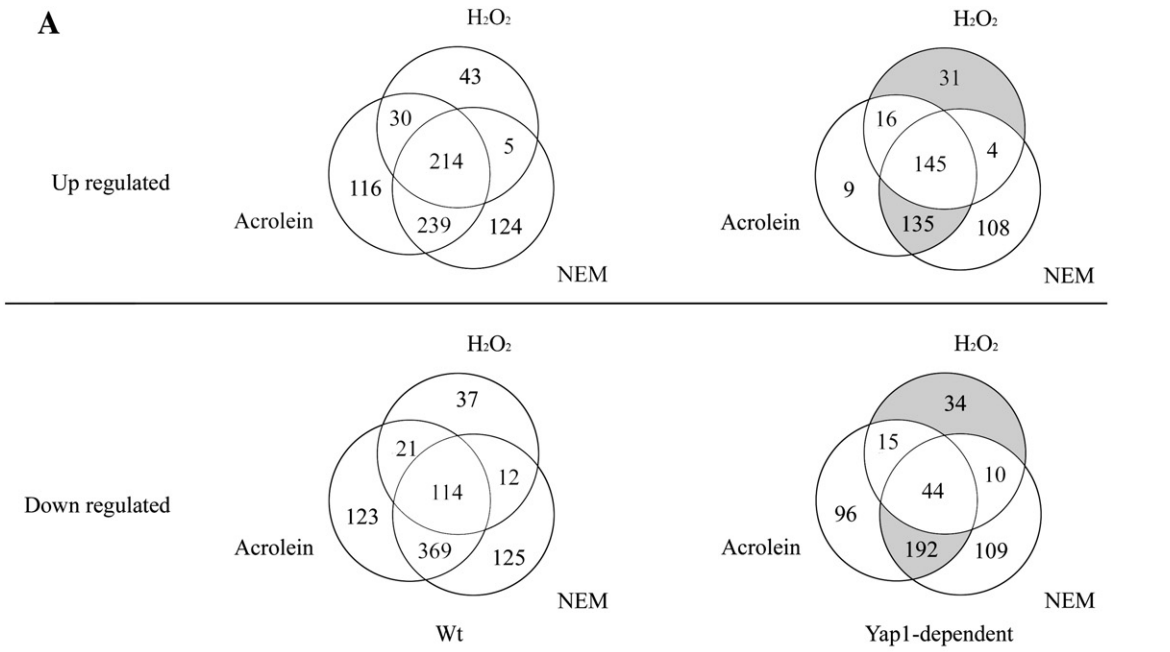
Data analysis

The microarray data were first processed by GC Robust Multi-array Average (GCRMA) to adjust background intensity levels and to normalize and combine the probe pair intensities into an estimate of gene expression for each gene in the dataset [28]. Affymetrix Microarray Suite 5.0 was used to detect the presence or absence of gene expression. Genes that were present in 75% of any treatment group were selected using a Perl program. The data were then filtered by fold-change, selecting genes that were up- or down-regulated by at least 1.5-fold in any treatment group compared to the corresponding control. Significant genes were identified using an exact nonparametric procedure previously described by our laboratory [29]. Multiple hypothesis testing correction was also applied [30]. Each gene was associated with a q value estimated at a positive false discovery rate of 0.05. Pairwise comparisons and gene clusters were identified as described previously [29]. Briefly, if a gene had a statistically significantly increased expression level after a treatment compared to the control sample, it was assigned a value of 2 for this treatment. If the expression level was lower, the value assigned was 0. A value of 1 was given when there was no statistically significant change in the expression level of a gene between the treated and the control samples. Thus each gene was associated with an expression pattern that summarized its response to H_2O_2, acrolein, and NEM treatments, relative to the control sample. Genes with similar response patterns were grouped into clusters. By comparison to the corresponding responses in the Δ*yap1* strain, the Yap1-dependent genes were identified.

Gene ontology analysis

To uncover similar and unique biological processes and molecular functions among the genes, functional analyses of the identified Yap1-dependent gene lists were performed. The yeast gene identifiers of each gene list were mapped to the gene ontology (GO) terms assigned by the *Saccharomyces* genome database (SGD; Department of Genetics,

