Cell Death and Autophagy under Oxidative Stress: Roles of Poly(ADP-Ribose) Polymerases and Ca^{2+}

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On the cellular level, oxidative stress may cause various responses, including autophagy and cell death. All of these outcomes involve disturbed Ca^{2+} signaling. Here we show that the nuclear enzymes poly(ADP-ribose) polymerase 1 (PARP1) and PARP2 control cytosolic Ca^{2+} shifts from extracellular and intracellular sources associated with autophagy or cell death. The different Ca^{2+} signals arise from the transient receptor potential melastatin 2 (TRPM2) channels located in the cellular and lysosomal membranes. They induce specific stress kinase responses of canonical autophagy and cell death pathways. Autophagy is under the control of PARP1, which operates as an autophagy suppressor after oxidative stress. Cell death is activated downstream of extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT, whereas cell survival correlates with the phosphorylation of p38, stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK), and cyclic AMP response element-binding protein (CREB) with its activating transcription factor (ATF-1). Our results highlight an important role for PARP1 and PARP2 in the epigenetic control of cell death and autophagy pathways.

On the cellular level, oxidative stress may induce pleiotropic responses ranging from autophagy to death. Little is known about the molecular switches between death and autophagy (10). Here we investigated the role of poly(ADP-ribose) polymerase 1 (PARP1) and PARP2, two nuclear enzymes, in regulating the outcome of oxidative stress. Oxidative stress induces DNA damage, activating PARP1 and PARP2. Activated PARP signals downstream into DNA repair processes and cell death pathways. It is therefore a key factor in the maintenance of genomic stability (11). PARP1 is the main enzyme, producing about 90% of poly(ADP-ribose) (PAR) (11, 12, 27). PARP2 shares many of the properties of PARP1 but contributes far less to PAR synthesis (38). PAR is immediately degraded by poly(ADP-ribose) glycohydrolase (PARG) to free PAR and finally to monomeric ADP-ribose (ADPR) (12). It has been shown *in vitro* and in a variety of different cell types that intracellular ADPR activates transient receptor potential melastatin 2 (TRPM2) channels (21, 24, 35). This Ca^{2+} channel operates in plasma membranes and lysosomes (16, 23, 30). It is well established that an insult-dependent cellular Ca^{2+} overload results in cytotoxic stress responses ranging from survival to autophagy to cell death (39).

This study focused on the types and sources of cytosolic Ca^{2+} shifts after low and high levels of oxidative stress in mouse embryonic fibroblasts (MEFs). Using gene knockout, RNA interference (RNAi), and chemical inhibitors, we determined the impact of PARP1 and PARP2 on distinct Ca^{2+} shifts and downstream consequences for cell death and autophagy. Our results show that PARP1 regulates Ca^{2+} shifts by plasma membrane TRPM2 and phosphatidylinositol 3-kinase (PI3K) at low levels of oxidative stress. The presence or absence of PARP1-mediated cytosolic Ca^{2+} is further translated into a different stress kinase pattern leading to cell death or survival responses. Therefore, H_2O_2 -provoked PARP1 activity is indicative of cell fate decisions.

In contrast, PARP2 induces Ca^{2+} shifts from other TRPM2 localizations, most likely lysosomes, under high-dose stress conditions, even when PARP1 is completely inactive. Downstream of Ca^{2+} signaling, PARP1 operates as a suppressor of the autophagy pathway.

MATERIALS AND METHODS

Cell culture. Immortalized MEFs from the *Parp1*^{+/+} (wild type [WT]) and *Parp1*^{-/-} backgrounds were used. MEFs were cultured at 37°C in a water-saturated (5% CO_2) atmosphere in complete Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 1 g/liter glucose and supplemented with 10% (vol/vol) fetal bovine serum and penicillin-streptomycin (Invitrogen).

RNAi. All transfection experiments were performed with siPORT Amine (Ambion) as the transfection reagent under conditions described earlier. Small interfering RNAs (siRNAs) against *Becn1* (siRNA-B1; 5'-AAAGCUA CCAUUGCAUGGUCA-3' [antisense sequence]), *Parp1* (siRNA-P1; 5'-AAGAGCGATGCCTATTACTGC-3' [antisense]) (7), *Parp2* (siRNA-P2; 5'-AAGATGATGCCAGAGGAAGTC-3' [antisense]) (7), and *Trpm2* (siRNA-T2; 5'-UACUCGACCACGUAGACUCCA-3' [antisense]) (3) were obtained from Applied Biosystems. For maximal silencing efficacy, we performed silencing of *Becn1* for 24 h and that of all others for 72 h.

H_2O_2 treatment. Cells were challenged with 1 part H_2O_2 (Sigma) diluted in OPTI-MEM I (Gibco) to the desired concentration. After 1 h, 3 parts complete DMEM was added. This treatment was maintained without any alterations until the end of the experiment.

Chemicals. 3-Aminobenzamide (3-AB) was from Alexis Biochemicals. 3-Methyladenine (3-MA), bafilomycin A1 (Baf A1), H_2O_2 , *N*-[[(phenylmethoxy)carbonyl]-L-valyl]-phenylalaninal (MDL 28170), and 2-(4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-1H-indol-5-ol (PP242) were from Sigma. *N*-(*p*-Amylcinnamoyl)-anthranilic acid (ACA) and *N*-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl ketone (Q-VD-OPh) were from Calbiochem. Hoechst 33342 was from Invitrogen. 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride (LY294002) was from BIAFFIN GmbH & Co. KG. 4-(4-Fluorophenyl)-2-(4-

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methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) was from Cell Signaling. 1-[6-[(17- β -3-Methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), methylthiohydantoin-DL-tryptophan (necrostatin 1), 2'-amino-3'-methoxyflavone (PD-98059), and 1,9-pyrazoloanthrone (SP600125) were from Enzo Life Sciences. All inhibitors were dissolved in dimethyl sulfoxide, except for U-73122, which was dissolved in chloroform, and 3-MA, which was directly dissolved in the culture medium. All other chemicals were from Applichem, Fluka, Merck, or Sigma. If not stated differently, all of the chemicals used as inhibitors were simultaneously administered with the H₂O₂ treatment.

Cellular subfractionation. Isolation of cell nuclei was performed as described before (7). Briefly, MEFs were harvested and washed twice with Tris-HCl (pH 7.0)–0.13 M NaCl–5 mM KCl–7.5 mM MgCl₂. After incubation in 3.5 mM Tris-HCl (pH 7.8)–3 mM NaCl–0.5 mM MgCl₂ for 5 min, cells were mechanically broken. Homogenate was mixed with 1/9 of the cell volume of 0.35 M Tris-HCl (pH 7.8)–0.2 M NaCl–50 mM MgCl₂ and spun for 3 min at 1,600 $\times$ g. The pellet (nuclei) was harvested directly in Laemmli buffer and subsequently analyzed by Western blotting. All procedures were performed on ice.

