



**HAL**  
open science

## **A scaffold protein that chaperones a cysteine-sulfenic acid in H<sub>2</sub>O<sub>2</sub> signaling**

Antoine Bersweiler, Benoît d'Autréaux, Hortense Mazon, Alexandre Kriznik, Gemma Belli, Agnès Delaunay-Moisan, Michel B Toledano, Sophie Rahuel-Clermont

► **To cite this version:**

Antoine Bersweiler, Benoît d'Autréaux, Hortense Mazon, Alexandre Kriznik, Gemma Belli, et al.. A scaffold protein that chaperones a cysteine-sulfenic acid in H<sub>2</sub>O<sub>2</sub> signaling. *Nature Chemical Biology*, 2017, 13 (8), pp.909 - 915. 10.1038/nChEMBio.2412 . hal-01652643

**HAL Id: hal-01652643**

**<https://hal.univ-lorraine.fr/hal-01652643>**

Submitted on 11 Jan 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21

Title

**A scaffold protein that chaperones a cysteine-sulfenic acid in H<sub>2</sub>O<sub>2</sub> signaling**

by

Antoine Bersweiler<sup>1</sup>, Benoît d'Autréaux<sup>2</sup>, Hortense Mazon<sup>1</sup>, Alexandre Kriznik<sup>1</sup>, Gemma Belli<sup>2,3</sup>, Agnès Delaunay-Moisan<sup>2</sup>, Michel B Toledano<sup>2\*</sup> & Sophie Rahuel-Clermont<sup>1\*</sup>

<sup>1</sup> UMR 7365 CNRS-Université de Lorraine IMoPA, Biopôle, campus Biologie-Santé,  
9 avenue de la Forêt de Haye, BP 20199, F-54505 Vandœuvre-lès-Nancy Cédex, France

<sup>2</sup> Institute for Integrative Biology of the Cell (I2BC), UMR9198, CNRS, CEA-Saclay, Université  
Paris-Saclay, iBiTecS/SBIGEM, Laboratoire Stress oxydant et Cancer, Bat 142, F-91198 Gif-  
sur-Yvette Cedex, France

<sup>3</sup> present address: Department of Basic Medical Sciences, IRB Lleida, University of Lleida,  
Lleida 25198 Spain

\* Corresponding authors

e-mail: [sophie.rahuel@univ-lorraine.fr](mailto:sophie.rahuel@univ-lorraine.fr), tel., +33 372 746 660

e-mail: [michel.toledano@cea.fr](mailto:michel.toledano@cea.fr), tel., +33 169 088 244

22 **ABSTRACT**

23 In *Saccharomyces cerevisiae*, Yap1 regulates an H<sub>2</sub>O<sub>2</sub>-inducible transcriptional response that  
24 controls cellular H<sub>2</sub>O<sub>2</sub> homeostasis. H<sub>2</sub>O<sub>2</sub> activates Yap1 by oxidation through the intermediacy of  
25 the thiol-peroxidase Orp1. Upon reacting with H<sub>2</sub>O<sub>2</sub>, Orp1 catalytic cysteine oxidizes to a sulfenic  
26 acid, which then engages either into an intermolecular disulfide with Yap1 that leads to Yap1  
27 activation, or an intramolecular disulfide that commits the enzyme into its peroxidatic cycle. Of  
28 these two competing reactions, how the former one, which is kinetically unfavorable, occurs? We  
29 show that the Yap1-binding-protein Ybp1 brings together Orp1 and Yap1 into a ternary complex  
30 that selectively activates condensation of the Orp1 sulfenylated cysteine with one of the six Yap1  
31 cysteines, while inhibiting Orp1 intramolecular disulfide formation. We propose that Ybp1 operates  
32 as a scaffold protein and as a sulfenic acid chaperone to provide specificity into the transfer of  
33 oxidizing equivalents by a reactive sulfenic acid species.

34

35

36

37           Reactive oxygen species (ROS), which include the superoxide anion ( $O_2^{\bullet-}$ ) and hydrogen  
38 peroxide ( $H_2O_2$ ), are toxic molecules that also operate in intracellular signaling. Both these effects  
39 are due to ROS chemical reactivity, which enables them to oxidize biological molecules, thereby  
40 causing either damage, or modifying the activity of regulatory proteins by covalent modification.  
41 Whereas the toxicity of ROS is intuitively perceived, their signaling function has been more difficult  
42 to grasp, owing to the paradox posed by the specificity required for signaling and the seeming  
43 indiscriminate reactivity of ROS<sup>1</sup>.

44           ROS-responsive pathways that regulate ROS intracellular homeostasis in microorganisms  
45 have provided, and still are, fundamental principles of how specificity can be achieved in ROS  
46 signaling<sup>1</sup>. These pathways make use of sensors that “measure” the intracellular concentration of  
47 ROS and proportionally set the expression of antioxidants, thereby maintaining ROS concentration  
48 below a toxic threshold<sup>2</sup>. One such pathway is the *S. cerevisiae*  $H_2O_2$ -responsive transcription factor  
49 Yap1, which activates most antioxidants, thiol-reductases, and other stress defense and detoxifying  
50 activities<sup>2</sup>. Yap1 is primarily controlled at the level of nucleo-cytoplasmic shuttling<sup>3</sup>.  $H_2O_2$  oxidizes  
51 Yap1 into two disulfides between Cys residues C303-C598 and C310-C629<sup>4-6</sup> (**Supplementary**  
52 **results, Supplementary Fig. 1**). The conformation change resulting from this oxidation conceals a  
53 nuclear export signal, which by inhibiting nuclear export promotes Yap1 accumulation into the  
54 nucleus<sup>4,7,8</sup>.

55           Yap1 oxidation by  $H_2O_2$  is mediated by the thiol-based Oxidant Receptor and Peroxidase  
56 Orp1<sup>9</sup> (**Fig. 1**). Reaction of Orp1 catalytic Cys residue (C36) with  $H_2O_2$  yields a sulfenic acid (Orp1—  
57 SOH) (reaction 1)<sup>10,11</sup>. Once formed, the C36-SOH can engage into either an intermolecular disulfide  
58 with Yap1 by condensation with Yap1 C598 yielding Orp1—S—S—Yap1 (reaction 2), which is then  
59 transposed into the first intramolecular disulfide of active Yap1, or into an Orp1 intramolecular  
60 disulfide by condensation with Orp1 resolving Cys residue (C82) (Orp1<sub>OxSS</sub>, reaction 3), which  
61 commits the enzyme into its peroxidatic cycle<sup>9</sup>. Of these two competing reactions, the unimolecular

62 one that produces Orp1<sub>oxSS</sub> should be kinetically favored over the bimolecular one<sup>12</sup> engaging Orp1  
63 and Yap1, which raises the question of the mechanism that enable the latter to occur. The Yap1-  
64 binding protein Ybp1 is required for the Orp1-dependent oxidation of Yap1 by H<sub>2</sub>O<sub>2</sub><sup>13-15</sup>. As it co-  
65 precipitates with Yap1, Ybp1 could favor the Orp1/Yap1 redox interaction by bringing these  
66 proteins together, and could also guide Orp1—SOH towards Yap1 C598, or prevent formation of the  
67 competing Orp1 C36-C82 disulfide<sup>2</sup>.

68 We sought elucidating Ybp1 function by measuring the kinetics of Orp1-dependent oxidation  
69 of Yap1 by H<sub>2</sub>O<sub>2</sub> upon reconstitution of the redox relay *in vitro*, and by probing the interactions  
70 between the redox relay partners. We found that Ybp1 brings together Orp1 and Yap1 into a ternary  
71 complex, in which Orp1—S—S—Yap1 intermolecular disulfide formation is activated, while  
72 Orp1<sub>oxSS</sub> intramolecular disulfide formation is impeded, hence favoring H<sub>2</sub>O<sub>2</sub> signaling at the  
73 expense of H<sub>2</sub>O<sub>2</sub> scavenging. Our data suggest a novel mechanism whereby by shielding and  
74 canalizing the reactivity of the Cys-sulfenic acid, Ybp1 operate as a “sulfenic acid chaperone”, an  
75 issue important in view of the emerging roles of thiol peroxidases<sup>16,17</sup> and sulfenic acid species<sup>17-19</sup>  
76 in redox biology.

## 77 RESULTS

### 78 Ybp1 brings Yap1 and Orp1 in a non-covalent 1:1:1 complex

79 We first assayed the interaction of Ybp1 and Yap1 *in vitro*. When alone in solution, Ybp1  
80 eluted from a size-exclusion column as a single peak with an apparent mass of 100 kDa, close to the  
81 calculated monomer mass (80 kDa), and Yap1 as a major peak of 470 kDa (calculated monomer  
82 mass = 73 kDa), which suggested that Yap1 oligomerizes in solution and/or adopts a non-globular,  
83 flexible conformation, in accordance with its sensitivity to proteolysis<sup>5</sup> (**Fig. 2a**). A small fraction of  
84 Yap1 also eluted within the column-void volume, likely reflecting protein aggregation. When  
85 present in an equimolar mix, the two proteins now eluted as one major species with an apparent  
86 mass of 307 kDa, confirming the Ybp1-Yap1 interaction shown by co-immunoprecipitation from  
87 crude lysates<sup>13,14</sup> and suggesting the presence of a 1:1 complex (calculated mass of 153 kDa) with a  
88 non-globular conformation, or a 2:2 complex (306 kDa). Doubling the concentration of Yap1 relative  
89 to Ybp1 produced an additional peak corresponding to free Yap1, thus confirming non-covalent  
90 complex formation with a stoichiometry of 1:1 or 2:2 (hereafter referred to as Ybp1·Yap1).  
91 Quantitative data of the Yap1-Ybp1 interaction were obtained from the change of fluorescence  
92 anisotropy of fluorochrome-labeled Ybp1 (AF-Ybp1) upon Yap1 titration. A saturable 11%  
93 anisotropy increase was observed, which after conversion to the fraction of bound AF-Ybp1, was  
94 fitted to a single-site binding equation, yielding a dissociation constant of  $1.6 \pm 0.3 \mu\text{M}$  (**Fig. 2b**).  
95 Using Yap1<sup>SSS SSS</sup>, a mutant with Ser substitution of all six Cys, gave a value close to the previous one  
96 ( $0.7 \pm 0.2 \mu\text{M}$ ). Titration of labeled Yap1 (AF-Yap1) by Ybp1 produced a saturable 22% fluorescence  
97 anisotropy increase and a dissociation constant of  $1.3 \pm 0.2 \mu\text{M}$ , very close to the value obtained  
98 using AF-Ybp1.

99 We next assayed the interaction of Orp1 with its two partners by isothermal titration  
100 calorimetry (ITC). Yap1<sup>SSS SSS</sup> was used here to avoid any redox interferences. No significant signal  
101 was observed when Orp1 was injected into a Yap1<sup>SSS SSS</sup> solution (**Fig. 2c**, left panel). In contrast,  
102 injection of Orp1 into a Ybp1 solution produced a binding isotherm that fitted a single-site model,

103 which yielded a dissociation constant of  $0.8 \pm 0.1 \mu\text{M}$  and a stoichiometry of  $0.8 \pm 0.1$ , indicative of a  
104 1:1 stoichiometry (**Fig. 2c**, central panel, **Supplementary Table 1**). Calorimetric titration of the  
105 Ybp1·Yap1 complex by Orp1 also produced an isotherm corresponding to a single-site binding  
106 interaction with a dissociation constant of  $0.7 \pm 0.2 \mu\text{M}$  and a stoichiometry of  $0.8 \pm 0.1$  (**Fig. 2c**,  
107 right panel, **Supplementary Table 1**), also indicative of a 1:1 stoichiometry, which indirectly  
108 confirmed a Ybp1-Yap1 1:1 association. In the latter experiment, Orp1 binding to Ybp1 could have  
109 dissociated the Ybp1·Yap1 complex, but predicted titrations that incorporated the Ybp1·Yap1 and  
110 Orp1·Ybp1 dissociation constants did not reach complete saturation under the conditions used. No  
111 significant signal was detected by titrating Ybp1 or the Ybp1·Yap1<sup>SSS SSS</sup> complex with Orp1 in the  
112 intramolecular disulfide form Orp1<sub>oxSS</sub> (**Supplementary Fig. 2**). TAP-Tag-affinity precipitation of  
113 Ybp1 from crude yeast lysates brought down both Yap1 and Orp1, thus establishing that the ternary  
114 Orp1·Ybp1·Yap1 complex is also formed *in vivo*, although we cannot totally rule out that Orp1  
115 coprecipitates with Ybp1 by virtue of its disulfide linkage to Yap1 (**Supplementary Fig. 3**).

