

Supplementary Information

A scaffold protein that chaperones a cysteine-sulfenic acid in H₂O₂ signaling

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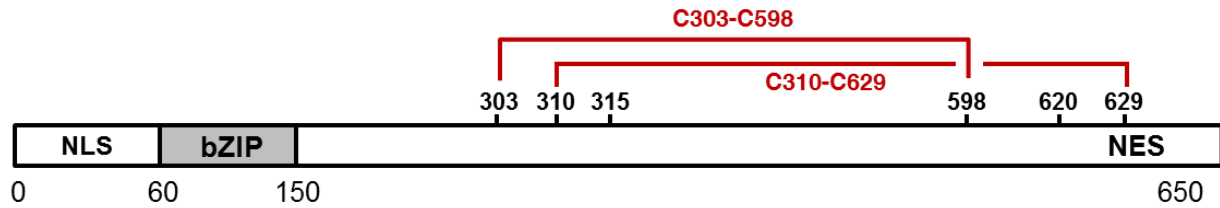
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Supplementary Results

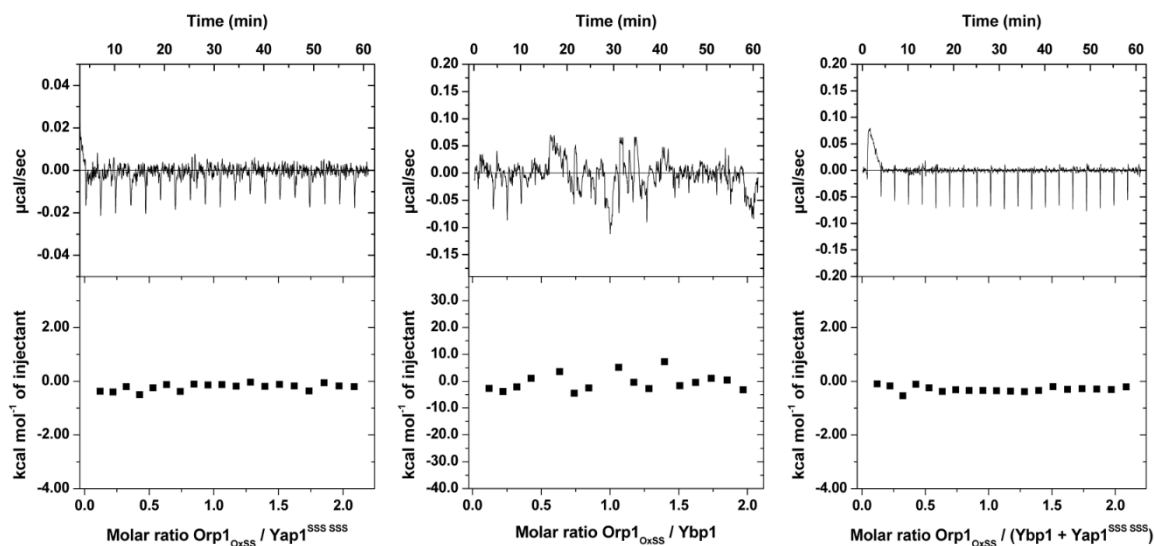


Supplementary Figure 1. Schematics of Yap1 with Cys residues position and disulfide bonds the oxidized active form of Yap1. NLS, nuclear localization sequence, NES, nuclear export sequence.

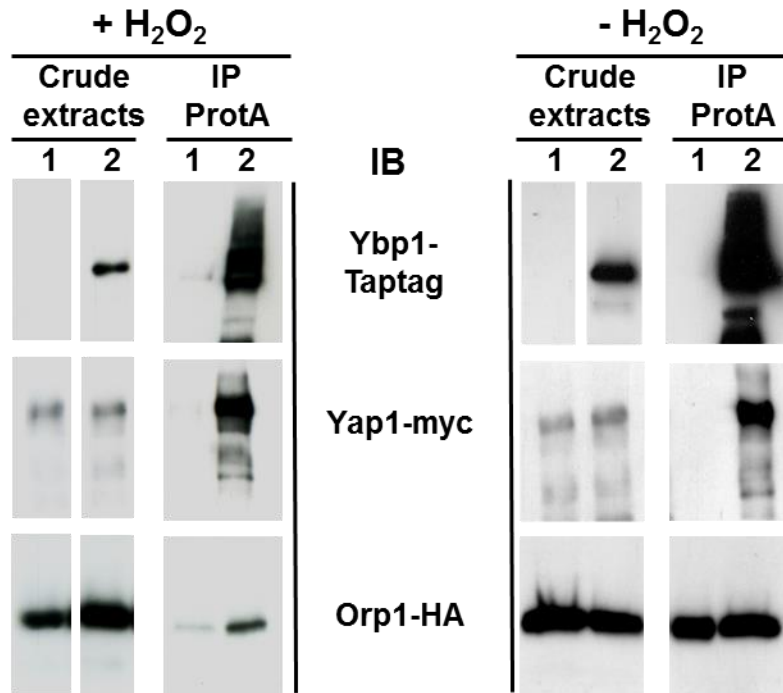
Supplementary Table 1. Thermodynamic parameters for Orp1 binding to Ybp1 and to the Yap1^{SSS}·Ybp1 complex obtained from ITC titrations^a

	Dissociation constant (μM)	Stoichiometry	ΔH cal mol^{-1}	$-\text{T}\Delta\text{S}$ cal mol^{-1}
Ybp1	0.8 ± 0.1	0.8 ± 0.1	-4890 ± 400	-3430
Ybp1·Yap1 ^{SSS}	0.7 ± 0.2	0.8 ± 0.1	-4210 ± 240	-4235