Antibodies for Western blot detection. The primary antibodies used were anti-pAKT (S473; R&D Systems; 1:4,000), anti-pAMPK α (Cell Signaling; 1:1,000), anti-Atg7 (Cell Signaling; 1:1,000), anti-Atg12 (D88H11 XP; Cell Signaling; 1:1,000), anti-BECN1 (H-300; Santa Cruz Biotechnology; 1:1,000), anti-CREB (48H2; Cell Signaling; 1:1,000), anti-pCREB (Ser133; Cell Signaling; 1:1,000), anti-ERK1/2 (R&D Systems; 1:1,000), anti-pERK1/2 [(T202/Y204)/(T185/Y187); R&D Systems; 1:2,000], anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; Ambion; 1:100,000), anti-inositol triphosphate (anti-IP3) receptor 1 (Millipore; 1:500), anti-LC-3B (G40; Cell Signaling; 1:1,000), anti-MSK1 (C27B2; Cell Signaling; 1:1,000), anti-pMSK1 (Cell Signaling; 1:1,000), anti-PARP1 (C2-10; Alexis; 1:5,000), anti-PARP2 (4G8; Alexis; 1:50), anti-pPERK (Thr 980; Cell Signaling; 1:1,000), anti-SAPK/JNK (56G8; Cell Signaling; 1:1,000), anti-pSAPK/JNK (Thr183/Tyr185 G9; Cell Signaling; 1:2,000), anti- α -tubulin (Cell Signaling; 1:50,000), anti-Topo-1 (BD Biosciences; 1:5,000), anti-p38 mitogen-activated protein kinase (MAPK; Cell Signaling; 1:1,000), anti-p-p38 MAPK (Thr180/Tyr182 3D7; Cell Signaling; 1:1,000), anti-p62 (Cell Signaling; 1:1,000), and anti-p-p70S6 kinase (Cell Signaling; 1:1,000). All secondary antibodies were from Sigma.

Immunoblot assays were performed as described previously (7). Equal quantities of protein were loaded into each lane for SDS-PAGE separation as controlled by the simultaneous use of internal protein standards.

Calcium measurements and visualization. Calcium measurements and visualization were performed as described before (3). Briefly, 20,000 cells/well in 96-well plates (Costar; Corning Incorporated) were washed twice with 49 parts of calcium-free Hanks balanced salt solution (HBSS; 0.49 mM MgCl₂, 0.41 mM MgSO₄, 5.33 mM KCl, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, 137 mM NaCl, 0.34 mM Na₂HPO₄, 5.56 mM dextrose) supplemented with 1 part 1 M HEPES (pH 7.2; assay buffer) containing or not containing CaCl₂. A 100- μ l volume of Fluo-4-NW dye mix from Molecular Probes (Invitrogen) was added, and the mixture was incubated for 30 min at 37°C and then incubated for 30 min in the dark at room temperature. Changes in the number of relative fluorescence units (Δ RFU) emitted by the Fluo-4-NW dye quantified alterations in cytosolic Ca²⁺ concentrations (excitation and emission wavelengths, 485 and 535 nm; slits, 10 and 15 nm) in an LS55 luminescence spectrometer (Perkin-Elmer) after H₂O₂ treatment. Ca²⁺ was monitored for 30 min with a 0.1-s interval between measurements. Every 50th time point was taken for analysis. For Ca²⁺ visualization, Fluo-4-loaded cells were analyzed 0.5 and 2 h after H₂O₂ treatment in a fluorescence microscope (Nikon).

Immunofluorescence. Cells were seeded onto coverslips (Thermo Scientific) in six-well plates (Costar; Corning Incorporated), washed twice with OPTI-MEM I, and then treated with H₂O₂. Cells were fixed with ice-cold methanol. Coverslips were subsequently washed with 1 $\times$ phosphate-buffered saline (PBS) and incubated with blocking buffer (1 $\times$ PBS, 3% milk powder, 1.5% bovine serum albumin) overnight at 4°C. The

primary antibodies (all diluted 1:200 in blocking buffer) used were anti-LC-3B (G40; Cell Signaling), anti-PAR (Alexis), and anti-PCNA (BD Biosciences). After incubation for 1 h at 37°C, coverslips were washed three times with blocking buffer and then subjected to a second antibody incubation (1:200) for 1 h at 37°C in the dark. Probes were washed once with blocking buffer and twice with 1 $\times$ PBS. If desired, Hoechst 33342 (stock, 1 mg/ml; 1:10,000 in PBS; 15 min) was added at room temperature. Probes were washed three times with 1 $\times$ PBS and dried afterward. Coverslips were processed further with the ProLong Antifade kit (Invitrogen) according to the manufacturer's protocol.

Visualization of acidic organelles. Cells were seeded into μ -Dishes (ibidi) and washed twice with 1 $\times$ PBS. Monolayers of cells were covered with 200 μ l of labeling solution from the GFP-Certified Lyso-ID Red Lysosomal detection kit (Enzo Life Sciences) and incubated for 30 min at 37°C in the dark. Cells were washed with 200 μ l of 1 $\times$ assay buffer and analyzed by fluorescence microscopy (Nikon).

Viability assay. Cells were seeded into 96-well plates (15,000 cells/well). Cells were treated at 50 μ l/well with H₂O₂ diluted in OPTI-MEM I for 1 h before 150 μ l DMEM was added. After 20 h, the medium was replaced with 200 μ l DMEM–10% (vol/vol) AlamarBlue (Biozol). After 4 h, fluorescence was monitored at an excitation wavelength of 530 nm and an emission wavelength of 590 nm in an LS55 luminescence spectrometer.

Statistical analysis. If not stated differently, all results are shown as the mean $\pm$ the standard deviation (SD) of the indicated number of independent experiments. The significance of differences was estimated by analysis of variance (ANOVA), and a *P* value of <0.05 was considered significant. All statistical analyses were calculated with InStat3 or Prism software 5.0b (GraphPad Software, San Diego, CA).

RESULTS

The activation of PARP1 by oxidant-induced DNA strand breaks plays a dominant role in H₂O₂-induced cytosolic Ca²⁺ shifts and cytotoxicity. MEFs lacking the *Parp1* gene (Fig. 1A) showed remarkable resistance to the cytotoxicity of 5 mM H₂O₂, in contrast to *Parp1*-carrying cells. While only 25% of the WT cells survived, more than 75% of the *Parp1*^{−/−} cells recovered 20 h after a challenge. To determine the type of stress pathway involved in H₂O₂ toxicity, we investigated three different cell death inhibitors (Fig. 1A). The caspase inhibitor Q-VD-OPh, the calpain inhibitor MDL 28170, and necrostatin 1 all failed to protect WT cells against oxidant-induced toxicity, suggesting that PARP1 controls a different cell death pathway.

