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Reversal of 2-Cys peroxiredoxin oligomerization by sulfiredoxin

Jeong Chan Moon [a], Gyeong Mi Kim [a], Eun-Kyung Kim [a], Hae Na Lee [a], Bin Ha [a], Sang Yeol Lee [b], Ho Hee Jang [a,*]

[a] Department of Molecular Medicine, Lee Gil Ya Cancer and Diabetes Institute, Gachon University, Incheon 406-799, Republic of Korea
[b] Division of Applied Life Sciences (BK21 Program), Gyeongsang National University, Jinju 660-701, Republic of Korea

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ABSTRACT

Hydrogen peroxide (H_2O_2) regulates the structure and function of 2-Cys peroxiredoxins (Prxs). Upon oxidation by excess H_2O_2, Prxs become overoxidized to a sulfinic acid of its peroxidatic cysteine residue, resulting in a structural change from a small oligomer with peroxidase function to a large oligomer with chaperone function. Then, sulfiredoxin (Srx) reduces the overoxidized Prxs by an ATP-dependent mechanism. Although Srx is known to repair the overoxidized forms of Prx, the role of Srx in the reversal of Prx oligomerization remains to be elucidated. Here we investigated whether Srx1 directly facilitates the dissociation of yeast Prx1 (YPrx1) from a high-molecular-weight (HMW) complex to a low-molecular-weight (LMW) complex *in vitro*. Srx1 reactivates the YPrx1 peroxidase activity that is inactivated by H_2O_2, whereas it decreases the chaperone activity enhanced by H_2O_2. We show that Srx1 dissociates the H_2O_2-induced HMW YPrx1 complex, and that the Srx1 Cys84 residue is critical for its dissociation. In contrast to wild-type Srx1, an inactive Srx1 mutant (Srx1-C84S) did not induce the reactivation of inactivated YPrx1 or dissociation of the HMW YPrx1 complex. We revealed that Srx1 interacts directly with YPrx1 in yeast cells using bimolecular fluorescence complementation. Taken together, these findings suggest that Srx1 regulates YPrx1 function and structure in yeast cells through a direct interaction.

1. Introduction

Peroxiredoxins (Prxs) constitute a family of peroxidases that reduce H_2O_2 and alkyl hydroperoxides to water and alcohol, respectively [1–3]. All Prx enzymes contain a conserved cysteine (Cys) residue at the amino-terminal region that is oxidized to a sulfenic acid (Cys-SOH) by H_2O_2 [4–7]. Prxs are divided into three subgroups (i.e. 2-Cys Prx, atypical 2-Cys Prx and 1-Cys Prx) based on their catalytic mechanism and the number of Cys residues that participate in catalysis [8–10]. Among them, 2-Cys Prx has an additional conserved Cys residue in the carboxyl-terminal region. In the normal catalytic cycle, the conserved amino-terminal Cys residue (peroxidatic Cys) of 2-Cys Prx is selectively oxidized to Cys-SOH by H_2O_2, which then reacts with the conserved carboxyl-terminal Cys-SH (resolving Cys) located in the other subunit of the homodimer to produce an intermolecular disulfide. The disulfide or sulfenic acid form of 2-Cys Prx is subsequently and specifically converted back to a reduced state by biological thiols, such as thioredoxin (Trx) [4,5]. Under conditions of extreme oxidative stress, increasing H_2O_2 flux leads to overoxidation of the amino-terminal Cys sulfenic acid (Cys-SOH) to sulfinic acid (Cys-SO_2H), resulting in enzyme inactivation. However, overoxidized 2-Cys Prx (Cys-SO_2H) is not reduced by biological thiols under physiological conditions [11–13]. Furthermore, 2-Cys Prxs overoxidation can promote the formation of high-molecular-weight (HMW) complexes, resulting in a switch in the enzymatic activity from a peroxidase to a molecular chaperone that can prevent stress-dependent aggregation of model substrates [14–17].

Sulfiredoxin (Srx), which can catalyze the reduction of overoxidized Prx to sulfenic Prx (Prx-SOH), was identified in yeast [18]; mammalian Srx was subsequently characterized extensively [19–24]. The reduction of overoxidized Prx by Srx requires the conserved Srx Cys residue, ATP hydrolysis, Mg^{2+}, and a thiol as an electron donor. The reduction reaction is first activated by formation of an anhydride bond with the γ-phosphate of ATP, leading to a phosphoryl sulfinic intermediate. The Srx catalytic Cys subsequently attacks the phosphoryl ester to produce a Srx-Prx thiosulfinate intermediate, which is resolved by the Trx sulfhydryl group or glutathione to generate Prx-SOH [25]. The latter is in turn reduced further to Prx-SH. In addition to its role in Prx overoxidation reversal, Srx possesses deglutathionylation activity [26].

