Supporting Information

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SI Materials and Methods

Preparation of Antibodies to 2-Cys Prx-SO_2H. The rabbit antibodies were prepared according to the procedure described before (1). Briefly, a peptide (DFTFVCPTEI), which corresponds to the active site of mammalian 2-Cys Prx (Prx I to IV), was oxidized by performic acid. The oxidized peptide was then purified by HPLCy on a Vydac C18 column. The major peak was subjected to analysis with a MALDI-TOF mass spectrometer to confirm the sulfonic oxidation state of the peptide. The sulfonylated Prx peptide (2 mg) was coupled to 10 mg of keyhole limpet hemocyanin (Pierce) using glutaraldehyde. The peptide-hemocyanin conjugate was mixed with incomplete Freund's adjuvant for the initial injection and with complete Freund's adjuvant for booster injections. After the initial injection with 1 mg of peptide, rabbits were subjected to two booster injections, each of 500 μg of peptide, administered (at multiple s.c. sites) at 4-wk intervals. Antisera were collected 1 wk after the second booster injection, and the IgG fraction was precipitated with 50% (wt/vol) ammonium sulfate. Antibodies that recognized the nonoxidized Prx peptide were removed by treating the IgG fraction with the thiol peptide coupled to Affi-Gel-15 affinity resin (Bio-Rad).

Purification of the 20S Proteasome Complex. The 20S proteasome complex was purified from 20 mL of mouse RBCs according to a modified version of a previously described method (2). In brief, RBCs were washed with PBS and then subjected to hypotonic lysis. The lysate was centrifuged at 35,000 × *g* for 1 h at 4 °C, and the resulting supernatant was diluted fivefold with a solution containing 20 mM Tris·HCl (pH 7.6), 1 mM DTT, and 1 mM $MgCl_2$ before centrifugation at 35,000 × *g* for 2 h at 4 °C. The resulting pellet was resuspended in 100 μL of a solution containing 30 mM Hepes-NaOH (pH 7.8), 75 mM NaCl, 2.5 mM DTT, and 7.5% (vol/vol) glycerol, and was then applied to a Superose 6 column (1 cm by 50 cm) that had been equilibrated with a solution containing 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, and 10% (vol/vol) glycerol. Proteins were eluted from the column at a flow rate of 0.12 mL/min for 120 min. Fractions (1.8 mL) were collected and assayed for 20S proteasome activity with the Suc-LLVY-AMC fluorogenic substrate of an assay kit. Fractions corresponding to the 20S proteasome peak (0.2 mg of protein eluting between 5.7 and 14.4 min) were pooled, divided into portions, and stored at –80 °C until use.

Degradation of PrxII–SO_2H by the 20S Proteasome. PrxII hyperoxidized to near completion was obtained by incubation of purified recombinant human PrxII (200 μg) in a 200-μL reaction mixture containing 20 μg of recombinant yeast thioredoxin 1, 1 mM EDTA, 10 mM DTT, 1 mM H_2O_2, and 50 mM Tris·HCl (pH 7.6), as described previously (3). DTT was included in the reaction mixture because it can support the redox cycle of thioredoxin in the absence of thioredoxin reductase and NADPH. To test the reactivity of the 20S proteasome toward hyperoxidized and nonhyperoxidized PrxII, we incubated an equal mixture of hyperoxidized and nonhyperoxidized PrxII (20 μg each) at 37 °C with 50 μL of the purified 20S proteasome fraction in a final volume of 200 μL containing 20S proteasome assay buffer with or without proteasome inhibitors (25 μM MG132 and 25 μM lactacystin). Portions (40 μL) of the reaction mixture were removed at 1-h intervals for 4 h and subjected to immunoblot analysis.

1. Woo HA, et al. (2003) Reversible oxidation of the active site cysteine of peroxiredoxins to cysteine sulfinic acid. Immunoblot detection with antibodies specific for the hyperoxidized cysteine-containing sequence. *J Biol Chem* 278(48): 47361–47364.
2. Shibatani T, et al. (2006) Global organization and function of mammalian cytosolic proteasome pools: Implications for PA28 and 19S regulatory complexes. *Mol Biol Cell* 17(12):4962–4971.
3. Woo HA, et al. (2005) Reduction of cysteine sulfinic acid by sulfiredoxin is specific to 2-cys peroxiredoxins. *J Biol Chem* 280(5):3125–3128.