Ca²⁺ shifts play an important role in cell death induction (39), and therefore we determined the role of PARP1 in regulating cytosolic Ca²⁺ shifts. H₂O₂ induced a biphasic elevation of cytosolic Ca²⁺, as determined in Fluo-4 Ca²⁺ assays (Fig. 1B). It started with an immediate peak (150 s), followed by a recovery period (minimal at 650 s) and a subsequent rise (up to 1,700 s). This final rise could be attributed to the PARP1/PARG-dependent formation of ADPR and its interaction with plasma membrane TRPM2 channels (3). The cytosolic Ca²⁺ influx originates from both extra- and intracellular Ca²⁺ sources (Fig. 1B). In contrast, when *Parp1*^{−/−} cells were challenged with 5 mM H₂O₂, the increase in cytosolic Ca²⁺ was only moderate and independent of extracellular Ca²⁺ (Fig. 1C). The Ca²⁺ shifts (or their absence in *Parp1*^{−/−} cells) were observed in all of the cells of the population and also after prolonged exposure to H₂O₂, as demonstrated by microscopy of Fluo-4 signals (Fig. 1D). Constitutive cytosolic Ca²⁺ is detectable in both cell types and is apparently not affected by *Parp1* ablation (see Fig. S1A to D in the supplemental material).

In order to elucidate the possible involvement of PARP2, the second PARP activated by DNA strand breaks (38), we silenced

FIG 1 Impact of H₂O₂ on cell survival, Ca²⁺ levels in WT and *Parp1*^{-/-} cells, and the role of PARP2 in WT cells. (A) WT cells were treated (additional 1 h pretreatment) with Q-VD-OPh (100 μM; *n* = 4), MDL 28170 (50 μM; *n* = 3) or necrostatin 1 (no pretreatment; 10 μM; *n* = 7) and 5 mM H₂O₂ (mean ± SD; not significant; *t* test). *Parp1*^{-/-} (P1^{-/-}) cells were also treated with 5 mM H₂O₂ (mean ± SD; *, *P* < 0.0001; *n* = 7; *t* test). Cell survival was monitored by AlamarBlue. Western blot analysis of PARP1 in untreated (UT) WT and *Parp1*^{-/-} cells. α-Tubulin was used as the control. (B) Fluo-4 assay of WT cells treated with 5 mM H₂O₂. Ca²⁺ determinations with and without extracellular Ca²⁺ ions were made directly after treatment with H₂O₂ for 30 min and were expressed as ΔRFU compared to the basal levels (mean ± SD; *, *P* < 0.005; *n* = 4; *t* test). (C) Ca²⁺ shifts after the treatment of *Parp1*^{-/-} cells with 5 mM H₂O₂ with and without extracellular Ca²⁺ ions (mean ± SD; not significant; *n* ≥ 5; *t* test). (D) Microscopic visualization of intracellular Ca²⁺ shifts after a challenge of WT and *Parp1*^{-/-} cells with 5 mM H₂O₂ (for 30 or 120 min) using the Ca²⁺ indicator Fluo-4. White light was used as the control. (E) Western blot analysis of WT cells with *Parp2* (P2) or a control (C) silenced. Topo-1 was the loading control. (F) PAR detection and Hoechst staining by immunofluorescence after treatment with 5 mM H₂O₂ (30 min) in control MEFs (WT) and cells treated overnight with 3 mM 3-AB or with *Parp2* silenced (P2). (G, left side) Comparison of Ca²⁺ levels at 1,700 s in WT cells with *Parp2* (siRNA-P2) or a control (siRNA-C) silenced after treatment with 5 mM H₂O₂ (mean ± SD; not significant; *n* = 4; *t* test) with extracellular Ca²⁺. (G, right side) Comparison of Ca²⁺ levels at 1,700 s in WT cells with *Parp2* (siRNA-P2) or a control (siRNA-C) silenced after treatment with 5 mM H₂O₂ (mean ± SD; not significant; *n* ≥ 4; *t* test) without extracellular Ca²⁺.

Parp2 in WT cells by RNAi as described previously (7). While *Parp2* silencing was effective (Fig. 1E), it did not have any effect on PAR levels (Fig. 1F), Ca^{2+} shifts (Fig. 1G), or H_2O_2 -induced cell death (see Fig. S2A in the supplemental material). Additionally, we saw similar effects in cells with abrogated TRPM2 (see Fig. S2B). Thus, when cells were challenged with 5 mM H_2O_2 for 30 min, PARP2 did not seem to play a role in cytosolic Ca^{2+} shifts involved in cell death signaling, demonstrating the predominant role of PARP1 (Fig. 1F). siRNA against *Parp2* or its controls did not affect cytosolic Ca^{2+} levels (see Fig. S1G to J in the supplemental material), nor did it affect Ca^{2+} shifts after H_2O_2 challenge (see Fig. S2C and D). To discriminate the sources of cytosolic Ca^{2+} shifts, we performed assays in the absence of extracellular Ca^{2+} . We found that Ca^{2+} released from intracellular compartments was not altered by RNAi against *Parp2* (Fig. 1G, right side; see Fig. S2D). These data confirmed the dominant role of PARP1 in oxidant-induced cell death.

PARP1 and PI3K orchestrate the cytosolic Ca^{2+} response to H_2O_2 and subsequent cell death. IP3 is a likely candidate for generating the first Ca^{2+} peak in H_2O_2 -induced cell death (13, 20, 28, 32). Therefore, IP3 synthesis was blocked by the phospholipase C (PLC) inhibitor U-73122 and cytosolic Ca^{2+} signals were determined with Fluo-4. While U-73122 (1 μM) suppressed the initial Ca^{2+} peak (Fig. 2A), which was evident only in WT cells, it did not affect basal levels of cytosolic Ca^{2+} (see Fig. S1E in the supplemental material). Interestingly, U-73122 had no effect on extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT, which occurred 30 min after H_2O_2 treatment (Fig. 2B). Figure 2C shows that IP3 receptor 1 was expressed at equal levels in WT and *Parp1*^{-/-} cells. We then investigated PI3K signaling, performing assays in the presence of the PI3K inhibitor LY294002 together with 5 mM H_2O_2 (Fig. 2D to F). In fact, 50 μM LY294002 suppressed the first cytosolic Ca^{2+} peak at 150 s (Fig. 2D). LY294002 alone had no effect on cytosolic Ca^{2+} kinetics (see Fig. S1F). The second rise in cytosolic Ca^{2+} was not altered by LY294002, indicating two distinct mechanisms, an early PI3K-mediated pathway (Fig. 2) and a late PARP1-derived pathway (Fig. 1 and 2). As expected, neither U-73122 nor LY294002 had any effect on cytosolic Ca^{2+} elevations in cells lacking *Parp1* (see Fig. S2E and F in the supplemental material).

LY294002 decreased downstream phosphorylation of ERK1/2 and AKT, which occurred 30 min after H_2O_2 treatment, in a dose-dependent manner (Fig. 2E). Moreover, wortmannin, another PI3K inhibitor, confirmed our findings (see Fig. S2G in the supplemental material). Finally, we tested the effect of LY294002 on the cytotoxicity of 5 mM H_2O_2 in WT cells (Fig. 2F). LY294002 increased the survival of cells from $24.6\% \pm 8.06\%$ to $43.0\% \pm 11.54\%$. The parallel incubation of 5 mM H_2O_2 -treated *Parp1*^{-/-} cells in LY294002 did not alter the survival rates (data not shown). Taken together, these results point to an early PI3K-dependent Ca^{2+} mobilization in PARP1-dependent cell death after H_2O_2 treatment. Both *Parp1*^{-/-} cells lacking the first immediate PI3K-controlled Ca^{2+} shift (Fig. 1C) and inhibition of its downstream signals in the WT background (Fig. 2A and D) led to elevated cell survival (Fig. 1A and 2F).

