Supporting Information

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SI Materials and Methods

Antibodies. Mouse monoclonal anti–ADP-ribosyl-acceptor hydrolase (ARH3) (A-7) and apoptosis-inducing factor (AIF) (B-9) antibodies, and rabbit polyclonal anti-Tom20 antibodies, were purchased from Santa Cruz; rabbit monoclonal anti-GAPDH (14C10), and Histone H3 (P1H2) antibodies from Cell Signaling; mouse monoclonal anti–poly (ADP ribose) polymerases (PARP) 1 (C-2-10) and tubulin (DM1A) antibodies from Sigma-Aldrich; mouse monoclonal anti–poly (ADP ribose) (PAR) (10H) antibodies from Enzo Life Sciences; mouse monoclonal anti-MnSOD antibodies from BD Biosciences; mouse monoclonal anti-DDK (DDK is identical to FLAG, which is a registered trademark of Sigma Aldrich) antibodies from Origene; fluorescence-conjugated secondary antibodies (Alexa Fluor 488- and 594-conjugated goat anti-mouse and anti-rabbit) from Invitrogen; and HRP-goat anti-mouse and rabbit IgG from Pierce.

Cell Culture. Mouse embryonic fibroblasts (MEFs) (from day 14.5 embryos) were incubated in high-glucose DMEM containing 10% (vol/vol) FBS, 100 units of penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. MEFs stably expressing plasmids encoding DDK-tagged mouse ARH3 (Origene) or shRNA plasmids targeting PARP1 (5′-GCCGCCTACTCTATCCTCAGCGAGGTCCA-3′) and PARG (5′-GTGGAAGATGAGAATGGTGAGCGAACTGC-3′) (Origene) and ARH3$^{-/-}$ MEFs transiently expressing ARH3 (D83N/D84N) inactive mutant were prepared by using Lipofectamine 2000 (Invitrogen). After a 10-min incubation at room temperature (RT) with plasmids and PLUS reagent (Invitrogen) in Opti-MEM reduced-serum medium (Invitrogen), Lipofectamine 2000 was added to the medium (20 min, RT), which was then added to each well; 2 h later, DMEM with 10% (vol/vol) FBS was added to each well. Cells stably transfected with DDK-tagged mouse ARH3 or shRNA plasmids were selected and grown in medium with 1 mg/mL G418 or 1 μg/mL puromycin, respectively.

Cell Viability Assays. Cells (3×10^3), seeded on 96-well plates, were incubated for 2 d before exposure (24 h) to the indicated concentrations (100–1,000 μM) of H_2O_2. PARP inhibitors and caspase-inhibitor were added for 1 h before H_2O_2 exposure. Cell numbers were determined by using CCK-8 (Dojindo) according to the manufacturer's instructions, by measuring absorbance at 450 nm (SpectraMax M5 Microplate Reader). The CCK-8 contains WST-8, which is reduced by cellular dehydrogenases and then produces a colored formazan. The amount of formazan is directly proportional to the number of living cells.

Immunocytochemistry. Cells (1×10^5), seeded on a glass-bottom dish (Mat-Tek), were incubated (2 d, 37 °C) in DMEM with 10% (vol/vol) FBS, fixed with 4% (vol/vol) paraformaldehyde (PFA; 20 min, 4 °C), and permeablized and blocked with 10% (vol/vol) FBS, 1% BSA, and 0.5% Triton X-100 (1 h, RT). After incubation (overnight, 4 °C) with primary antibodies, cells were treated (1 h, RT) with either Alexa 488-conjugated goat anti-rabbit IgG or Alexa 594-conjugated goat anti-mouse IgG (1:500), then washed three times with PBS and incubated (5 min, RT) with 300 nM DAPI (Invitrogen) to stain nuclei. No significant reactivity of cells without primary antibody was seen. Mitochondria were stained with 200 nM deep-red mitotracker (Invitrogen) before fixation with PFA. Cells were imaged with a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss) equipped with an oil-immersion objective (40×, numerical aperture = 1.3), an Ar laser (488 nm) to excite GFP and Alexa 488, a He-Ne laser (594 nm) to excite Alexa 594, and a 405 diode (405 nm) to excite DAPI. Fluorescence data were processed with ImageJ 1.37a (National Institutes of Health).