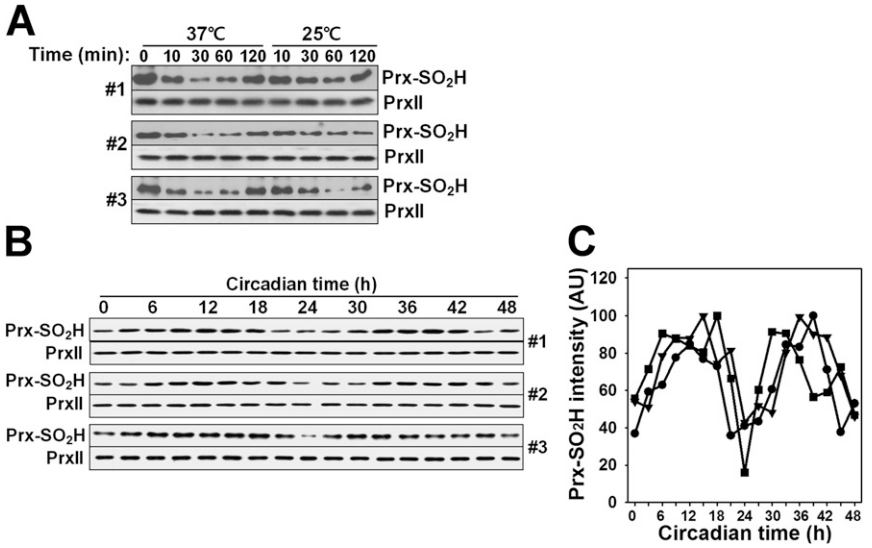

Fig. S1. Changes in the extent of Prx hyperoxidation during incubation of mouse RBCs under optimized conditions (*A*), and Circadian oscillation of Prx–SO_2H in RBCs at 32 °C (*B* and *C*). (*A*) RBCs isolated from three mice (#1, #2, and #3) were incubated in modified DMEM in the dark at 37 °C or 25 °C and under room atmosphere. At the indicated times, the cells were subjected to immunoblot analysis with antibodies to the sulfinic form of 2-Cys Prxs and to PrxII. (*B* and *C*) RBCs isolated from three mice (#1, #2, and #3) were incubated and analyzed for Prx hyperoxidation as in Fig. 1, with the exception that the incubation was performed at 32 °C.