We then investigated the PARP1 dependence of Ca^{2+} signaling at early (0.25 and 0.5 h) and late (1, 2, 4, and 6 h) time points. Phosphorylation of the ERK1/2 and AKT proteins after treatment with 5 mM H_2O_2 (Fig. 2G and H) strongly correlated with IP3-mediated Ca^{2+} in WT but not in *Parp1*^{-/-} mutant cells. In gen-

eral, phosphorylation of ERK1/2 and AKT was faster and more abundant in WT than in *Parp1*^{-/-} cells. In WT cells, a maximal level of phosphorylated ERK1/2 and AKT already occurred between 0.25 and 1 h. Concomitant incubation of H_2O_2 with the inhibitors LY294002 and wortmannin suppressed the phosphorylation of ERK1/2 and AKT at 0.5 h (Fig. 2E; see Fig. S2G in the supplemental material) and its mediated cytosolic Ca^{2+} signal dose dependently (Fig. 2A and D). Thus, there is a functional link between PARP1 and Ca^{2+} -mediated stress signaling. Remarkably, inhibition of ERK1/2 phosphorylation downstream of IP3, using the MEK inhibitor PD-98059 (Fig. 2J), could not rescue cell viability (Fig. 2I). Thus, IP3 is an upstream signal for oxidant-induced cell death.

The role of PARP1 in Ca^{2+} -independent stress signaling. Stress response kinases are activated in a Ca^{2+} -independent manner (2, 17, 22, 37). Therefore, we analyzed the phosphorylation of p38, stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK), and their related transcription factors cyclic AMP response element-binding protein (CREB) and its activating transcription factor (ATF-1) in WT and *Parp1*^{-/-} cells (Fig. 3A to C). P38 phosphorylation was faster (1 h) in 5 mM H_2O_2 -challenged *Parp1*^{-/-} cells than in WT cells (0.25 h) (Fig. 3A). Moreover, activation of SAPK/JNK was detectable only in *Parp1*^{-/-} cells, at 2, 4, and 6 h posttreatment with a maximum at 4 h, and not in WT cells (Fig. 3B). As expected, the phosphorylation of SAPK/JNK led to the subsequent phosphorylation of their downstream transcription factors CREB and ATF-1 (Fig. 3C). In the next set of experiments, we investigated the effects of JNK and p38 inhibitors on cell survival in H_2O_2 -treated *Parp1*^{-/-} cells (Fig. 3D to H). First, we included the inhibitor SP600125, which is selective for JNK1, -2, and -3, to evaluate a possible survival function of phosphorylated SAPK/JNK in H_2O_2 -treated *Parp1*^{-/-} cells (Fig. 3D and F). SAPK/JNK phosphorylation was inhibited (Fig. 3D), but this had no impact on *Parp1*^{-/-} cell death (Fig. 3F). Likewise, we analyzed the effect of SB203580, an inhibitor of p38 signaling (Fig. 3E and G). As expected, SB203580 reduced the phosphorylation of the p38 downstream factor MSK1 in a dose-dependent manner without any effect on the phosphorylation status of p38 itself (Fig. 3E). The parallel application of SB203580 in H_2O_2 -challenged *Parp1*^{-/-} cells did not interfere with the overall survival rates (Fig. 3G), and also combined treatment with SP600125 and SB203580 did not alter the response of H_2O_2 -treated *Parp1*^{-/-} cells (Fig. 3H).

In conclusion, we identified two distinct cellular stress responses to H_2O_2 : a PARP1- and PI3K-dependent response involving Ca^{2+} signaling and phosphorylation of ERK1/2 and AKT leading to cell death and a PARP1-independent response involving the preferential phosphorylation of p38, SAPK/JNK, and CREB/ATF-1 leading to survival.

PARP1 and Ca^{2+} is involved in autophagy signaling. Autophagy is an organized clearance mechanism that delivers damaged cell structures to lysosomes for subsequent degradation (18, 26, 34). H_2O_2 induces autophagy, and this is associated with lysosomal Ca^{2+} release (5, 25, 33). The results in Fig. 4A show that H_2O_2 induces early autophagy markers in WT and *Parp1*^{-/-} cells (15). While beclin-1 was expressed constitutively, Atg7 and an Atg12-Atg5 complex were induced by H_2O_2 in WT cells and with some delay in *Parp1*^{-/-} cells. However, the autophagy marker microtubule-associated protein 1 light chain (Map1lc3b; LC-3B-II) was evident only in *Parp1*^{-/-} cells (Fig. 4A to C), suggesting

FIG 2 PI3K acts upstream in oxidant-induced cell death. (A) Cytosolic Ca^{2+} shifts after treatment with 5 mM H_2O_2 in control (– U-73122) WT cells and those treated with 1 μM U-73122 (+ U-73122). Results represent the mean $\pm$ SD (*, $P < 0.0005$; $n \geq 3$; t test). (B) Western blot analysis of pAKT and pERK1/2 in WT cells treated with different doses of U-73122 as indicated (pretreatment for 5 min) and 5 mM H_2O_2 for 30 min. GAPDH was used as the control. (C) Western blot analysis of IP3 receptor 1 in WT and *Parp1*^{−/−} (P1^{−/−}) cells shown. Topo-1 was the loading control. (D) Cytosolic Ca^{2+} shifts after treatment with 5 mM H_2O_2 in control (– LY) WT cells and those treated with 50 μM LY294002 (+ LY) (pretreatment for 30 min). Results represent the mean $\pm$ SD (*, $P < 0.0001$; $n \geq 3$; t test). (E) Western blot analysis of pAKT and pERK1/2 in WT cells treated with different doses of LY294002 as indicated (pretreatment for 30 min) and 5 mM H_2O_2 for 30 min. GAPDH was used as the control. (F) Cytotoxicity assay of WT cells treated or not treated with LY294002 (1 h pretreatment, 50 μM) and 5 mM H_2O_2 (mean $\pm$ SD; *, $P < 0.05$; $n = 4$; t test). (G) Western blot analysis of phosphorylated ERK1/2 (pERK1/2). ERK1/2 was used as the control for WT and *Parp1*^{−/−} cells treated with 5 mM H_2O_2 for the times indicated. (H) Western blot analysis of phosphorylated AKT (pAKT) in 5 mM H_2O_2 -treated WT and *Parp1*^{−/−} MEFs at the indicated time points. α -Tubulin was the loading control. (I) Cytotoxicity assay of WT cells treated or not treated with PD-98059 (pretreatment overnight) and 5 mM H_2O_2 (mean $\pm$ SD; not significant; $n = 3$; t test). (J) Western blot analysis of pERK1/2 in WT cells treated with PD-98059 as indicated (pretreatment overnight) and 5 mM H_2O_2 for 1 h. α -Tubulin was the loading control. UT, untreated.