Fig. 2. Yap1 cysteine 620 and cysteine 629 are required for acrolein- and NEM-induced activation. (A) Schematic of the GFP-*YAP1* fusion gene construct. The Yap1 bZIP domain, the n-CRD, and the c-CRD are indicated. (B) (Left) Patch analysis of NEM adaptive response. Wt and Δ*yap1* cells carrying the GFP gene or GFP-*YAP1* fusion gene were pretreated with 0.016 mM NEM before challenge with NEM at the indicated concentrations. (Right) Patch analysis of acrolein adaptive response. Wt and Δ*yap1* cells carrying the GFP gene or GFP-*YAP1* fusion gene were pretreated with 0.05 mM acrolein before challenge with acrolein at the indicated concentrations. (C) Cellular localization of GFP-Yap1 or GFP-Yap1-cysteine substitution mutants. Δ*yap1* cells expressing GFP-Yap1, GFP-Yap1$^{C303S,\ C310S,\ C315S}$, GFP-Yap1$^{C598S,\ C620S}$, GFP-Yap1$^{C598S,\ C629T}$, GFP-Yap1$^{C620S,\ C629T}$, GFP-Yap1^{C620S}, or GFP-Yap1^{C629T}, as indicated, were analyzed for localization of intracellular GFP fluorescence 30 min after treatment with acrolein (0.05 mM) or NEM (0.016 mM). (D) The cell samples shown in (C) were analyzed to calculate the percentage of the cell population with nuclear accumulation of fluorescence signal by counting approximately 100 cells in each of five fields. All of the cells that had green fluorescence in a field of view of the microscope were counted. Shown are the means and standard deviations of five independent measurements. *Significant difference in GFP nuclear localization between the GFP-Yap1 control strain and the indicated strain compared within chemical treatment. (E) (Left) Δ*yap1* cells expressing the indicated mutant GFP-Yap1 proteins were tested for NEM adaptive responses as described for (B). (Right) Δ*yap1* cells expressing indicated mutant GFP-Yap1 proteins were tested for acrolein adaptive responses as described for (B).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Up regulated
Down regulated
H2O2
Acrolein
NEM
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30
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5
116
239
124
31
16
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15
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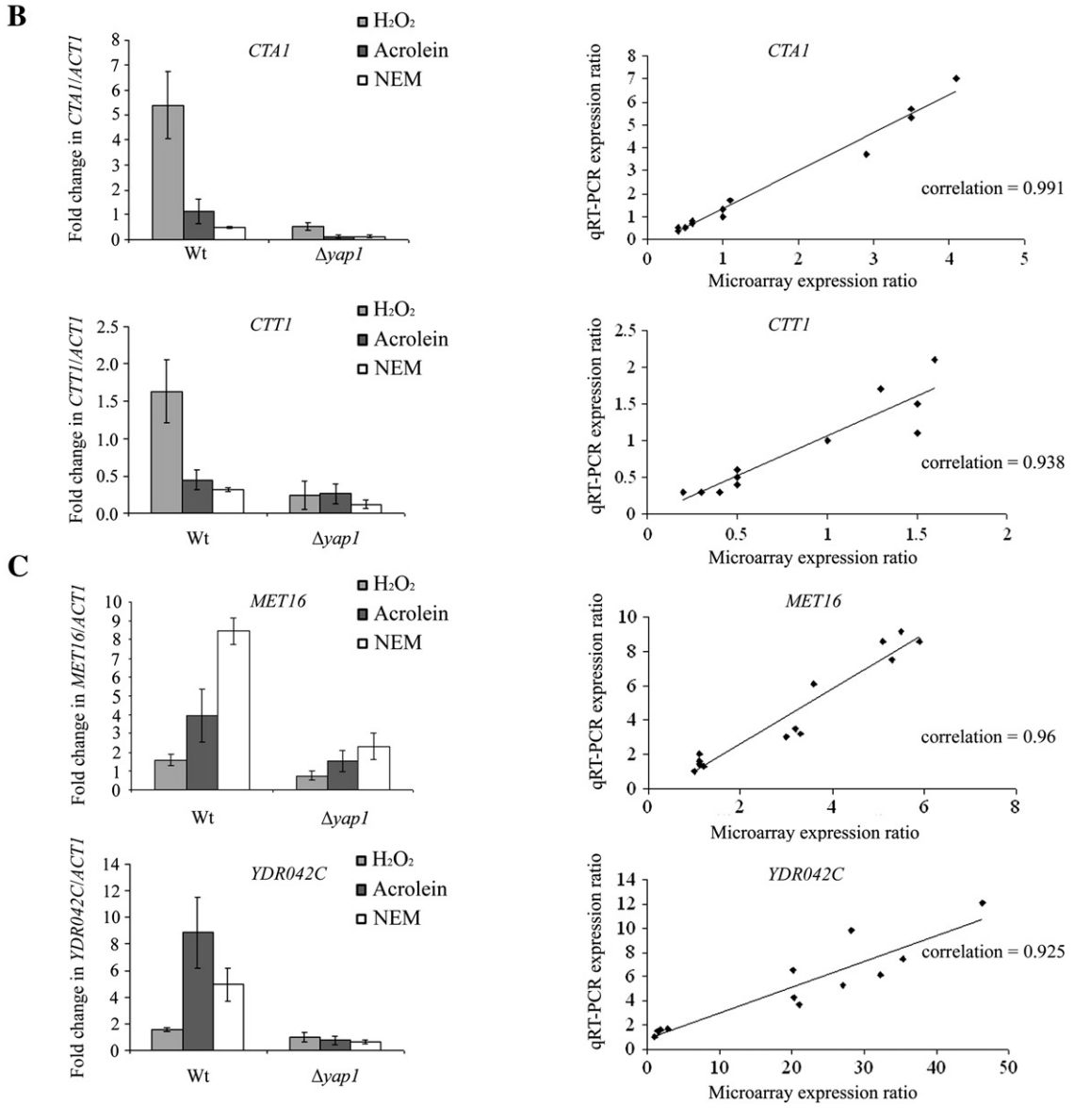
10
96
192
109
Wt
Yap1-dependent


B
C
CTA1
CTT1
MET16
YDR042C
Fold change in CTA1/ACT1
Fold change in CTT1/ACT1
Fold change in MET16/ACT1
Fold change in YDR042C/ACT1
Wt
Δyap1
qRT-PCR expression ratio
Microarray expression ratio
correlation = 0.991
correlation = 0.938
correlation = 0.96
correlation = 0.925

Stanford University School of Medicine, Stanford, CA, USA), using the GO Term Finder provided on the SGD Website (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl), and sorted into GO term groups representing major biological processes or molecular functions. The GO Term Finder program was run at the default setting ($p<0.01$).

Quantitative real-time PCR (qRT-PCR)

cDNAs were synthesized from 500 ng of total RNA by reverse transcription using an 18-mer oligo(dT) primer (IDT, Coralville, IA, USA). qRT-PCR was carried out on a Bio-Rad iCycler with the primer pairs indicated in Supplementary Table S2. The transcription level of the β-actin gene *ACT1* was used as the internal reference in calculating target gene expression ratios between control and treated samples [31].

Fluorescence microscopy

The fluorescence microscopy slides were prepared as described [32], with modifications. Yeast were fixed by immersion in electron microscopy-grade aqueous paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) at a final concentration of 2% (w/v) for 10 min at 25 °C with inversion. Cells were pelleted and washed in 0.1 M potassium phosphate buffer, pH 7.0, for 10 min at 25 °C before being resuspended in fresh 0.1 M potassium phosphate buffer, pH 7.0. A 2.3-μl aliquot of the cell suspension was mounted on each slide and fluorescence images were taken using a Nikon Eclipse 800 confocal microscope with a Nikon digital camera DXM1200F. For quantitative analyses, five fields (60× objective) for each strain were counted and the data were expressed as the ratio of cells showing nuclear localization divided by the total number of cells per field. Statistical analysis was performed using ANOVA followed by pairwise comparison within each chemical treatment for each strain compared to the GFP-Yap1 control by the Bonferroni multiple comparison test ($p<0.05$).