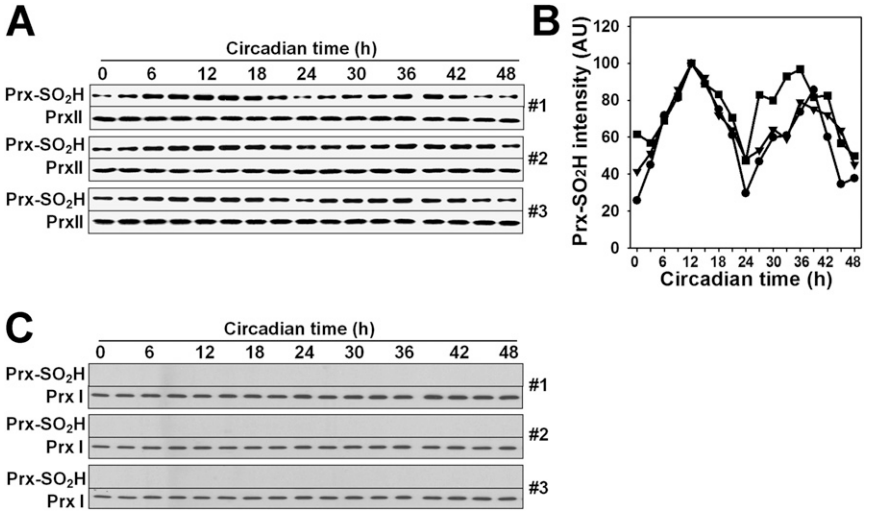

Fig. S2. Analysis of circadian oscillation of Prx–SO_2H in RBCs from $PrxI^{-/-}$ or $PrxII^{-/-}$ mice. (*A* and *B*) RBCs isolated from three $PrxI^{-/-}$ mice (#1, #2, and #3) were incubated and analyzed for Prx hyperoxidation as in Fig. 1. (*C*) RBCs isolated from three $PrxII^{-/-}$ mice (#1, #2, and #3) were also incubated and analyzed as in Fig. 1*A*, with the exception that antibodies to PrxI were used instead of those to PrxII.

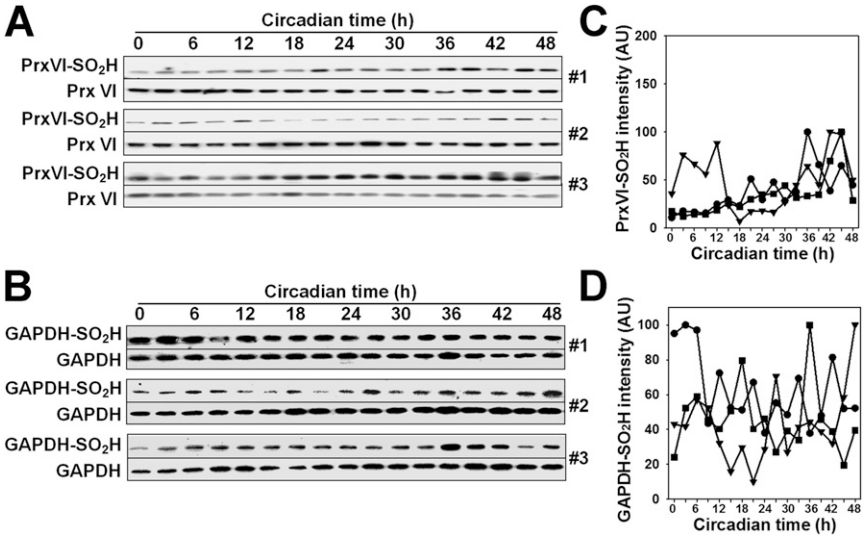

Fig. S3. Hyperoxidation state of PrxVI and GAPDH in RBCs incubated under conditions permissive for circadian oscillation of PrxII–SO_2H. RBCs isolated from three mice (#1, #2, and #3) were incubated and analyzed as in Fig. 1*A*, with the exception that immunoblot analysis was performed with antibodies to PrxVI–SO_2H and to PrxVI (*A*) or to GAPDH–SO_2H and to GAPDH (*B*). (*C* and *D*) The intensities of the PrxVI–SO_2H in *A* and GAPDH–SO_2H bands in *B* were normalized by that of the corresponding PrxVI and GAPDH bands, respectively, and then expressed in arbitrary units (AU) relative to the corresponding maximum value, with ●, ▼, and ■ representing mice #1, #2, and #3, respectively.