that PARP1 suppresses late-stage autophagy. In fact, LC-3B lipidization is a marker of autophagosome formation. It is activated by the Atg-7-mediated conjugation of phosphatidylethanolamine. Figure 4B shows the course of LC-3B-II formation in *Parp1*^{−/−} cells and the virtual absence of this marker in WT cells. Nevertheless, while WT cells failed to display LC-3B-II in response to H_2O_2 , they retained the expected LC-3B-II formation in star-

vation-induced autophagy (see Fig. S3C in the supplemental material). Likewise, LC-3B-II could also be induced with bafilomycin A1 (4, 14, 19), a canonical lysosomal Ca^{2+} releaser (see Fig. S3A and B). LC-3B-II induction in *Parp1*^{−/−} cells was sensitive to the autophagy inhibitor 3-MA (Fig. 4C). Microscopic analysis demonstrated the distribution of LC-3B-II immunofluorescence dots exclusively in H_2O_2 -treated *Parp1*^{−/−} cells and not in untreated

FIG 3 The role of PARP1 in Ca²⁺-independent stress signaling. (A) Western blot analysis of p-p38 in WT and *Parp1*^{-/-} cells treated with 5 mM H₂O₂ for the times indicated. Unphosphorylated p38 was the loading control. (B) Western blot analysis of pSAPK/JNK in WT and *Parp1*^{-/-} cells treated with 5 mM H₂O₂ for the times indicated. Unphosphorylated SAPK/JNK was the loading control. (C) Western blot analysis of pCREB and pATF-1 in WT and *Parp1*^{-/-} cells treated with 5 mM H₂O₂ for the times indicated. Unphosphorylated CREB was the loading control. (D) Western blot analysis of pSAPK/JNK in *Parp1*^{-/-} cells treated with increasing doses of SP600125 (1 h pretreatment) as indicated and 5 mM H₂O₂ for 4 h. GAPDH was the loading control. (E) Western blot analysis of p-p38, p-38, p-MSK1, and MSK1 in *Parp1*^{-/-} cells treated with 5 mM H₂O₂ and increasing doses of SB203580 as indicated for 2 h. GAPDH was the loading control. (F) Survival was monitored in *Parp1*^{-/-} cells treated with 50 μM SP600125 (1 h pretreatment) and 5 mM H₂O₂ (mean ± SD; not significant; *n* = 3; *t* test). (G) Survival was monitored in *Parp1*^{-/-} cells treated with 10 μM SB203580 (1 h pretreatment) and 5 mM H₂O₂ (mean ± SD; not significant; *n* = 4; *t* test). (H) Survival was monitored in *Parp1*^{-/-} cells treated with 50 μM SP600125 together with 10 μM SB203580 (1 h pretreatment) and 5 mM H₂O₂ (mean ± SD; not significant; *n* = 4; *t* test). UT, untreated.

FIG 4 PARP1 is involved in autophagy signaling after H₂O₂ treatment. (A) Western blot analysis of beclin-1, Atg7, Atg12-Atg5, and LC-3B-II in WT or *Parp1*^{-/-} cells after H₂O₂ treatment as indicated. α-Tubulin was the loading control. (B) Statistical analysis of LC-3B-II Western blot signals of 5 mM H₂O₂-treated WT or *Parp1*^{-/-} cells at the indicated time points. Results are shown as arbitrary units (AU) normalized to GAPDH or α-tubulin (mean ± SD; **, *P* < 0.01; ***, *P* < 0.001; *n* ≥ 4; two-way ANOVA with Bonferroni multiple-comparison posttest). (C) Western blot analysis of LC-3B-II in *Parp1*^{-/-} cells pretreated for 1 h with increasing doses of 3-MA before a challenge with 5 mM H₂O₂ for 2 h. (D, top) LC-3B-II and PCNA staining by immunofluorescence in WT and *Parp1*^{-/-} cells treated with 5 mM H₂O₂ after 2 h. (D, bottom) Quantification of LC-3B-II dots in WT (*n* = 142) and *Parp1*^{-/-} cells (*P1*^{-/-}; *n* = 94) after 2 h of H₂O₂ treatment (mean ± standard error of the mean; *, *P* < 0.025; *t* test). Only LC-3B-II-positive cells were considered. (E) Visualization of acidic organelles with Lyso-ID Red dye in WT and *Parp1*^{-/-} cells after 2 h of 5 mM H₂O₂ treatment or after 2 h of 300 μM CQ treatment with Hoechst staining as the control. UT, untreated.

FIG 5 H₂O₂ induction of mTOR-mediated autophagy and role of beclin-1 in H₂O₂-induced PARP1-dependent cell death. (A) Western blot analysis of phosphorylated AMPK (pAMPK) in WT and *Parp1*^{-/-} cells at the indicated time points after treatment with 5 mM H₂O₂. The levels of phosphorylated p70S6K (p-p70S6K) are also shown, as well as p62. α -Tubulin was the loading control. (B) *Becln1* mRNA levels of WT and *Parp1*^{-/-} cells with the *Becln1* gene silenced (siRNA-B1) or not silenced (siRNA-C) as determined by RT-PCR normalized to GAPDH (mean $\pm$ SD; *, $P < 0.0001$; $n = 3$; t test). (C) Western blot analysis of WT and *Parp1*^{-/-} (P1^{-/-}) cells with *Becln1* (B1) or a control (C) silenced. PARP1 and GAPDH were controls. (D) Assay of cytotoxicity of treatment with 5 mM H₂O₂ of WT ($n = 4$) and *Parp1*^{-/-} cells ($n = 4$) with *Becln1* (siRNA-B1) or a control (siRNA-C) silenced (mean $\pm$ SD; not significant in WT; *, $P < 0.025$ in *Parp1*^{-/-} cells; t test). UT, untreated.

and WT cells (Fig. 4D). The fusion of lysosomes with the autophagosome is the final step in autophagy. To monitor this, we determined lysosomal activity by using the acidity-selective dye Lyso-ID in WT and *Parp1*^{-/-} cells challenged with 5 mM H₂O₂ for 2 h. Figure 4E shows massively induced lysosomal activity in *Parp1*^{-/-} but not in WT cells. The lysosome-perturbing agent chloroquine (CQ), which regulates changes in lysosome number, was used as a positive control (Fig. 4E). From these data, we conclude that PARP1 is a suppressor of H₂O₂-induced autophagy.

