Supplementary Information

Materials and methods

Materials. Antibodies and their sources were as follows: anti-caveolin-1 IgG (pAb N-20), anti-TrxR1 IgG (pAb) and anti-Trx1 IgG (pAb FL-105) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA-tag IgG (mAb HA.11) was from Covance (Emeryville, CA). Anti-6XHis-tag IgG (mAb) was from Clontech (Mountain View, CA). All other biochemicals used were of the highest purity available and were obtained from regular commercial sources.

Cell culture, oxidative stress, and transient transfection. WI38 cells (from ATCC) were grown in Minimum Essential Eagle's Medium (MEM) supplemented with glutamine, antibiotics (penicillin and streptomycin) and 10% fetal bovine serum. NIH 3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine, antibiotics (penicillin and streptomycin) and 10% donor bovine calf serum. A549 cells were grown in RPMI-1640 supplemented with glutamine, antibiotics (penicillin and streptomycin) and 10% fetal bovine serum. Oxidative stress was induced by subcytotoxic levels of hydrogen peroxide (150μM) for 2 hours. Cells were then recovered in normal medium for different periods of time (see text for details). 3T3 cells (30-50% confluent) were transfected using calcium-phosphate precipitation, WI-38 and A549 cells (80% confluent) were transfected using Lipofectamine 2000 (Invitrogen).

Immunoblotting. Cells were collected in boiling sample buffer. Cellular proteins were resolved by SDS-PAGE (12.5% acrylamide) and transferred to BA83 nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Blots were incubated for 2 h in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 2% powdered skim milk and 1% bovine serum albumin (BSA). After three washes with TBST, membranes were incubated for 2 h with the primary antibody and for 1 h with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG. Bound antibodies were detected using an ECL detection kit (Pierce, Rockford, IL).

Preparation of caveolae-enriched membrane fractions. Cells were scraped into 2 ml of Mes-buffered saline containing 1% (vol/vol) Triton X-100. Homogenization was carried out with 10 strokes of a loose fitting Dounce homogenizer. The homogenate was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in Mes-buffered saline and placed at the bottom of an ultracentrifuge tube. A 5-30% linear sucrose gradient was formed above the homogenate and centrifuged at 45,000 rpm for 16-20 h in a SW60 rotor (Beckman Coulter, Fullerton, CA). A light-scattering band confined to the 15–20% sucrose region was observed that contained endogenous caveolin-1 but excluded most of other cellular proteins. From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 11 fractions. Fractions 4-6, representing caveolar membranes, and fractions 9-11, representing non-caveolar membranes, were pooled together. An equal amount of protein from each of the two groups was separated by SDS-PAGE and subjected to immunoblot analysis.

Co-Immunoprecipitation. Cells were washed twice with PBS and lysed for 30 min at 4 °C in a buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, and 60 mM octyl glucoside. Samples were precleared for 1 h at 4°C using protein A-Sepharose (20 μl; slurry, 1:1) and subjected to overnight immunoprecipitation at 4 °C using the intended antibody and protein A-Sepharose (30 μl; slurry, 1:1). After three washes with the immunoprecipitation buffer, samples were separated by SDS-PAGE (12.5% acrylamide) and transferred to nitrocellulose. Then, blots were probed with the intended antibody.

Immunofluorescence Microscopy. Cells grown on glass coverslips were washed three times with PBS w/Ca^{++}/Mg^{++} and fixed for 30 min at room temperature with 2% paraformaldehyde in PBS w/Ca^{++}/Mg^{++}. Fixed cells were rinsed with PBS w/Ca^{++}/$Mg^{++}$and permeabilized with 0.1% Triton X-100, 0.2% bovine serum albumin for 10 min. Then cells were treated with 25 mM NH_4Cl in PBS w/Ca^{++}/Mg^{++} for 10 min at room temperature to quench free aldehyde groups. Cells were rinsed with PBS w/Ca^{++}/Mg^{++} and incubated with the primary antibody (diluted in PBS with 0.1% Triton X-100, 0.2% bovine serum albumin) for 2h at room temperature. After three washes with PBS w/Ca^{++}/Mg^{++} (10 min each), cells were incubated with the secondary antibody for 1h at room temperature: lissamine rhodamine B sulfonyl chloride-conjugated goat anti-rabbit antibody (5 μg/ml) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (5 μg/ ml). Finally, cells were washed three times with PBS w/Ca^{++}/Mg^{++} (10 min each wash) and slides were mounted with slow-Fade anti-fade reagent (Molecular

Probes, Inc., Eugene, OR) and observed using a Zeiss Confocal Microscope (LSM 5 Pascal).

GST fusion proteins pull-down assay. The GST-caveolin-1 (GST-Cav-1) fusion protein constructs were transformed into *Escherichia coli* (BL21 strain; Novagen, Inc.). After induction of expression through addition of 5 mM isopropyl-β-D-galactoside (Sigma), GST-Cav-1 constructs were affinity purified on glutathione-agarose beads, using the detergent Sarcosyl for initial solubilization. GST-Cav-1 and GST alone (bound to glutathione-agarose beads) were washed 3 times with TNET buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing protease inhibitors. SDS-PAGE followed by Comassie staining was used to determine the concentration of GST-Cav-1 per 100 μl of packed bead volume. Pre-cleared cell lysates were diluted in buffer A (10 mM Tris, pH 8.0, 0.1% Tween 20) and added to approximately 100 μl of equalized bead volume for overnight incubation at 4 °C. After binding, the beads were extensively washed with phosphate-buffered saline (6 times). Finally, the beads were resuspended in 3X sample buffer and subjected to SDS-PAGE.