FITC-Conjugated Annexin-V Staining. Cells (1×10^5), seeded on a glass-bottom dish, were incubated (2 d, 37 °C) in DMEM with 10% (vol/vol) FBS before assessment of viability by using the FITC Annexin-V/Dead Cell Apoptosis Kit (Invitrogen), according to the manufacturer's instructions.

Western Blotting. Cells (3×10^5), seeded on a 6-well plate, were incubated (2 d, 37 °C), in DMEM with 10% (vol/vol) FBS. Cell lysates were prepared with 2% (vol/vol) SDS in 20 mM Tris·HCl at pH 7.4 containing a protease inhibitor mixture (Roche). After adjustment of protein concentration by using a bicinchoninic acid (BCA) assay kit (Thermo Scientific), cell lysates were subjected to Bis-Tris or Tris-acetate SDS/PAGE (Invitrogen) and then transferred to nitrocellulose membranes (Invitrogen). The blots were blocked with 5% (vol/vol) nonfat dry milk (Bio-Rad) in Tris-buffered saline for 1 h at RT and then incubated with primary antibody. The ECL system (Fujifilm Las-3000; Fujifilm) was used for detection.

Quantitative RT-PCR. Cells (5×10^3), seeded on a 96-well plate, were incubated (2 d, 37 °C) in DMEM with 10% (vol/vol) FBS before mRNA extraction and preparation of cDNA by reverse transcription using a Taqman expression assay kit with a 7900HT Fast Real-Time PCR system (Invitrogen). PARG mRNA level was normalized to that of GAPDH mRNA. 6-carboxyfluorescein-labeled probes and predesigned primers (PARG, Mm00449464_m1; GAPDH, Mm99999915_g1) were purchased from Invitrogen.

Measurement of β-NAD Concentration. Cells (5×10^5), seeded on a 10-cm dish, were incubated (2 d, 37 °C) in DMEM with 10% (vol/vol) FBS. After washing with PBS, 1 M $HClO_4$ (300 μL) was added and the incubation continued on ice for 10 min. The reactants were spun at 14,000 × *g*, and the supernatant (240 μL) was neutralized with 3 M K_2CO_3 (80 μL). After centrifugation, samples were loaded on a reverse-phase high-performance liquid chromatography (HPLC) system (series 1100, with a diode array spectrophotometric detector set at 259 nm; Hewlett-Packard) equipped with an LC-18T column (5 μm particle size, 4.6–250 mm; Sigma-Aldrich). Isocratic elution (1 mL/min) with 100% buffer A (50 mM KPO_4) from 0 to 2 min was followed by a linear gradient to 100% buffer B (methanol) from 2 to 10 min.

Subcellular Fractionation. Cells (3×10^5), seeded in a six-well plate, were incubated (2 d, 37 °C) in DMEM with 10% (vol/vol) FBS, before washing three times with PBS and fractionation by using a Subcellular Proteome Extraction Kit (Calbiochem), according to the manufacturer's instructions.

Measurement of Recombinant SIRT1 Activity. Recombinant SIRT1 protein (25 units, 6.1 μg) was incubated with 100 μM [adenine-^{14}C]-β-NAD (2,500 cpm/pmol; PerkinElmer Life Sciences) and acetylhistone H3 peptide (100 μg) for 4 h at 30 °C with 50 mM Tris·HCl at pH 7.0 buffer containing 2.7 mM KCl, 1 mM $MgCl_2$, and 0.2 mg of BSA without or with 10 μM PJ34. ADP ribose, β-NAD, and *O*AADPr were separated on a Vydac C18 column (4.6–250 mm; W. R. Grace & Co.). Isocratic elution (1 mL/min) with 100% buffer A [0.05% (vol/vol) TFA in water] from 0 to 5 min

was followed by a linear gradient to 60% buffer A and 40% buffer B [0.05% (vol/vol) TFA in acetonitrile] from 5–45 min. Fractions (1 mL/min) were collected for quantification of radioactivity by using a liquid scintillation counter (TriCarb 1600TR; PerkinElmer Life Sciences).

Immunoprecipitation of poly-ADP Ribosylated PARP1. Cells (6×10^5), seeded in a six-well plate, were incubated (2 d, 37 °C) in DMEM with 10% (vol/vol) FBS. After H_2O_2 exposure for indicated times, cell lysates were prepared with 50 mM Tris·HCl at pH 8, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, containing protease inhibitor mixture and 10 μM PJ34 and 2 μM ADP-HPD (PARG and ARH3 inhibitor). Immunoprecipitation experiments were performed by using magnetic Dynabeads covalently coupled to Protein G (Invitrogen). Two micrograms of anti-PAR antibody were mixed with 50 μg of Dynabeads in PBS at RT for 1 h to establish the complex. Five hundred micrograms of cell lysates were incubated with anti-PAR antibody-labeled Dynabeads at 4 °C for 1 h. After washing three times with PBS, PAR–protein complexes were collected with an elution buffer.