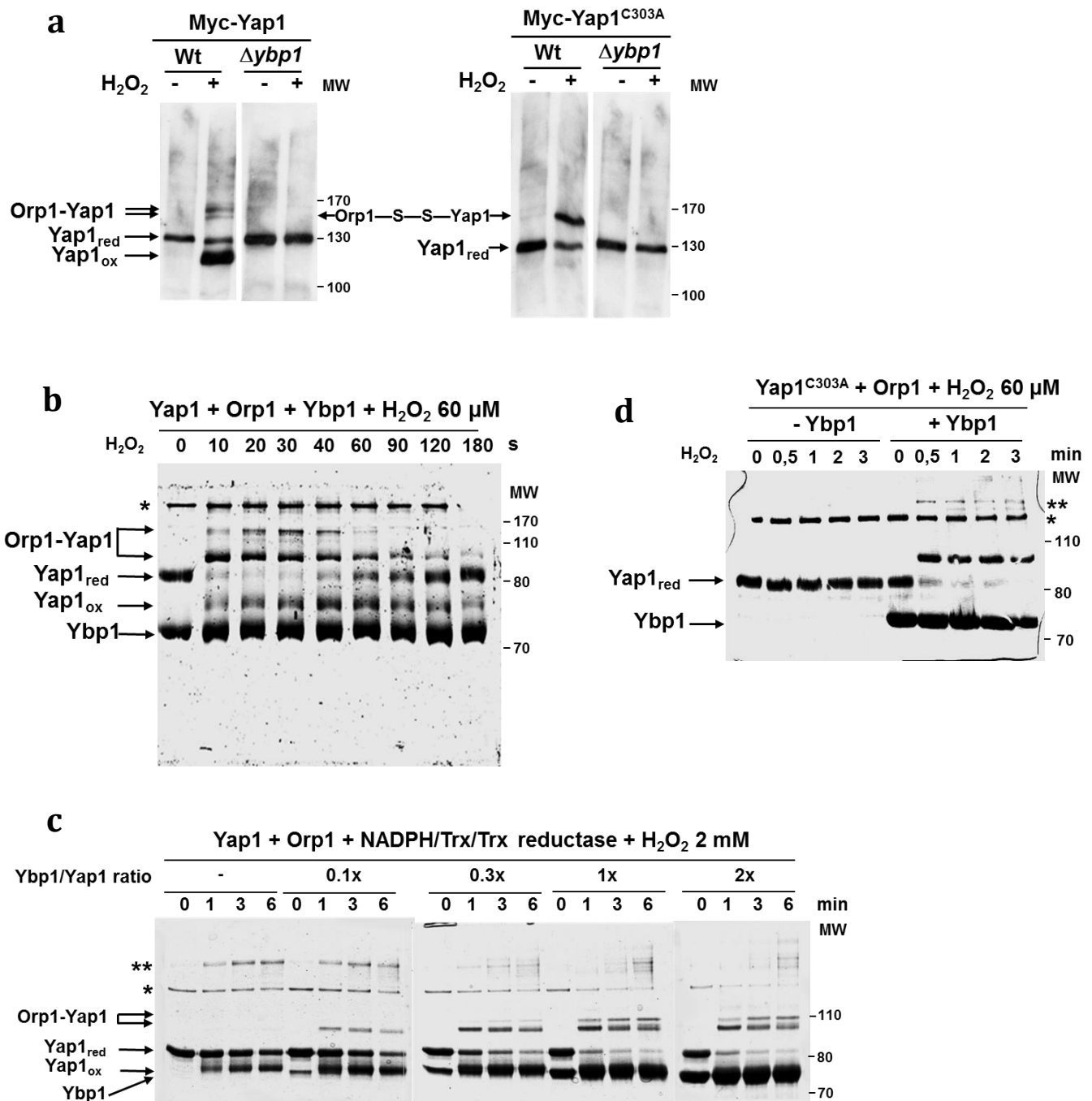
^aParameters derived from the binding isotherms shown in **Fig. 2c**. Data are the mean value from $n=2$ independent experiments performed on distinct protein samples \pm s.d..



Supplementary Figure 2. Related to Fig. 2c. Orp1_{OxSS} does not bind to Yap1, Ybp1 or to the Ybp1·Yap1 complex. ITC titration of Yap1^{SSS} (50 µM, left), Ybp1 (20 µM, center) and both (50:50 µM, right) by Orp1_{OxSS} at 25°C. In the experiment of the right panel, 85 % of Ybp1 and Yap1^{SSS} are bound together.