116 Ybp1 thus brings together Yap1 and reduced Orp1, but not Orp1<sub>oxSS</sub>, into a non-covalent  
117 ternary Orp1·Ybp1·Yap1 complex of 1:1:1 stoichiometry.

118

### 119 ***In vitro* reconstitution of the Orp1-Yap1 redox relay**

120 To address the molecular function of Ybp1, we reconstituted the redox relay *in vitro* with  
121 purified Yap1, Orp1, Ybp1, thioredoxin (Trx), Trx reductase and NADPH, and monitored the kinetics  
122 of Yap1 oxidation by H<sub>2</sub>O<sub>2</sub> by the differential migration of Yap1 redox conformers by non-reducing  
123 SDS-PAGE. As previously shown<sup>9</sup>, Myc-Yap1 from untreated cells migrates as a unique band  
124 (Yap1<sub>red</sub>); minutes after H<sub>2</sub>O<sub>2</sub> exposure, the C36-C598-linked Orp1—S—S—Yap1 complex and Yap1  
125 intramolecular disulfide forms (Yap1<sub>ox</sub>) are both seen as two distinct bands that migrate above and  
126 below reduced Yap1, respectively (**Fig. 3a**, **Supplementary Fig. 4**). A third H<sub>2</sub>O<sub>2</sub>-inducible Myc-  
127 immunoreactive band was also seen just above Orp1—S—S—Yap1, which also contains Orp1 and  
128 possibly leads to formation of the second Yap1 intramolecular disulfide. Analysis of the

129 reconstituted relay similarly identified reduced Yap1 and Ybp1 prior to addition of H<sub>2</sub>O<sub>2</sub>, and then  
130 three new Yap1 electrophoretic forms upon addition of H<sub>2</sub>O<sub>2</sub>, which correspond to the Yap1-  
131 intramolecular disulfide forms and to the two Orp1-Yap1 disulfide-linked complexes (**Fig. 3b**). Yap1  
132 oxidation was fast, occurring within seconds, and lasted a few minutes after which Yap1 returned to  
133 its reduced form, closely reproducing the *in vivo* kinetics of Yap1 oxidation<sup>9</sup>.

134

### 135 **Orp1 and Ybp1 are required for Yap1 oxidation *in vitro***

136 We next probed each of the components of the redox relay on Yap1 oxidation. When alone,  
137 Yap1 (5 μM) became oxidized, but at very high doses of H<sub>2</sub>O<sub>2</sub>, much higher than those effective in  
138 the fully reconstituted system (H<sub>2</sub>O<sub>2</sub> > 500 μM vs < 100 μM), with appearance of Yap1 high  
139 molecular weight disulfide-linked complexes, indicative of non-native disulfide bond formation  
140 (**Supplementary Fig. 5a**). Excluding the Trx system from the reaction did not alter Yap1 oxidation,  
141 except for a slower kinetics, likely due to the trapping of Orp1 in the Orp1<sub>oxSS</sub> form (**Supplementary**  
142 **Fig. 5b**). Excluding Orp1 however prevented Yap1 oxidation by H<sub>2</sub>O<sub>2</sub> at 100 μM (**Supplementary**  
143 **Fig. 5c**), thus confirming Orp1 absolute requirement for Yap1 oxidation<sup>9,13</sup>. Similarly, excluding  
144 Ybp1 prevented Yap1 oxidation by H<sub>2</sub>O<sub>2</sub> at 100 μM (**Supplementary Fig. 5b**). Only at a very high  
145 dose of H<sub>2</sub>O<sub>2</sub> (2 mM), a slow and weak oxidation of Yap1 was observed (**Fig. 3c**), which was partially  
146 dependent upon Orp1 (10 μM), as indicated the presence of a very weak band corresponding to the  
147 Orp1-Yap1 disulfide complex. Introducing Ybp1 led to a concentration-dependent stimulation of  
148 Yap1 oxidation, and appearance of one, and then of the two Orp1-Yap1 disulfide-linked complexes  
149 (**Fig. 3c**). The effect of Ybp1 reached a maximum at a Ybp1:Yap1 ratio of 1:1, thus confirming the  
150 results of **Fig. 2**.

151

### 152 **Ybp1 is required for the formation of Orp1—S—S—Yap1**

153 As previously observed *in vivo* (**Fig. 3a**), Ala substitution of Yap1 C303 (Yap1<sup>C303A</sup>) stabilizes  
154 the Orp1-Yap1 C36-C598 intermolecular disulfide by preventing its transposition into the C303-



155 C598 intramolecular Yap1 disulfide<sup>9</sup>. In  $\Delta ybp1$  cells that expressed Yap1<sup>C303A</sup> however, the Orp1—  
156 S—S—Yap1 complex did not form (**Fig. 3a**). Similarly, in the reconstituted system, Yap1<sup>C303A</sup> formed  
157 a stable Orp1—S—S—Yap1 complex with Orp1 in the presence, but not in the absence of Ybp1 (**Fig.**  
158 **3d**). The peroxiredoxin Tsa1 was proposed to replace Orp1 in Yap1 oxidation in cells lacking  
159 Ybp1<sup>20</sup>, but no band corresponding to a Tsa1-Yap1 adduct was detected in  $\Delta ybp1$ , as also reported<sup>20</sup>.

160 The reconstituted system thus faithfully reproduces the *in vivo* setting, thereby confirming  
161 the requirement of both Orp1 and Ybp1 for efficient and sensitive Yap1 oxidation by H<sub>2</sub>O<sub>2</sub>.  
162 Furthermore, both *in vivo* and *in vitro* data indicate that Ybp1 is required for the initial Orp1-Yap1  
163 redox interaction (reaction 2, see **Fig. 1**).

164

### 165 **Ybp1 activates formation of Orp1—S—S—Yap1**

166 The Orp1-Yap1 redox relay can be deconstructed into three single reactions: formation of the  
167 Orp1—SOH (reaction 1, rate constant  $k_{SOH}$ ); condensation of the former with Yap1 C598 (reaction 2)  
168 or with Orp1 C82 (reaction 3, rate constant  $k_{SS}$ ) (see **Fig. 1**). To test whether Ybp1 modulates  
169 reactions 2, or 3 or both, we assessed its effect on their kinetics using a quench flow apparatus.

170 Reaction 2 was evaluated with an Orp1 mutant only retaining C36 (Orp1 C64S, C82A, Orp1<sup>CSA</sup>)  
171 to eliminate reaction 3, and a Yap1 mutant only retaining C598 (Yap1<sup>SSS CSS</sup>), to avoid unwanted  
172 redox reactions. In the absence of Ybp1, these two mutants engaged in the stable Orp1—S—S—  
173 Yap1 disulfide by lack of Yap1 C303 (**Supplementary Fig. 6**, also showing that Orp1<sup>C82S</sup> behaves as  
174 Orp1<sup>CSA</sup>). We also used H<sub>2</sub>O<sub>2</sub> at concentrations low enough to avoid non-specific oxidation (100  $\mu$ M)  
175 which would still ensure fast enough Orp1—SOH formation, for the latter not to be rate limiting for  
176 the overall reaction ( $k_{SOH} = 1.6 \cdot 10^5 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$ , which gives a rate constant of 16 s<sup>-1</sup> at 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>,  
177 see **Supplementary Fig. 7**). Reactions were started by mixing H<sub>2</sub>O<sub>2</sub> to a mixture containing excess  
178 Orp1<sup>CSA</sup> and either Yap1<sup>SSS CSS</sup> or the Ybp1·Yap1<sup>SSS CSS</sup> complex, and were stopped by acid quenching.  
179 Prior to H<sub>2</sub>O<sub>2</sub> addition, reverse-phase chromatograms of the reaction mixtures lacking Ybp1  
180 identified Yap1<sup>SSS CSS</sup> (peak 1) and Orp1<sup>CSA</sup> (peak 4) (**Supplementary Fig. 8** and **Supplementary**

181 **Table 2).** Upon H<sub>2</sub>O<sub>2</sub> addition, the Yap1 and Orp1<sup>CSA</sup> peaks gradually decreased over time, and three  
182 additional peaks appeared with masses compatible with Orp1—S—S—Yap1 (peak 2), disulfide-  
183 linked homodimeric Orp1 (C36—S—S—C36) (peak 5), and oxidized Orp1<sup>CSA</sup> in the sulfenylamide  
184 form (peak 3) (**Supplementary Fig. 8** and **Supplementary Table 2**). The latter species likely forms  
185 by Orp1—SOH intramolecular cyclization upon attack of the neighboring Gly37 amide nitrogen at  
186 acidic pH<sup>21-23</sup>. Chromatograms of reaction mixtures containing Ybp1 yielded the same products as  
187 those observed in its absence (**Fig 4a**).

188 The kinetics of Orp1—S—S—Yap1 formation in the absence and presence of Ybp1 were  
189 deduced from the evolution of peak-2 area, yielding respective observed rate constants of  $0.3 \pm 0.03$   
190 and  $2.6 \pm 0.4 \text{ s}^{-1}$ , which indicated a significant stimulatory effect of Ybp1 (**Fig. 4b**). Reacting H<sub>2</sub>O<sub>2</sub> in a  
191 mixture containing wild-type (Wt) Orp1 and the Ybp1·Yap1<sup>SSS CSS</sup> complex led again to a decrease of  
192 Yap1<sup>SSS CSS</sup> (peak 1) and Orp1 (peak 8), and the appearance of Orp1<sub>oxSS</sub> (peak 7) and of a species with  
193 mass compatible with Orp1—S—S—Yap1 (peak 6) (**Fig. 4c, Supplementary Table 2**). The latter  
194 appeared at an observed first-order rate constant of  $4.6 \pm 0.2 \text{ s}^{-1}$  (**Fig. 4d**). This value did not change  
195 by increasing Orp1 concentration up to near saturation (10 to 80 μM) (mean  $5.6 \pm 1.0 \text{ s}^{-1}$ ), further  
196 demonstrating that Orp1 operates within the Orp1·Ybp1·Yap1 ternary complex (**Fig. 4d, inset**), and  
197 hence that the reaction should obey saturation kinetics, in contrast to the bimolecular one measured  
198 in the absence of Ybp1 (Orp1 and Yap1 do not form a pre-complex).

199 The kinetics of Orp1—S—S—Yap1 formation in the absence of Ybp1 (**Supplementary Fig. 8,**  
200 **Fig. 4b**) was analyzed by directly fitting data to a two-step model, which comprises reaction 1 ( $k_{\text{SOH}}$   
201 =  $1.6 \cdot 10^5 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$ , **Supplementary Fig. 7**) followed by reaction 2 (**Supplementary Fig. 9a**). This  
202 analysis yielded a second order rate constant  $k_{\text{bimolecular}}$  for reaction 2 of  $(7.4 \pm 4.9)10^3 \text{ M}^{-1}\text{s}^{-1}$ . To  
203 interpret the kinetics obtained in presence of Ybp1 (**Fig. 4a,b**), we directly fitted data to a similar  
204 reaction scheme that was now preceded by the formation of the ternary complex, assumed to be a  
205 rapid equilibrium characterized by the  $K$  affinity constant between Ybp1·Yap1<sup>SSS CSS</sup> and Orp1 of 0.7  
206 μM (**Supplementary Fig. 9b**). This analysis yielded a first order rate constant for reaction 2 in the

207 complex  $k_{\text{complex}}$  of  $3.5 \pm 0.7 \text{ s}^{-1}$ , close to the observed value of  $2.6 \text{ s}^{-1}$ . We then compared the value of  
208  $\frac{k_{\text{complex}}}{K}$  ( $5.0 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$  for Orp1<sup>CSA</sup>) to the  $k_{\text{bimolecular}}$  value of  $7.4 \cdot 10^3 \text{ M}^{-1}\text{s}^{-1}$  obtained in the absence of  
209 Ybp1. The 700-fold ratio between these two parameters reveals the actual impact of Ybp1 on  
210 reaction 2, which becomes particularly significant when considering the low *in vivo* concentrations  
211 of Orp1 and Yap1 (**Supplementary Fig. 9c**). Reacting oxidized Orp1 (Orp1<sub>oxSS</sub>) under the same  
212 conditions yielded an observed rate constant of  $0.026 \pm 0.006 \text{ s}^{-1}$ , nearly 200-times slower relative  
213 to the reaction with reduced Orp1 (**Fig. 4e,f**).