Statistical Analysis. Statistical analysis was performed by using Prism. Significance was determined by using paired *t* tests, Student's *t* test for pairwise comparison or a two-way ANOVA with post hoc Bonferroni test. Data are means ± SEM of values from the indicated number of experiments. *P* values <0.05 were considered significant. All representative experiments were repeated three times.

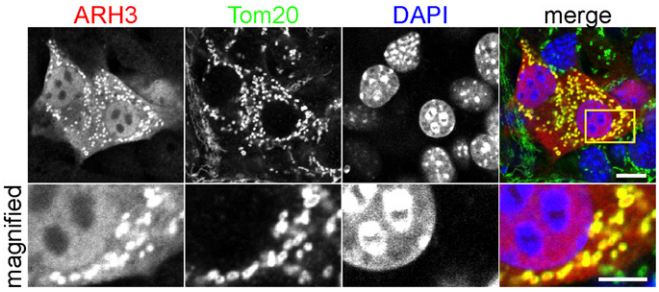

Fig. S1. Microscopic localization of overexpressed DDK-tagged ARH3 in ARH3$^{-/-}$ MEFs. DDK-ARH3 transfected in ARH3$^{-/-}$ MEFs was detected by immunoreaction with anti-DDK antibody (red in the merged image). Nuclei were stained with DAPI (blue in the merged image) and mitochondria with anti-Tom20 antibodies (green in the merged image). Lower images are magnifications indicated by rectangle in the merged image. These representative data have been replicated three times with similar results. (Scale bar: *Upper*, 20 μm; *Lower*, 5 μm.)