Results

Two Yap1-activation mechanisms lead to distinct adaptive responses

To confirm that Yap1 is responsible for the induced resistance to H_2O_2, the H_2O_2 adaptive response was measured in the wild-type yeast strain BY4741 and its isogenic *YAP1* deletion strain (Δ*yap1*). Patch assays as well as quantitative survival analyses were performed. The wild-type strain pretreated with 0.3 mM H_2O_2 for 1 h acquired higher resistance to challenge with increasing concentrations of H_2O_2, showing fairly strong growth after treatment with 10 mM H_2O_2. In contrast, nonpretreated cells were mostly killed after treatment with 6 (Fig. 1A) or 4 mM (Fig. 1G) H_2O_2. This adaptive response to H_2O_2 was absent in the yeast strain lacking Yap1 (Δ*yap1*). The H_2O_2-pretreated Δ*yap1* cells were even more sensitive to H_2O_2 than the nonpretreated Δ*yap1* cells (Fig. 1A), which was statistically significant ($p<0.05$; Fig. 1G), indicating that the adaptive response to H_2O_2 in yeast is greatly dependent on Yap1.

A previous study showed that the Δ*yap1* yeast strain is hypersensitive to the cysteine thiol-reactive Michael acceptor NEM, which induces the nuclear accumulation of the Yap1 protein via a Gpx3-independent mechanism [22]. These observations imply that yeast may be capable of mounting a Yap1-dependent adaptive response against NEM. Consistent with this idea, a patch assay and quantitative survival analysis showed that the wild-type yeast strain was much more resistant to NEM after pretreatment with a nonlethal concentration of 0.01 mM NEM, compared to nonpretreated cells (Figs. 1B and H). In the Δ*yap1* strain, the same pretreatment provided essentially no protection (Figs. 1B and H). When the wild-type strain was pretreated with 0.3 mM H_2O_2, which was sufficient to activate Yap1 for an adaptive response to H_2O_2, no cross-protection against NEM was observed (Figs. 1B and H). These results suggest that although Yap1 mediates the adaptive responses to both H_2O_2 and NEM, the H_2O_2 pretreatment does not induce the same response as the NEM pretreatment.

Yap1 has been reported to regulate the adaptive response to malondialdehyde, a highly reactive aldehyde and lipid peroxidation product [7]. This prompted us to test whether another reactive aldehyde and lipid peroxidation product, acrolein, can induce a Yap1-mediated adaptive response. Like NEM, acrolein is a strong electrophile and acts as a Michael acceptor to cysteine thiol nucleophilic attack of certain cysteine residues within reactive proteins [33]. The similarity between NEM and acrolein also raises the possibility that these two chemicals may activate Yap1 through the same mechanism, thus leading to the same downstream gene induction response. This hypothesis was first tested by measuring the cross-protection between acrolein and NEM in wild-type and Δ*yap1* yeast strains. Consistent with the above hypothesis, by patch assay and quantitative survival analysis, pretreatment of the BY4741 strain with either 0.016 mM NEM or 0.05 mM acrolein (nonlethal doses) caused increased tolerance to both chemicals compared to the nonpretreated control (Figs. 1C, D, H, and I). In contrast, the Δ*yap1* strain showed no adaptive response to either chemical (Figs. 1C, D, H, and I). Moreover, the 0.3 mM H_2O_2 pretreatment, which did not trigger protection to NEM in wild-type yeast, also failed to induce higher tolerance against acrolein (Figs. 1E and I). Correspondingly, acrolein or NEM pretreatment did not induce a significant protective response against H_2O_2 (Figs. 1F and G). These data indicate that acrolein and NEM induce similar Yap1-mediated adaptive responses that are distinct from the response induced by H_2O_2.

To explain these results, we proposed that the differences observed in these adaptive responses stem from distinct mechanisms of Yap1 activation by these chemicals. In this scheme (Fig. 1J), acrolein and NEM activate Yap1 through the same Gpx3-independent mechanism, leading to an adaptive response that protects against either chemical, but not against H_2O_2. In contrast, H_2O_2 activates Yap1 via a Gpx3-dependent mechanism that results in an adaptive response against H_2O_2, but does not protect against either NEM or acrolein. Our model makes the following predictions: (i) the same set of cysteine residues in Yap1 are essential for both NEM- and acrolein-induced activations; (ii) different subsets of Yap1-dependent genes are activated upon pretreatment with H_2O_2 versus NEM or acrolein; (iii) among each subset of genes, there are genes whose protein products protect against the corresponding inducers. Although there might be a partial overlap between these two subsets of genes, the lack of cross-protection between H_2O_2 and NEM or acrolein indicates that such overlapping genes are unlikely to play an important role in selectively protecting yeast against any of the three chemicals. The following work was done to test each of these predictions.

Fig. 3. Activation of Yap1 by H_2O_2 or the thiol-reactive chemicals NEM and acrolein leads to the expression of distinct and common sets of Yap1-dependent genes. (A) (Left) Venn diagrams of the microarray results showing the number of unique or overlapping genes up- or down-regulated by H_2O_2, acrolein, or NEM in wild-type yeast. (Right) Comparison of the responses between isogenic wild type (Wt) and Δ*yap1* strains identified the Yap1-dependent genes. The Yap1-dependent genes that specifically responded to H_2O_2 or to NEM and acrolein treatments (highlighted in gray) were further studied. (B) (Left) qRT-PCR validation of microarray results for the Yap1-dependent H_2O_2-specific genes *CTA1* and *CTT1*. (Right) The correlations between the qRT-PCR and the microarray expression ratios are shown for each of the individual samples. (C) (Left) qRT-PCR validation of microarray results for the Yap1-dependent NEM/acrolein-specific genes *MET16* and *YDR042C*. (Right) The correlations between the qRT-PCR and the microarray expression ratios are shown for each of the individual samples.