214 These data indicate a strong increasing effect of Ybp1 on the rate of reaction of Yap1 C598  
215 with Orp1—SOH, akin to enzyme catalysis, which is accounted for by Ybp1-dependent pre-complex  
216 formation and possibly also by a chemical activation of the reaction within the Orp1·Ybp1·Yap1  
217 ternary complex. They also confirm that Orp1<sub>oxSS</sub> is not competent for Yap1 oxidation.

218

#### 219 **Orp1<sub>oxSS</sub> formation is impeded in the ternary complex**

220 We next explored whether Ybp1 could also favor reaction 2 by inhibiting reaction 3. We first  
221 evaluated the effect of Ybp1 and Yap1 on Orp1 peroxidase activity, as measured by the rate of  
222 NADPH consumption under multiple turnovers, *i.e.* in the presence of Trx and Trx reductase. In the  
223 presence of Yap1<sup>SSSSS</sup>—used here and below to prevent reaction 2— or Ybp1, Orp1 activity was  
224 not altered. In contrast, in their presence in equimolar amounts, Orp1 peroxidase activity was  
225 strongly inhibited up to 88% and 97%, at 1 and 20  $\mu\text{M}$  of added proteins, respectively  
226 (**Supplementary Fig. 10a**). This effect was not due to inhibition of the Trx system since Yap1 and  
227 Ybp1 did not alter Trx-dependent recycling of a peroxiredoxin and methionine sulfoxide reductase  
228 (**Supplementary Fig 10b**).

229 Inhibition of the Orp1 peroxidatic cycle might occur at reaction 1 or 3 (**Fig. 1**). We thus  
230 evaluated the impact of Ybp1·Yap1<sup>SSSSS</sup> on reaction 1, using Orp1<sup>CSA</sup>, which accumulates as a  
231 sulfenamide at acidic pH upon conversion of the Orp1—SOH form (see above), and in the absence  
232 of Trx. Reaction products of Orp1 with excess  $\text{H}_2\text{O}_2$  were analyzed as in **Fig. 4**. The major

233 chromatographic peaks observed were those of Yap1<sup>SSSSSS</sup> (peak 9), the Orp1<sup>CSA</sup> sulfenylamide (peak  
234 3) and reduced (peak 4) forms (**Fig. 5a**). Evolution of peak 3 area yielded first-order rate constants  
235 of  $29 \pm 7 \text{ s}^{-1}$  in the absence of Yap1<sup>SSSSSS</sup> and Ybp1, and of  $32 \pm 7$  and  $24 \pm 7 \text{ s}^{-1}$  in their presence at a  
236 1:1 ratio, and at 15 and 75  $\mu\text{M}$  (**Fig. 5b**), respectively, which indicated that Orp1—SOH formation is  
237 not altered within the ternary complex (see also **Supplementary Fig 7d**).

238 We similarly measured the effects of the Ybp1·Yap1 complex on reaction 3, using Wt Orp1,  
239 and setting its catalytic cycle to single turnover to accumulate Orp1<sub>oxSS</sub>, i.e. in the absence of the Trx  
240 system. Chromatographic analysis of the reaction mixtures identified Yap1<sup>SSSSSS</sup> (peak 9), Orp1<sub>oxSS</sub>  
241 (peak 7), reduced Orp1 (peak 8), and a small amount of a species with a mass that could not be  
242 determined (peak 10) (**Fig. 5c, Supplementary Table 2**). With Orp1 alone, evolution of peak 7 gave  
243 an observed rate constant of  $32 \pm 6 \text{ s}^{-1}$ , close to the value measured for Orp1—SOH formation,  
244 which indicates that reaction 1 is limiting Orp1<sub>oxSS</sub> formation under these conditions (**Fig. 5d**). In  
245 contrast, in the presence of a 1:1 ratio of Yap1<sup>SSSSSS</sup> and Ybp1, evolution of peak 7 area now yielded a  
246 value of  $10 \pm 2 \text{ s}^{-1}$  at 15  $\mu\text{M}$ , and of  $6 \pm 2 \text{ s}^{-1}$  at 75  $\mu\text{M}$  of added proteins, indicating that in the ternary  
247 complex the rate-limiting step for Orp1<sub>oxSS</sub> formation switches from reaction 1 to reaction 3 (**Fig.**  
248 **5d**). To further evaluate this effect, we determined reaction 3 intrinsic rate constant ( $k_{SS}$ ) by  
249 monitoring the change of enzyme Trp fluorescence associated with C36-C82 disulfide formation,  
250 under single turnover conditions. We increased the concentration of H<sub>2</sub>O<sub>2</sub> up to levels ensuring that  
251 reaction 1 is not rate-limiting relative to reaction 3 (**Supplementary Fig. 7**). The deduced value of  
252  $k_{SS}$  for Orp1 alone,  $507 \pm 4 \text{ s}^{-1}$ , was 80-fold higher than the  $6 \text{ s}^{-1}$  measured in the presence of Ybp1  
253 and Yap1<sup>SSSSSS</sup>. Accordingly, reaction 3 rate constant ( $k_{SS}$ ) is > 1000-fold higher than the observed  
254 value of reaction 2 in the absence of Ybp1 ( $0.3 \text{ s}^{-1}$ ) (**Fig. 4**). In the presence of Ybp1 alone, the value  
255 of  $k_{SS}$  decreased to  $22 \text{ s}^{-1}$ , indicating a large contribution of Ybp1 to the slowdown of reaction 3  
256 within the ternary complex (**Supplementary Fig. 7d**).

257 We therefore enquired whether eliminating the Orp1 resolving Cys residue would make Ybp1  
258 dispensable for Yap1 oxidation. In cells that express Orp<sup>C82S</sup>, oxidation of Yap1 by H<sub>2</sub>O<sub>2</sub> is not

259 altered<sup>9</sup>. In  $\Delta ybp1$  cells, Orp1<sup>C82S</sup> did not allow Yap1 oxidation, even when overexpressed, although  
260 in the latter condition a very faint band corresponding to the Orp1-Yap1 disulfide-linked complex  
261 could now be seen (**Fig. 6a,b, Supplementary Fig. 11**). *In vitro* also, Orp1<sup>C82S</sup> did not allow Yap1  
262 oxidation in the absence of Ybp1, but instead totally shifted Yap1 into several high molecular weight  
263 (HMW) species (**Fig. 6c**). These species were formed by the independent attachment of more than  
264 three Orp1<sup>C82S</sup> molecules to Yap1, as deduced from the presence of a unique such attachment when  
265 Orp1<sup>CSA</sup> was reacted with Yap1<sup>SSSCSS</sup> (**Supplementary Fig. 6a**). These Orp1-Yap1 HMW species  
266 decreased upon reintroducing Ybp1 (**Fig. 6c**), with the very few remaining caused by excess free  
267 Orp1 that had reacted with Yap1 in an Ybp1-independent manner, as shown by the titration of  
268 Ybp1·Yap1 by Orp1 (**Supplementary Fig. 6b**).

269 We lastly enquired whether glutathione (GSH) at physiological concentrations would compete  
270 with Yap1 for condensation with Orp1—SOH. In the absence of Ybp1, GSH totally prevented the  
271 reaction between Orp1<sup>CSA</sup> and Yap1<sup>SSSCSS</sup>, and in its presence, it prevented this reaction only when  
272 Orp1<sup>CSA</sup>, but not Wt Orp1 was used (**Supplementary Fig. 12**). This effect of GSH most probably  
273 occurs on the free unassembled fraction of Yap1. Hence, a competition between GSH and Yap1 for  
274 condensation with Orp1—SOH C598 is prevented by ternary complex formation, which explains  
275 why GSH does not interfere with Yap1 activation *in vivo*, and why the Orp1<sup>C82S</sup> mutant has a Wt  
276 phenotype with regards to Yap1 activation<sup>9</sup>.

277 Hence, within the ternary complex, reaction of the Orp1—SOH with one (C598) out of the five  
278 others Yap1 Cys residues is specified, which adds to the activation of reaction 2 and to the  
279 impediment of reaction 3 within this complex, and the accessibility of GSH is prevented. In  
280 conclusion, the different levels at which Ybp1-dependent ternary complex formation improves H<sub>2</sub>O<sub>2</sub>  
281 signaling from Orp1 to Yap1 explain why Ybp1 cannot be made dispensable under any conditions.

282 **DISCUSSION**

283 Since the identification of Orp1 as the H<sub>2</sub>O<sub>2</sub> receptor in Yap1 activation<sup>9</sup>, other thiol-  
284 peroxidases-based redox relays have been recognized<sup>16,17,24-26</sup>. Due to the high reactivity of its  
285 peroxidatic Cys residue, Orp1 confers to the Yap1 redox relay H<sub>2</sub>O<sub>2</sub> specificity and sensitivity, i.e. the  
286 ability to detect very low doses of peroxide and to remain unreactive to other oxidants<sup>2</sup>. How then is  
287 achieved the functional coupling between Orp1 and Yap1 and what are the mechanisms ensuring  
288 absolute precision in the transfer of H<sub>2</sub>O<sub>2</sub> oxidizing equivalents? Formation of a sulfenic acid at the  
289 Orp1 catalytic Cys residue upon its reaction with H<sub>2</sub>O<sub>2</sub> initiates the first step of the redox relay, i.e.  
290 formation of the Orp1—S—S—Yap1 species. Based on estimates of the cellular concentrations of  
291 Orp1 of ~ 0.5 μM, and of Yap1 below 0.1 μM<sup>27,28</sup>, and on the bimolecular rate constant measured  
292 here (7.4 10<sup>3</sup> M<sup>-1</sup>.s<sup>-1</sup>), formation of the Orp1—S—S—Yap1 complex would require > 10 min, which  
293 is not consistent with Yap1 fast oxidation *in vivo*. Furthermore, Orp1 peroxidatic turnover involves  
294 formation of an intramolecular disulfide between the same Cys-sulfenic acid and the Orp1 resolving  
295 Cys residue at a rate that should totally impede formation of the Orp1-Yap1 intermolecular  
296 disulfide. We show here, that Ybp1, a protein identified as a Yap1-binding partner with no  
297 recognizable functional domains, and then as required for its oxidative activation<sup>13</sup>, enables the  
298 redox coupling between Orp1 and Yap1 at several levels. By recruiting these two proteins within a  
299 ternary complex, Ybp1 raises their local concentration, thereby increasing the rate constant for their  
300 reaction from the predicted value in the 10<sup>-3</sup> s<sup>-1</sup> to the 1 s<sup>-1</sup>, even for the estimated low *in vivo*  
301 concentrations of the three partners (see details in **Supplementary Fig. 9**). *In vitro* reconstitution  
302 also shows that by enabling the recruitment of the peroxide-sensitive Orp1 factor, Ybp1 decreases  
303 by more than 50-fold the concentration of H<sub>2</sub>O<sub>2</sub> needed to oxidize Yap1 (100 μM vs. >5 mM,  
304 **Supplementary Fig. 5**).

305 The previously described Orp1-dependent proximity-based oxidation of the redox protein  
306 roGFP2 by H<sub>2</sub>O<sub>2</sub> was made possible by the fusion between these two proteins<sup>29</sup>. Ybp1 similarly  
307 enables Orp1-Yap1 redox coupling by ternary complex formation, but may also improve its

308 efficiency. Ternary complex formation might create a molecular environment that either stabilizes  
309 the C598 thiolate and/or provide the means of acid catalysis to activate the release of a water  
310 molecule, which is akin to an enzyme active site. In the absence of Ybp1, the Orp1-SOH could react  
311 with any of the Yap1 Cys residues (see **Fig. 6c**, and **Supplementary Fig. 6b**), which indicates that  
312 within the ternary complex both selection of C598 over the other five Yap1 Cys residues and its  
313 alignment with C36—SOH might also be favored. In addition, ternary complex formation appears  
314 also to prevent the attack of glutathione on the C36—SOH by impeding access of GSH to the sulfenic  
315 acid (**Supplementary Fig. 12**).