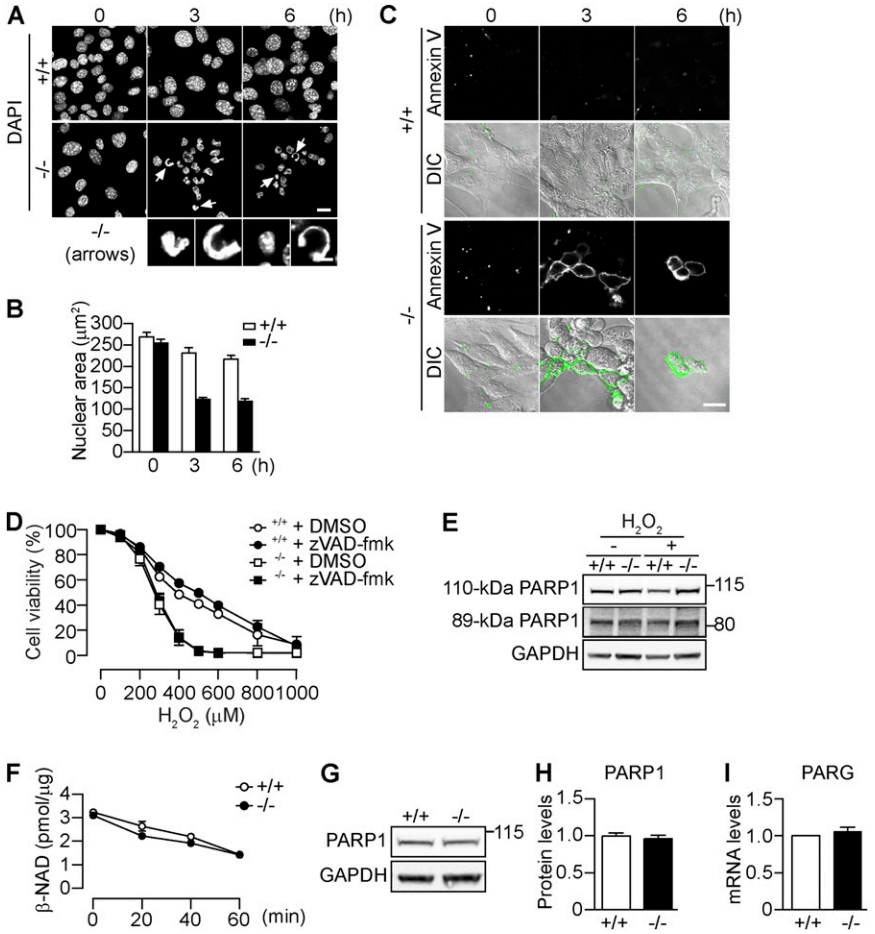

Fig. S2. Cell death in ARH3$^{-/-}$ MEFs resulted from caspase-independent apoptosis. (*A*) Change of nuclear morphology after 3- or 6-h exposure to 300 μM H_2O_2. Cells were stained with DAPI. Magnified images (*Bottom*) are nuclei with condensed chromatin indicated by arrows in images of ARH3$^{-/-}$ MEFs. (*B*) Summarized nuclear areas (square micrometers). Nuclear areas were assessed by measuring areas of DAPI fluorescence (means ± SEM, n = 78–149 cells). (*C*) Annexin V-positive cells after 3- or 6-h exposure to 300 μM H_2O_2. Cells were reacted with FITC-conjugated annexin V. Lower images are overlay of FITC fluorescence and differential interference contrast (DIC) images. (*D*) Effect of caspase inhibitor on H_2O_2-induced cell death. Ten micromolar zVAD-fmk was treated for 1 h before 24-h exposure to H_2O_2 at indicated concentrations (means ± SEM, n = 3). (*E*) Fragments (89 kDa) of proteolyzed PARP1 after 3-h exposure to 300 μM H_2O_2. Cells were subjected to Western blotting by using anti-PARP1 antibody to detect 89-kDa fragment (*Middle*) generated from 110-kDa PARP1 (*Top*). (*F*) β-NAD concentration after 300 μM H_2O_2 exposure. β-NAD concentration (picomoles per microgram) was measured by reverse-phase HPLC using an LC18T column (means ± SEM, n = 3). (*G*) PARP1 expression. Cells were subjected to Western blotting by using anti-PARP1 antibody. (*H*) Quantification of PARP1 expression levels. The amount of PARP1 protein was normalized to that of GAPDH (means ± SEM, n = 3). (*I*) Quantification of PARG mRNA levels. PARG mRNA was normalized to GAPDH mRNA (means ± SEM, n = 3). These representative data (*A*, *C*, *E*, and *G*) have been replicated three times with similar results. (Scale bar: *A Middle* and *C*, 20 μm; *A Bottom*, 5 μm.)

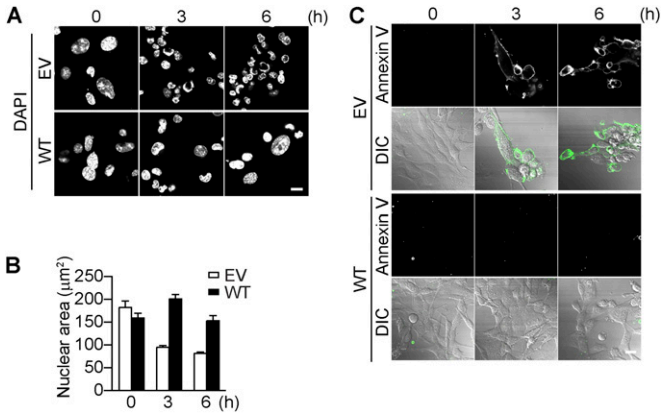

Fig. S3. ARH3 expression in ARH3$^{-/-}$ MEFs suppressed H_2O_2-induced nuclear shrinkage and phosphatidylserine exposure on cell membrane. (*A*) Change of nuclear morphology after 3- or 6-h exposure to 300 μM H_2O_2. Cells were stained with DAPI. (*B*) Summarized nuclear areas (square micrometers) (means ± SEM, n = 59–157 cells). (*C*) Annexin V-positive cells after 3- or 6-h exposure to 300 μM H_2O_2. Cells were reacted with FITC-conjugated annexin V. Lower images are overlay of FITC fluorescence and DIC images. These representative data (*A* and *C*) have been replicated three times with similar results. (Scale bars: 20 μm.)