Table 1
Gene ontology analysis of H_2O_2-up-regulated genes

GO term	Frequency	% of total 48 genes	*p* value
Biological process	5	10.4	0.00096
Iron ion homeostasis			
Molecular function			
Catalase activity	2	4.2	0.00255
Iron ion transmembrane transporter activity	3	6.2	0.0026
Heme binding	2	4.2	0.00761

Acrolein- and NEM-induced Yap1 activation and adaptation require the same set of Yap1 c-CRD cysteines

A GFP-*YAP1* fusion gene under control of the *YAP1* promoter was constructed (Fig. 2A). A second construct containing only the GFP gene controlled by the *YAP1* promoter was made as the negative control. Each construct was inserted into the yeast plasmid pYES2 and transformed into the Δ*yap1* strain. The Δ*yap1* cells carrying the GFP-*YAP1* fusion gene regained wild-type adaptive responses to NEM (Fig. 2B) or acrolein (Fig. 2C), whereas the Δ*yap1* cells with only the GFP gene remained unable to mount an adaptive response to either chemical. These data demonstrate that the Yap1 in the GFP-*YAP1* fusion is fully functional in mediating NEM- or acrolein-induced adaptive responses.

Yap1 has six cysteine residues located in two CRDs: Cys303, Cys315, and Cys320 in the n-CRD and Cys598, Cys620, and Cys629 in the c-CRD (Fig. 2A). To identify which cysteine residues are essential for Yap1 activation by NEM and acrolein, site-directed mutations were introduced into the GFP-*YAP1* fusion gene to systematically substitute the cysteine residues (C) with serine (S) or threonine (T). The wild-type GFP-*YAP1* and mutant GFP-*YAP1* genes were transformed individually into the Δ*yap1* strain, and the cellular distribution of the fusion proteins was tracked by microscopic subcellular localization of the green fluorescent signal. The wild-type GFP-Yap1 protein was cytoplasmic in control cells and became nuclear in cells treated with 0.016 mM NEM or 0.05 mM acrolein for 30 min (Fig. 2C, first row). All six of the mutant GFP-Yap1 proteins were cytoplasmic in untreated cells. After NEM and acrolein treatments, the n-CRD triple mutant GFP-Yap1$^{C303S,\ C310S,\ C315S}$ and c-CRD double mutants GFP-Yap1$^{C589S,\ C620S}$ and GFP-Yap1$^{C598S,\ C629T}$ showed the same nuclear localization as the wild type (Fig. 2C, second to fourth rows). However, the other c-CRD double mutant, GFP-Yap1$^{C620S,\ C629T}$, remained cytoplasmic (Fig. 2C, fifth row). Consistent with previous work [22], the n-CRD cysteines are not required in NEM-induced Yap1 activation. Further, these n-CRD cysteines are not required for acrolein-induced activation. Mutating Cys620 and Cys629 abolished acrolein- or NEM-induced nuclear localization of the Yap1 protein, whereas keeping either one of these two cysteines was sufficient to retain the response to either chemical (Fig. 2C, third to seventh rows). These data indicate that the same set of c-CRD cysteines (Cys620 or Cys629) is required for both the acrolein- and the NEM-induced Yap1 activation mechanism.

Table 2
Gene ontology analysis of NEM- and acrolein-up-regulated genes

GO term	Frequency	% of total 134 genes	*p* value
Biological process			
Ribosome biogenesis	33	24.6	3.38×10^{-11}
Ribonucleoprotein complex biogenesis	35	26.1	4.77×10^{-11}
RNA processing	34	25.4	6.71×10^{-8}
Nitrogen compound metabolic process	66	49.3	1.43×10^{-6}
Sulfur metabolic process	10	7.5	0.00334

Table 3
List of yeast single-gene-deletion strains that were tested for a functional phenotype[a]

Chemical subset	Identifier	Gene name	Source of deletion strain
H_2O_2-selective	YGR088W	*CTT1*	ATCC
H_2O_2-selective	YDR256C	*CTA1*	ATCC
H_2O_2-selective	YOR028C	*CIN5*[b]	ATCC
H_2O_2-selective	YLR205C	*HMX1*[b]	ATCC
NEM/acrolein-selective	YDR042C	Unknown	ATCC
NEM/acrolein-selective	YPR167C	*MET16*[c]	Invitrogen
NEM/acrolein-selective	YDR227W	*SIR4*[b]	ATCC
NEM/acrolein-selective	YCR016W	Unknown[b]	ATCC
NEM/acrolein-selective	YMR167W	*MLH1*[c]	ATCC
NEM/acrolein-selective	YDR369C	*XRS2*[c]	ATCC

[a] These are genes identified in Fig. 3A as Yap1-dependent and selectively up-regulated in response to H_2O_2 or acrolein and NEM.
[b] The single-gene-deletion strain lacking this gene did not show a decreased adaptive response to the corresponding chemical inducers, indicating that this gene does not convey a protective effect.
[c] The single-gene-deletion strain lacking this gene showed decreased adaptive response to NEM only, indicating that this gene does not protect against acrolein.

Quantification of nuclear accumulation of the GFP-Yap1 mutants after treatment with NEM or acrolein (Fig. 2D) clearly demonstrated that the double mutant GFP-Yap1$^{C620S,\ C629T}$ was not able to respond to NEM or acrolein and accumulate in the nucleus. Cys620, present in the Yap1 nuclear export signal, seemed to be especially important for nuclear localization in response to NEM (Fig. 2D). However, the fact that these experiments were performed at a single concentration of the respective chemicals precludes further speculation about the importance of this observation. The Δ*yap1* cells transformed with these mutant fusion genes were also tested for their adaptive responses to NEM and acrolein (Fig. 2E). Cells carrying the n-CRD triple mutant GFP-Yap1$^{C303S,\ C310S,\ C315S}$ retained a wild-type adaptive response to NEM or acrolein. The cells carrying the c-CRD double mutant GPF-Yap1$^{C589S,\ C620S}$ or GFP-Yap1$^{C598S,\ C629T}$ showed a NEM adaptive response comparable to that of wild type (Fig. 2E, second and third row, respectively). Although the acrolein adaptive response seen in these two double mutants was weaker than in the wild type, these mutants maintained sufficient inducible protection (Fig. 2E, right, second and third rows). However, the cells carrying the double mutant GFP-Yap1$^{C620S,\ C629T}$ did not show increased resistance to NEM or acrolein after pretreatment, indicating the loss of an adaptive response (Fig. 2E, fourth row). Single mutations did allow an adaptive response after pretreatment with either NEM or acrolein (Fig. 2E, fifth and sixth rows). Thus, the yeast adaptive response and cellular localization results for the GFP-Yap1 fusion proteins are consistent, demonstrating that either Cys620 or Cys629 is sufficient for these effects.