316         Lastly, our data indicate that the rate of attack of the resolving C82 thiolate on the Orp1—  
317 SOH that leads to Orp1<sub>oxSS</sub> formation is decreased by 20-fold within the Orp1·Ybp1 binary complex,  
318 and by > 80-fold within the Orp1·Ybp1·Yap1 complex, which are values in the range of those of  
319 reaction 2 (6 vs 5 s<sup>-1</sup>). Therefore, within the ternary complex, the reaction between the Orp1—SOH  
320 and Yap1 C598 is activated, and the competing reaction between the Orp1-SOH and Orp1 C82  
321 inhibited, which also favors the former. The failure of the Orp1 C82S mutant to activate Yap1 in the  
322 absence of Ybp1 underscores the fact that the inhibition of Orp1 intramolecular disulfide formation  
323 is just one of the means by which Ybp1-dependent ternary complex formation enables the redox  
324 relay. The Orp1 domain that comprises the C82 residue is highly flexible<sup>30</sup>, which might allow it to  
325 be constrained within the ternary complex into a conformation preventing its reaction with the C36-  
326 SOH. Structural reorganization of Orp1 upon its interaction with Ybp1·Yap1 and Ybp1 is consistent  
327 with the significant entropic change associated with complex formation, as measured by ITC  
328 (**Supplementary Table 1**), and with the inability of Orp1<sub>oxSS</sub> to interact with Ybp1·Yap1 and Ybp1.  
329 Since Orp1 C82 residue is dispensable for Yap1 activation and since Orp1 peroxidase activity does  
330 not contribute to cellular H<sub>2</sub>O<sub>2</sub> tolerance<sup>9</sup>, why has evolution maintained the C82 residue? Based on  
331 the *in vivo* protein concentration estimates and on the dissociation constants for Orp1·Ybp1·Yap1  
332 complex formation, a large fraction of Orp1 exists as a free protein. Therefore, free Orp1 fast  
333 disulfide formation might shield the reactivity of the Orp1—SOH, a hypothesis that is corroborated

334 by the *in vivo* identification of disulfide crosslinks between Orp1<sup>C82S</sup> and several proteins<sup>31</sup>, while  
335 ensuring the availability of reduced Orp1 for Yap1 activation upon recycling of Orp1<sub>oxSS</sub> by Trx.

336 In summary we propose that Ybp1 acts as signaling scaffold, akin to the signaling scaffolds of  
337 GTPases, protein kinases and phosphatases<sup>32</sup>, since it binds to two signaling proteins and helps  
338 guiding the flow of information between them. By shielding a protein Cys—SOH, and selectively  
339 activating its condensation into a disulfide with one over other thiolates, Ybp1 scaffolding function  
340 also incorporates the function of a “sulfenic acid chaperone”, principles that may apply to other  
341 sulfenic acid-dependent signaling pathways.

342

### 343 **ACKNOWLEDGMENTS**

344 We gratefully acknowledge G. Branlant for his essential input to initiate the project. We also thank A.  
345 Gruez for assistance in protein stability optimization, S. Boschi-Muller and F. Talfournier for fruitful  
346 discussions and help with quench flow experiments, J.M. Alberto for support with use of NGERE  
347 U954-INSERM chromatographic facility, J. Charbonnel and G. Palais for excellent technical support.  
348 Microcalorimetry and mass spectrometry were performed respectively at SCBIM (Fédération de  
349 Recherche 3209 BMCT) and SCMS platforms of Université de Lorraine. This work was supported by  
350 grants from Ligue contre le Cancer to SRC, and from ANR ERRed, InCA PLBIO INCA\_5869 to MBT.  
351 A.B. was supported by a PhD fellowship from the French research minister.

352

### 353 **AUTHOR CONTRIBUTIONS**

354 A.B., B.D.A., M.B.T. and S.R.C. designed the experiments and analyzed the data. A.B., A.K. and B.D.A.  
355 produced and purified the proteins, A.B. and B.D.A. performed *in vitro* reconstitution assays, B.D.A.  
356 on gels kinetics and A.B. all other kinetics, H.M. and A.B. chromatography and mass spectrometry  
357 analyses, A.B. and A.K. protein interaction experiments. G.B., B.D.A. and A.D.M. carried out *in vivo*  
358 experiments. S.R.C, B.D.A. and M.B.T wrote the manuscript.

359



360 **REFERENCES**

- 361 1. D'Autréaux, B. & Toledano, M. B. ROS as signalling molecules: mechanisms that generate  
362 specificity in ROS homeostasis. *Nat. Rev. Mol. Cell Biol.* **8**, 813–824 (2007).
- 363 2. Toledano, M. B., Delaunay, A., Monceau, L. & Tacnet, F. Microbial H<sub>2</sub>O<sub>2</sub> sensors as archetypical  
364 redox signaling modules. *Trends Biochem. Sci.* **29**, 351–357 (2004).
- 365 3. Kuge, S. Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J.* **16**,  
366 1710–1720 (1997).
- 367 4. Delaunay, A., Isnard, A.-D. & Toledano, M. B. H<sub>2</sub>O<sub>2</sub> sensing through oxidation of the Yap1  
368 transcription factor. *EMBO J.* **19**, 5157–5166 (2000).
- 369 5. Wood, M. J., Andrade, E. C. & Storz, G. The redox domain of the Yap1p transcription factor  
370 contains two disulfide bonds. *Biochemistry* **42**, 11982–11991 (2003).
- 371 6. Wood, M. J., Storz, G. & Tjandra, N. Structural basis for redox regulation of Yap1 transcription  
372 factor localization. *Nature* **430**, 917–921 (2004).
- 373 7. Kuge, S., Toda, T., Iizuka, N. & Nomoto, A. Crm1 (Xpo1) dependent nuclear export of the budding  
374 yeast transcription factor yAP-1 is sensitive to oxidative stress. *Genes Cells* **3**, 521–532 (1998).
- 375 8. Yan, C., Lee, L. H. & Davis, L. I. Crm1p mediates regulated nuclear export of a yeast AP-1-like  
376 transcription factor. *EMBO J.* **17**, 7416–7429 (1998).
- 377 9. Delaunay, A., Pflieger, D., Barrault, M. B., Vinh, J. & Toledano, M. B. A thiol peroxidase is an H<sub>2</sub>O<sub>2</sub>  
378 receptor and redox-transducer in gene activation. *Cell* **111**, 471–481 (2002).
- 379 10. Ma, L.-H., Takanishi, C. L. & Wood, M. J. Molecular mechanism of oxidative stress perception by  
380 the Orp1 protein. *J. Biol. Chem.* **282**, 31429–31436 (2007).
- 381 11. Paulsen, C. E. & Carroll, K. S. Chemical dissection of an essential redox switch in Yeast. *Chem.*  
382 *Biol.* **16**, 217–225 (2009).
- 383 12. Jencks, W. P. *Catalysis in Chemistry and Enzymology*, Ch 1 (Courier Corporation, 1987).

- 384 13. Veal, E. A., Ross, S. J., Malakasi, P., Peacock, E. & Morgan, B. A. Ybp1 is required for the hydrogen  
385 peroxide-induced oxidation of the Yap1 transcription factor. *J Biol. Chem.* **278**, 30896–904  
386 (2003).
- 387 14. Gulshan, K., Lee, S. S. & Moye-Rowley, W. S. Differential oxidant tolerance determined by the key  
388 transcription factor Yap1 is controlled by levels of the Yap1-binding protein, Ybp1. *J. Biol. Chem.*  
389 **286**, 34071–34081 (2011).
- 390 15. Patterson, M. J. *et al.* Ybp1 and Gpx3 signaling in *Candida albicans* govern hydrogen peroxide-  
391 induced oxidation of the Cap1 transcription factor and macrophage escape. *Antioxid. Redox*  
392 *Signal.* **19**, 2244–2260 (2013).
- 393 16. Fomenko, D. E. *et al.* Thiol peroxidases mediate specific genome-wide regulation of gene  
394 expression in response to hydrogen peroxide. *Proc. Natl. Acad. Sci.* **108**, 2729–2734 (2011).
- 395 17. Sobotta, M. C. *et al.* Peroxiredoxin-2 and STAT3 form a redox relay for H<sub>2</sub>O<sub>2</sub> signaling. *Nat. Chem.*  
396 *Biol.* **11**, 64–70 (2015).
- 397 18. Gupta, V. & Carroll, K. S. Sulfenic acid chemistry, detection and cellular lifetime. *Biochim.*  
398 *Biophys. Acta BBA - Gen. Subj.* **1840**, 847–875 (2014).
- 399 19. Devarie-Baez, N. O., Silva Lopez, E. I. & Furdui, C. M. Biological chemistry and functionality of  
400 protein sulfenic acids and related thiol modifications. *Free Radic. Res.* **50**, 172–194 (2016).
- 401 20. Tachibana, T. *et al.* A major peroxiredoxin-induced activation of Yap1 transcription factor is  
402 mediated by reduction-sensitive disulfide bonds and reveals a low level of transcriptional  
403 activation. *J. Biol. Chem.* **284**, 4464–4472 (2008).
- 404 21. Haynes, A. C., Qian, J., Reisz, J. A., Furdui, C. M. & Lowther, W. T. Molecular basis for the  
405 resistance of human mitochondrial 2-Cys peroxiredoxin 3 to hyperoxidation. *J. Biol. Chem.* **288**,  
406 29714–29723 (2013).
- 407 22. Salmeen, A. *et al.* Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-  
408 amide intermediate. *Nature* **423**, 769–773 (2003).

- 409 23. Lim, J. C., You, Z., Kim, G. & Levine, R. L. Methionine sulfoxide reductase A is a stereospecific  
410 methionine oxidase. *Proc. Natl. Acad. Sci.* **108**, 10472–10477 (2011).
- 411 24. Jarvis, R. M., Hughes, S. M. & Ledgerwood, E. C. Peroxiredoxin 1 functions as a signal peroxidase  
412 to receive, transduce, and transmit peroxide signals in mammalian cells. *Free Radic. Biol. Med.*  
413 **53**, 1522–1530 (2012).
- 414 25. Calvo, I. A. *et al.* Dissection of a redox relay: H<sub>2</sub>O<sub>2</sub>-dependent activation of the transcription  
415 factor Pap1 through the peroxidatic Tpx1-thioredoxin cycle. *Cell Rep.* **5**, 1413–1424 (2013).
- 416 26. Winterbourn, C. C. & Hampton, M. B. Redox biology: Signaling via a peroxiredoxin sensor. *Nat.*  
417 *Chem. Biol.* **11**, 5–6 (2015).
- 418 27. Ghaemmaghami, S. *et al.* Global analysis of protein expression in yeast. *Nature* **425**, 737–741  
419 (2003).
- 420 28. Webster, M. T., McCaffery, J. M. & Cohen-Fix, O. Vesicle trafficking maintains nuclear shape in  
421 *Saccharomyces cerevisiae* during membrane proliferation. *J. Cell Biol.* **191**, 1079–1088 (2010).
- 422 29. Gutscher, M. *et al.* Proximity-based protein thiol oxidation by H<sub>2</sub>O<sub>2</sub>-scavenging peroxidases. *J.*  
423 *Biol. Chem.* **284**, 31532–31540 (2009).
- 424 30. Zhang, W.-J. *et al.* Crystal structure of glutathione-dependent phospholipid peroxidase Hyr1  
425 from the yeast *Saccharomyces cerevisiae*. *Proteins Struct. Funct. Bioinforma.* **73**, 1058–1062  
426 (2008).
- 427 31. Lee, P. Y. *et al.* Interactome analysis of yeast glutathione peroxidase 3. *J. Microbiol. Biotechnol.*  
428 **18**, 1364–1367 (2008).
- 429 32. Langeberg, L. K. & Scott, J. D. Signalling scaffolds and local organization of cellular behaviour.  
430 *Nat. Rev. Mol. Cell Biol.* **16**, 232–244 (2015).

431

432

433

434

## FIGURE LEGENDS

435

436 **Figure 1 | Schematics of the dual function of Orp1 as the H<sub>2</sub>O<sub>2</sub> receptor of the Yap1 regulator**  
437 **and as a peroxidase** (adapted from ref. 1). The Orp1-Yap1 redox relay is depicted at the right:  
438 reaction of Orp1 catalytic Cys (C36) with H<sub>2</sub>O<sub>2</sub> leads to reduction of the latter into H<sub>2</sub>O and to  
439 formation of the C36-sulfenic acid (C36-SOH) (reaction 1), which condenses with Yap1 C598 to  
440 produce the Orp1—S—S—Yap1 intermediate (reaction 2). The latter is transposed into a Yap1  
441 intramolecular disulfide by attack of Yap1 C303. Yap1 full activation requires further oxidation  
442 reactions. Once formed, the C36-SOH condenses with Orp1 C82 into an intramolecular disulfide  
443 (Orp1<sub>oxSS</sub>) (reaction 3), which is then reduced by thioredoxin (Trx), completing the cycle. Reaction 2  
444 and 3 are in competition.