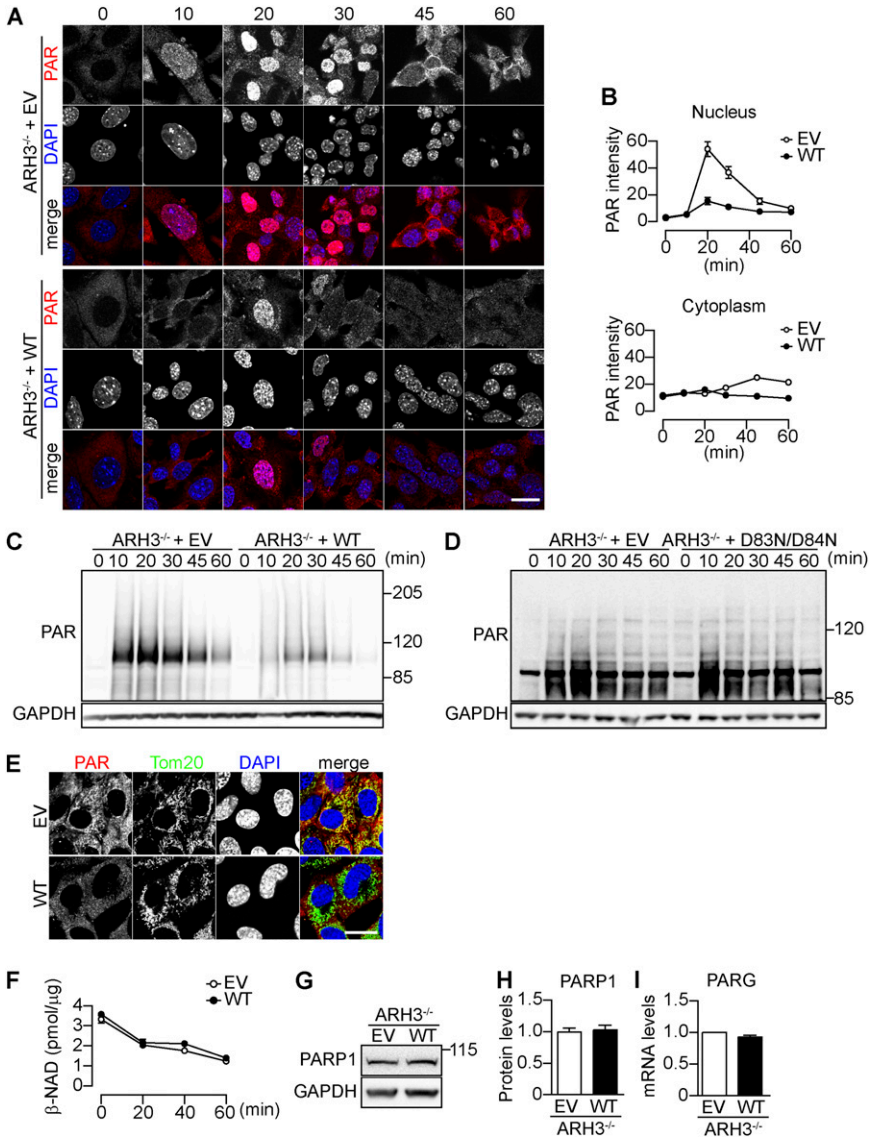

Fig. S4. ARH3 expression in ARH3$^{-/-}$ MEFs suppressed H_2O_2-induced PAR accumulation. (*A*) Time-dependent localization change after exposure to 300 μM H_2O_2 for indicated times. Cells were subjected to immunocytochemistry by using anti-PAR antibody (red in merged images) and DAPI staining (blue in merged images). (*B*) Mean PAR fluorescence in nuclei and cytoplasm (means ± SEM, $n = 40$ cells). (*C*) Time course of H_2O_2-induced PAR accumulation in ARH3$^{-/-}$ MEFs expressing EV or ARH3 after exposure to 300 μM H_2O_2 for indicated time. Cells were subjected to Western blotting by using anti-PAR antibody. GAPDH was used as a loading control. (*D*) Time course of H_2O_2-induced PAR accumulation in ARH3$^{-/-}$ MEFs expressing EV or ARH3 (D83N/D84N) inactive mutant after exposure to 300 μM H_2O_2 for indicated time. Cells were subjected to Western blotting by using anti-PAR antibody. GAPDH was used as a loading control. (*E*) PAR localization after 2-h exposure to 300 μM H_2O_2. Cells subjected to immunocytochemistry using anti-PAR (red in merged images) and Tom20 antibodies (green in merged images) and DAPI staining (blue in merged images). (*F*) β-NAD concentration after 300 μM H_2O_2 exposure. β-NAD concentration (picomoles per microgram) was measured by reverse-phase HPLC using a LC18T column and normalized to the protein concentration (means ± SEM, $n = 3$). (*G*) PARP1 expression. Cells were subjected to Western blotting by using anti-PARP1 antibody. (*H*) Quantification of PARP1 expression levels. The amount of PARP1 protein was normalized to that of GAPDH (means ± SEM, $n = 3$). (*I*) Quantification of PARG mRNA levels. PARG mRNA was normalized to GAPDH mRNA in the same sample (means ± SEM, $n = 3$). These representative data (*A*, *C*–*E*, and *G*) have been replicated three times with similar results. (Scale bars: 20 μm.)