Distinct subsets of Yap1-dependent genes are induced by H_2O_2 versus NEM or acrolein

We have shown that the Yap1-mediated adaptive response to NEM or acrolein is different from the adaptive response to H_2O_2. To identify the subsets of Yap1-regulated genes that underlie this difference, we performed microarray analysis of RNA isolated from buffer control-treated wild-type or Δ*yap1* strains (control) and from these strains treated with 0.3 mM H_2O_2, 0.05 mM acrolein, or 0.016 mM NEM. In our initial analysis, we focused on gene response levels of expression that were increased 1.5-fold or higher in treated samples compared to controls and whose responses were statistically significant. For the up-regulated genes, this analysis revealed 43 genes specifically responding to H_2O_2, 239 genes responding to both NEM and acrolein, and 214 genes responding to all three chemicals (Fig. 3A, left). By comparison to the corresponding responses in the Δ*yap1* strain, the Yap1-dependent genes were identified (Fig. 3A, right). (See Supplementary Tables S3 and S4 for the lists of Yap1-dependent genes

up- or down-regulated, respectively, by the treatments and the expression profile of each gene.) Of note, we also identified 22 genes that we termed "special case" genes. These genes exhibited statistically significant responses to each of the chemicals, but the response to H_2O_2 was in the direction opposite to the response to NEM and acrolein (Supplementary Table S5). The observed microarray results (Fig. 3A, right) clearly support our hypothetical scheme (Fig. 1J) and show that distinct subsets of Yap1-dependent genes were activated by H_2O_2 versus NEM or acrolein.

The Yap1-dependent up-regulated gene lists specific for the H_2O_2 versus the NEM and acrolein responses were functionally analyzed using GO analysis of biological process and molecular function (Tables 1 and 2). For the H_2O_2-up-regulated genes, GO analysis identified iron homeostasis as an enriched biological process and catalase activity, iron ion transmembrane transporter activity, and heme binding as enriched molecular functions. For NEM- and acrolein-up-regulated genes, GO analysis identified ribosome biogenesis, ribonucleoprotein complex biogenesis, RNA processing, nitrogen compound metabolic process, and sulfur metabolic process as enriched biological processes and no enriched molecular functions.

The following criteria were then used to prioritize candidate genes for further functional analysis: (i) genes with known functions that might relate to protection against the corresponding inducer chemical, especially those genes present in the enriched GO terms; (ii) genes in the "biological process unknown" GO term with relatively high fold change in expression levels after chemical treatment(s); and (iii) the availability of haploid single-gene-deletion strains. By these criteria, 10 genes (Table 3) were selected as candidates for functional analysis.

The expression profiles of four candidate genes, *CTA1* and *CTT1* from the H_2O_2-specific response group and *MET16* and *YDR042C* from the NEM- and acrolein-specific response group, were examined by qRT-PCR as a validation step for the microarray result (Figs. 3B and C, left). For each tested gene, the expression ratio of each treated sample over untreated control was plotted against the corresponding expression ratio value calculated from the microarray data. All four genes showed a correlation greater than 0.9 correlation between the microarray and the qRT-PCR data (Figs. 3B and C, right), indicating a good consistency between the two methods.

Functional analysis identifies Yap1-dependent genes that afford chemical-selective protection

To determine whether the candidate genes actually provide protection against the chemical inducers, the BY4741 isogenic single-deletion strains lacking each of these genes were tested for their adaptive response to the corresponding chemicals by patch analysis and quantitative survival analysis (Table 3; Fig. 4). Initially, we focused on up-regulated genes because the functional importance of such genes could be easily tested using the available single-gene-deletion yeast strains. However, functional studies of chemical-selective adaptive responses using single-gene-deletion strains can be difficult to interpret because the gene deletion can affect both the basal sensitivity and the adaptive response of the strain. Further complicating these assessments, the qualitative patch assay is based on approximately 1000 cells per treatment and colonies derived from a small percentage of these cells can appear significant. Thus to aid in this effort, genes identified as having a functional effect in the patch analysis (Figs. 4A–C) were subjected to further quantitative analyses of adaptive response and cell viability (Figs. 4D–F).

Four Yap1-dependent H_2O_2-selective candidate genes were analyzed, *CTT1, CTA1, CIN5,* and *HMX1* (Table 3). Patch analysis indicated that neither Δ*cin5* nor Δ*hmx1* had any effect on the adaptive response of each strain to challenge with H_2O_2 (Table 3). The strain lacking *CIN5* retained an adaptive response to H_2O_2 that was comparable to that of wild type and consistent with the results of others [8]. In contrast, the deletion of *CTA1* (Δ*cta1*) reduced resistance to H_2O_2 in the nonpretreated cells, yet retained the adaptive protective response against H_2O_2, although this response was significantly diminished in comparison to the wild-type cells (Figs. 4A and D). The strain lacking *CTT1* (Δ*ctt1*) showed a slight decrease in response to H_2O_2 in the nonpretreated cells and did not demonstrate a significant adaptive response to H_2O_2 (Figs. 4A and D). The importance of *CTA1* and *CTT1* in selectively protecting against H_2O_2 toxicity is demonstrated in Figs. 4B, C, E, and F, in that deletion of either of these genes did not affect the adaptive response to NEM or acrolein. Interestingly, the deletion of *CTA1* or *CTT1* actually increased the resistance to acrolein in nonpretreated cells.

Five Yap1-dependent NEM and acrolein-selective candidate genes were analyzed, including two unknown genes, YDR042C and YCR016W, as well as *MET16, SIR4, MLH1,* and *XRS2*. Patch analysis indicated that neither Δ*sir4* nor Δ*ycr016w* had any effect on the adaptive responses of these strains to challenges with either NEM or acrolein, whereas Δ*met16,* Δ*mlh1,* and Δ*xrs2* strains showed decreased adaptive responses to NEM, but not to acrolein (Table 3).