Although the biochemical reaction, reversible overoxidation, catalyzed by Srx has been extensively characterized, 2-Cys Prxs reversible function and structure that is regulated by Srx is unclear.

* Corresponding author. Fax: +82 32 899 6318.
E-mail address: hhjang@gachon.ac.kr (H.H. Jang).

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Here we aimed to characterize the regulation of function and structure of Prx1 by Srx1 in yeast.

2. Materials and methods

2.1. Materials, yeast and culture media

Saccharomyces cerevisiae (BY4741; *MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) was cultured in Yeast Extract Peptone Dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose). To investigate the role of Srx in YPrx1 functional regulation, Srx1-deficient mutant yeast (Δ*srx1*) was obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). Malate dehydrogenase (MDH), H_2O_2, and NADPH were obtained from Sigma (St. Louis, MO). Rabbit antibodies against YPrx1, Srx1 and YPrx1-$SO_{2/3}$ were produced as described previously [27]. Xpert protease and phosphatase inhibitor cocktail were purchased from GenDEPOT (Barker, TX).

2.2. Cloning and protein expression in Escherichia coli

YPrx1, Srx1, Trx and Trx reductase (TR) genes were cloned from a *S. cerevisiae* cDNA library, and point-mutated Srx1 (Srx1-C84S) DNA was generated by polymerase chain reaction (PCR)-mediated mutagenesis, as described [28]. Using a pGEX expression vector, the recombinant proteins were expressed in *E. coli* BL21 (DE3) and purified as described [15].

2.3. Peroxidase and chaperone activity assays

Trx-dependent peroxidase activity of YPrx1 linked to NADPH oxidation was measured by a decrease in absorbance at 340 nm (A_{340}), and the chaperone activity was measured using MDH as a substrate, as described [16,29]. Turbidity due to substrate aggregation, indicating a lack of chaperone activity, was monitored in a DU800 spectrophotometer equipped with a thermostatic cell holder (Beckmann, Fullerton, CA, USA).

2.4. Polyacrylamide gel electrophoresis (PAGE) and immunoblotting

To prepare samples for native- and sodium dodecyl sulfate (SDS)–PAGE, yeast cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol, Xpert protease inhibitor cocktail, and Xpert phosphatase inhibitor cocktail) by vortexing using glass beads. After removing insoluble materials by centrifugation at 12,000 rpm for 15 min, the supernatants were obtained. Protein concentration was measured using Bradford's method [30]. Equal amounts of proteins were subjected to 10% native- or 12% SDS–PAGE. For immunoblotting, proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane, which was incubated with an anti-YPrx1, anti-YPrx-$SO_{2/3}$ or anti-Srx1 antibody.

2.5. Protein interaction assay using bimolecular fluorescence complementation (BiFC)

N- and C-terminal regions of YFP cloned from pSAT4-nEYFP-C1 (GenBank accession no. DQ168994) and pSAT4-cEYFP-C1-B (GenBank accession no. DQ168997) were fused with Srx1 and YPrx1 by PCR, respectively. The fused DNA constructs, NYFP-Srx1 (Srx1-YN) and CYFP-YPrx1 (YPrx1-YC), were ligated into the pYES2 and pTS911 yeast vectors, respectively. The constructs were sequenced to confirm the absence of sequence errors. Unfused N- and C-terminal YFP clones were used as negative controls. Transformation was performed in wild-type yeast cells by the lithium acetate method as described [31], and then the yeast cells were cultured in standard yeast nitrogen (YNB) minimal media lacking leucine and uracil. Fused proteins were induced by adding 2% galactose (w/v) as a carbon source. Expression of the fused proteins was verified by immunoblotting. Protein–protein interactions were visualized by confocal laser scanning microscopy (Leica, Heidelberg, Germany).