Direct binding assays. WT-TrxR1 and ϕ→A-TrxR1 were cloned into the pET-22b(+) vector (from Novagen) to generate WT-TrxR1-His and ϕ→A-TrxR1-His. NIH 3T3 cells were transiently transfected with WT-TrxR1-His and ϕ→A-TrxR1-His. WT-TrxR1-His and ϕ→A-TrxR1-His were affinity purified from cell lysates using Ni-NTA agarose beads. Affinity-purified WT-TrxR1-His and ϕ→A-TrxR1-His were then incubated with affinity-purified GST alone or GST fused to residues 82-101 (Cav-1 (82-101)-GST)

immobilized on glutathione-agarose beads. The beads were then subjected to immunoblotting analysis with anti-6xHis IgGs to detect TrxR1 binding.

Establishment of NIH 3T3 cell lines stably overexpressing WT and mutant TrxR1. NIH 3T3 cells were co-transfected with either pCMV-WT-TrxR1-HA or pCMV-Φ→A-TrxR1-HA and with a plasmid containing hygromycin resistance (pCB7) using a modified calcium phosphate precipitation protocol. NIH 3T3 cells were also transfected with the empty vector pCMV-HA as a control. Resistant clones were selected using hygromycin B (200 μg/ml). Individual clones were isolated using cloning rings. Stably transfected NIH 3T3 cells were screened by immunoblot analysis using anti-HA IgGs.

Acid β-galactosidase staining. Cells were subjected to acid β-galactosidase staining using the Senescence-Associated β-galactosidase Staining Kit (Cell Signaling), according to the manufacturer's recommendations. Briefly, cells were washed twice with PBS and fixed with the fixative solution for 15 minutes. Then, cells were washed twice with PBS and incubated overnight at 37^{o}C with the staining solution. Cells were then examined for the development of blue color. Cells were photographed at low magnification (x10) using a BX50WI Olympus Optical light microscope (Tokyo, Japan).

Intracellular ROS measurement. ROS levels were monitored using carboxy-2',7'-dichlorodihydro-fluorescein diacetate (carboxy-H_2DCFDA) (Molecular Probe, Eugene, OR). H_2DCFDA is converted into carboxy-H_2DCF, a non-fluorescent derivate, by intracellular esterase and is trapped within the cell. Carboxy-H_2DCF is oxidized to the

fluorescent derivate carboxy-DCF by intracellular ROS. After the indicated treatment, cells were washed with PBS and incubated with 5μM carboxy-H_2DCFDA in PBS for 1 hour at 37^{o}C. Cells were then washed twice with PBS, made into single cell suspension and analyzed by flow cytometry. Values were expressed as the mean carboxy-DCF fluorescence intensity ± S.E.M. from three independent experiments.

Supplemental Figure 1. *In vitro* binding assays.

(A) *Co-immunoprecipitation studies*. Cell lysates of WI-38 cells were immunoprecipitated with a polyclonal anti-caveolin-1 antibody. Then, immunoprecipitates were subjected to SDS-PAGE and immunoblotting analysis using a monoclonal antibody probe specific for TrxR1. Total expression of caveolin-1 and TrxR1 before immunoprecipitation (TOT) is shown in the two lower panels.

(B) *Expression of HA-tagged TrxR1*. TrxR1 was cloned into the expression vector pCMV-HA (from Clontech). TrxR1-HA was transiently transfected in NIH 3T3 cells and its expression detected by immunoblotting analysis using anti-HA IgGs.

(C) *Generation and expression of caveolin-1 deletion mutants fused to GST*. Amino acids 1-101 and 82-101 of caveolin-1 were fused to GST. After expression in bacteria, affinity-purified GST alone or GST-caveolin-1 deletion mutants were subjected to SDS-PAGE and the gel was stained with Ponceau S.

(D) and (E) *Generation and expression of Φ→A TrxR1 mutant*. (D) Residues 454-463 of wild type human TrxR1 (WT-TrxR1) is shown in the upper lane. A TrxR1 mutant (ϕ→A-TrxR1) in which the five aromatic residues were mutated to alanines is shown in

the lower lane. (E) HA-tagged wild type TrxR1 (WT-TrxR1-HA) and ϕ→A-TrxR1-HA were transiently transfected into NIH 3T3 cells. Forty eight hours after transfection, cell lysates were subjected to immunoblotting analysis using anti-HA IgGs.

Supplemental Figure 2. Inhibition of TrxR activity by caveolin-1.

(A) and (B) *Over-expression of caveolin-1 and measurement of TrxR activity in 3T3 cells*. NIH 3T3 cells were transiently transfected with pCAGGS-Cav-1 and pCAGGS alone. Forty eight hours after the transfection, cells were collected and the expression of caveolin-1 and TrxR1 detected by immunoblotting analysis using anti-caveolin-1 and anti-TrxR1 IgGs, respectively (A). Quantification of caveolin-1 expression is shown in (B). Values represent means ± SEM. $^{*}P<0.001$.