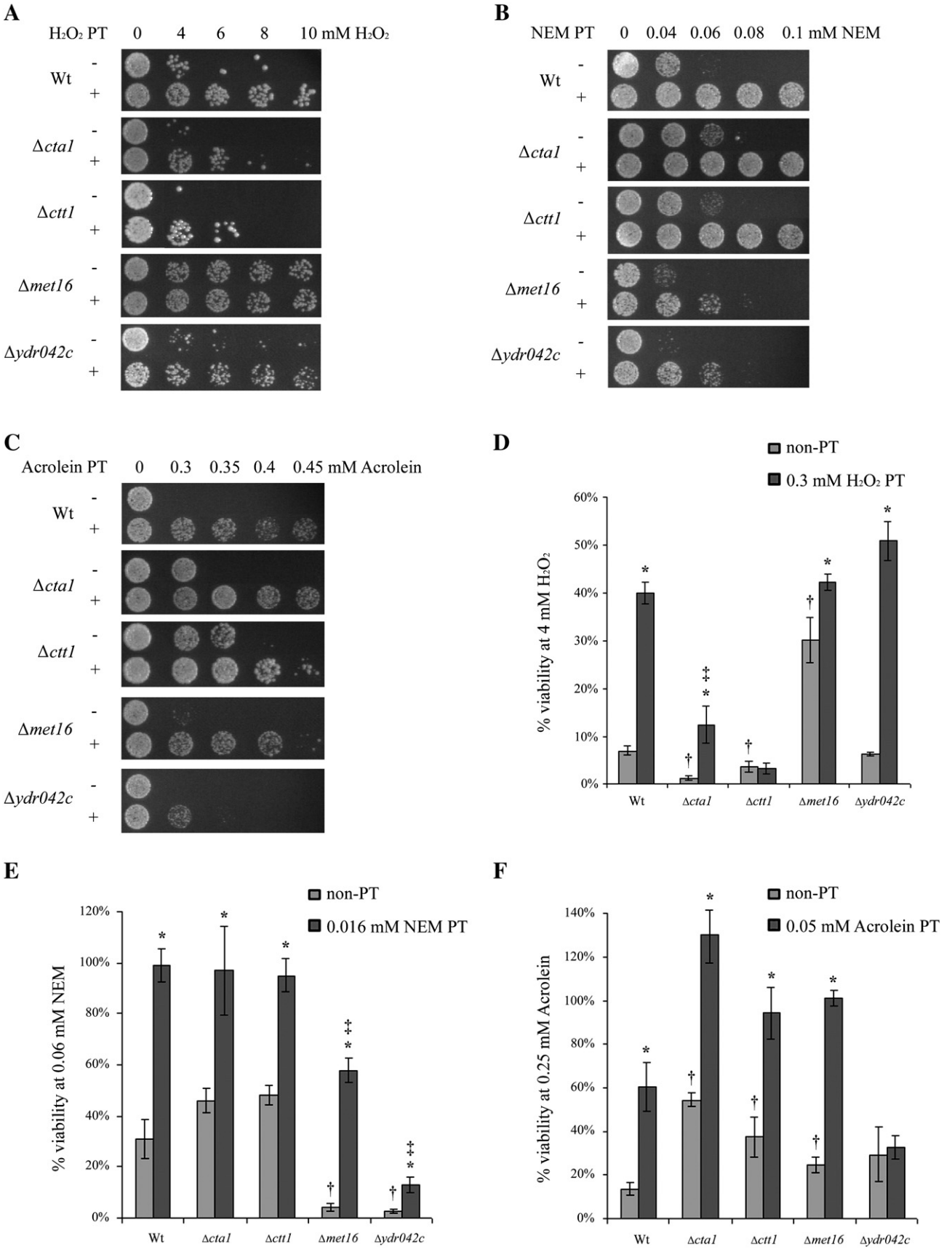
The single-gene-deletion yeast strains lacking *MET16* (Δ*met16*) or *YDR042C* (Δ*ydr042c*) were more sensitive to NEM without pretreatment, yet were able to mount some degree of increased tolerance to NEM after pretreatment, although neither reached the same level of resistance that was observed in the pretreated wild-type strain (Figs. 4B and E). These results indicate that each of these genes provides partial protection against NEM and imply that more than one protective mechanism is involved in the adaptive response to NEM. Whereas the Δ*ydr042c* strain showed an adaptive response to H_2O_2 that was similar to that of the wild-type strain, a deletion of *MET16* increased the resistance to H_2O_2 in the nonpretreated (Figs. 4A and D).

The *YDR042C* gene plays an important role in acrolein adaptive resistance, as the Δ*ydr042c* strain was no longer able to mount an adaptive response against acrolein, yet was not more sensitive under nonpretreatment conditions (Fig. 4F). Deletion of *MET16* did not impair the sensitivity or adaptive response to acrolein, indicating that this gene is not involved in resistance to acrolein (Figs. 4C and F). The specificity of *YDR042C* to protect against NEM and acrolein toxicity and not H_2O_2 is demonstrated in Figs. 4A and D, as Δ*ydr042c* showed a response to H_2O_2 that was very similar to the response of the wild-type strain. So far, *YDR042C* is the only gene identified in this research that is capable of protecting against both NEM and acrolein. The function of this gene remains unknown.

Discussion

Although both H_2O_2- and thiol-reactive chemical-induced adaptive responses are mediated by the Yap1 transcription factor, pretreatment with H_2O_2 or cysteine thiol-reactive chemicals failed to cross-protect against a subsequent challenge with the other class of inducer. To explain this selectivity, we have proposed a model of Yap1 transcriptional activation in which the specific cysteine reactivity of Yap1 leads to distinct active conformations of the transcription factor that recognize and regulate different subsets of Yap1-dependent genes that afford unique mechanisms of protection against the corresponding inducing chemical(s) (Fig. 1J). In support of this model, we demonstrated that the same cysteine residues in Yap1 were essential for NEM- and acrolein-induced activation, yet distinct from those required by H_2O_2. An earlier study showed that mutating all three c-CRD cysteines eliminated the Yap1-mediated response to NEM [12]. NEM was found to form adducts on all three cysteine residues [22], and it is very likely that acrolein can do the same because it has strong thiol reactivity and is smaller than NEM. However, the capability of reacting with NEM and acrolein does not demonstrate that a particular cysteine residue is essential for Yap1

activation by the chemicals. Our data from all three possible double mutations of the c-CRD cysteines indicated that Cys620 or Cys629 is sufficient for the NEM- and acrolein-induced activation, whereas Cys598 is dispensable for this effect. Furthermore, our results showed that Cys620 and Cys629 affect not only the cellular localization of Yap1, but also its function to activate a protective response.