445

446 **Figure 2 | Ybp1 brings together Yap1 and Orp1 into a ternary complex. (a)** Yap1 (30 μM, solid  
447 line), Ybp1 (30 μM, dashed line), or both at a 1:1 (dotted line) or 2:1 (dashed-dotted line)  
448 stoichiometry were resolved on a Superdex 200 gel filtration column equilibrated in 20 mM  
449 Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl buffer pH 7. **(b)** Fluorescence anisotropy titration of AF-Ybp1 by Yap1  
450 (square), AF-Yap1 by Ybp1 (circle) or AF-Ybp1 by Yap1<sup>SSSSS</sup> (triangle). The fraction of bound-  
451 labeled protein is plotted against unlabeled protein concentration and fitted to a single-site binding  
452 equation (solid, dashed and dotted lines, respectively). The TCEP reducing agent had no effect on  
453 the dissociation constant. **(c)** Isothermal titration calorimetry of Yap1<sup>SSSSS</sup> (50 μM, left), or Ybp1 (20  
454 μM, center) or both at 1:1 stoichiometric amounts (50:50 μM, right) by Orp1. In the latter  
455 experiment, 85 % of Ybp1 and Yap1<sup>SSSSS</sup> are bound together. Thermodynamic parameters  
456 describing these interactions are in **Supplementary Table 1**. Data are representative of n=2  
457 independent experiments using different proteins samples.

458

459 **Figure 3 | Ybp1 is required for Orp1—S—S—Yap1 intermediate formation. (a)** Extracts from  
460 Wt or  $\Delta ybp1$  cells carrying Myc-Yap1 or Myc-Yap1<sup>C303A</sup> and exposed or not to H<sub>2</sub>O<sub>2</sub> (400 μM) for 15

461 min, as indicated, were resolved by non-reducing SDS-PAGE followed by anti-Myc immunoblotting.  
462 Yap1-Orp1 mixed disulfide (Orp1—S—S—Yap1), reduced (Yap1<sub>red</sub>) and intramolecular disulfide  
463 (Yap1<sub>ox</sub>) Yap1 species are indicated by arrows. **(b)** *In vitro* reconstitution of the Orp1/Yap1 redox  
464 relay. Coomassie-stained non-reducing SDS-PAGE of reaction mixtures containing Orp1 (10 μM),  
465 Yap1 (4 μM), Ybp1 (4 μM), Trx (4 μM), Trx reductase (0.5 μM) and NADPH (0.4 mM) incubated for  
466 the indicated time with H<sub>2</sub>O<sub>2</sub> (60 μM). **(c)** As in (b) with reaction mixtures containing Orp1 (10 μM),  
467 Yap1 (2 μM), Trx (10 μM), Trx reductase (0.5 μM) and NADPH (1 mM) in the absence or presence of  
468 Ybp1 at the indicated amount relative to Yap1, incubated for the indicated time with H<sub>2</sub>O<sub>2</sub> (2 mM).  
469 **(d)** As in (b) with reactions mixture containing Orp1 (10 μM) and Yap1<sup>C303A</sup> (2 μM), in the absence  
470 or presence of Ybp1 (2 μM) incubated for the indicated time with H<sub>2</sub>O<sub>2</sub> (60 μM). Orp1 migrate  
471 outside gels in (b,c,d). MW, molecular weight (kDa). Data representative of n=2 or n=3 independent  
472 experiments. Complete gels are shown in **Supplementary Fig. 4**.

473  
474 **Figure 4 | Ybp1 activates Orp1—S—S—Yap1 formation.** **(a,c)** Orp1<sup>CSA</sup> (a) or Orp1 (c) (50 μM),  
475 incubated with Ybp1 (10 μM) and Yap1<sup>SSS CSS</sup> (10 μM), were reacted with H<sub>2</sub>O<sub>2</sub> (100 μM). **(e)** Orp1<sub>oxSS</sub>  
476 (50 μM) incubated with Ybp1 (10 μM) was reacted with Yap1<sup>SSS CSS</sup> (10 μM). Products were analyzed  
477 by reverse-phase chromatography after acidic quenching. Masses of products eluted are in  
478 **Supplementary Table 2**. Ybp1 was eluted at a higher acetonitrile ratio and does not appear on the  
479 chromatograms. **(b,d,f)** Evolution of the area of peak 2 (in a) or peak 6 (in c,e) corresponding to  
480 Orp1—S—S—Yap1 (squares) analyzed by a first-order mono-exponential kinetic model (solid line);  
481 data for Orp1<sup>CSA</sup> in the absence of Ypb1 (b, circles). **(d, inset)** Dependence of the rate constant of  
482 Orp1—S—S—Yap1 formation on Orp1 concentration analyzed as in (c). Data are representative of  
483 n=2 or n=3 independent experiments performed with different proteins samples.

484  
485 **Figure 5 | The Ybp1·Yap1 complex inhibits Orp1 intramolecular disulfide formation.** **(a,c)**  
486 Orp1<sup>CSA</sup> (a) or Orp1 (c) (15 μM), incubated with Yap1<sup>SSS SSS</sup> (15 μM) and Ybp1 (15 μM) were reacted

487 with H<sub>2</sub>O<sub>2</sub> (100 μM) and products were analyzed as in Fig. 4. Masses of the products eluted in each  
488 peak are collected in **Supplementary Table 2**. **(b,d)** Evolution of the area of peak 3 (Orp1  
489 sulfenylamide) (in b) or peak 7 (Orp1<sub>oxSS</sub>) (in d) (circles) analyzed by a first-order mono-  
490 exponential kinetic model (dashed line); data obtained in the absence (squares, solid line) and  
491 presence of Yap1<sup>SSSSSS</sup> (75 μM) and Ybp1 (75 μM) (triangles, dotted line). In the latter condition,  
492 90% of Orp1 is associated with the ternary complex. The experiment performed in the absence and  
493 presence of Yap1<sup>SSSSSS</sup> and Ybp1 gave similar chromatographic profiles as in a and c, except for the  
494 lack of peak 9 (Yap1<sup>SSSSSS</sup>) when using Orp1 alone. Data are representative of n=2 or n=3  
495 independent experiments performed with different protein samples.

496

497 **Figure 6 | Lack of Orp1 resolving C82 does not make Ybp1 dispensable for Yap1 oxidation.**

498 **(a,b)** Lysates of  $\Delta yap1$  or  $\Delta yap1\Delta ybp1$  or  $\Delta yap1\Delta ybp1\Delta orp1$  cells, as indicated, expressing Myc-Yap1  
499 (a,b) and overexpressing Orp1<sup>C82S</sup> (b), and treated with H<sub>2</sub>O<sub>2</sub> (400 μM) during the indicated time,  
500 were resolved by non-reducing SDS-PAGE followed by western-blot with an anti-Myc antibody.  
501 Yap1 redox conformers are indicated by arrows. **(c)** Coomassie-stained non-reducing SDS-PAGE of  
502 reactions mixture containing Orp1<sup>C82S</sup> (10 μM) and Yap1 (2 μM), and Ybp1 (2 μM), as indicated,  
503 incubated for the indicated time with H<sub>2</sub>O<sub>2</sub> (100 μM). HMW, high molecular weight complexes. MW,  
504 molecular weight (kDa). Data are representative of n=2 or n=3 independent experiments. Complete  
505 gels are shown in **Supplementary Fig. 11**.

506 **ONLINE METHODS**

507 **Chemicals**

508 All chemicals were reagent grade and were used without additional purification. KCl,  
509 Ethylene diamine tetra acetic acid (EDTA), acetonitrile and Benzonase nuclease were purchased  
510 from Merck. Glucose and lactose were from Fisher Chemical. NaCl, sodium phosphate dibasic  
511 ( $\text{Na}_2\text{HPO}_4$ ) were obtained from Carlo Erba Reagents. Hepes free acid was from MP Biomedicals. Tris,  
512 ammonium sulfate, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), chloramphenicol, ampicillin and  
513 kanamycin were from Euromedex. Trifluoroacetic acid (TFA) and  $\text{H}_2\text{O}_2$  were obtained from Acros  
514 Organics. Sodium dodecyl sulfate (SDS) was obtained from AppliChem GmbH. N-ethylmaleimide  
515 (NEM), iodoacetamide (IAM), tris(2-carboxyethyl)phosphine (TCEP), mes, imidazole, betaine and  
516 phenylmethanesulfonylfluoride (PMSF), *E. coli* thioredoxin 2 (Trx) were from Sigma-Aldrich. *E. coli*  
517 thioredoxin reductase (Trx reductase) was from Sigma-Aldrich or prepared following experimental  
518 procedures described previously<sup>33</sup>. *E. coli* thioredoxin 1 (Trx1)<sup>34</sup>, Methionine sulfoxide reductase  
519 (MsrA)<sup>35</sup> and *S. cerevisiae* peroxiredoxin Tsa1<sup>36</sup> were prepared following experimental procedures  
520 described previously.

521

522 **Plasmids and Recombinant Protein Purification**

523 The plasmids pET28bHTYap1 and pET28bHTYbp1 encoding the N-terminal His tag fusion of  
524 *S. cerevisiae* Yap1 (670 residues) and Ybp1 (674 residues), referred to as Yap1 and Ybp1  
525 respectively, were obtained by cloning the Yap1 and Ybp1 ORFs amplified from *S. cerevisiae*  
526 genomic DNA by PCR into the pET28b and pET22b vectors, using the NdeI and SacI or NotI and SacI  
527 restriction sites, respectively. The plasmid pET22bOrp1 encoding *S. cerevisiae* Orp1 (163 residues)  
528 was obtained similarly by cloning the Orp1 ORF into the pET22b vector, using the NdeI and SacI  
529 sites. All mutant of Yap1 and Orp1 were generated by standard PCR site-directed mutagenesis and  
530 sequenced to confirm that no mutations had been introduced in the amplification reactions.

531 Wt and mutant Yap1 and Ybp1 were overexpressed in *E. coli* Rosetta 2(DE3) strain  
532 (Novagen) transformed by the pET28bHTYap1 and pET28bHTYbp1, respectively. For Yap1  
533 production, cells were grown for 5 h at 37° C in Luria-Bertani (LB) medium supplemented by and  
534 0.5% glucose, followed by addition of 1:1 volume of cold LB medium containing 0.6% lactose, 20  
535 mM Hepes pH 7, 1 mM IPTG, kanamycin (50 mg.l<sup>-1</sup>) and chloramphenicol (50 mg.l<sup>-1</sup>) and further  
536 incubation for 18 h at 20° C. For Yap1 purification, cells pellets were suspended in 20 mM Na<sub>2</sub>HPO<sub>4</sub>  
537 pH8 buffer, 400 mM NaCl (buffer A), supplemented by 1 mM PMSF, Roche protease EDTA free  
538 inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and 5 U.ml<sup>-1</sup> Benzonase nuclease and  
539 lysed by sonication. Yap1 contained in the soluble fraction was purified on Ni<sup>2+</sup>-sepharose column  
540 (GE Healthcare) equilibrated with buffer A with 60 mM imidazole, connected to a fast protein liquid  
541 chromatography (FPLC) system (ÄKTA Avant, GE Healthcare), and eluted with a linear 0.06 to 0.5 M  
542 imidazole gradient. Fractions containing Yap1 were dialyzed against 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM NaCl,  
543 pH 8 containing 20 mM DTT, and further purified on a anion exchange Q-sepharose column (GE  
544 Healthcare) equilibrated in the same buffer, using a linear gradient of 0.1 to 1 M NaCl.

545 For Ybp1 production, cells were grown in LB medium containing kanamycin (50 mg.l<sup>-1</sup>),  
546 chloramphenicol (50 mg.l<sup>-1</sup>) for 3 h at 37°C, followed by addition of 0.5 M NaCl and 2 mM betaine  
547 and incubation for 1h at 47°C (adapted from **ref. 37**<sup>37</sup>). Cells were then cooled to 20°C, and Ybp1  
548 expression was induced by addition of 1 mM IPTG and incubation for 18h at 20°C. The same  
549 procedure as for Yap1 was used for Ybp1 purification on Ni<sup>2+</sup>-sepharose column, except that buffer  
550 A was replaced by 20 mM Hepes, 400 mM NaCl, pH 8.5 buffer. Fractions containing Ybp1 were  
551 directly concentrated using an Amicon Ultra 30kDa concentrator before further purification by gel  
552 filtration on a Superdex 200 column (GE Healthcare) equilibrated in the same buffer.