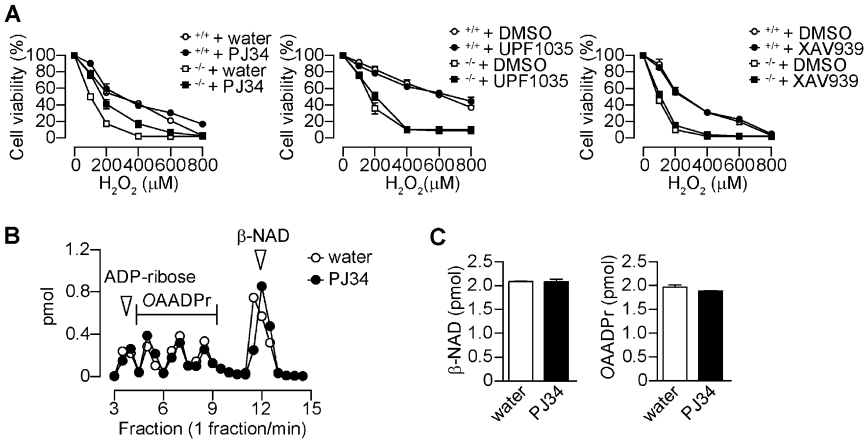

Fig. S5. PARP inhibitor prevented H_2O_2-induced cell death in ARH3$^{-/-}$ MEFs. (*A*) Effect of PARP inhibitors on H_2O_2-induced cell death. PARP inhibitors [10 μM PJ34 (PARPs, *Left*), 1 μM UPF1035 (PARP2, *Center*), or 1 μM XAV939 (tankyrase, *Right*)] was treated for 1 h before 24-h exposure to indicated concentrations of H_2O_2 and measurement of cell viability (means ± SEM, $n = 3$). (*B*) Effect of PJ34 on OAADPr production and β-NAD consumption by SIRT1 activity. Products and the substrate were separated and collected with a Vydac C18 column by using reverse-phase HPLC, and their radioactivities were measured by a liquid scintillation counter. These representative data have been replicated three times with similar results. (*C*) Amounts (picomoles) of [adenine-^{14}C]-labeled products (OAADPr) and substrate (β-NAD) (means ± SEM, $n = 3$).