(C-E) *Over-expression of caveolin-1 and measurement of TrxR activity in MCF7 cells*. MCF7 breast cancer epithelial cells were infected with an adenoviral vector carrying the caveolin-1 cDNA (Ad-Cav-1). Infection with an adenoviral vector expressing GFP (Ad-GFP) was done as control. Seventy two hours after the infection, cells were collected and the expression of caveolin-1 and TrxR1 detected by immunoblotting analysis using anti-caveolin-1 and anti-TrxR1 IgGs, respectively (C). Quantification of caveolin-1 expression is shown in (D). Cells were also collected and TrxR activity measured as described in Experimental Procedures (E). Values represent means ± SEM. $^{*}P<0.001$.

(F) *Effect of disruption of caveolar membranes on TrxR activity.* NIH 3T3 cells were treated with either DMSO or methyl-β-cyclodextrin (MβC) for 48 hours. Cells were then

collected and TrxR activity measured as described in Experimental Procedures. Values represent means ± SEM. $^{*}P<0.01$.

Supplemental Figure 3. Caveolin-1 negatively regulates TrxR activity *in vivo*.

Lungs were derived from wild type (WT Lung), caveolin-1 transgenic (Cav-1 Tg Lung), and caveolin-1 null (Cav-1 KO Lung) mice. In (A), lung tissues were lysed and immunoprecipitated with an antibody probe specific for TrxR1. Immunoprecipitates were then subjected to immunoblotting analysis with anti-caveolin-1 IgGs. Total expression of TrxR1 and caveolin-1 before immunoprecipitation (TOT) is shown in the lower panels. In (B), TrxR activity was measured as described in Experimental Procedures. Values represent means ± SEM. $^{*,\#}P<0.001$.

Supplemental Figure 4. Exit of TrxR1 from caveolar membranes results in activation of TrxR enzymatic activity.

(A) *Isolation of caveolar membranes*. WI-38 cells were subjected to sub-cytotoxic oxidative stress for 2 hours. Cells were washed and cultured in normal medium for different periods of time (24, 48, and 72 hours). Untreated cells were used as control. Cells were then collected and caveolar membranes isolated by equilibrium sucrose gradient centrifugation. In this fractionation scheme, immunoblotting with anti caveolin-1 IgGs can be used to track the position of caveolae-derived membranes within this

bottom-loaded sucrose gradient. Immunoblotting with an antibody probe specific for TrxR1 was used to determine its caveolar localization.

(B) *TrxR activity assay.* WI-38 cells were treated as in (A). Cells were then collected and TrxR activity measured as described in Experimental Procedures. Values represent means ± SEM. $^{*}P<0.001$; $^{\#}P<0.005$.

Supplemental Figure 5. Effect of a mutant form of TrxR1 that can not be inhibited by caveolin-1 on TrxR activity, activation of p53 and premature senescence.

HA-tagged wild type and Φ→A mutant TrxR1 were stably expressed in NIH 3T3 cells. NIH 3T3 cells transfected with the HA expression vector only were used as controls. Cells were treated with 150μM H_2O_2 for 2 hours, washed and cultured in normal medium for the indicated period of time. Untreated cells (-H_2O_2) were used as control. In (A), TrxR activity was measured as described in Experimental Procedures. Values represent means ± SEM. $^{*,\#}P<0.001$. 100% activity corresponds to 1.5 units/ml. In (B), cells were transiently transfected with 2 μg of the luciferase reporter plasmid pTA-p53RE, which contains a p53 responsive element. Twenty four hours post-transfection, cells were treated with 150 μM H_2O_2 for 2 hours. Cells were washed and cultured in complete medium for 24 hours. Cells were then lysed and the luciferase activity was measured. Values represent means ± SEM. $^{*}P<0.005$. In (C), five days after oxidative stress, cells were subjected to senescence-associated β-galactosidase activity staining. Representative images are shown.

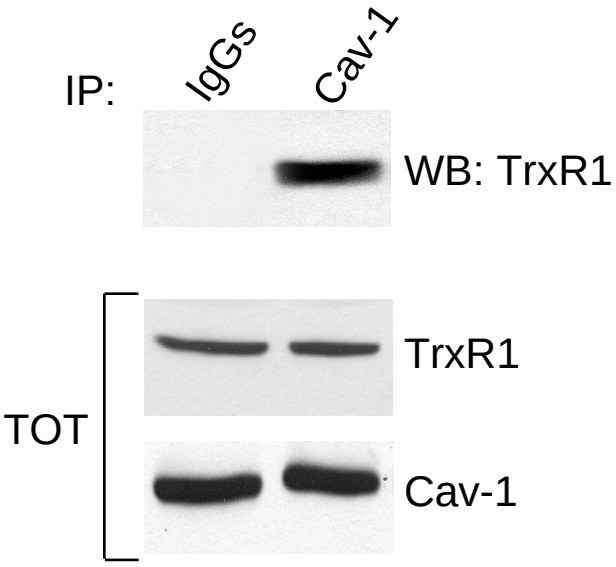
Supplemental Figure 6. Over-expression and down-regulation of caveolin-1 in A549 cells.