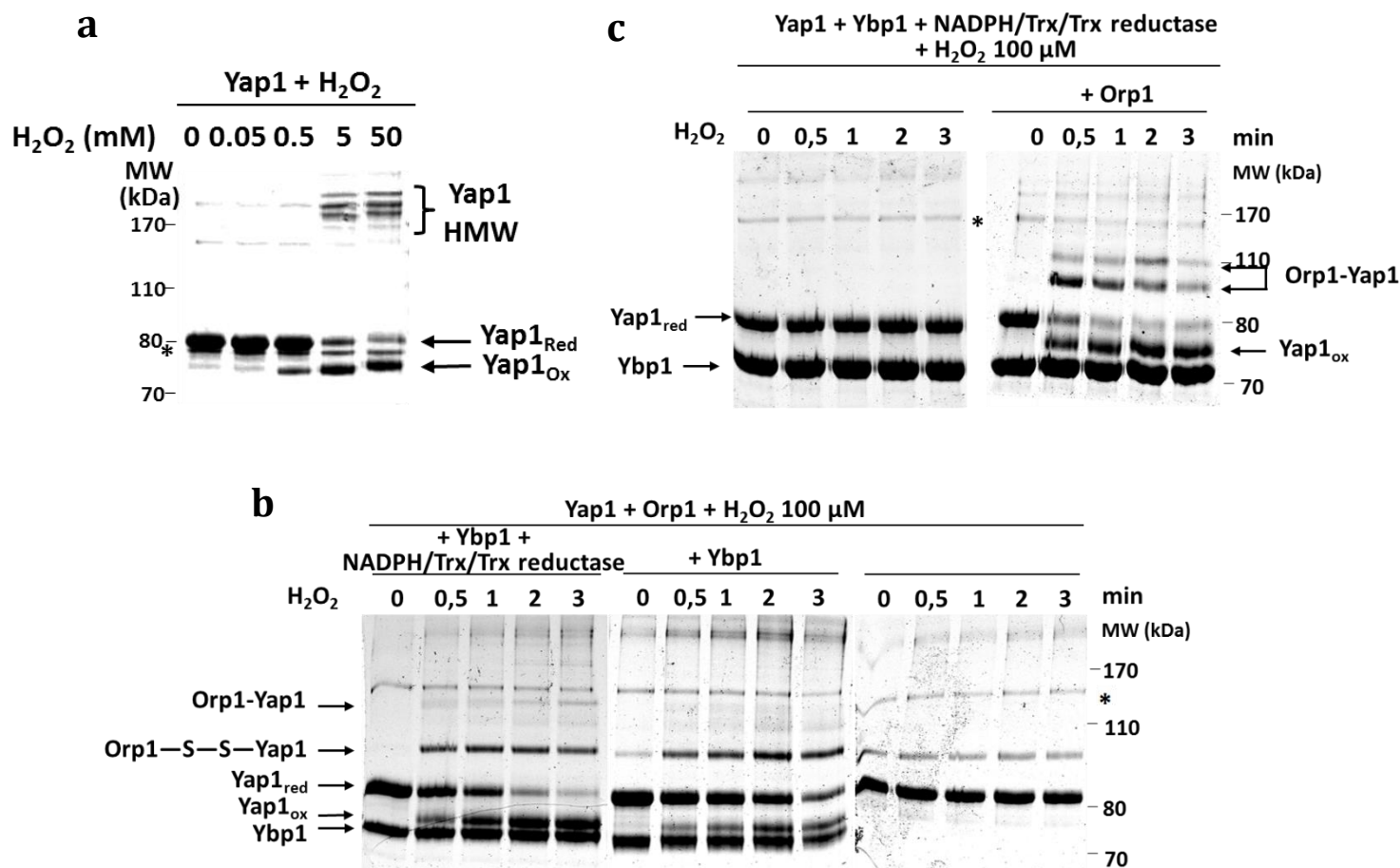


Supplementary Figure 3. Orp1 and Yap1 interact with Ybp1 *in vivo*. Exponentially growing $\Delta yap1\Delta orp1$ not carrying (1) or carrying a *YBP1-TAPTAG* gene fusion chromosomally integrated at the *YBP1* locus (2) and expressing Yap1-Myc and Orp1-HA from centromeric plasmids (1,2) were exposed or not to H₂O₂ (400 μ M) for 2 min, and broken in a cell disruptor (CellD). The soluble fraction was incubated at 4°C for 4h with Protein A sepharose beads in 10 mM Tris-Cl pH 8, 150 mM NaCl, 0.1% NP40, 200 μ M EDTA, 1 mM PMSF. After discarding the supernatant, beads were washed twice with the same buffer, then resuspended in Laemmli buffer, and loaded for SDS-PAGE. Proteins were revealed by western blot, using the 9E10 anti-Myc, anti-HA and PAP antibodies. Under H₂O₂-induced conditions, Myc-Yap1 and Orp1-HA are thus co-immunoprecipitated with Ybp1-TAPtag. The co-immunoprecipitation yield is much higher with Yap1 than with Orp1, relative to the abundance of these proteins in crude extracts, likely due to Orp1_{oxSS} not interacting with Ybp1. Under non-induced conditions, Yap1 coprecipitates with Ybp1, indicating that this interaction is not modulated by H₂O₂. However, although it coprecipitates with Ybp1, Orp1 is also brought down in cells lacking Ybp1. We therefore cannot totally rule out that in the induced extracts, Orp1 coprecipitates with Ybp1 by virtue of its disulfide linkage with Yap1.



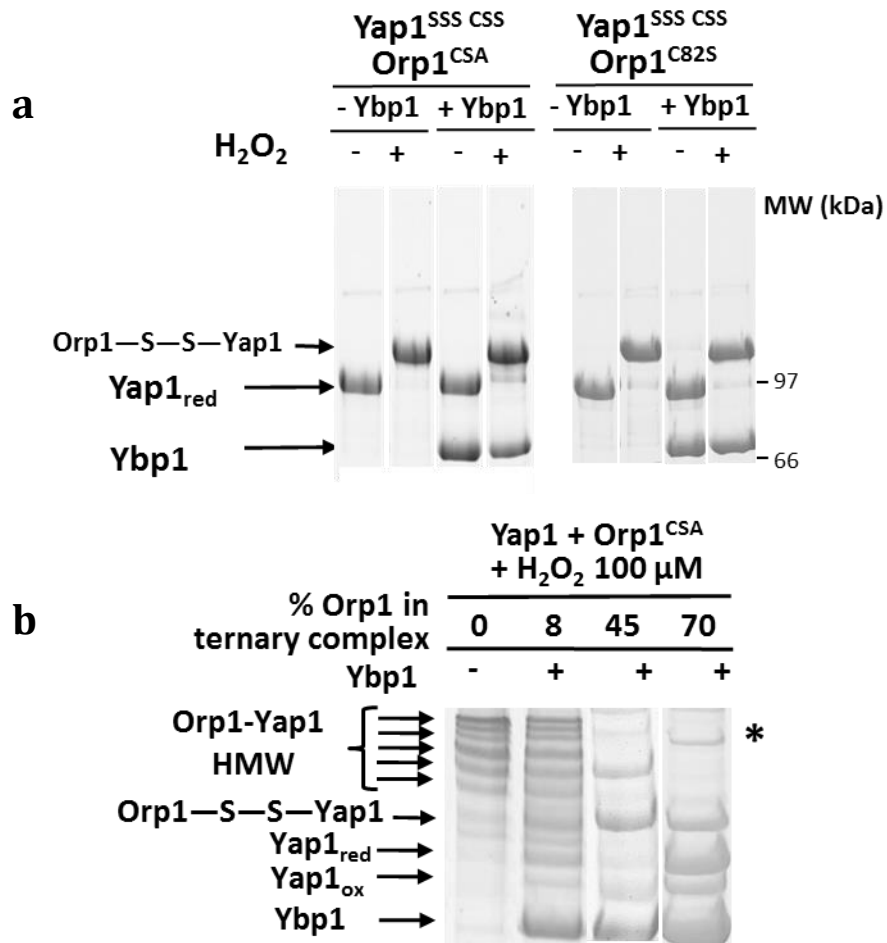
Supplementary Figure 4. Full gels from Fig. 3