553 Wt and mutants Orp1 were overexpressed in *E. coli* C41(DE3) strain transformed by the  
554 pET22bOrp1 plasmid by overnight culture at 37°C in the autoinducible media ZYM-5052  
555 supplemented with ampicillin (200 mg.l<sup>-1</sup>)<sup>38</sup>. For Orp1 purification, cells pellets were suspended in  
556 50 mM Tris, 2 mM EDTA buffer pH 8 supplemented by 1 mM PMSF, 5 U/mL Benzonase Nuclease, 20



557 mM DTT and lysed by sonication. Orp1 contained in the soluble fraction was precipitated by  
558 ammonium sulfate at 65% saturation, followed by gel filtration on an Ultrogel AcA54 column  
559 (Biosepra, Pall Corporation). Fractions containing Orp1 were buffer exchanged by 20 mM Mes, 2  
560 mM EDTA, pH 6.1 and further purified on a cation exchange SP-Sepharose column (GE Healthcare)  
561 equilibrated in the same buffer and eluted using a 0 to 1 M KCl gradient.

562 The identity and purity of Yap1, Ybp1 and Orp1 proteins were confirmed by Coomassie  
563 stained sodium dodecyl sulfate-polacrylamide gel electrophoresis (SDS-PAGE) and by electrospray  
564 mass spectrometry analyses. Purified proteins were stored at -80°C. Protein molar concentrations  
565 were determined spectrophotometrically using molar absorption coefficient at 280 nm of 30 285 M-  
566 <sup>1</sup>.cm<sup>-1</sup>, 69 680 M<sup>-1</sup>.cm<sup>-1</sup> and 19 300 M<sup>-1</sup>.cm<sup>-1</sup> for Yap1, Ybp1 and Orp1 respectively. Immediately  
567 before use, proteins were reduced by 20 mM DTT for 30 min at 4°C, followed by desalting against 20  
568 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, pH7 (buffer B) degassed under nitrogen. Orp1<sub>oxSS</sub> was obtained by  
569 addition of 1.2 molar excess of H<sub>2</sub>O<sub>2</sub> in Orp1 solution.

570

### 571 ***S. cerevisiae* protein extracts and redox Western blots**

572 Myc-Yap1, a N-terminal Yap1 fusion with a Myc<sub>9</sub> epitope and its mutant alleles inserted into  
573 a pRS315 vector were previously described<sup>4</sup>. The *S. cerevisiae* strain YPH98<sup>39</sup> (MATa, ura3-52, lys2-  
574 801amber, and ade2-101ochre trp1-Δ1 leu2-Δ1) and isogenic derivatives were used in all  
575 experiments. The Δ*ybp1* strain was derived from the WT strains by replacing the entire ORF by a  
576 KAN selection cassette. Δ*ybp1* strain transformed with pRS315-Myc-Yap1<sup>C303A</sup> were grown at 30°C  
577 in YPD [1% yeast extracts, 2% bactopectone, and 2% glucose]. For overexpression of the Orp1<sup>C82S</sup>  
578 mutant, the Δ*ybp1* strain was transformed with pRS315-Myc-Yap1 and pRS426-Orp1<sup>C82S</sup> and the  
579 cells were grown in SD medium (0.67% Yeast Nitrogen Base without amino acid and 2% Glucose)  
580 with the required amino acids. For analysis of the *in vivo* Yap1 redox state, extracts were prepared  
581 by the TCA acid lysis method as described<sup>4</sup>. Precipitated proteins were solubilized in buffer  
582 containing 100 mM Tris-HCl pH 8, 1% SDS, 1 mM EDTA, and 50 mM NEM at 30°C for 1h (cysteine-

583 trapping method). Extracts were resolved by non-reducing 8% SDS-PAGE as indicated and analyzed  
584 by immunoblotting with the anti-Myc monoclonal antibody (9E10) used at 1/2000°.

585

### 586 **SDS-PAGE analyses**

587 Reaction mixtures containing Yap1, Orp1 and their respective mutants, Ybp1, Trx, Trx  
588 reductase, NADPH and H<sub>2</sub>O<sub>2</sub> were incubated in Hepes 50 mM, NaCl 50 mM, EDTA 1 mM, pH = 7.5 at  
589 room temperature and quenched with 100 mM IAM and 1% SDS at room temperature for 1 h.  
590 Proteins were loaded onto 10% non-reducing SDS-PAGE gel (30:0.4 acrylamide:bisacrylamide),  
591 followed by colloidal Coomassie staining (adapted from **ref. 40**<sup>40</sup>).

592

### 593 **Fluorescence anisotropy titrations**

594 The N-terminal amine of Ybp1 and Yap1 was derivatized in 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl,  
595 pH 8 by incubation with a tenfold molar excess of the fluorophore Alexa Fluor 488  
596 sulfodichlorophenol Ester (Molecular probes, Life technologies) for 30 min at room temperature in  
597 the dark. The labeled protein (AF-Yap1 or AF-Ybp1) was separated from excess dye by a Econo-Pac  
598 10DG Desalting column (Bio-Rad). The protein concentration and the degree of labeling (typically  
599 0.7-0.8 mol Alexa Fluor per mol of protein) were determined by UV-VIS spectroscopy.

600 Fluorescence intensity and anisotropy measurements were carried out on a  
601 spectrofluorimeter SAFAS Flx-Xenius equipped with dual monochromators. The dissociation  
602 constant of the Yap1-Ybp1 complex in buffer B at 25°C in the presence of 2 mM TCEP was deduced  
603 from anisotropy titrations of 100 nM of AF-Ybp1 of AF-Ybp1 with increasing concentrations of Yap1  
604 or Ybp1, respectively, recorded using 494 nm excitation and 523 nm emission wavelengths and 10  
605 nm slits. The instrument computed anisotropy data from samples illuminated with vertically  
606 polarized light  $A = (I_{VV} - G \cdot I_{VH}) / (I_{VV} + 2G \cdot I_{VH})$  where  $I_{VV}$  is the fluorescence intensity recorded with  
607 excitation and emission polarization in vertical position, and  $I_{VH}$  is the fluorescence intensity  
608 recorded with the emission polarization aligned in horizontal position. The G-factor of the

609 spectrofluorometer represents the ratio of the sensitivities of the detection system for vertically and  
610 horizontally polarized light and was calculated by the instrument for each individual sample.

611 Data obtained were expressed as the fraction of labeled protein in complex, taking into  
612 account the effect of complex formation on the fluorescence intensity according to equation (1),

613

$$614 \text{ Fraction of labeled protein in complex} = (A - Af) / ((Ab - A) \times (Ib/I_f) + (A - Af)) \quad (1)$$

615

616 where Af, If and Ab, Ib represent the anisotropy and fluorescence intensity of free and complexed  
617 labeled protein, respectively. Plots of the fraction of labeled protein in complex vs. unlabeled protein  
618 concentration were fit to a single-site binding model to deduce Yap1·Ybp1 dissociation constant.

619 Dissociation constants are reported as the mean value obtained from n=2 independent experiments  
620 performed on distinct protein productions  $\pm$  standard deviation.

621

### 622 **Isothermal Titration Calorimetry**

623 ITC titration were performed using an iTC200 system from Microcal (GE Healthcare) at 25°C  
624 in buffer B. Orp1 or Orp1<sub>0xSS</sub> (200 to 500  $\mu$ M) contained in the syringe was injected into the sample  
625 cell containing Yap1<sup>SSSSS</sup>, Ybp1 or an equimolar mix of both proteins (20 or 50  $\mu$ M, as indicated), in  
626 20 injections of 2  $\mu$ L separated by delay of 180 s. Cell stirring speed was 750 rpm. Reference  
627 injections of protein into buffer and buffer into protein were performed and subtracted from the  
628 integrated data prior to curve fitting. Data were fit to a single-site binding model using Origin 7.0  
629 software (Origin Labs) to determine the dissociation constant, enthalpy of binding ( $\Delta$ H), and  
630 stoichiometry. These parameters are reported as the mean value obtained from n=2 independent  
631 experiments performed on distinct protein productions  $\pm$  standard deviation.

632

### 633 **Stopped flow and quench flow rapid kinetics**

634 The reaction of Orp1 with H<sub>2</sub>O<sub>2</sub> in single turnover conditions was followed by the increase of  
635 Orp1 intrinsic fluorescence intensity when oxidized to the Orp1<sub>oxSS</sub> disulfide form. Emission  
636 fluorescence was recorded at 25°C in buffer B on an SX19MV-R stopped flow apparatus (Applied  
637 Photophysics) fitted for fluorescence measurements, with excitation wavelength set at 295 nm, and  
638 emitted light collected above 320 nm using a cutoff filter. One syringe contained Orp1 (2 μM, final  
639 concentration after mixing), the other syringe contained H<sub>2</sub>O<sub>2</sub>. Equal volumes of each syringe were  
640 rapidly mixed to start the reaction. An average of at least three runs was recorded for each  
641 concentration of H<sub>2</sub>O<sub>2</sub>. The data set obtained at variable H<sub>2</sub>O<sub>2</sub> concentrations was fitted globally to a  
642 two step process using Kintek Global Kinetic Explorer software to extract the rate constants  $k_{SOH}$  for  
643 Orp1—SOH formation and  $k_{SS}$  for Orp1<sub>oxSS</sub> formation.

644 To monitor the formation of the Orp1—S—S—Yap1 species, Yap1<sup>SSS CSS</sup>, Orp1 in the presence  
645 or absence of Ybp1 were rapidly mixed with 100 μM H<sub>2</sub>O<sub>2</sub> (final concentration after mixing) at room  
646 temperature in buffer B and incubated for defined reaction times (2.5 ms to 200 s) on a RQF3  
647 chemical quenched-flow apparatus (Kintek corp., Austin, Texas). The reaction was then quenched  
648 by addition of 1 volume of 1% TFA and reaction products were analyzed by reversed-phase liquid  
649 chromatography (UltiMate 3000 Standard, Dionex) on an Vydac C8 300A (C8) column, 4,6x250 mm,  
650 5 μm (Grace) equilibrated in 0.1% TFA. Proteins were eluted using a 20 min linear gradient of 35%  
651 to 41% acetonitrile at a flow rate of 2 ml.min<sup>-1</sup>. Each species was identified by mass spectrometry.  
652 The rate constant of Orp1—S—S—Yap1 formation was deduced from the evolution of amount of  
653 product proportional to the area of the chromatographic peak. The rate constant of Orp1—S—S—  
654 Yap1 formation by reaction of Orp1<sub>oxSS</sub> was measured similarly by rapidly mixing Orp1<sub>oxSS</sub> and with  
655 Yap1<sup>SSS CSS</sup> in the presence of Ybp1. Rate constants are reported as the mean value obtained from  
656 n=2 or n=3 independent experiments performed on distinct protein productions ± standard  
657 deviation.

658 The kinetics of formation of Orp1<sub>oxSS</sub> and Orp1—SOH were monitored by the same protocol  
659 using either Wt Orp1 or Orp1<sup>CSA</sup>, respectively, in the presence of equimolar mix of Yap1<sup>SSS SSS</sup> and  
660 Ybp1 at the indicated concentrations.

661

### 662 **Steady-state kinetics**

663 Orp1 peroxidase activity was measured by using the Trx/Trx reductase coupled (0.5 μM  
664 NTR, 250 μM NADPH, 50 μM Trx1), with 100 μM H<sub>2</sub>O<sub>2</sub> and was started by addition of 0.05 μM Orp1  
665 in buffer B at 25°C. Initial rate measurement were carried out on a UV mc2 spectrophotometer  
666 (Safas, Monaco) by following the decrease of absorbance at 340 nm due to the oxidation of NADPH.  
667 A blank measurement recorded in the absence of Orp1 was systematically deduced from the assay  
668 to account for non-specific oxidation of Trx or Trx reductase. Initial rate measurements were  
669 carried with Orp1 alone and in the presence of increasing concentrations of the proteins Yap1<sup>SSS CSS</sup>,  
670 Ybp1 or an equimolar mix of both as indicated.

671 The Tsa1 peroxidase and the MsrA methionine sulfoxide reductase activities were measured  
672 by the same coupled assay using 100 μM H<sub>2</sub>O<sub>2</sub> and 100 mM oxidized Met as substrate, respectively.  
673 Concentrations of the Trx recycling system were: 1 μM Trx reductase, 200 μM NADPH, 50 μM Trx1  
674 and 0.5 μM Tsa1, and 4.8 μM Trx reductase, 200 μM NADPH, 100 μM Trx1 and 1 μM MsrA.