(A) A549 cells were stably transfected with vector alone (pCAGGS) or caveolin-1 (Cav-1). Cells were then treated with 150 μM H_2O_2 for 2 hours. Untreated cells were used as control. Cells were washed, cultured in normal medium for seven days and subjected to senescence-associated β-galactosidase activity staining. Representative images are shown.

(B) A549 cells were transfected with scrambled siRNA (CTL siRNA) or caveolin-1 siRNA (Cav-1 siRNA). Forty eight hours after transfection, cells were collected and cell lysates were subjected to immunoblotting analysis using a polyclonal antibody probe specific for caveolin-1.

Volonte et al.,
Suppl. Figure 1A, B, C

A



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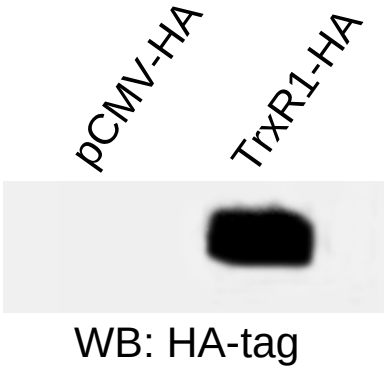

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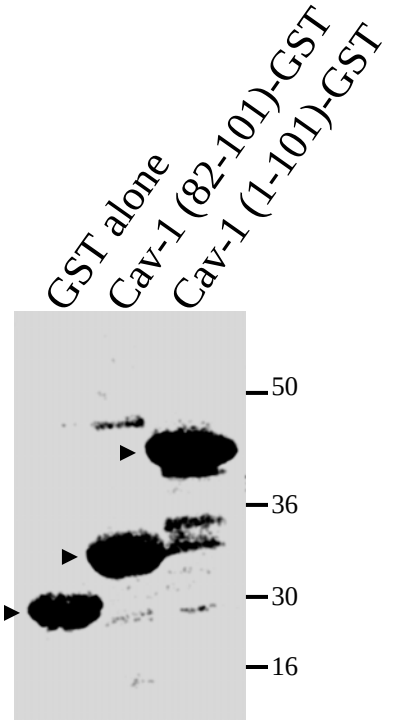


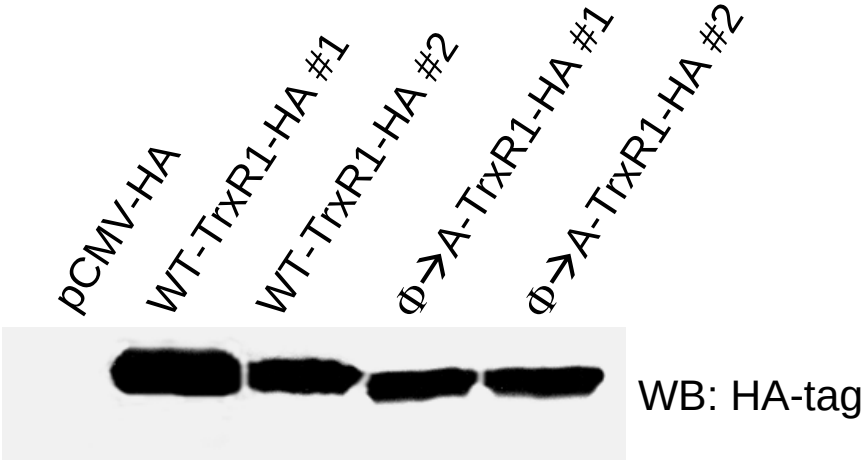
Volonte et al.,
Suppl. Figure 1D, E

D

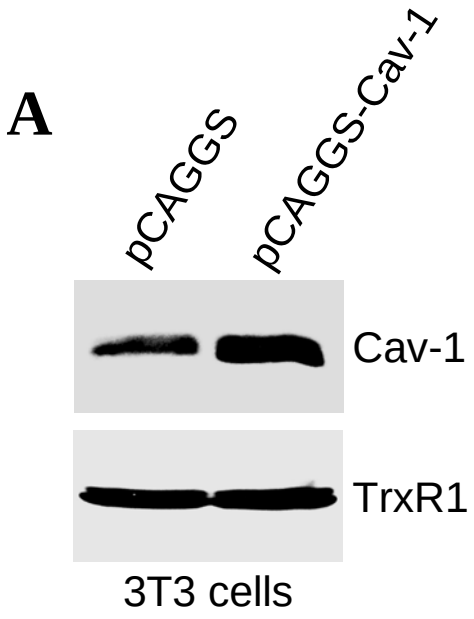
WT-TrxR1: $_{454}$**Y** (2X) **YFW** (3X) **W**$_{463}$