Autophagy plays an integrative role in PARP1-dependent cell death after H₂O₂ treatment. The mammalian target of rapamycin (mTOR) complex is an integrative negative regulator in autophagy signaling and senses growth factors, nutrients, and energy availability (18, 26, 34). First, we analyzed phosphorylated AMP-activated protein kinase (pAMPK), one of the upstream inhibitors of mTOR, in WT and *Parp1*^{-/-} cells after H₂O₂ treatment (Fig. 5A). AMPK phosphorylation was already observed after 0.25 h and maintained until 2 h, regardless of *Parp1* status. However, phosphorylated AMPK declined markedly between 2 and 6 h in WT cells but not in *Parp1*^{-/-} cells. Second, H₂O₂ initiated mTOR-mediated dephosphorylation of p70S6K in WT and

Parp1^{-/-} cells, but it was delayed in the latter (Fig. 5A). Independent of PARP1, phosphorylation of p70S6K was restored to basic levels within 6 h posttreatment, suggesting its dynamic regulation. Remarkably, 1 and 2 h after oxidative damage, phosphorylation of p70S6K was lowest in *Parp1*^{-/-} cells. A similar pattern was observed in the degradation of p62, a second factor downstream of mTOR activity. This strongly correlates with maximal LC-3B-II conversions in these cells (Fig. 4B). The observed (de)activation of AMPK, p70S6K, and p62 clearly indicates a direct involvement of mTOR signaling in H₂O₂-induced autophagy. The AMPK and p70S6K expression levels in the two cell types were similar (data not shown). Control experiments using serum starvation showed that mTOR signaling was intact in both of the cell types analyzed (see Fig. S3C in the supplemental material). To determine effects of H₂O₂-induced autophagy on cell survival, we performed experiments with the autophagy inhibitor 3-MA in *Parp1*^{-/-} cells. However, no effects were seen, even at an inhibitor concentration of up to 20 mM (see Fig. S3D in the supplemental material). Moreover, the mTOR inhibitor PP242 could enhance H₂O₂-induced autophagy in *Parp1*^{-/-} cells as assessed by p-p70S6K, p62, and LC-3B-II conversion 2 h after H₂O₂ treatment (see Fig. S3E). In-

FIG 6 Treatment with 10 mM H₂O₂ induces PAR formation and cytosolic Ca²⁺ shifts in *Parp1*^{-/-} cells. (A) WT and *Parp1*^{-/-} cells were treated with increasing concentrations of H₂O₂ as indicated. Cell survival was determined ($n \geq 4$). (B) PAR detection in *Parp1*^{-/-} cells by immunofluorescence after 30 min of treatment with 10 mM H₂O₂. White light was used as the control. (C) Fluo-4 assay of *Parp1*^{-/-} cells treated with 10 mM H₂O₂ with and without extracellular Ca²⁺. Results represent the mean $\pm$ SD (*, $P < 0.0001$; $n = 4$; t test). (D, top) Cytosolic Ca²⁺ shifts of *Parp1*^{-/-} cells with *Parp2* (siRNA-P2) or a control (siRNA-C) silenced after treatment with 10 mM H₂O₂ in Ca²⁺-containing buffer (mean $\pm$ SD; *, $P < 0.05$; $n = 4$; t test). (D, bottom) Same as before but in the absence of extracellular Ca²⁺ (mean $\pm$ SD; *, $P < 0.005$; $n = 4$; t test). (E) Comparison of cytosolic Ca²⁺ levels at 1,700 s after treatment with 10 mM H₂O₂ in *Parp1*^{-/-} control and 3-AB treated cells (3 mM 3-AB overnight) in the presence (mean $\pm$ SD; *, $P < 0.005$; $n \geq 3$; t test) or absence of extracellular Ca²⁺ (mean $\pm$ SD; *, $P = 0.0005$; $n = 5$; t test).

terestingly, parallel treatment of PP242 together with H₂O₂ did not alter the overall cell survival rate (see Fig. S3F).

In a more targeted analysis, RNAi silencing of the autophagy upstream regulator *Becn1* in *Parp1*^{-/-} and WT cells (Fig. 4A) was effective at the mRNA (Fig. 5B) and protein levels (Fig. 5C) but did not alter the cell death response in WT cells treated with 5 mM H₂O₂ (Fig. 5D, left side). Nevertheless, the autophagy-proficient *Parp1*^{-/-} cells displayed a higher survival rate when challenged with 5 mM H₂O₂ (Fig. 5D, right side), suggesting that beclin-1-dependent autophagy does not rescue cells from cell death.

PARP2 as a back-up mechanism for PAR synthesis and Ca²⁺ signaling in *Parp1*^{-/-} cells. *Parp1*^{-/-} cells were markedly more resistant to H₂O₂ than WT cells were (Fig. 6A and 1A). An H₂O₂ concentration (5 mM) 10 times higher than in WT cells (0.5 mM)

was needed to induce a cell death rate of 25% in *Parp1*^{-/-} cells. A 5 mM H₂O₂ concentration led to 75% cell death in WT cells. A 10 mM H₂O₂ concentration induced equivalent death rates in *Parp1*^{-/-} cells (Fig. 6A), and this caused substantial PAR synthesis by PARP2 (Fig. 6B; see Fig. S4A in the supplemental material). A rapid increase in cytosolic Ca²⁺ to more than 100 RFU was observed with almost linear kinetics up to 30 min (Fig. 6C). This was double the amount observed in WT cells challenged with equivalent doses of H₂O₂ (Fig. 1B).

RNAi silencing was used to address the role of PARP2 in Ca²⁺ signaling in *Parp1*^{-/-} cells. siRNA-P2 reduced *Parp2* mRNA to 36.4% $\pm$ 10.65% of control levels (see Fig. S4B in the supplemental material). As a functional consequence, PAR formation by PARP2 was reduced in *Parp1*^{-/-} cells after treatment with 10 mM

FIG 7 Intracellular TRPM2 channels release Ca^{2+} in *Parp1*^{-/-} cells after H_2O_2 treatment. (A, left side) Fluo-4 assays with extracellular Ca^{2+} in *Parp1*^{-/-} cells with *Trpm2* (siRNA-T2) or a control (siRNA-C) silenced after treatment with 10 mM H_2O_2 (mean $\pm$ SD; *, $P < 0.00025$; $n = 4$; t test). (A, right side) Fluo-4 assays done as before but without extracellular Ca^{2+} (mean $\pm$ SD; *, $P < 0.05$; $n \geq 3$; t test). (B) Comparison of cytosolic Ca^{2+} levels at 1,700 s after treatment with 10 mM H_2O_2 in *Parp1*^{-/-} control and ACA-treated cells (pretreatment for 5 min; 5 μM) with extracellular Ca^{2+} (mean $\pm$ SD; *, $P < 0.0001$; $n \geq 3$; t test) and without Ca^{2+} (mean $\pm$ SD; *, $P = 0.0001$; $n = 3$; t test). (C) Cytosolic Ca^{2+} shifts in *Parp1*^{-/-} cells with both *Parp2* and *Trpm2* (siRNA-P2T2) or a control (siRNA-C) silenced after treatment with 10 mM H_2O_2 in the absence of extracellular Ca^{2+} (mean $\pm$ SD; *, $P < 0.0025$; $n \geq 3$; t test).