By microarray analysis, we identified subsets of Yap1-dependent genes associated with H_2O_2 treatment or with NEM and acrolein treatment. An essential prediction of our model requires that each of these subsets of Yap1-dependent genes contains genes whose products confer selective protection against the corresponding class of chemical inducer. We have identified such genes by bioinformatic-based prioritization followed by functional testing using isogenic single-deletion-yeast strains.

In the Yap1-dependent H_2O_2-responsive gene subset, the two yeast catalase genes, *CTA1* and *CTT1,* protected against H_2O_2 toxicity in an adaptive response. *CTA1* encodes peroxisome catalase A and *CTT1* encodes cytosolic catalase T. Whereas both catalases were previously considered functionally redundant for the GSH system [34], our results confirm an earlier study [35] indicating that they provide nonredundant protection in the adaptive response to H_2O_2, as either single-gene-deletion strain was less able to mount an adaptive response to H_2O_2 than the wild-type yeast. In contrast to the report by Izawa et al. [35] that showed that the tolerance of the Δ*cta1* yeast to H_2O_2 was similar to that of the wild-type strain, our results indicate that both the Δ*cta1* and Δ*ctt1* strains are more sensitive to H_2O_2 than the wild-type strain. Of interest, the Δ*met16* strain was much less sensitive to H_2O_2. Repression of genes involved in sulfate assimilation, including *MET16,* has been shown to ameliorate the oxidative stress of zinc deficiency [36].

In the NEM- and acrolein-response group, the *MET16, MLH1,* and *XRS2* genes showed protection against NEM only. The *MET16* gene encodes 3′-phosphoadenylsulfate reductase [37], an enzyme essential for sulfate assimilation and sulfur amino acid biosynthesis [38]. In addition to *MET16,* several other genes encoding components of the sulfur amino acid biosynthesis pathway were also represented in this response group, including *MET2, MET8, MET14,* and *MET10* (Supplementary Table S3), highlighting the likely importance of this pathway in the protection against NEM. Two genes important to DNA repair, *MLH1,* which is essential for DNA mismatch repair [39], and *XRS2,* which is involved in the repair of double-stranded breaks [40], are critical to the adaptive response to NEM, indicating that DNA damage contributes to the cytotoxicity of this chemical. NEM most probably causes DNA damage indirectly, as it usually reacts with cysteine residues in proteins, not DNA. Several studies have shown that NEM and other thiol-reactive compounds inhibit DNA polymerases α, γ, and δ [41]. DNA polymerases α and δ are involved in normal DNA base replication and proofreading and DNA polymerase δ is also associated with mismatch repair [42]. Furthermore, reduced levels of DNA polymerases α and δ have been shown to induce chromosome fragile-site instability in yeast, owing to stalled replication forks. In addition, NEM and other thiol-reactive compounds are thought to alkylate DNA topoisomerase II, leading to increased DNA strand breaks [43]. Therefore, there are several reported mechanisms by which NEM could indirectly increase DNA damage. Our observations demonstrate that the adaptive response to NEM is dependent on *MLH1* and *XRS2,* indicating that the repair of DNA damage is a critical factor in the adaptive response to NEM.

Such Yap1-mediated effects on sulfate assimilation, sulfur amino acid biosynthesis, and DNA damage repair emphasize the fact that Yap1 not only regulates cellular responses to changes in the redox status of the cell, but also acts to integrate complex physiological responses important to cellular homeostasis, as well as survival. Further examples of the contribution of Yap1 to broad integration of basic cell physiology are seen in its contribution to the cellular response to another stressor, heat shock [44], and its role as a regulator of cell proliferation under conditions of normal aerobic as well as H_2O_2-stress conditions [45].

The *YDR042C* gene, also in the NEM- and acrolein-responsive subset, showed a significant protective role against NEM toxicity and in the adaptive response to NEM or acrolein. Previously identified in a high-throughput experiment as a gene whose deletion confers resistance to rapamycin and wortmannin [46], the function and regulation of this gene are essentially unknown. To our knowledge, our results are the first to demonstrate Yap1-dependent regulation of *YDR042C* and its protective function against electrophilic chemicals. However, the details of how this gene protects against both NEM and acrolein remain to be elucidated.

Collectively, these results show that the Yap1 transcription factor is capable of specifying downstream gene expression profiles based on the chemical nature of the activating agent. Various types of oxidative stress inducers, although sharing the similarity of being electrophilic, are distinctly reactive and probably produce distinct cellular damage, requiring specific cellular mechanisms of detoxification and/or repair. A finely tuned transcriptional response would avoid activation of irrelevant, and perhaps energy-wasting, protective pathways, thus allowing the yeast to cope with environmental challenges more efficiently. The physiological necessity of having a regulated adaptive response to Michael acceptors is apparent because of the distribution of chemicals such as acrolein in nature. Unlike NEM and diamide, which are effective laboratory tools, acrolein is widely present, with sources including incomplete combustion of organic materials and intracellular processes such as lipid peroxidation [47,48]. As the strongest electrophile among the α,β-unsaturated aldehydes, acrolein is highly toxic to the cell because of its ability to form DNA and protein adducts and to disrupt gene expression [33,47,49].