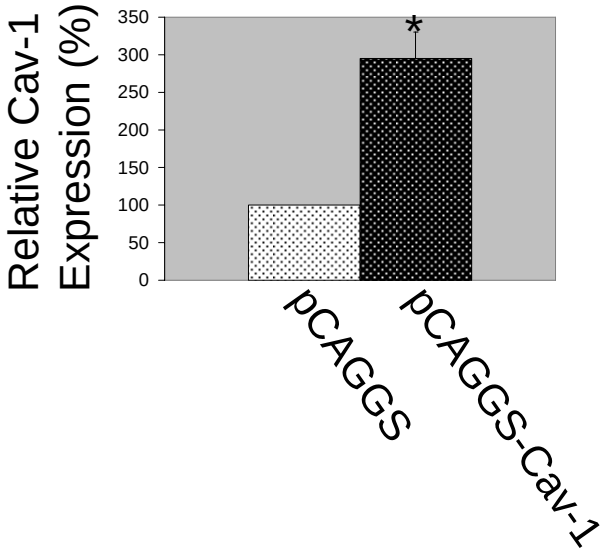
Φ→A-TrxR1: **A** (2X) **AAA** (3X) **A**

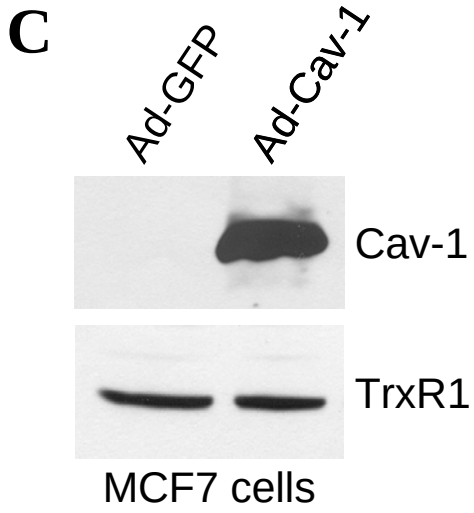
E

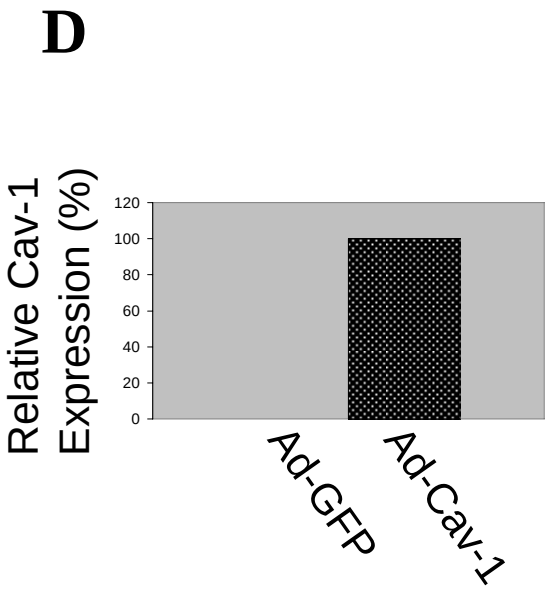


Volonte et al.,
Suppl. Figure 2A, B, C, D



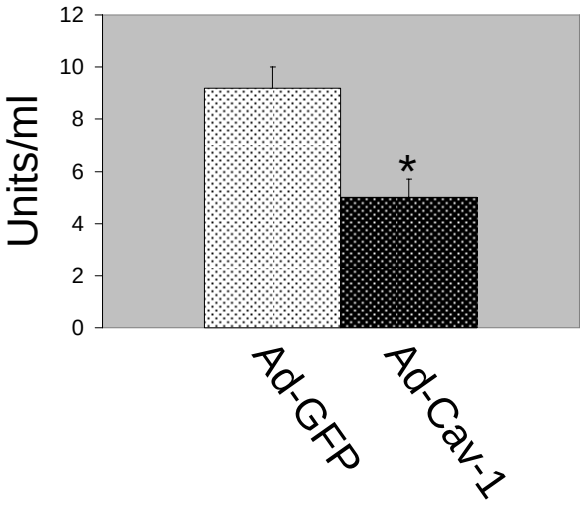






Volonte et al.,
Suppl. Figure 2E, F

E



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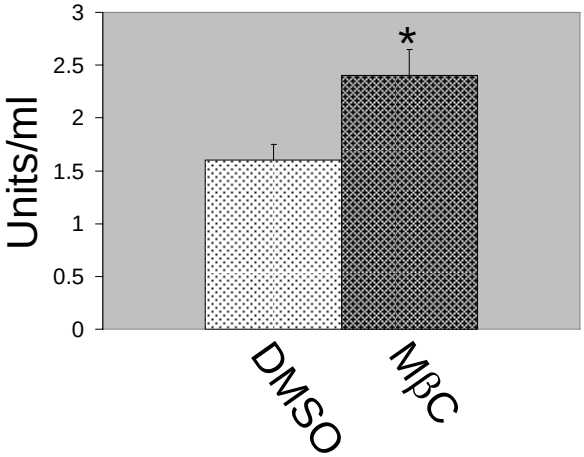
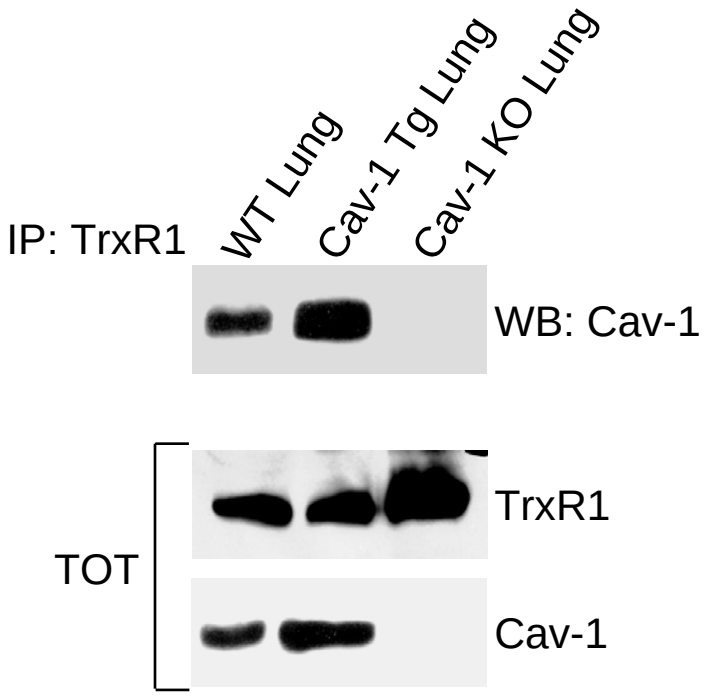