3. Results and discussion

3.1. Srx1 reactivates YPrx1 peroxidase activity previously inactivated by H_2O_2

Overoxidation of Prx peroxidatic cysteine to sulfinic acid induces a functional and structural switch in mammalian and yeast cells [15,16]. Srx1 was able to reduce the overoxidized form of Prxs in an ATP-dependent reaction [18]. To investigate whether Srx1 reverses overoxidized Prxs function, we examined the peroxidase activity of yeast 2-Cys Prx, YPrx1. Trx-dependent peroxidase activity of YPrx1 decreased gradually with increasing H_2O_2 concentration, and peroxidase activity was almost completely inhibited by 500 μM H_2O_2 (Fig. 1A). When Srx1 protein was added to a reaction mixture containing H_2O_2-inactivated YPrx1, YPrx1 peroxidase activity was reactivated in a concentration- and time-dependent manner (Fig. 1B and C). However, active-site mutation of Srx1 (Srx1-C84S) did not induce reactivation of YPrx1 peroxidase activity (Fig. 1B).

3.2. Srx1 reduces H_2O_2-enhanced YPrx1 chaperone activity

2-Cys Prx chaperone function was enhanced by overoxidation of the peroxidatic Cys residue [14]. To determine whether Srx1 regulated YPrx1 chaperone activity, we measured the capacity to suppress thermal aggregation using MDH as a substrate. YPrx1 completely prevented the heat-induced aggregation of MDH at a molar ratio of YPrx1 to substrate of 2:1, but was similar to the control at a molar ratio of YPrx1 to MDH of 0.5:1. When 0.5 μM YPrx1 was treated with H_2O_2 in the presence of a Trx system containing NADPH, TR, and Trx, YPrx1 chaperone activity increased greatly with increasing H_2O_2 concentration (Fig. 2A). YPrx1 exposed to 400 μM H_2O_2 had about fourfold higher chaperone activity as compared with YPrx1 before H_2O_2 treatment. We next assessed whether Srx1 reduced the YPrx1 chaperone activity increased by H_2O_2. YPrx1 proteins were treated with 400 μM H_2O_2 for 10 min and incubated with Srx1 protein for 30 min. The capacity of YPrx1 to protect against MDH aggregation was decreased to half. After 60-min Srx1 incubation, the YPrx1 chaperone activity was decreased to its initial level; i.e. prior to H_2O_2 treatment (Fig. 2B). These results suggest that Srx1 reverses the chaperone activity of YPrx1 enhanced by H_2O_2.

3.3. Srx1 dissociates the H_2O_2-mediated HMW YPrx1 complex

YPrx1 function was related to its structure, and its functional switching from a peroxidase to chaperone was induced by oligomerization [14]. Since Srx1 regulates the peroxidase and chaperone activities of YPrx1, the effect of Srx1 on YPrx1 structure was assessed by native-PAGE. As shown in Fig. 3A, recombinant YPrx1 proteins exist as multiple oligomers of various sizes, but mainly as low-molecular-weight (LMW) complexes under native conditions. However, YPrx1 was shifted to a HMW complex by H_2O_2 in the presence of a Trx system *in vitro* (Fig. 3A, lane 2). Under this condition, recombinant Srx1 time-dependently dissociated YPrx1 from higher oligomeric forms into lower oligomeric forms in an