Such a sophisticated mechanism of regulation of the Yap1 transcription factor, based on multiple active conformations, has been previously suggested from biochemical studies of the H_2O_2-responsive *E. coli* OxyR. In that study, Cys199, essential for OxyR activation, was subjected to various types of modifications including disulfide bond formation, S-nitrosylation, S-hydroxylation, and S-glutathionylation, leading to significantly different conformations of OxyR, each with varying DNA binding affinity toward the promoters of the OxyR target genes *katG* and *oxyS* [50]. However, the various binding affinities did not predict the transactivating potency, indicating that in OxyR, the effects of cysteine modification on promoter binding and transcriptional activation are independent. Nonetheless, functional studies of OxyR mutants did demonstrate that various cysteine residues contribute differently to the transcriptional activation of selected target genes. The $OxyR^{C208S}$ mutant retained wild-type activity to activate transcription of the *oxyS* gene, whereas transcription of the *katG* gene was weakened. In contrast, the $OxyR^{C199S}$ mutant was unable to activate transcription of either

Fig. 4. Functional analysis of inducer-specific Yap1-dependent genes. (A) Patch analysis of H_2O_2 adaptive responses in single-deletion yeast strains. Wild type (Wt), Δ*cta1,* Δ*ctt1,* Δ*met16,* and Δ*ydro42c* yeast cells were pretreated (PT) with 0.3 mM H_2O_2 before challenge with H_2O_2 at the indicated concentrations. (B) Patch analysis of NEM adaptive responses in single-deletion yeast strains. The same strains as for (A) were pretreated with 0.016 mM NEM before challenge with NEM at the indicated concentrations. (C) Patch analysis of acrolein adaptive response in the single-deletion yeast strains. The strains were pretreated with 0.05 mM acrolein before challenge with acrolein at the indicated concentrations. (D–F) Shown are the means and standard deviations of three independent measurements. The significance of difference in survival rates was determined by ANOVA ($p<0.05$). As these tests were significant, post hoc Student *t* tests ($p<0.05$) were performed using a Bonferroni multiple-significance-test correction. *Significant difference in survival between the nonpretreated and the pretreated samples within a yeast strain; †significant difference in survival between nonpretreated Wt and nonpretreated isogenic deletion strain; ‡significant difference in survival between the pretreated Wt and the pretreated isogenic deletion strain. (D) Quantitative analysis of H_2O_2 adaptive responses in the same set of single-deletion yeast strains. The yeast cells were pretreated with 0.3 mM H_2O_2 before challenge with 4 mM H_2O_2. The survival rates of nonpretreated (non-PT) and pretreated cells were measured. (E) Quantitative analysis of NEM adaptive responses in single-gene-deletion yeast strains. The yeast strains were pretreated with 0.016 mM NEM before challenge with 0.6 mM NEM, and the survival rates of nonpretreated and pretreated cells were measured. (F) Quantitative analysis of acrolein adaptive responses in single-gene-deletion yeast strains. The yeast strains were pretreated with 0.05 mM acrolein before challenge with 0.25 mM acrolein, and the survival rates of nonpretreated and pretreated cells were measured.

gene [50]. Theoretically, if OxyR can adopt a conformation that mimics the effect of the $OxyR^{C208S}$ mutation, it could show transcriptional selectivity toward *oxyS* relative to *katG*. To our knowledge, such results have not yet been reported.

Another interesting antioxidant defense system with strong similarities to Yap1 is the mammalian Keap1–Nrf2 complex [51,52]. Although it regulates the response of mammalian cells to oxidative stress, this system is now recognized to have primitive origins with homologues found in the invertebrate species *Caenorhabditis* and *Drosophila* [53]. Like Yap1, Nrf2 is a transcription factor known to regulate numerous genes with antioxidant functions [53]. In addition, Nrf2 is increasingly recognized as playing important roles in the integration of complex physiological processes including the response to heat shock, cell survival, macrophage homeostasis, and wound repair [53]. In contrast to Yap1 protein, which contains the cysteine residues necessary for response to reactive oxygen species as well as thiol-reactive chemicals, the Nrf2 system is more complex [51,52]. In this system, Keap1, which binds to and regulates Nrf2 degradation, contains a multitude of cysteine residues that have recently been demonstrated to distinctly sense and respond to at least six classes of Nrf2 activators that block Keap1-dependent Nrf2 ubiquitination [51]. Parallel to Yap1, H_2O_2 has recently been shown to oxidize Keap1, leading to an intermolecular disulfide formation at Keap1 Cys151. This modification was found to stabilize Nrf2 and is thus an activating reaction of this system [52].

Although the mechanism(s) of selective Yap1-dependent gene expression has not yet been elucidated, the current understanding of the yeast Yap family of bZIP transcription factors indicates that the formation of distinct heterodimeric forms is unlikely. Eleven of the 14 proteins in this family are known to act as homodimers, including Yap1 [54]. To achieve the observed chemical selective patterns of Yap1-dependent gene expression, each active Yap1 conformation may recognize different DNA sequences specific to a distinct set of target genes or bind to additional transcription factors and/or coactivator proteins to activate transcription. Of the few reports on transcriptional regulation of target genes of Yap1, one report, following up on the observation that Yap1 and a second transcription factor, Skn7, regulate genes responding to oxidative stress [26], showed the transcriptional dependence of two H_2O_2-responsive genes, *TSA1* and *CTT1,* on novel DNA binding sites for Yap1 and Skn7 [55]. This altered Yap1 response element was enriched in 179 genes responding to H_2O_2 [55], indicating that additional studies of this response element and the interaction of Yap1 and Skn7 in potentially mediating the selective response to H_2O_2 are warranted. A related idea to such unique binding sites is half-site spacing between the core binding sites. Although this has not been specifically addressed for Yap1 DNA binding, one study of Gcn4 binding implicated half-site spacing as a potential source of selective Gcn4 DNA binding at DNA recognition sites [56]. This concept, as well as the idea of selective transcription factor/coactivator/corepressor recruitment, deserves further investigation as a means to understanding the selectivity of the yeast adaptive responses to oxidative stress.

In summary, the results presented here indicate that Yap1 affords protection to H_2O_2 or thiol-reactive chemicals by distinct CRD-mediated transcriptional mechanisms, resulting in differential gene expression that is necessary for protection against each of these chemicals. This finely tuned response at the level of transcription allows the cell to efficiently respond to oxidative stress induced by a variety of chemicals.

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2010.10.697.

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