* impurity; ** Yap1 high molecular weight disulfide-linked intermolecular complexes formed nonspecifically upon H₂O₂ exposure (see **Supplementary Fig. 5a**).

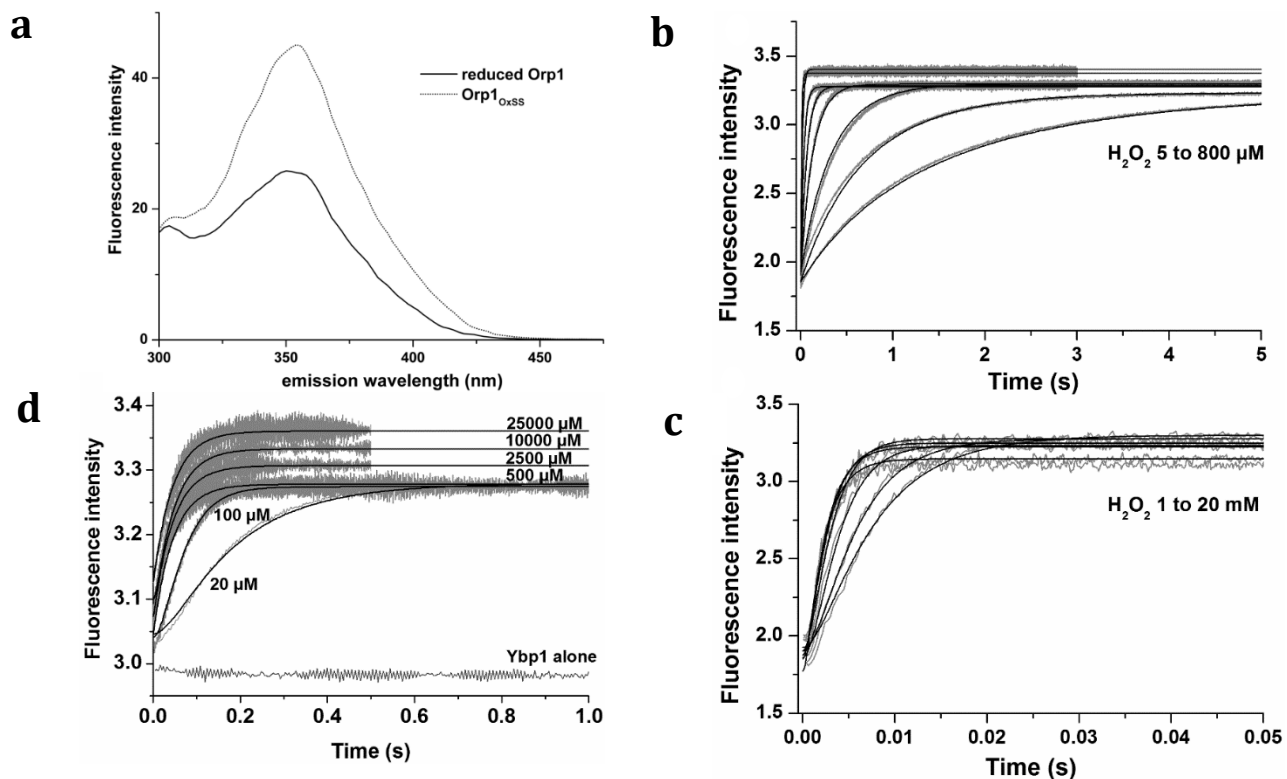


Supplementary Figure 5. Related to Fig. 3. Analysis of the influence of the different components of the redox relay on Yap1 oxidation in the *in vitro* reconstituted system

(a) Coomassie-stained non-reducing SDS-PAGE of a Yap1 solution (5 μM) incubated with increasing amounts of H₂O₂, as indicated, for 5 min. HMW, high molecular weight disulfide-linked Yap1 intermolecular complexes. (b) Coomassie-stained non-reducing SDS-PAGE of reaction mixtures containing Yap1 (2 μM) and Orp1 (10 μM), in the presence or absence of Ybp1 (2 μM), Trx (10 μM), Trx reductase (0.5 μM) and NADPH (1.0 mM), as indicated, incubated for the indicated time with H₂O₂ (100 μM). In the absence of Trx, Trx reductase and NADPH, reactions products were identical to those observed in their presence, except, for a slower reaction kinetics, likely due to trapping of Orp1 in the oxidized Orp1_{oxSS} form. (c) Coomassie-stained non-reducing SDS-PAGE of reactions mixture containing Yap1 (2 μM), Ybp1 (2 μM), Trx (10 μM), Trx reductase (0.5 μM) and NADPH (1.0 mM), and incubated for the indicated time with H₂O₂ (100 μM) in the presence or absence of Orp1 (2 μM). (*) impurities.