H_2O_2 (see Fig. S4A in the supplemental material). *Parp2* silencing reduced the overall cytosolic Ca^{2+} response from 99.0 ± 5.85 RFU (siRNA-C) to 77.9 ± 14.59 RFU (siRNA-P2) at 1,700 s (Fig. 6D, top) in the presence of extracellular Ca^{2+} . Interestingly, the silencing effect on intracellular Ca^{2+} release was greater (Fig. 6D, bottom), suggesting that PARP2 regulates Ca^{2+} release from intracellular stores. The PARP inhibitor 3-AB also reduced Ca^{2+} shifts (Fig. 6E; see Fig. S4C and D in the supplemental material), indicating that PARP2 catalytic activity is involved. The peak levels of Ca^{2+} abrogation obtained with siRNA-P2 and 3-AB were similar (21 versus 25 RFU at 1,700 s). More importantly, this effect was also observed in *Parp1*^{-/-} cells incubated with 3-AB in the absence of extracellular Ca^{2+} (Fig. 6E; see Fig. S4D). *Parp2* silencing or incubation with 3-AB did not alter the baseline cytosolic Ca^{2+} levels of unchallenged cells (see Fig. S1M, N, Q, and T in the supplemental material). Taken together, these observations suggest that PARP2 activity controls an intracellular Ca^{2+} release mechanism.

PARP2 activity mobilizes intracellular TRPM2 channels in a *Parp1*^{-/-} background. We tested the hypothesis that PARP2-sensitive intracellular Ca^{2+} release after H_2O_2 treatment in *Parp1*^{-/-} cells could be mediated by TRPM2. RNAi silencing of *Trpm2* in *Parp1*^{-/-} cells challenged with 10 mM H_2O_2 reduced cytosolic Ca^{2+} responses (Fig. 7A, left side). Repeating these experiments in the absence of extracellular Ca^{2+} again suggested an intracellular source of Ca^{2+} (Fig. 7A, right side). To verify this, we used the broad-spectrum inhibitor of TRP channels ACA (1) (Fig. 7B; see Fig. S4E and F in the supplemental material). As expected, treatment with ACA reduced the cytosolic Ca^{2+} response to H_2O_2

in *Parp1*^{-/-} cells when extracellular Ca^{2+} was present (Fig. 7B; see Fig. S4E), as well as in the absence of extracellular Ca^{2+} (Fig. 7B; see Fig. S4F). In fact, Ca^{2+} release from intracellular sources was the same in siRNA-C-transfected cells (45.5 ± 12.59 RFU, Fig. 7A, right side) and *Parp1*^{-/-} cells without ACA (49.6 ± 5.03 RFU, Fig. 7B; see Fig. S4F). In addition, blocking TRPM2 activity by either siRNA-T2 (Fig. 7A, right side) or ACA (Fig. 7B; see Fig. S4F) reduced this level to 24.3 ± 7.01 or 23.3 ± 4.99 RFU in the absence of extracellular Ca^{2+} , respectively. *Trpm2* silencing or ACA incubation did not induce cytosolic Ca^{2+} differences of unchallenged cells (see Fig. S1K, L, O, and P in the supplemental material). Moreover, double silencing of *Parp2* and *Trpm2* (siRNA-P2T2) could not additionally promote this effect (Fig. 7C; see Fig. S1U). Thus, Ca^{2+} release from intracellular compartments is an integral part of the H_2O_2 response of *Parp1*^{-/-} cells and is under the control of PARP2 (Fig. 6D and E; see Fig. S4C and D) and TRPM2 (Fig. 7A to C; see Fig. S4E and F).

DISCUSSION

Oxidative stress induces DNA damage, Ca^{2+} mobilization, autophagy, and cell death (Fig. 8). It is not well understood how these stress responses are interconnected and regulated. Here we found that Ca^{2+} imbalances, primarily gated by two membrane-bound TRPM2 channels, are under the control of two DNA nick-sensing enzymes, PARP1 and PARP2. Using a combination of genetic knockout, silencing, and chemical inhibition, we found that plasma membrane TRPM2 channels are under the control of PARP1 under moderate-stress conditions. Lysosomal TRPM2 seems to be the channel for high-stress conditions, activated by

FIG 8 Roles of PARP1 and PARP2 in the oxidative stress response leading to cell death, autophagy, or cell survival. (A) Summary of PARP1- and PARP2-driven, TRPM2-mediated cytosolic Ca²⁺ responses after H₂O₂-induced DNA damage. (B) A moderate stress level (5 mM H₂O₂) mobilizes Ca²⁺ via activation of PARP1. PARP1 produces the activating signal (ADPR) for the plasma membrane TRPM2 channel in conjunction with PARG (14). This, in combination with the phosphorylation of ERK1/2 and AKT, causes cell death in WT MEFs. A moderate stress level (5 mM H₂O₂) in a *Parp1*^{-/-} background involves the phosphorylation of p38, SAPK/JNK, and CREB/ATF-1 and activates autophagy markers, leading to cell survival. PARP1 operates as a checkpoint between cell survival and death or autophagy. A high level of oxidative stress (10 mM H₂O₂) triggers late autophagy steps and leads to the activation of PARP2 activity in a *Parp1*^{-/-} background. This ends in Ca²⁺ mobilization from lysosomes, the only other known localization of TRPM2, leading to cell death.

PARP2. Both channels respond to ADPR generated by one of the PARPs in conjunction with PARG, which converts PAR into ADPR (3).

PARP1 plays the dominant role in H₂O₂-induced Ca²⁺ mobilization when cells are navigating toward cell death. The cytosolic Ca²⁺ shifts originated from both PI3K-mediated and TRPM2-gated Ca²⁺ sources. Whereas abrogation of PLC and PI3K abolished the first Ca²⁺ peak at 150 s after H₂O₂ treatment (Fig. 1 and 2), the subsequent rise in cytoplasmic Ca²⁺ was not affected (Fig. 2). These findings indicate that there are two different and independent mechanisms by which PARP1 controls the cytosolic Ca²⁺ stress response. Indeed, *Parp1* ablation led to a different stress kinase pattern, resulting in cell survival after exposure to 5 mM H₂O₂, a dose that killed their WT counterparts (Fig. 1 to 3). Our data also show that PARP1 does not control the entire intracellular Ca²⁺ mobilization process since there is a substantial amount that could come from the endoplasmic reticulum (ER), which contains around 500 μ M Ca²⁺ (31). We found a substantial ER stress response, i.e., protein kinase-like ER kinase phosphorylation (pPERK), regardless of the *Parp1* proficiency of the cells (see Fig. S3G in the supplemental material).