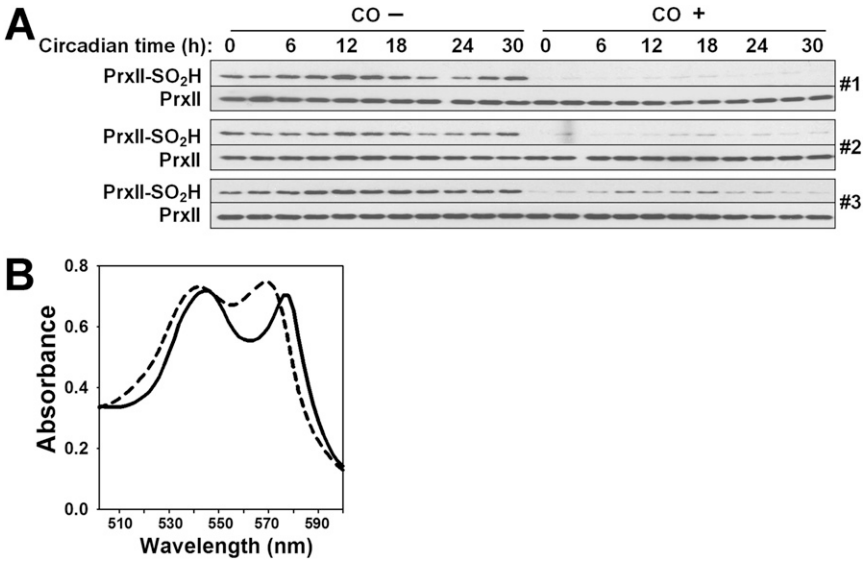

Fig. S4. Effect of CO on PrxII hyperoxidation in RBCs. (*A*) RBCs isolated from three mice (#1, #2, and #3) were suspended in modified DMEM and exposed to 100% CO or room air for 30 min before incubation and analysis of PrxII hyperoxidation as in Fig. 1*A*. Immunoblot intensities of the PrxII–SO_2H bands are shown in Fig. 2. (*B*) Absorption spectra for RBCs from mouse #1 in *A* exposed to CO (dotted line) or to room air (solid line), showing the distinct spectra of carboxyhemoglobin and oxyhemoglobin, respectively.

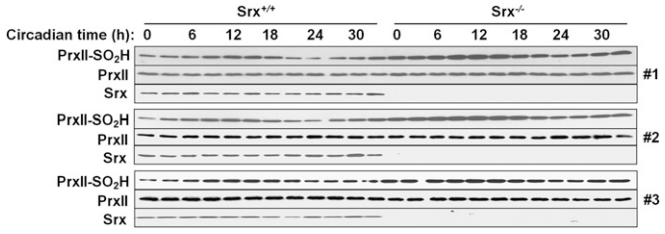

Fig. S5. Comparison of circadian oscillation of PrxII–SO_2H in RBCs from $Srx^{+/+}$ or $Srx^{-/-}$ mice. RBCs isolated from three $Srx^{+/+}$ or $Srx^{-/-}$ mice (#1, #2, and #3) were incubated and analyzed for PrxII hyperoxidation as in Fig. 1*A*. The blots were also probed with antibodies to Srx. Immunoblot intensities of the PrxII–SO_2H bands are shown in Fig. 3.

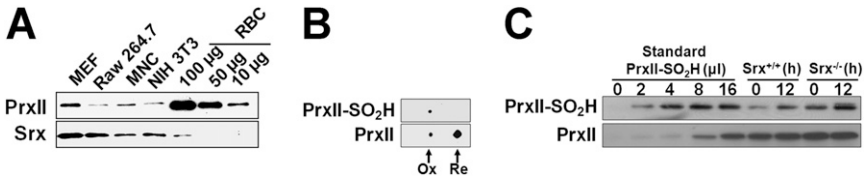

Fig. S6. Comparison of the amounts of PrxII and Srx in mouse RBCs with those in mouse embryonic fibroblasts (MEFs), RAW 264.7 cells, NIH 3T3 cells, and MNCs (*A*) and measurement of the extent of PrxII hyperoxidation in RBCs (*B* and *C*). (*A*) Lysates (100 μg of protein) of MEFs, RAW 264.7 cells, mouse MNCs, and NIH 3T3 cells as well as the indicated amounts of mouse RBC lysates were subjected to immunoblot analysis with antibodies to PrxII and to Srx. (*B*) A lysate of RBCs that had been exposed to an H_2O_2-generating medium (containing glucose plus glucose oxidase) was subjected to 2D PAGE followed by immunoblot analysis with antibodies to the sulfinic form of 2-Cys Prxs and to PrxII. Determination of the intensity of the hyperoxidized (Ox) and reduced (Re) PrxII spots indicated that ~10% of PrxII in the lysate was hyperoxidized. This lysate was then used as a standard for measurement of PrxII hyperoxidation. (*C*) Lysates (4 μL) of RBCs that had been isolated from $Srx^{+/+}$ or $Srx^{-/-}$ mice and incubated for 0 or 12 h as in Fig. 1, together with the indicated amounts of the standard RBC lysate, were subjected to immunoblot analysis with antibodies to the sulfinic form of 2-Cys Prxs and to PrxII. Comparison of the immunoblot intensities for $Srx^{+/+}$ or $Srx^{-/-}$ RBC lysates with those for the standard lysate indicated that ~0.7% and ~1.6% of PrxII in $Srx^{+/+}$ RBCs was hyperoxidized at 0 and 12 h, respectively, and that ~1.7% and ~2.8% of PrxII in $Srx^{-/-}$ RBCs was hyperoxidized at 0 and 12 h, respectively.