Volonte et al.,
Suppl. Figure 3A, B

A



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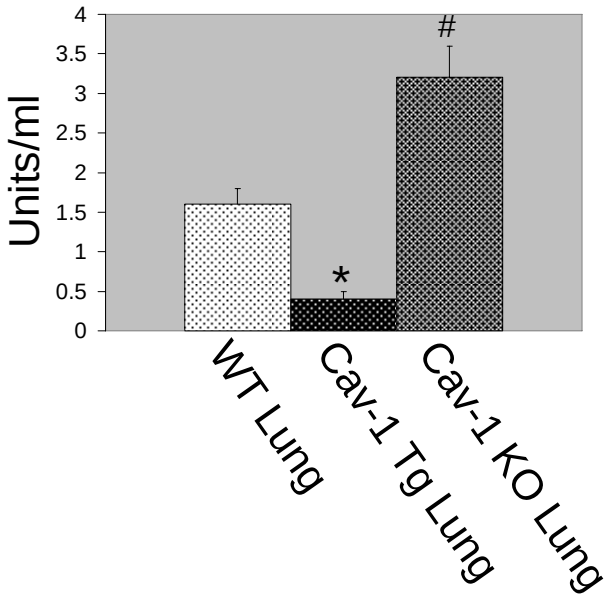
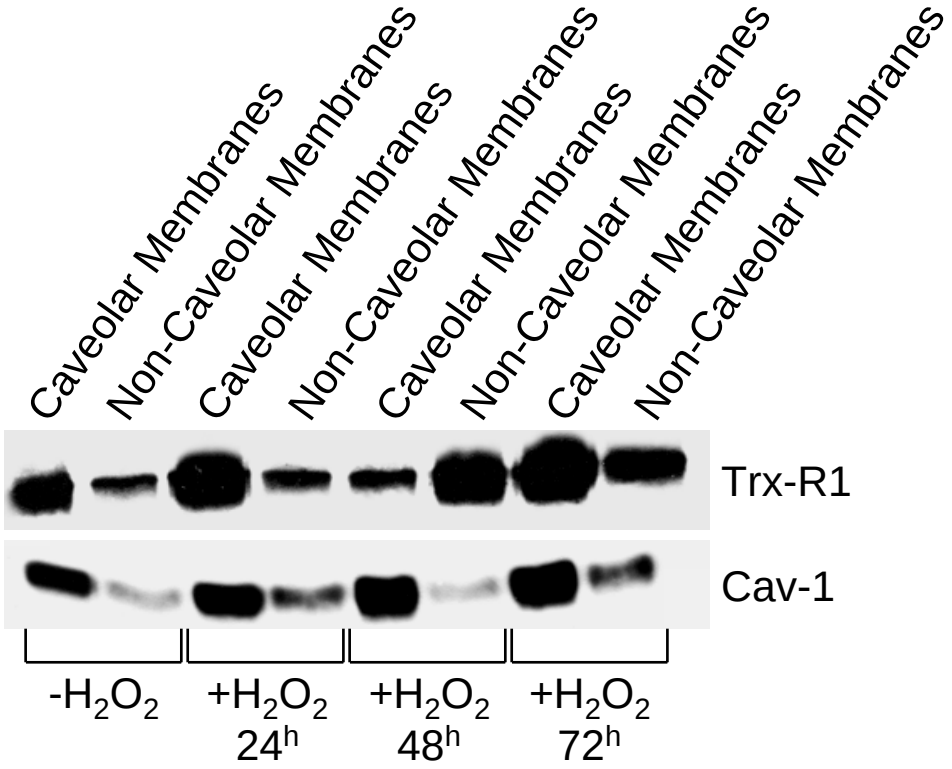