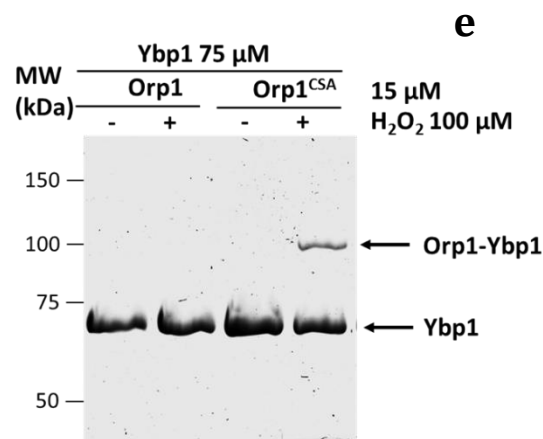
Supplementary Figure 6. (a) Coomassie-stained non-reducing SDS-PAGE analyses of 1 min-reaction mixture containing Orp1^{CSA} or Orp1^{C82S} (10 μM) and Yap1^{SSS CSS} (2 μM), in the absence or presence of 2 μM Ybp1 before and after addition of 100 μM H₂O₂. **(b)** Formation of HMW products is linked to the fraction of Orp1^{CSA} associated with the ternary complex. Reaction mixtures containing Yap1, Ybp1 and Orp1^{CSA} concentrations of respectively 2, 0 and 10 μM (% Orp1 in ternary complex = 0%), 2, 2, 10 μM (8%), 5, 5, and 3 μM (45%) and 10, 10 and 2 μM (70%) were incubated 1 min with H₂O₂ (100 μM) and resolved by non-reducing Coomassie-stained SDS-PAGE. The concentration of the Orp1·Ybp1·Yap1 complex is similar in all conditions including Ybp1, based on the dissociation constants determined Fig. 2. * indicates contaminant in Yap1 preparation.

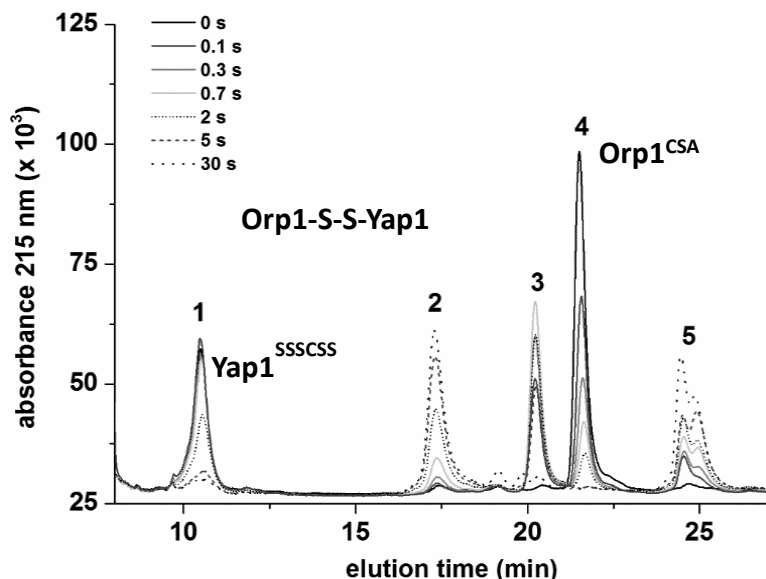


Supplementary Figure 7. Kinetics of Orp1 peroxidase activity under single-turnover conditions.

(a) Intrinsic fluorescence emission spectrum of reduced Orp1 (solid) and Orp1_{oxSS} (dots), with excitation wavelength set at 282 nm. Orp1_{oxSS} was obtained by adding H₂O₂ to Orp1 (1.5:1). **(b,c)** Kinetics of Orp1 peroxidase reaction monitored by intrinsic tryptophan fluorescence. Final concentrations: Orp1 (2 μM), H₂O₂ (5, 10, 20, 50, 100, 200, 400 and 800 μM) (b) and H₂O₂ (1, 1.5, 3, 5, 7.5, 10, 15 and 20 mM) (c). Reactants were rapidly mixed in a stopped flow apparatus at 25 °C. The

complete data set (grey lines) was analyzed by global fitting according to a two-step mechanism comprising reaction 1, yielding a second order rate constant k_{SOH} of $1.6 \cdot 10^5 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$, followed by reaction 3, yielding a very fast first order rate constant k_{SS} of $507 \pm 4 \text{ s}^{-1}$ (black solid lines). **(d)** Orp1 (2 μM) and Ybp1 (10 μM) were rapidly mixed with indicated concentrations of H₂O₂ in a stopped flow apparatus at 25 °C. In the presence of Ybp1 k_{SOH} slightly increased to $3.3 \cdot 10^5 \pm 0.2 \text{ M}^{-1}\text{s}^{-1}$, which indicates that the kinetics of Orp1—SOH formation is not altered within the binary complex Orp1-Ybp1, and k_{SS} decreased to a value of $22 \pm 1 \text{ s}^{-1}$. **(e)** Coomassie-stained non-reducing SDS-PAGE of reactions mixture containing Ybp1 (75 μM), Orp1 or Orp1^{CSA} (15 μM) incubated during 10 s with H₂O₂ (100 μM). A non-specific Orp1^{CSA}-Ybp1 adduct can be seen, but which does not alter the kinetics of reaction 1.