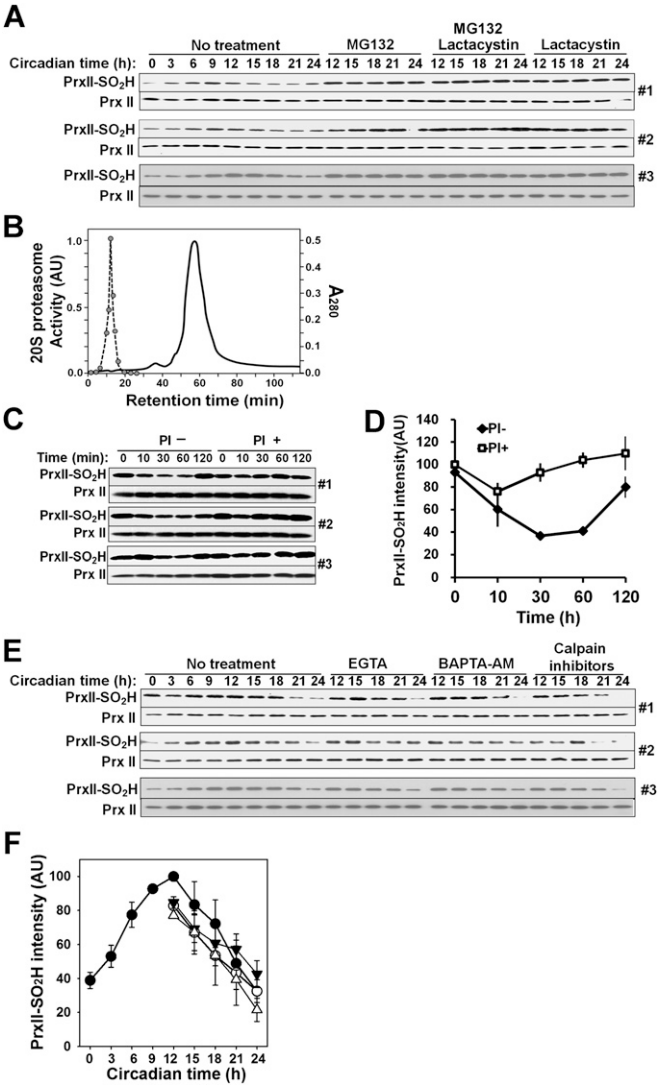

Fig. S7. Prevention of the decay phase of PrxII–SO_2H oscillation in RBCs by proteasome inhibitors (*A*), purification of a 20S proteasome fraction from mouse RBCs (*B*), effects of proteasome inhibitors on the gradual decrease of Prx II-SO_2H observed during the first 30 min incubation in the modified DMEM (*C* and *D*), and effects of the inhibition of Ca^{2+}-dependent proteases on circadian oscillation of PrxII–SO_2H in RBCs (*E* and *F*). (*A*) RBCs from three mice (#1, #2, and #3) were incubated and analyzed as in Fig. 1*A*, with the exception that the cells were incubated in the absence or presence of the proteasome inhibitors MG132 or lactacystin, or both agents, each at a final concentration of 25 μM, beginning at circadian time 9.5 h. Immunoblot intensities of the PrxII–SO_2H bands are shown in Fig. 4*A*. (*B*) RBC proteins were subjected to gel filtration chromatography, protein elution was monitored by measurement of absorbance at 280 nm (A_{280}, solid line), and the resulting fractions were assayed for 20S proteasome activity (dashed line). (*C* and *D*) RBCs from three mice (#1, #2, and #3) were incubated in modified DMEM in the dark at 37 °C in the absence or presence of MG132 and lactacystin and analyzed as Fig. 1. Data in *D* are means ± SD for cells from the three mice (*C* and *D*). (*E* and *F*) RBCs from three mice (#1, #2, and #3) were incubated and analyzed as in Fig. 1, with the exception that the cells were incubated in the absence (●) or presence of 1 mM EGTA (▼), 25 μM 1,2-bis (*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) (○), or a mixture of 25 μM each of calpain inhibitor I and calpain inhibitor II (△) (BioVision) beginning at circadian time 9.5 h. Data in *F* are means ± SD for cells from the three mice.