Volonte et al.,
Suppl. Figure 4A, B

A



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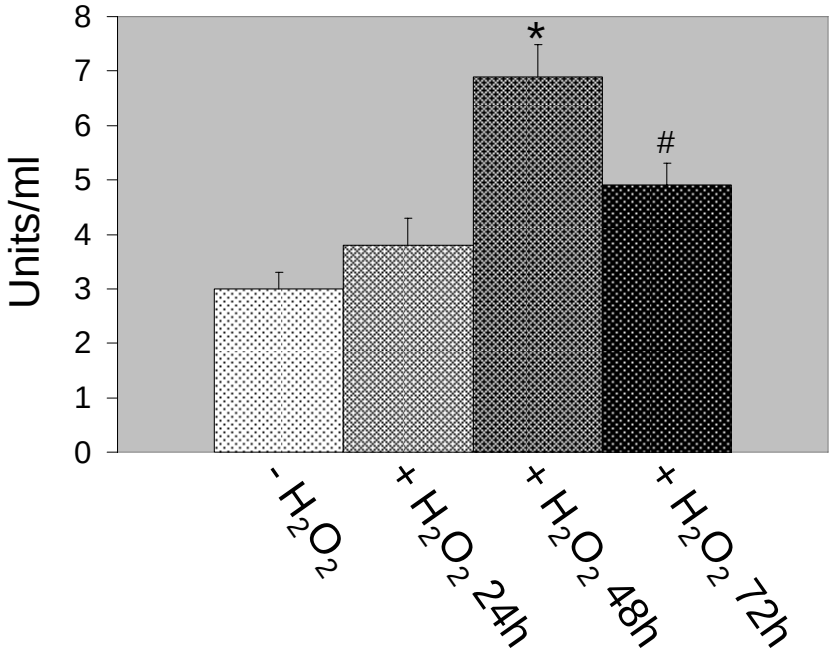
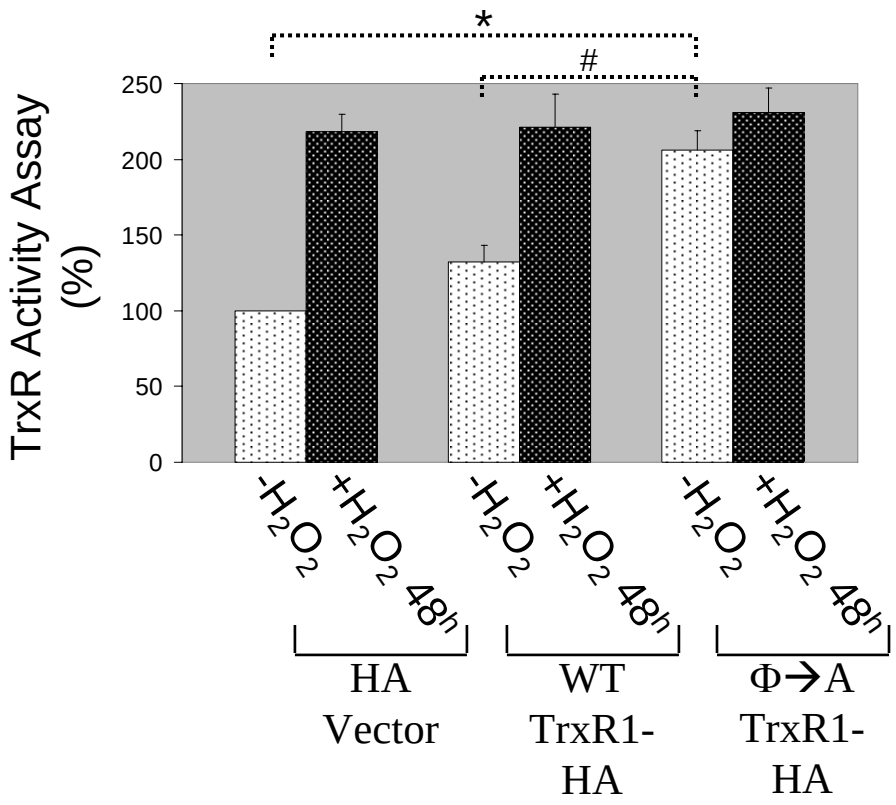