675

### 676 **Mass spectrometry**

677 Electrospray ionization mass spectrometry measurements were performed on a MicrOTOF-  
678 Q instrument (Bruker Daltonics) as described previously<sup>41</sup>.

679

### 680 **Data availability**

681 The datasets generated and analysed during the current study are available from the  
682 corresponding authors on reasonable request.

683

684

- 685 33. Mulrooney, S. B. Application of a single-plasmid vector for mutagenesis and high-level  
686 expression of thioredoxin reductase and its use to examine flavin cofactor incorporation.  
687 *Protein Expr. Purif.* **9**, 372–378 (1997).
- 688 34. Mössner, E., Huber-Wunderlich, M. & Glockshuber, R. Characterization of *Escherichia coli*  
689 thioredoxin variants mimicking the active-sites of other thiol/disulfide oxidoreductases.  
690 *Protein Sci. Publ. Protein Soc.* **7**, 1233–1244 (1998).
- 691 35. Boschi-Muller, S. *et al.* A sulfenic acid enzyme intermediate is involved in the catalytic  
692 mechanism of peptide methionine sulfoxide reductase from *Escherichia coli*. *J. Biol. Chem.* **275**,  
693 35908–35913 (2000).
- 694 36. Roussel, X. *et al.* Evidence for the formation of a covalent thiosulfinate intermediate with  
695 peroxiredoxin in the catalytic mechanism of sulfiredoxin. *J. Biol. Chem.* **283**, 22371–22382  
696 (2008).
- 697 37. Oganessian, N., Ankoudinova, I., Kim, S.-H. & Kim, R. Effect of osmotic stress and heat shock in  
698 recombinant protein overexpression and crystallization. *Protein Expr. Purif.* **52**, 280–285  
699 (2007).
- 700 38. Studier, F. W. Protein production by auto-induction in high density shaking cultures. *Protein*  
701 *Expr Purif* **41**, 207–34 (2005).
- 702 39. Sikorski, R. S. & Hieter, P. A system of shuttle vectors and yeast host strains designed for  
703 efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27 (1989).
- 704 40. Neuhoff, V., Arold, N., Taube, D. & Ehrhardt, W. Improved staining of proteins in polyacrylamide  
705 gels including isoelectric focusing gels with clear background at nanogram sensitivity using  
706 Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**, 255–262 (1988).
- 707 41. Boukhenouna, S. *et al.* Evidence that glutathione and the glutathione system efficiently recycle  
708 1-Cys sulfiredoxin in vivo. *Antioxid. Redox Signal.* **22**, 731–743 (2014).

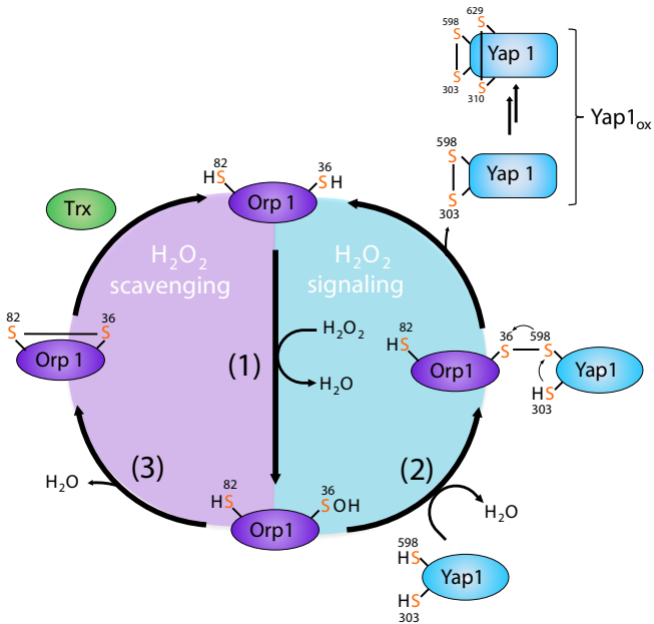
709

710

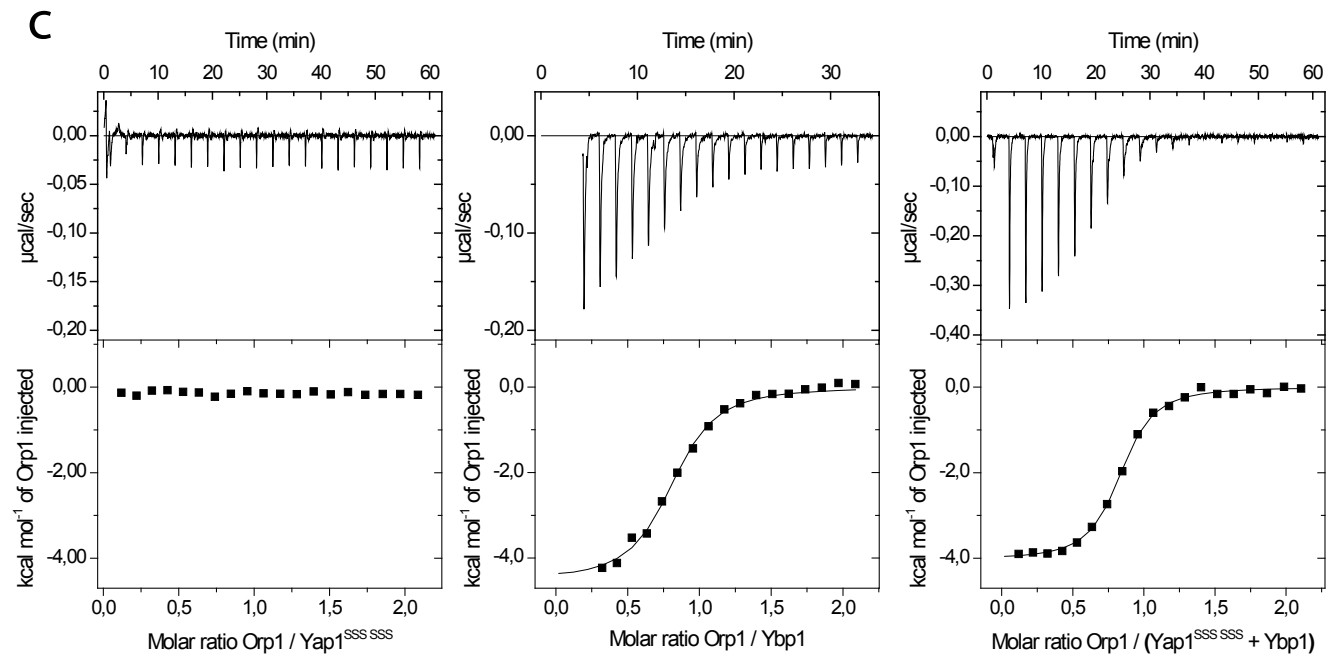
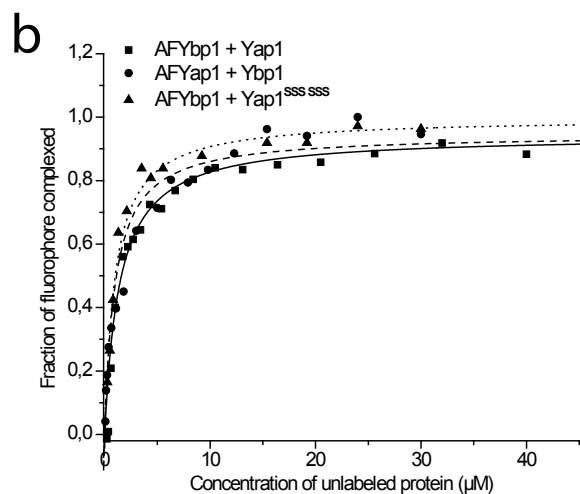
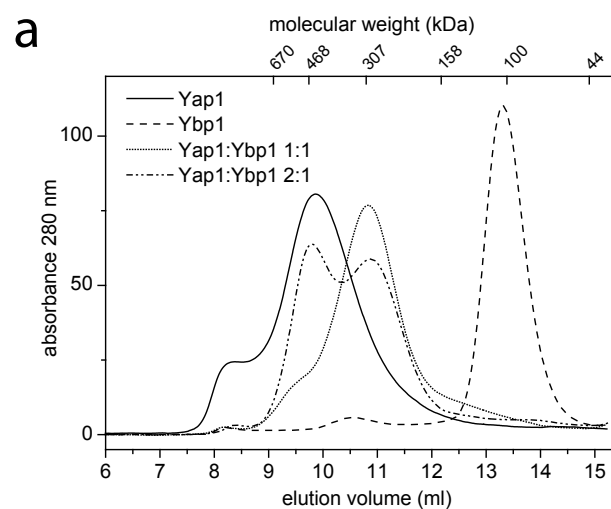
711

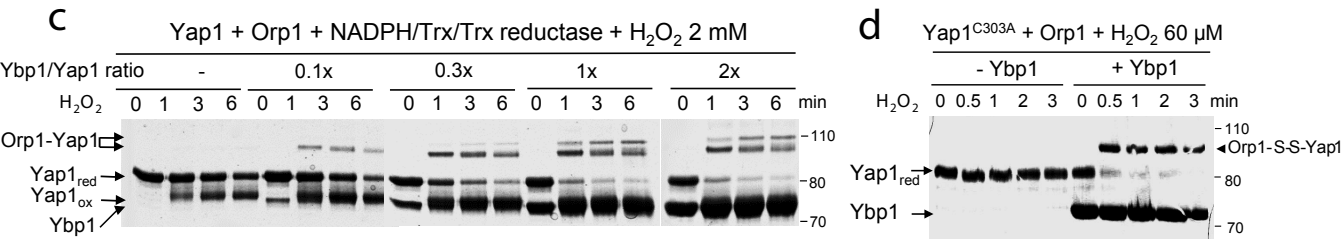
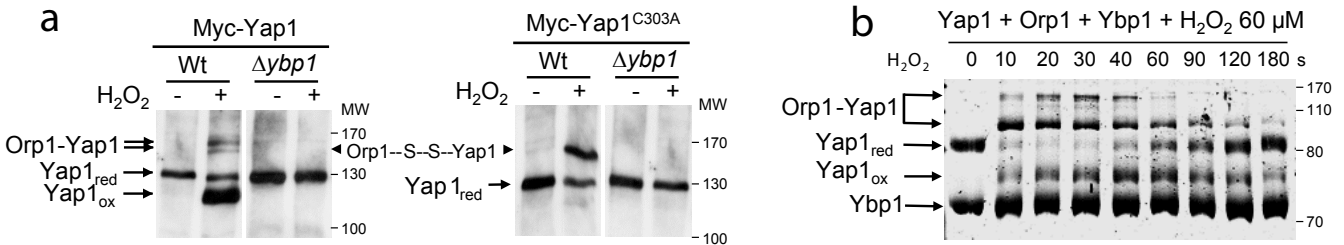
712 **COMPETING FINANCIAL INTERESTS**