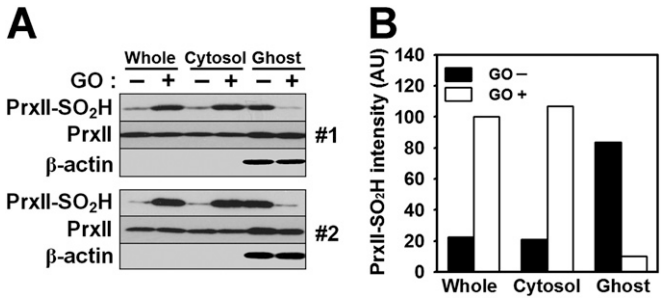

Fig. S8. Effect of external H_2O_2 on the abundance of ghost membrane-associated PrxII–SO_2H. (*A*) RBCs isolated from two mice (#1 and #2) were incubated in modified DMEM in the absence or presence of 1 mU of glucose oxidase (GO) for 1 h at 37 °C, after which the cells were separated by centrifugation, lysed, and fractionated into cytosolic and ghost membrane fractions for immunoblot analysis with antibodies to the sulfinic form of 2-Cys Prxs, to PrxII, and to β-actin (ghost marker). The volume of samples loaded onto the gel was adjusted to represent a cell ratio of 1:1:30 for the whole lysate, cytosolic fraction, and ghost membranes, respectively. (*B*) Immunoblot intensities of PrxII–SO_2H bands in *A* were normalized by the corresponding value for the whole lysate of cells incubated with glucose oxidase, are expressed in arbitrary units, and are means of values from the two mice.

Table S1. Comparison of experimental conditions used for the study by O'Neil and Reddy (1) and current study

Experimental condition	Study by O'Neil and Reddy	Current study
RBC source	human	mouse
RBC concentration	unknown number of cells/100 μL medium	1.5×10^7 cells/50 μL medium
Culture tube	0.2 mL PCR tube (Thermal)	1.5 mL tube (Eppendorf)
Incubation media	Krebs–Hanseleit based media (mg/L) osmolarity 280 mOsm/L; pH 7.4; D-glucose 2000; $MgSO_4$ (anhydrous) 141; KH_2PO_4 160; KCl 350; NaCl 6900; $CaCl_2$ (anhydrous) 373; $NaHCO_3$ 2100	DMEM based media (mg/L) osmolarity 330 mOsm/L; pH 7.3; D-glucose 4500; $MgSO_4$ (anhydrous) 96.67; $NaH_2PO_4{\cdot}H_2O$ 125; KCl 400; NaCl 6400; $CaCl_2$ (anhydrous) 200; $NaHCO_3$ 3700; sodium pyruvate 110; 20 amino acids and micronutrients
Incubator	37 °C at a thermal cycler (Bio-Rad Tetrad)	37 °C temperature controlled incubator under an atmosphere of 5% CO_2 and 20% O_2 in air
Entrainment	Temperature cycles of 12 h at 32 °C, 12 h at 37 °C	media change
Immunoblot analysis	nonreducing gels	reducing gels
Oligomeric state of sulfinic Prx detected by immunoblot analysis	mainly dimeric form of Prx	monomeric form of Prx II

1. O'Neill JS, Reddy AB (2011) Circadian clocks in human red blood cells. *Nature* 469(7331):498–503.