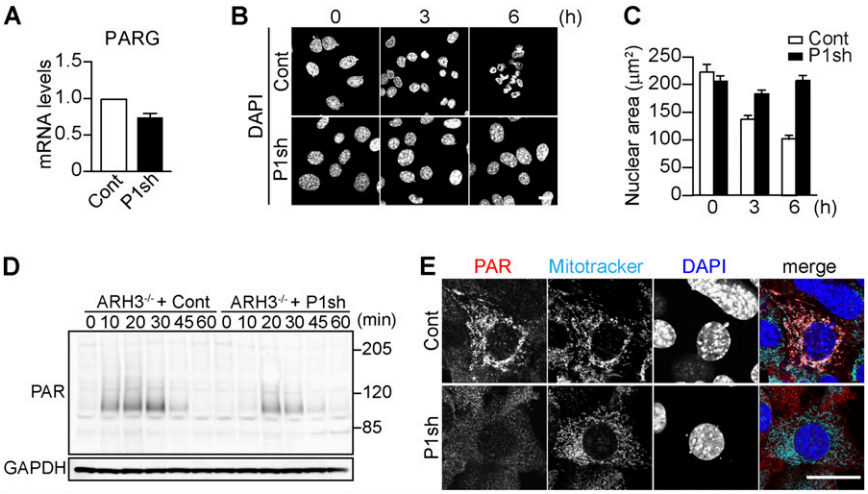

Fig. S6. Depletion of PARP1 protein suppressed nuclear shrinkage and PAR synthesis in response to H_2O_2 in ARH3$^{-/-}$ MEFs. (*A*) Quantification of PARG mRNA levels. PARG mRNA was normalized to GAPDH mRNA (means ± SEM, $n = 3$). (*B*) Effects of PARP1 depletion on nuclear morphology of ARH3$^{-/-}$ MEFs after 3- or 6-h exposure to 300 μM H_2O_2. Nuclei were stained with DAPI. (*C*) Summarized nuclear areas (square micrometers) (means ± SEM, $n = 81$–130 cells). (*D*) Time course of H_2O_2-induced PAR accumulation after exposure to 300 μM H_2O_2 for indicated times. Cells were subjected to Western blotting by using anti-PAR antibody. GAPDH was used as a loading control. (*E*) PAR localization after 2-h exposure to 300 μM H_2O_2. Cells were subjected to immunocytochemistry by using anti-PAR antibody (red in merged images) and staining with mitotracker (light blue in merged images) and DAPI (blue in merged images). (Scale bars: 20 μm.) These representative data (*B*, *D*, and *E*) have been replicated three times with similar results.

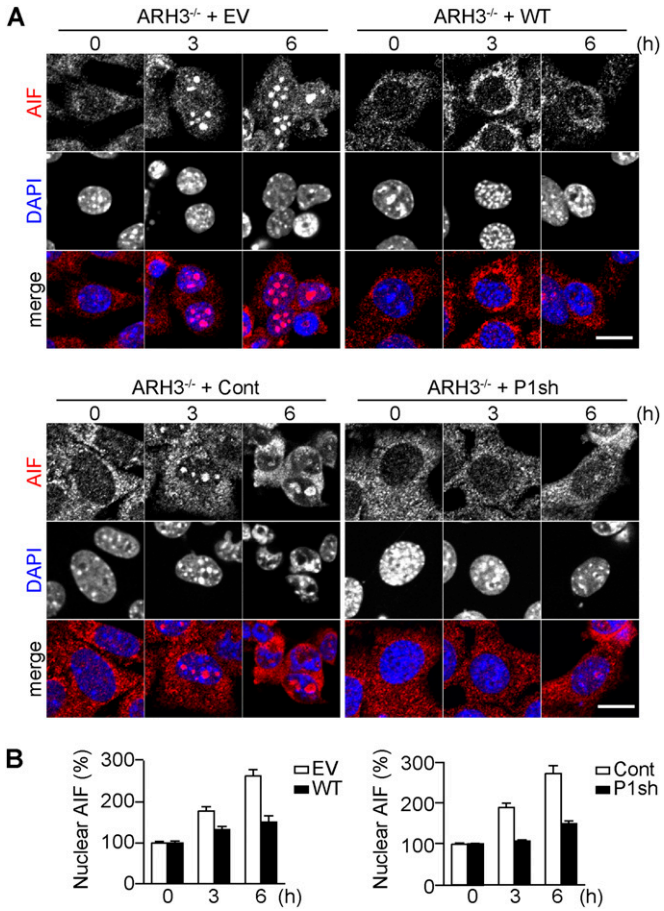

Fig. S7. ARH3 expression or depletion of PARP1 protein suppressed AIF translocation to the nuclei following H_2O_2 exposure. (*A*) AIF translocation after 3- or 6-h exposure to 300 μM H_2O_2. Cells were subjected to immunocytochemistry by using anti-AIF antibody and DAPI staining. Immunoreactive AIF (red in merged images) and nuclei stained with 300 nM DAPI (blue in merged images) were monitored by confocal microscopy. (Scale bar: 10 μm.) These representative data have been replicated three times with similar results. (*B*) Mean AIF fluorescence in nuclei (means ± SEM, n = 40–70 cells).