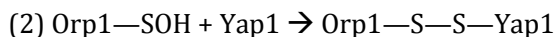
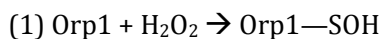
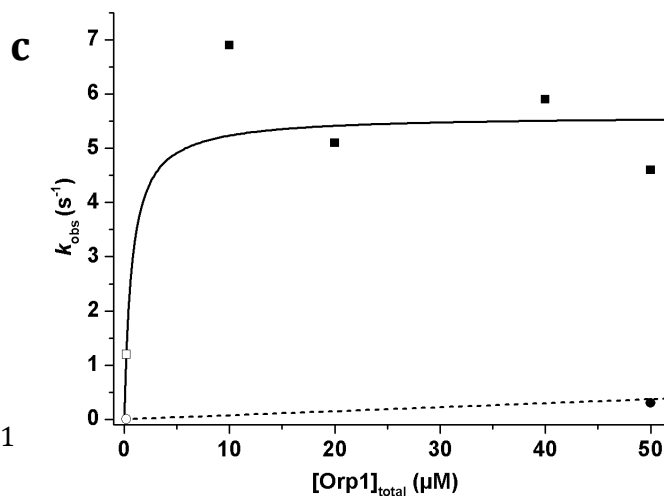
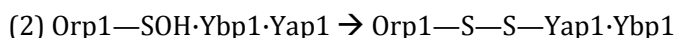
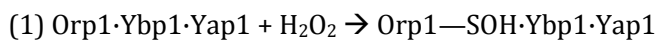
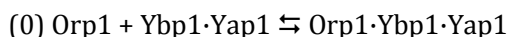


Supplementary Figure 8. Related to Fig. 4a,b. Reverse-phase analysis of the reaction products of Orp1^{CSA} and Yap1^{SSSCSS} in the absence of Ybp1. Reaction between Yap1^{SSSCSS} (10 μ M) and Orp1^{CSA} (50 μ M) was initiated by rapid mixing with H₂O₂ (100 μ M) at 25°C in a quench flow apparatus for the indicated time and stopped by acid quenching. Masses of products eluted in each peaks are in **Supplementary Table 2**.

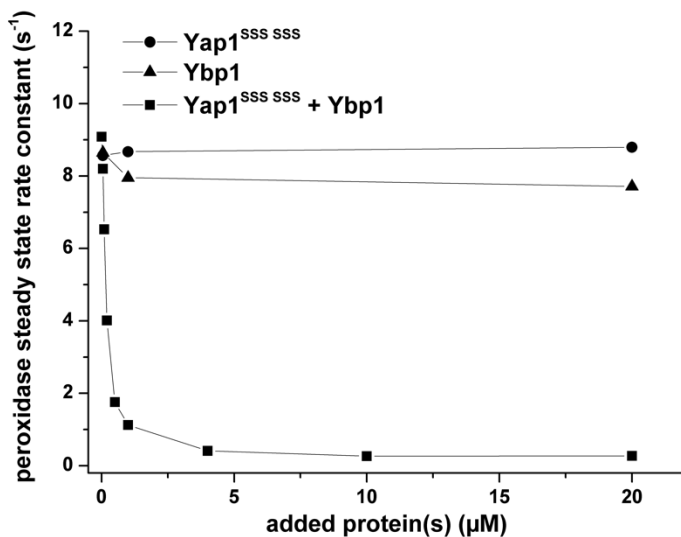
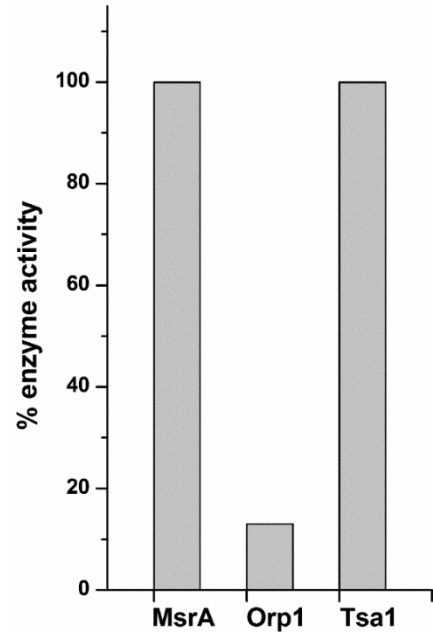
Supplementary Table 2. Theoretical and observed masses of species observed in reverse-phase chromatographic analyses

Peak	Observed mass, Da ^c	Species ^a (theoretical mass ^b , Da)
1	74507.0 \pm 10.6	Yap1 ^{SSSCSS} (74498.6)
2	92961.1 \pm 12.9	Yap1 ^{SSSCSS} —S—S—Orp1 (92958.7)
3	18460.0 \pm 0.5	Orp1 ^{CSA} sulfenylamide form (18460.1)
4	18461.8 \pm 0.4	Orp1 ^{CSA} reduced (18462.1)
5	36922.2 \pm 0.5	Orp1 ^{CSA} oxidized disulfide dimer (36922.2)
6	93008.6 \pm 8.0	Yap1—S—S—Orp1 (93006.9)
7	18508.1 \pm 0.4	Orp1 _{oxSS} (18508.2)
8	18510.0 \pm 0.3	Orp1 reduced (18510.2)
9	74486.9 \pm 2.6	Yap1 ^{SSSSS} (74482.6)