Volonte et al.,
Suppl. Figure 5A, B

A



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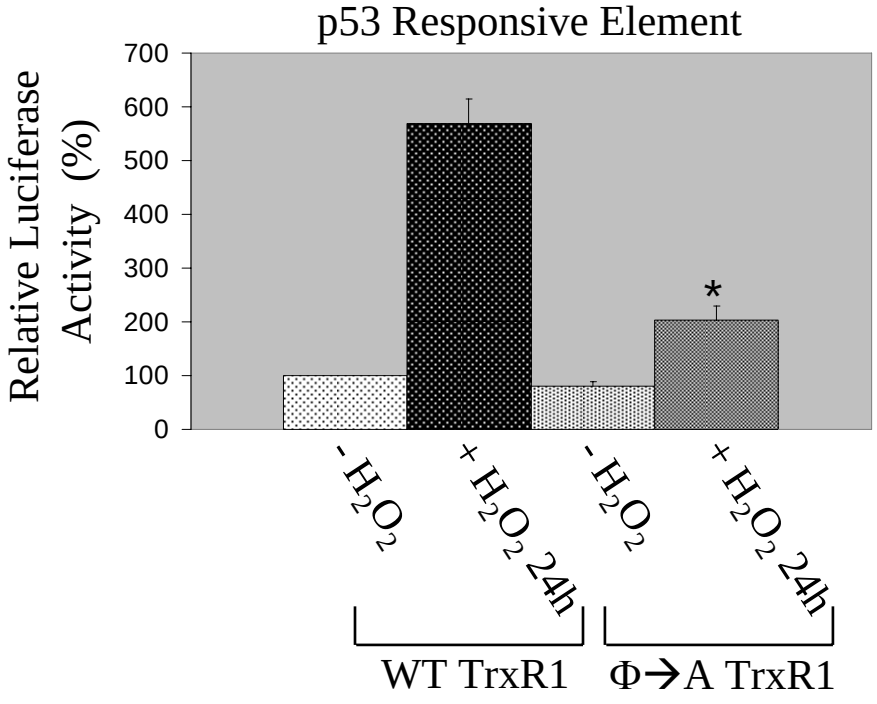
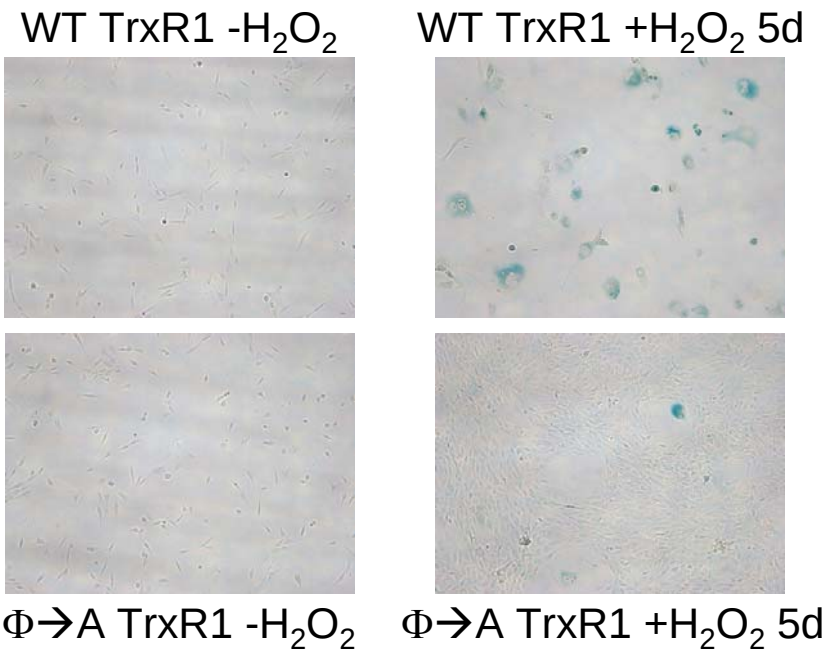


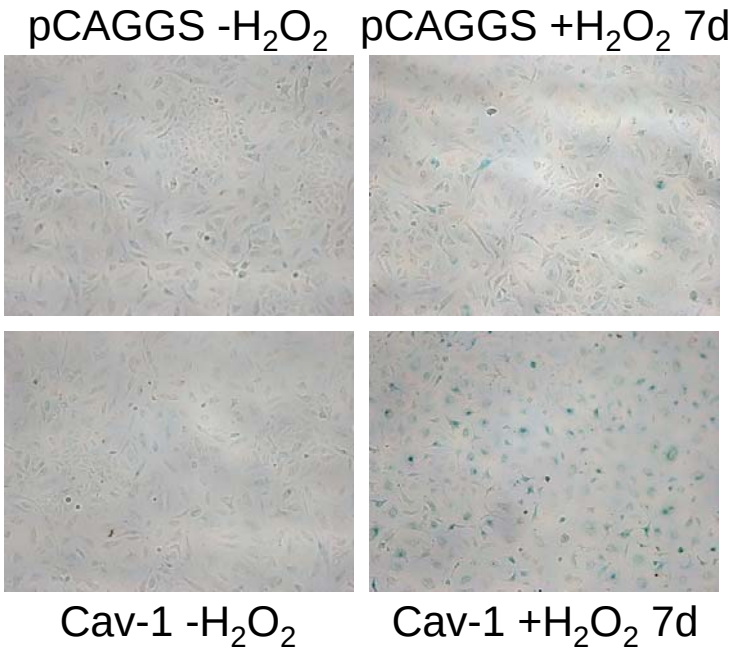
Volonte et al.,
Suppl. Figure 5C

C



Volonte et al.,
Suppl. Figure 6A, B

A



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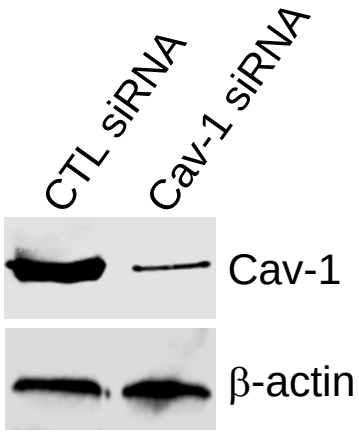

Table I. Quantification of TrxR1 localization in caveolar (C. M.) and non-caveolar (N. C. M.) membranes in WI-38 cells before and after sub-cytotoxic oxidative stress.

	C. M.	N. C. M.
- H_2O_2	79%	21%
+H_2O_2 24h	81%	19%
+H_2O_2 48h	20%	80%
+H_2O_2 72h	65%	35%