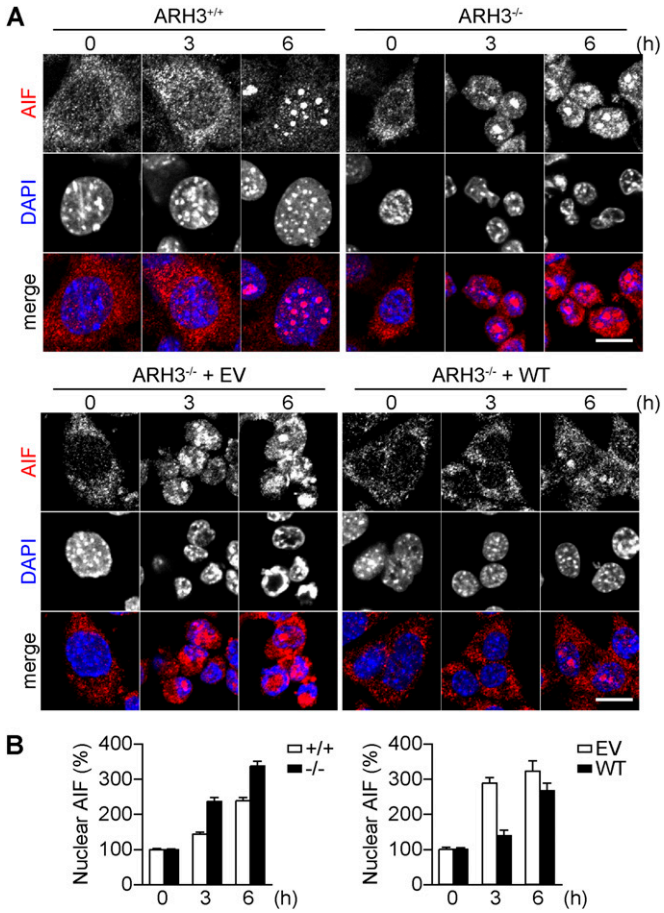

Fig. S8. Greater exposure to H_2O_2 caused AIF translocation to nuclei of WT MEFs. (*A*) AIF translocation to nuclei after 3- or 6-h exposure to 600 μM H_2O_2. Immunoreactive AIF (red in merged images) and nuclei stained with 300 nM DAPI (blue in merged images) were monitored by confocal microscopy. (Scale bar: 10 μm.) These representative data have been replicated three times with similar results. (*B*) Mean AIF fluorescence in nuclei (means ± SEM, n = 29–70 cells).

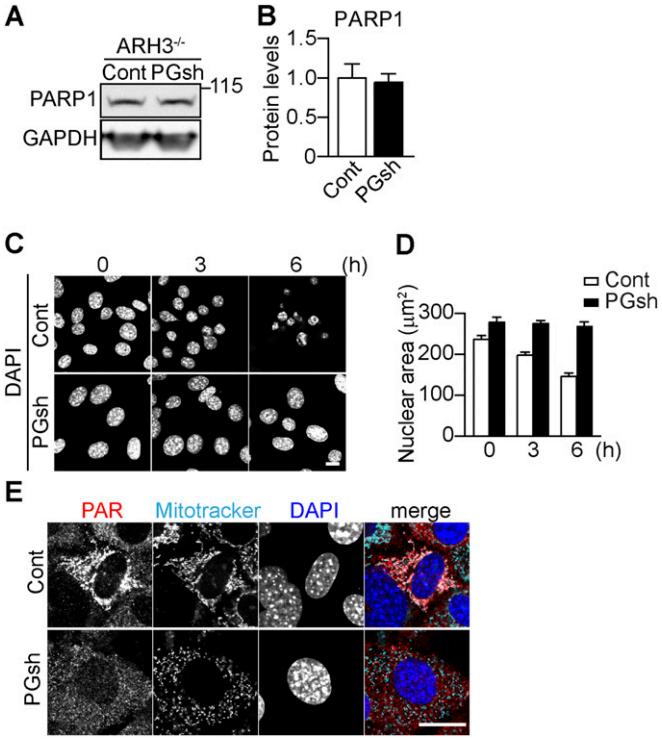

Fig. S9. Depletion of PARG proteins by shRNA-inhibited AIF translocation to the nucleus and PAR association with mitochondria. (*A*) PARP1 expression. Cells were subjected to Western blotting by using anti-PARP1 antibody. (*B*) Quantification of PARP1 expression levels. The amount of PARP1 protein was normalized to that of GAPDH (means ± SEM, $n = 3$). (*C*) Effects of PARG depletion on nuclear morphology of ARH3$^{-/-}$ MEFs after 3- or 6-h exposure to 300 μM H_2O_2. Nuclei were stained with DAPI. (*D*) Summarized nuclear areas (square micrometers) (means ± SEM, $n = 70$–140 cells). (*E*) PAR localization after 2-h exposure to H_2O_2. Cells were subjected to immunocytochemistry by using anti-PAR antibody (red in merged images) and staining with mitotracker (light blue in merged images) and DAPI (blue in merged images). These representative data (*A*, *C*, and *E*) have been replicated three times with similar results. (Scale bars: 20 μm.)