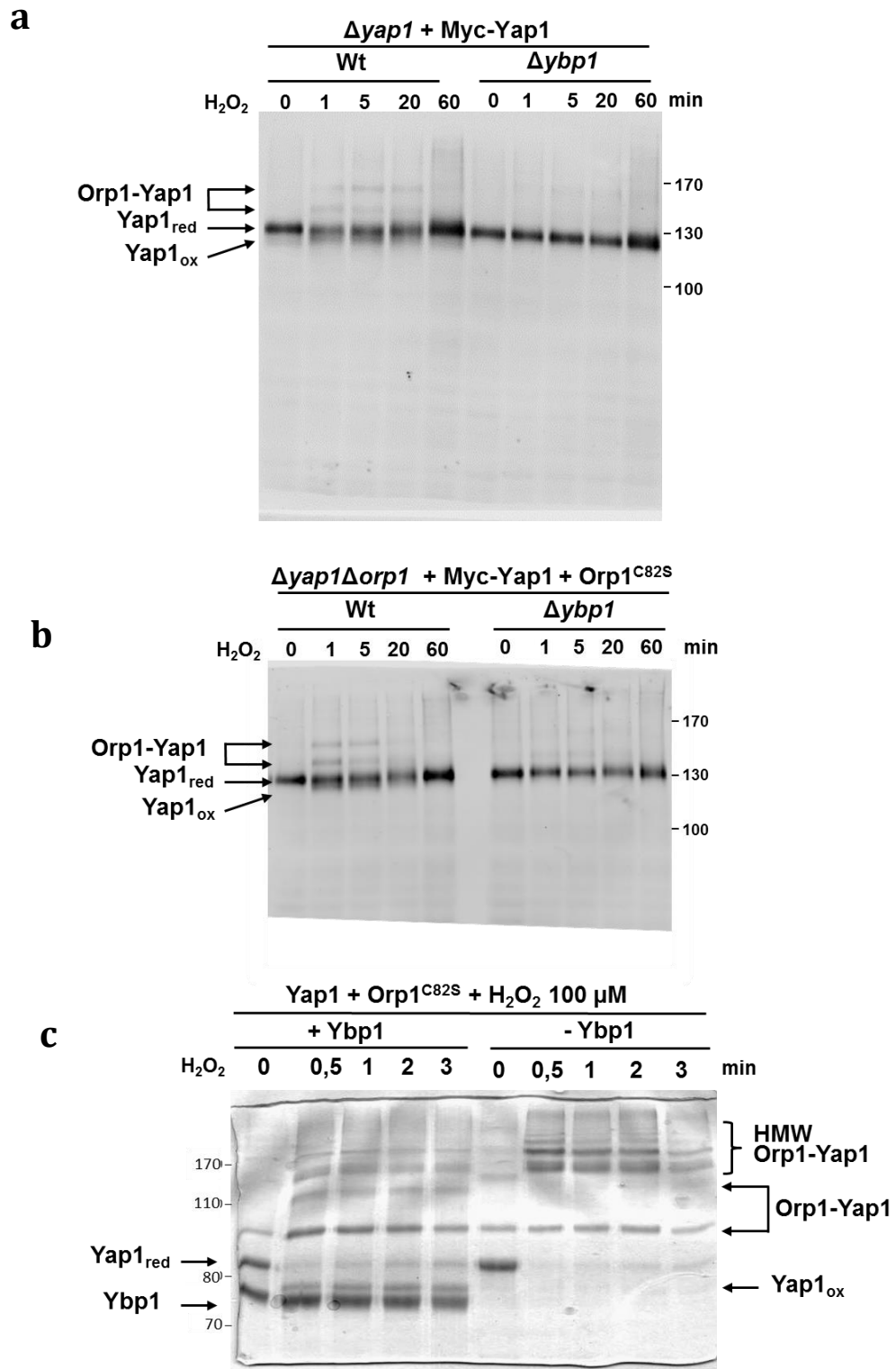
^aSpecies of **Fig. 4 and 5** chromatographic analyses. ^bTheoretical mass without N-terminal methionine. ^cMasses are the mean value of at least 8 m/z values of the mass spectrum \pm s.d.. Mass precision for Yap1 and Yap1—S—S—Orp1 is lower relative to Orp1, due to their poor stability in the gas phase and mass spectra lower quality.

a**b****Supplementary Figure 9. Related to Fig. 4. Interpretation of the Orp1/Yap1/H₂O₂ reaction kinetics**