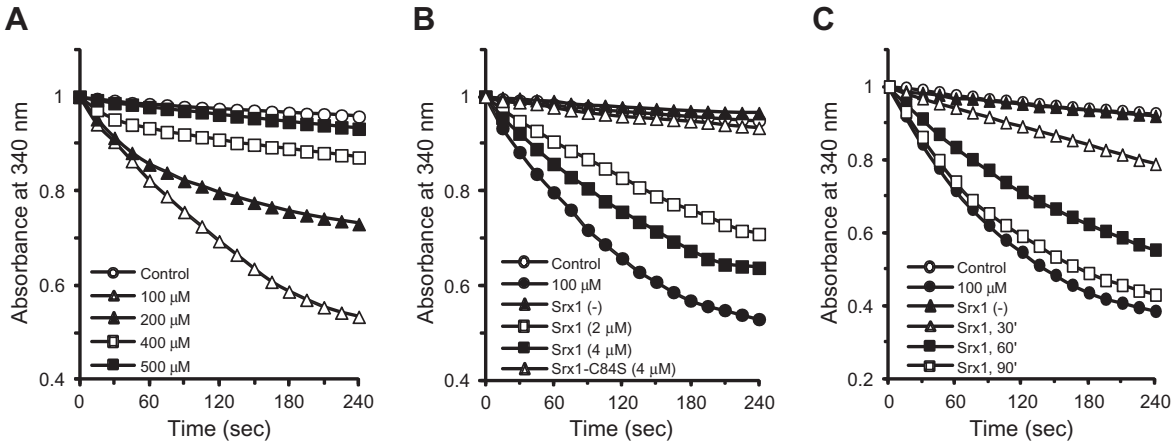

Fig. 1. Srx1 reactivates YPrx1 peroxidase activity inactivated by H_2O_2. (A) YPrx1 peroxidase activity was measured in the absence (○-○) or presence of 100 μM H_2O_2 (△-△), 200 μM H_2O_2 (▲-▲), 400 μM H_2O_2 (□-□), or 500 μM H_2O_2 (■-■) in the reaction mixtures containing NADPH, TR, and Trx. (B) YPrx1 was treated with 100 μM H_2O_2 for 10 min and then incubated without (▲-▲) or with 2 μM Srx1 (□-□), 4 μM Srx1 (■-■) or 4 μM Srx1-C84S (△-△) for 60 min in the presence of ATP and $MgCl_2$; YPrx1 peroxidase activity was then measured using 100 μM H_2O_2 as in panel A. (C) After 100 μM H_2O_2 treatment, YPrx1 was incubated with 4 μM Srx1 for the indicated time and peroxidase activity was measured.

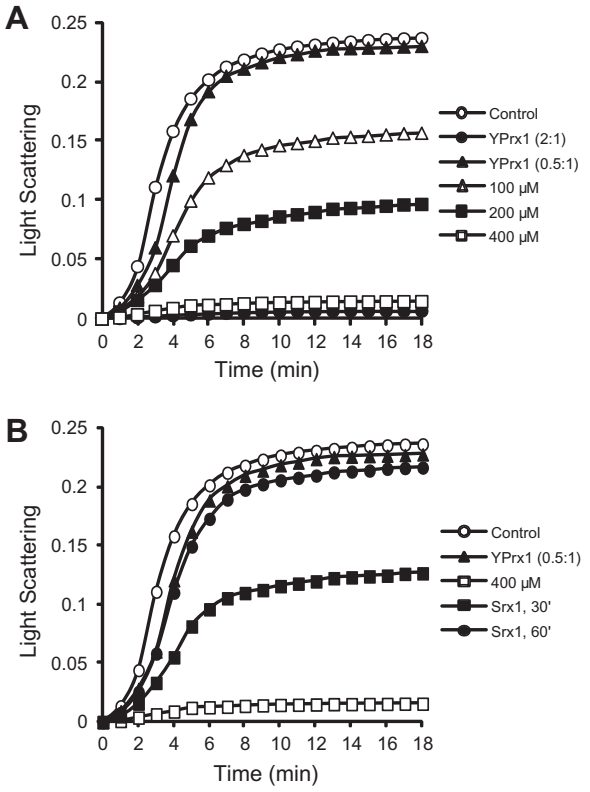

Fig. 2. Srx1 inactivates the YPrx1 chaperone activity increased by H_2O_2. YPrx1 chaperone activity was measured using MDH as substrate. (A) MDH (1 μM; ○-○) in 50 mM HEPES, pH 7.0, was incubated in a spectrophotometer at 45 °C with 0.5 μM (▲-▲) or 2 μM (●-●) YPrx1. YPrx1 (0.5 μM) was incubated with 100 μM (△-△), 200 μM (■-■), or 400 μM (□-□) H_2O_2 for 10 min, and then its protection against substrate aggregation was monitored by measuring light scattering at A_{360}. (B) YPrx1 (0.5 μM) was treated with 400 μM H_2O_2 for 10 min and then incubated with Srx1 for 30 min (■-■) or 60 min (●-●) in the presence of ATP and $MgCl_2$; YPrx1 chaperone activity was then measured.

ATP-dependent reaction (Fig. 3A, lane 3–5). In the absence of ATP and Mg^{2+}, Srx1 did not catalyze YPrx1 dissociation (Fig. 3A, lane 6). We next assessed the redox status of YPrx1 by SDS–PAGE and Western blotting using an anti-YPrx1-$SO_{2/3}$ antibody. Overoxidized YPrx1 was decreased gradually by Srx1 in a time-dependent manner (Fig. 3A, middle panel). In addition, Srx1 Cys84 was critical for dissociation of HMW forms of YPrx1 and reduction of YPrx1 overoxidation (Fig. 3B).