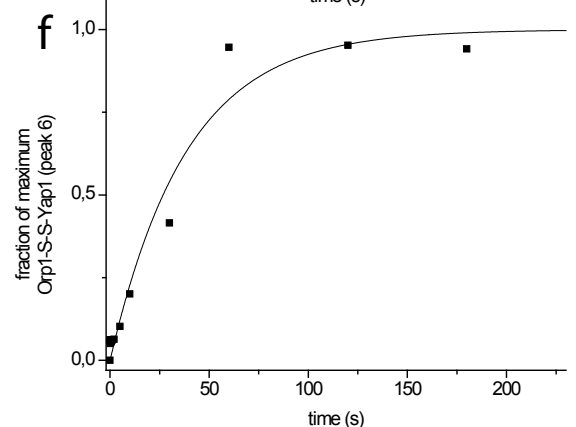
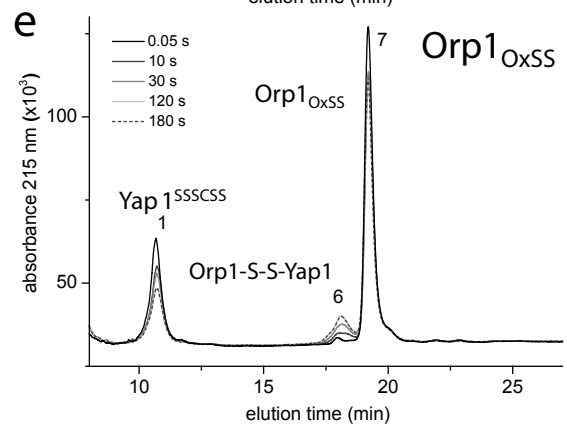
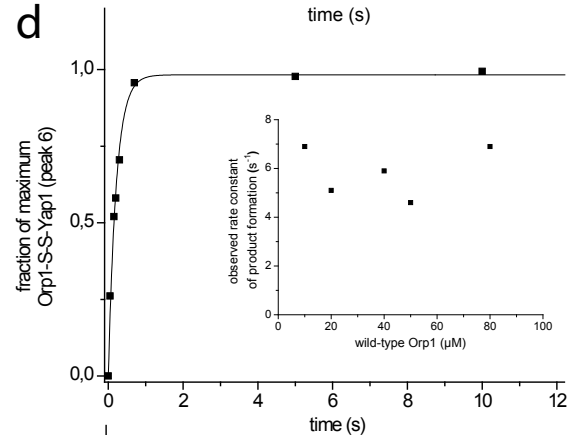
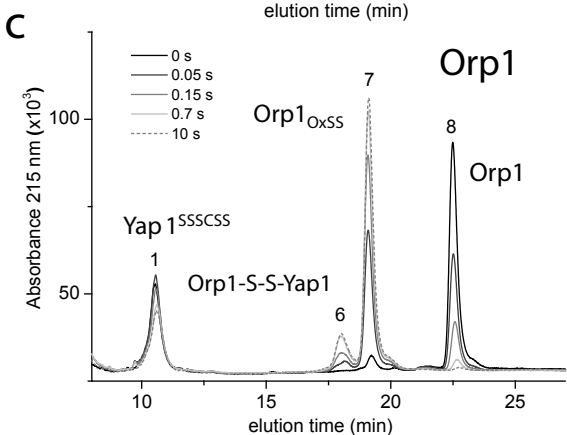
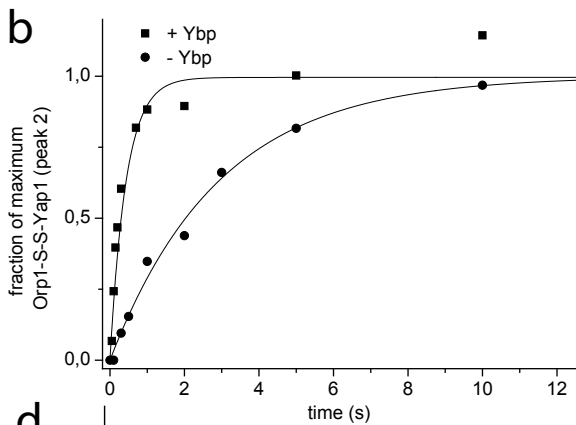
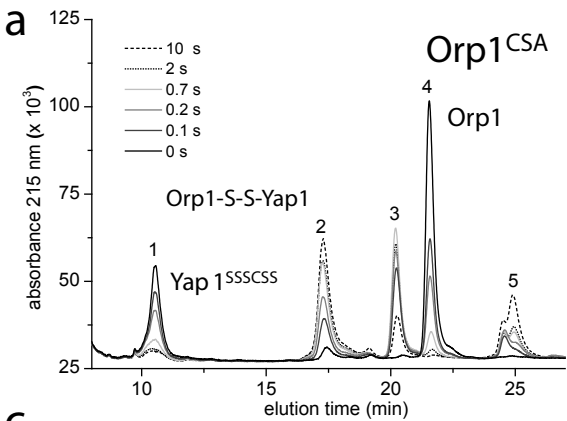
713 The authors declare no competing financial interests.

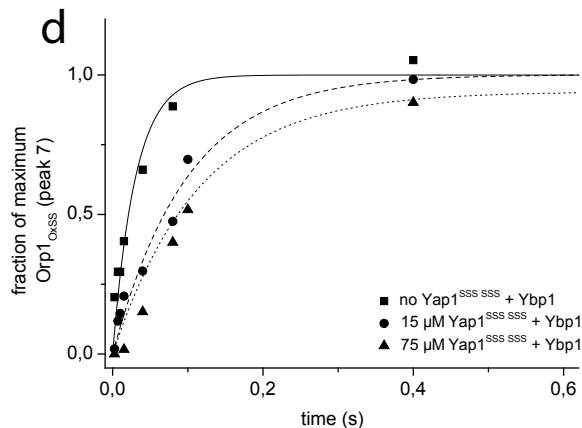
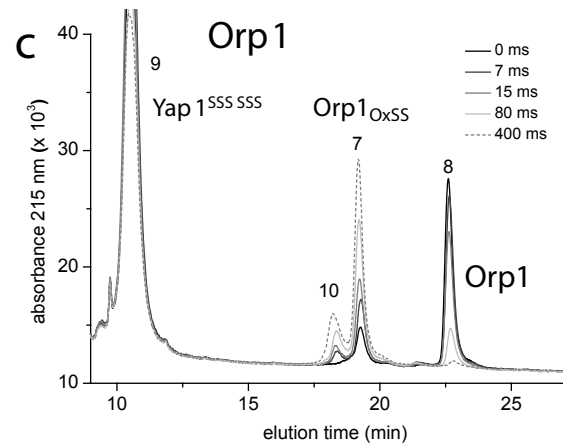
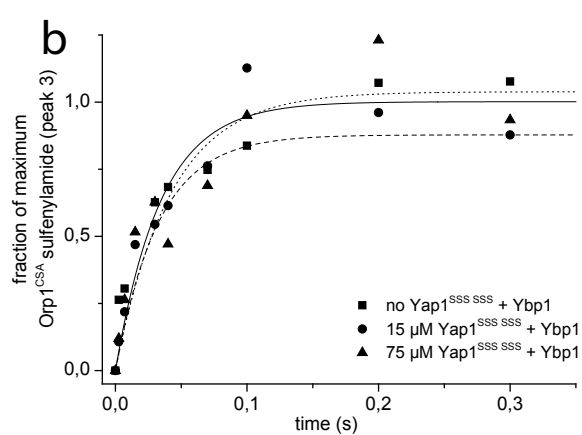
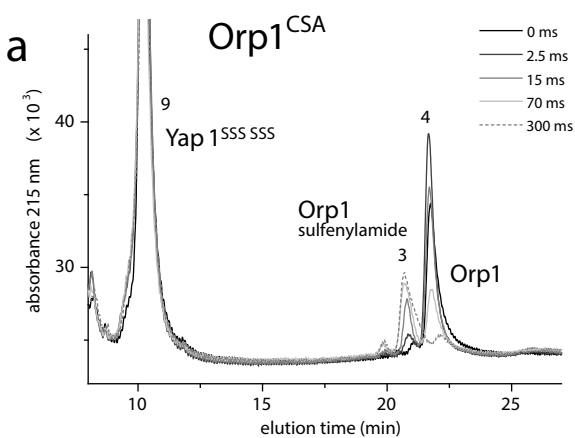


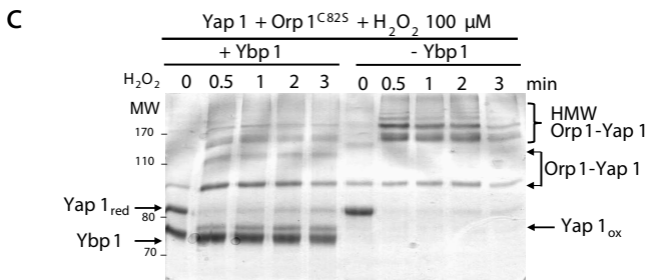
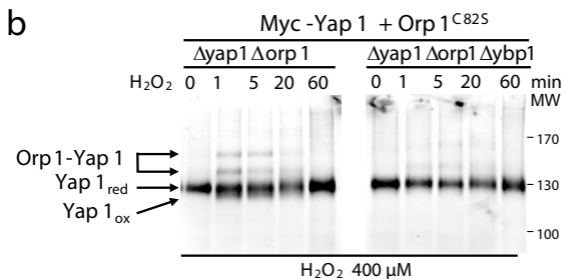
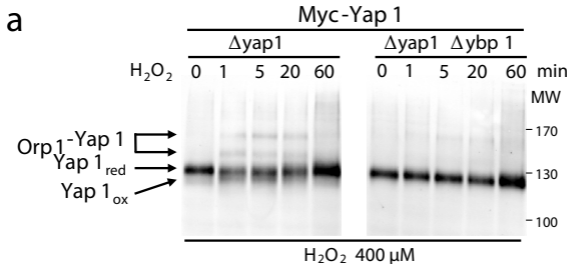


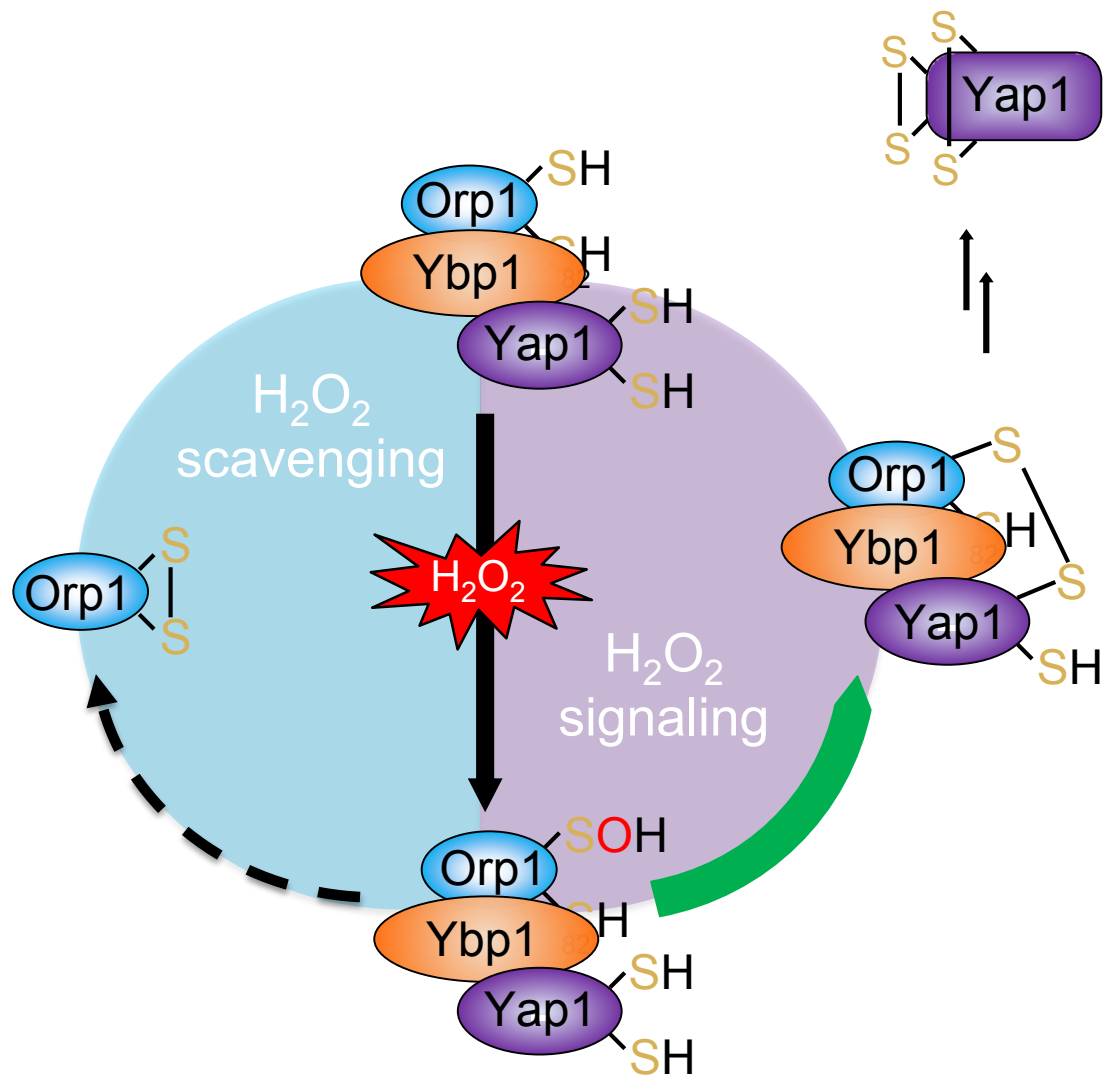












*S. cerevisiae*  
 $H_2O_2$  response