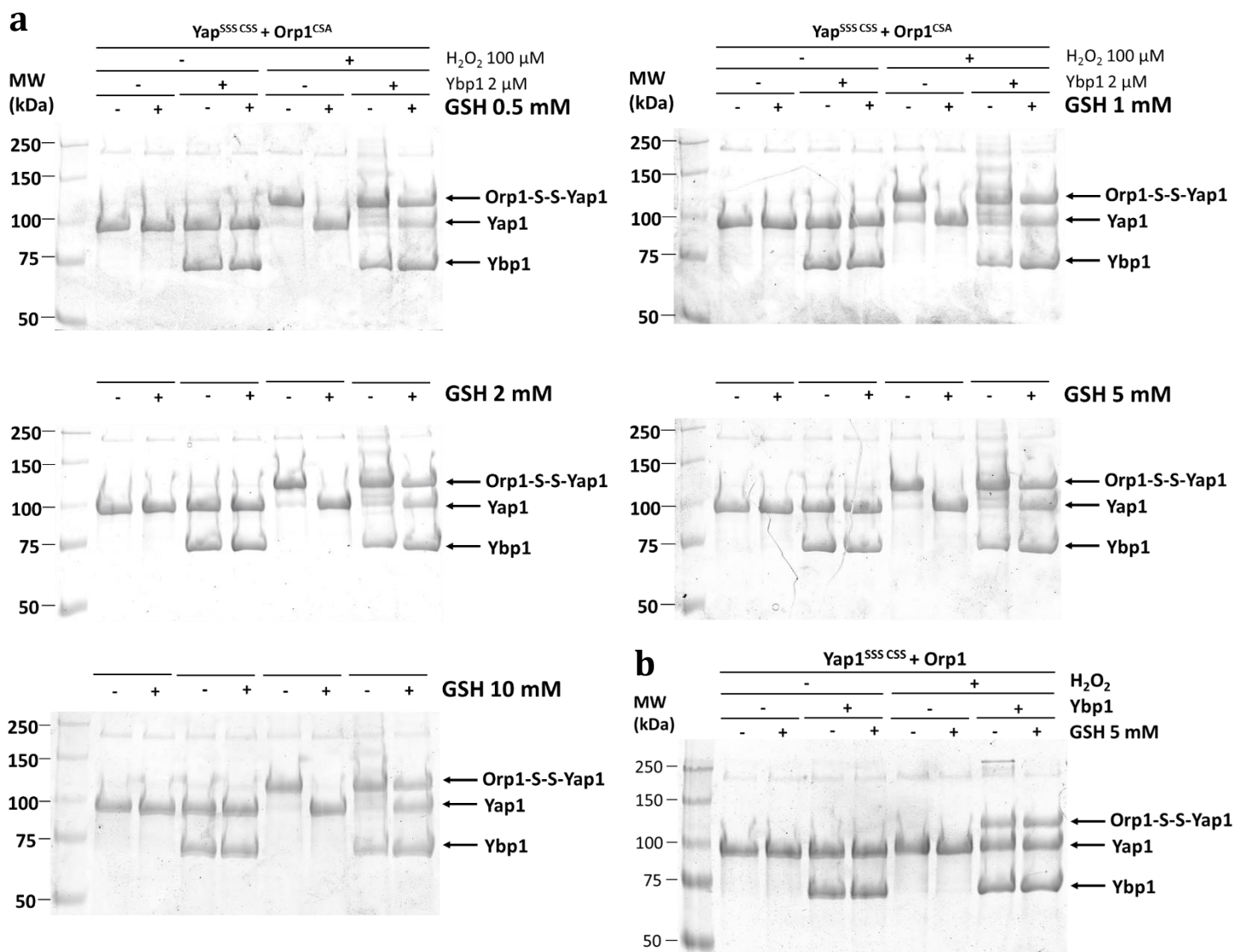
(a) Bimolecular mechanism used to interpret the reaction kinetics between Orp1^{CSA}, Yap1^{SSS CSS} and H₂O₂ shown in Supplementary Fig. 7. Reaction numbers match with those defined in Fig. 1. Rate constants for reaction 1 and 2 are k_{SOH} ($1.6 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$) and $k_{\text{bimolecular}}$, respectively. **(b)** Ternary complex mechanism used to interpret the reaction kinetics between Orp1^{CSA}, Ybp1·Yap1^{SSS CSS} and H₂O₂ of Fig. 4a. Reaction 0 is assumed as a rapid equilibrium; rate constants for reaction 1 and 2 are k_{SOH} ($3.3 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$, corresponding to the value measured in the presence of Ybp1 in Supplementary Fig. 4d), and k_{complex} , respectively. This model is analogous to the classical Michaelis-Menten mechanism, with the catalytic constant corresponding to k_{complex} and the Michaelis constant to the apparent dissociation constant of Orp1 from the Orp1·Ybp1·Yap1 complex, assumed to be equal to $0.7 \mu\text{M}$ in rapid equilibrium conditions. The parameter $\frac{k_{\text{complex}}}{K}$ can be compared to $k_{\text{bimolecular}}$, and reflects the actual impact of Ybp1 on reaction 2. **(c)** Kinetics of Orp1—S—S—Yap1 formation in the bimolecular (dash) and ternary complex (solid) schemes were simulated using $k_{\text{obs}} = k_{\text{bimolecular}} [\text{Orp1}]_{\text{total}}$ and $k_{\text{obs}} = \frac{k_{\text{complex}} [\text{Orp1}]_{\text{total}}}{K + [\text{Orp1}]_{\text{total}}}$, respectively, with values of $7.4 \cdot 10^3 \text{ M}^{-1}\text{s}^{-1}$, 5.6 s^{-1} and $0.7 \mu\text{M}$ for $k_{\text{bimolecular}}$, k_{complex} (the k_{obs} value obtained at saturating concentrations of Orp1, Fig. 5d) and K , respectively. Experimental data from Fig. 5b (filled circle) and Fig. 5d (filled squares) and predicted values corresponding to $0.2 \mu\text{M}$ Orp1 are shown ($\sim 10^{-3}$ and $\sim 1 \text{ s}^{-1}$ in the absence and presence of Ybp1, open symbols). Thus, even if reaction 2 proceeds one or two orders of magnitude slower *in vivo*, due to very low ternary complex concentration (predicted $< 0.01 \mu\text{M}$ based on Yap1 and Ybp1 concentrations, refs. 27, 28), it would still be compatible with the kinetics observed *in vivo*, i.e. Yap1 oxidation is completed in seconds (Ref. 4).

a**b**

Supplementary Figure 10. Related to Fig. 5. Impact of the Ybp1 and Yap1^{SSSSSS} on the Trx recycling system. (a) Orp1 peroxidase activity (50 nM) was measured using the Trx (50 μM), Trx reductase (0.5 μM), NADPH (200 μM) coupled assay, in the presence of Yap1^{SSSSSS} (circle), Ybp1 (triangle) or Yap1^{SSSSSS} and Ybp1 together (1:1) (square). The reaction was initiated with 100 μM H₂O₂. **(b)** Tsa1 peroxidase and MsrA methionine sulfoxide reductase activities measured using the Trx coupled assay in the presence of both Ybp1 and Yap1 (1 μM each). The reaction was initiated with 100 μM H₂O₂ or 100 mM methionine sulfoxide as substrate, respectively. Concentrations of the Trx recycling system used were: 1 μM Trx reductase, 200 μM NADPH, 50 μM Trx and 0.5 μM Tsa1, and 4.8 μM NTR, 200 μM NADPH, 100 μM Trx and 1 μM MsrA. Data are plotted as % of enzyme activity in the absence of Yap1 and Ybp1. Orp1 data are from (a).



Supplementary Figure 11. Full gels and blots from Fig. 6



Supplementary Figure 12. The reaction of Glutathione with the Orp1—SOH is restricted within the ternary complex. Effect of glutathione on the reaction products formed in mixtures containing Yap1^{SSS CSS} (2 μM) and **(a)** Orp1^{CSA} (10 μM) and GSH (0.5, 1, 2, 5 and 10 mM) or **(b)** Orp1 (10 μM) and GSH (5 mM), in the absence or presence of Ybp1 (2 μM), as indicated. Mixtures were incubated 1 min after addition of 100 μM H₂O₂, followed by Coomassie-stained non-reducing SDS-PAGE.