To confirm the dissociation of YPrx1 oligomeric structure by Srx1 *in vivo*, the oligomeric states of YPrx1 in wild-type cells were compared with those of Δ*srx1* cells and Δ*srx1* cells carrying wild type Srx1 or Srx1-C84S (Fig. 3C). In wild-type yeast, YPrx1 oligomerization from LMW to HMW complexes by H_2O_2-induced overoxidation is reversibly catalyzed by Srx1. However, HMW forms of YPrx1 were not dissociated in Δ*srx1* yeast cells, indicating that Srx1 induces a YPrx1 structural change from HMW to LMW complexes. To further confirm these findings, we determined the YPrx1 oligomeric states in Δ*srx1* cells expressing wild-type Srx1 or Srx1-C84S. The dissociation of YPrx1 HMW complexes was detected in Δ*srx1* cells complemented with wild-type Srx1, but not Srx1-C84S. These results suggest that Srx1 catalyzes not only the dissociation of a HMW complex, but also that reversal of YPrx1 overoxidation and the Srx1 Cys84 residue are essential for these processes.

3.4. Srx1 interacts with YPrx1 in yeast cells

Srx1 was initially found in yeast as a YPrx1-interacting protein [18]. Thus, we explored the interaction between Srx1 and YPrx1 *in vivo* using a BiFC technique. Two DNA constructs, one encoding Srx1 fused to the N-terminal fragment of YFP (Srx1-YN) and the other encoding YPrx1 fused to the C-terminal fragment (YPrx1-YC), were expressed under the control of the Gal1 promoter in yeast cells. As shown in Fig. 4A, expression of Srx1-YN and YPrx1-YC proteins was induced by galactose. Complementation of the two YFP halves revealed that Srx1 interacts with YPrx1 protein, as indicated by the significant proportion of YFP-positive fluorescent cells (Fig. 4B). No fluorescence was observed in cells that expressed either Srx1-YN or YPrx1-YC. These results in living cells show that Srx1 interacts directly with YPrx1, which is in accordance with the immunoprecipitation findings [18].

3.5. Summary

Here we demonstrated that YPrx1 functional and structural changes are regulated directly by Srx1. YPrx1 peroxidase activity inactivated by H_2O_2 was reactivated by Srx1 in a concentration- and time-dependent manner. Furthermore, the YPrx1 chaperone activity increased by H_2O_2 was reduced by Srx1. The YPrx1 HMW complex induced by H_2O_2 was dissociated by Srx1 *in vitro* and

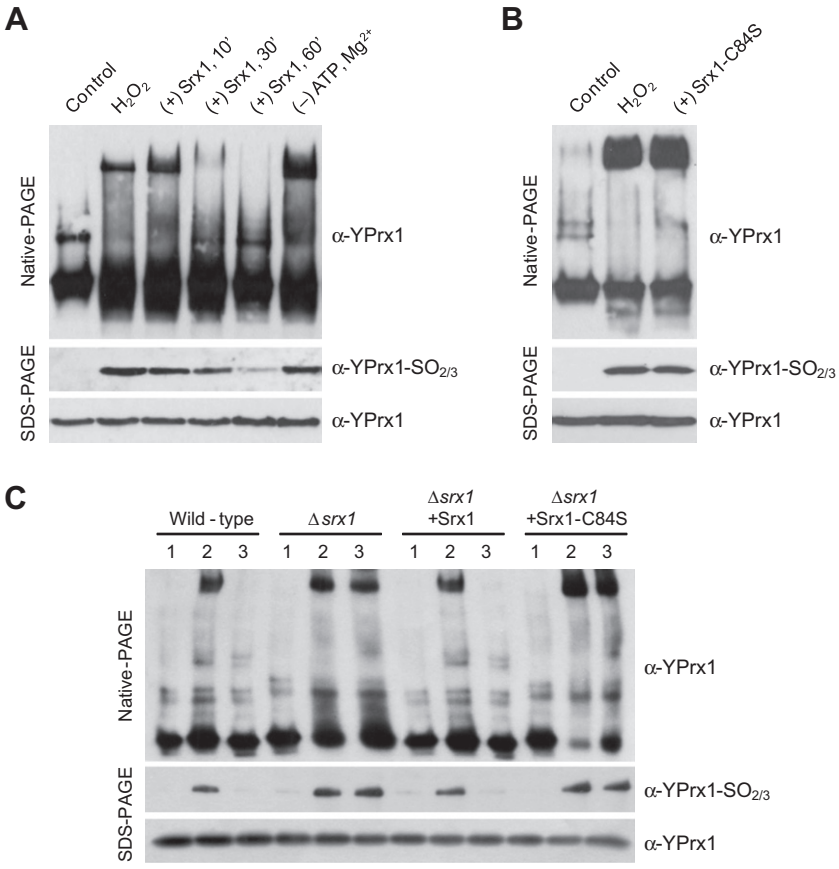

Fig. 3. Srx1 dissociates the H_2O_2-mediated HMW YPrx1 complex. (A) Recombinant YPrx1 proteins were treated without (lane 1) or with (lane 2) H_2O_2 for 10 min in the presence of a Trx system. Then, the proteins were incubated with Srx1 for the indicated time in the presence (lane 3–5) or absence of ATP and $MgCl_2$ (lane 6). YPrx1 structural changes and redox status were assessed by immunoblotting with anti-YPrx1 and anti-YPrx1-$SO_{2/3}$ antibodies, respectively. (B) YPrx1 was treated with H_2O_2 for 10 min and then incubated with Srx1-C84S for 60 min. YPrx1 structural changes and redox states are shown in panel A. (C) Yeast cells were grown to exponential phase and divided into three equal portions. The cells were treated without (lane 1) or with 0.3 mM H_2O_2 (lane 2) for 10 min, and then incubated in fresh H_2O_2-free culture medium for 30 min (lane 3). Crude proteins extracted from wild type, Δ*srx1*, and Δ*srx1* cells expressing wild-type Srx1 or Srx1-C84S were separated on native- and SDS–PAGE and subjected to immunoblotting with anti-YPrx1 and anti-YPrx1-$SO_{2/3}$ antibodies.

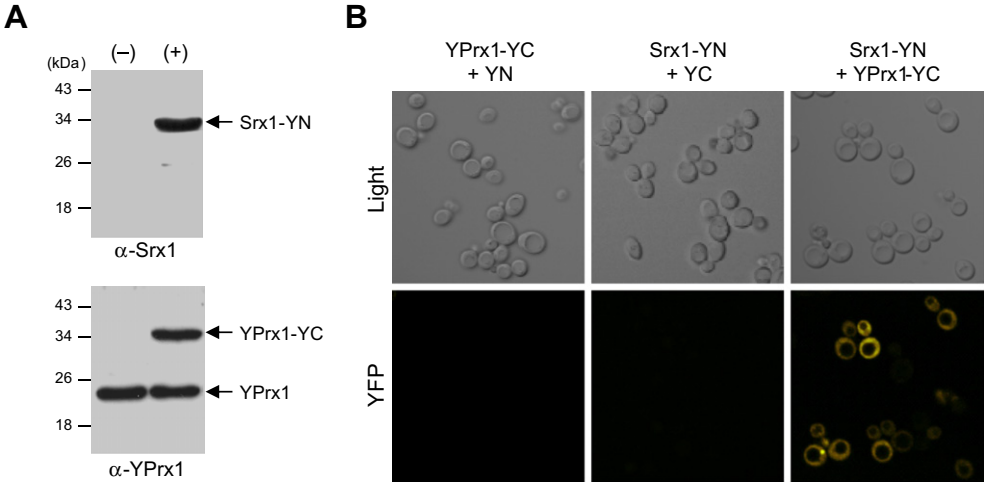

Fig. 4. Srx1 interacts directly with YPrx1 in yeast cells. (A) After exponential growth of yeast cells harboring Srx1 fused with the YFP N-terminal half domain (Srx1-YN), and YPrx1 fused with the YFP C-terminal half domain (YPrx1-YC), proteins were induced without (–) or with (+) galactose. Protein expression was assessed by immunoblotting with anti-YPrx1 and Srx1 antibodies. Detection of endogenous YPrx1 was observed in lower panel (YPrx1) but endogenous Srx1 did not detected due to its low expression level in upper panel. (B) YFP fluorescence in yeast cells harboring Srx1-YN and YPrx1-YC was visualized by confocal microscopy. Empty YC and YN vectors were used as negative controls.

in vivo. Direct interaction between Srx1 and YPrx1 in yeast cells was visualized by BiFC. Taken together, these data suggest that Srx1 regulates YPrx1 functional and structural changes through direct interaction in yeast cells.

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