PARP2 operates as a backup mechanism under high-stress conditions (10 mM H₂O₂) in the absence of PARP1. It activates PAR synthesis and subsequent cytosolic Ca²⁺ shifts (Fig. 6 and 7). As PARP2 generates PAR to a lesser extent than PARP1 does (38), an equitoxic concentration (10 versus 5 mM) is required to advance *Parp1*^{-/-} cells into the cell death pathway. Moreover, *Parp1*^{-/-} cell death shows PARP2- and TRPM2-dependent cytosolic Ca²⁺ responses. In contrast to dying WT cells, PARP2/TRPM2-mediated Ca²⁺ shifts originate from intracellular compartments (Fig. 6 and 7). TRPM2 localizes to lysosomes, the only other membrane system described, expressing TRPM2 channels (16, 30). TRPM2 channels in lysosomes are associated with cellular functions different from plasma membrane localization, e.g., the differentiation and maturation of dendritic cells (29). Lysosomes represent a substantial pool of 400 to 600 μ M Ca²⁺ (6, 9, 19).

Another major finding was that PARP1 is not required for the late autophagic response. In fact, only *Parp1*^{-/-} cells showed autophagy markers (Fig. 4A and B) and related autophagy phenotypes (Fig. 4D and E). This suggests that *Parp1* is an autophagy suppressor gene. Moreover, H₂O₂ treatment stimulated the mTOR complex, a negative regulator of autophagy. Phosphorylated AMPK and dephosphorylated p70S6K and p62, all indicative of mTOR signaling, were observed solely in *Parp1*^{-/-} cells (Fig. 5A). This correlated closely with maximal LC-3B-II conversion in these cells (Fig. 4B). Moreover, the autophagy-proficient *Parp1*^{-/-} cells with *Becn1* silenced displayed a higher survival rate after an oxidative insult. Therefore, beclin-1-dependent autophagy seemed not to rescue these cells from death. In general, beclin-1 exists in a complex with bcl-2 (B-cell lymphoma 2) in the cells and becomes liberated from this complex because of phosphorylation of JNK or p38 kinase to process autophagy (8, 36). Both stress kinases are activated in H₂O₂-treated *Parp1*^{-/-} cells (Fig. 3). Therefore, we analyzed inhibitors of JNK and p38. However, none of the chemicals applied or their combination was sufficient to modulate the amount of surviving cells after H₂O₂ treatment. Nevertheless, both inhibitors successfully repressed their corresponding downstream signaling factors.

In conclusion, this study describes three different stress re-

sponse scenarios under the control of PARP1 and PARP2 (Fig. 8A and B). First, 5 mM H₂O₂ causes cell death in the WT background. This cell death is characterized by PARP1 activation with concomitant plasma membrane TRPM2- and PI3K-dependent cytosolic Ca²⁺ elevations and subsequent ERK1/2 and AKT phosphorylation (Fig. 1 and 2). In contrast, 5 mM H₂O₂ does not affect cell viability in the absence of PARP1. This type of stress response involves the phosphorylation of p38, MSK1, SAPK/JNK, and CREB/ATF-1 and, beyond that, activates autophagy markers, leading finally to cell survival (Fig. 3 to 5). Third, 10 mM H₂O₂ is an equitoxic killing dose in the *Parp1*^{-/-} background compared to in WT cells (Fig. 8B). Here, PARP2 generates TRPM2-dependent cytosolic Ca²⁺ shifts from an intracellular source, most likely lysosomal, triggering a parallel autophagy pathway leading to cell death (Fig. 6 and 7).

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REFERENCES

1. Bari MR, et al. 2009. H₂O₂-induced Ca²⁺ influx and its inhibition by *N*-(*p*-amylcinnamoyl) anthranilic acid in the beta-cells: involvement of TRPM2 channels. *J. Cell Mol. Med.* 13:3260–3267.
2. Blanc A, Pandey NR, Srivastava AK. 2003. Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H₂O₂ in vascular smooth muscle cells: potential involvement in vascular disease (review). *Int. J. Mol. Med.* 11:229–234.
3. Blenn C, Wyrsch P, Bader J, Bollhalder M, Althaus FR. 2011. Poly(ADP-ribose)glycohydrolase is an upstream regulator of Ca²⁺ fluxes in oxidative cell death. *Cell. Mol. Life Sci.* 68:1455–1466.
4. Bowman EJ, Siebers A, Altendorf K. 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. U. S. A.* 85:7972–7976.
5. Cheng WT, et al. 2009. Oxidative stress promotes autophagic cell death in human neuroblastoma cells with ectopic transfer of mitochondrial PPP2R2B (Bbeta2). *BMC Cell Biol.* 10:91. doi:10.1186/1471-2121-10-91.
6. Christensen KA, Myers JT, Swanson JA. 2002. pH-dependent regulation of lysosomal calcium in macrophages. *J. Cell Sci.* 115:599–607.
7. Cohausz O, Blenn C, Malanga M, Althaus FR. 2008. The roles of poly(ADP-ribose)-metabolizing enzymes in alkylation-induced cell death. *Cell. Mol. Life Sci.* 65:644–655.
8. De Chiara G, et al. 2006. Bcl-2 phosphorylation by p38 MAPK: identification of target sites and biologic consequences. *J. Biol. Chem.* 281:21353–21361.
9. Dong XP, Wang X, Xu H. 2010. TRP channels of intracellular membranes. *J. Neurochem.* 113:313–328.
10. Green DR, Galluzzi L, Kroemer G. 2011. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. *Science* 333:1109–1112.
11. Hassa PO, Haenni SS, Elser M, Hottiger MO. 2006. Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol. Mol. Biol. Rev.* 70:789–829.
12. Heeres JT, Hergenrother PJ. 2007. Poly(ADP-ribose) makes a date with death. *Curr. Opin. Chem. Biol.* 11:644–653.
13. Joseph SK, Hajnoczky G. 2007. IP3 receptors in cell survival and apoptosis: Ca²⁺ release and beyond. *Apoptosis* 12:951–968.
14. Kinnear NP, Boittin FX, Thomas JM, Gialione A, Evans AM. 2004. Lysosome-sarcoplasmic reticulum junctions. A trigger zone for calcium signaling by nicotinic acid adenine dinucleotide phosphate and endothelin-1. *J. Biol. Chem.* 279:54319–54326.
15. Klionsky DJ, et al. 2010. A comprehensive glossary of autophagy-related molecules and processes. *Autophagy* 6:438–448.

16. Lange I, et al. 2009. TRPM2 functions as a lysosomal Ca^{2+} -release channel in beta cells. *Sci. Signal.* 2:ra23. doi:10.1126/scisignal.2000278.
17. Lee B, et al. 2009. The CREB/CRE transcriptional pathway: protection against oxidative stress-mediated neuronal cell death. *J. Neurochem.* 108:1251–1265.
18. Levine B, Mizushima N, Virgin HW. 2011. Autophagy in immunity and inflammation. *Nature* 469:323–335.
19. Lloyd-Evans E, et al. 2008. Niemann-Pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. *Nat. Med.* 14:1247–1255.
20. Marks AR. 1997. Intracellular calcium-release channels: regulators of cell life and death. *Am. J. Physiol.* 272:H597–H605.
21. Naziroğlu M. 2011. TRPM2 cation channels, oxidative stress and neurological diseases: where are we now? *Neurochem. Res.* 36:355–366.
22. Nishina H, Wada T, Katada T. 2004. Physiological roles of SAPK/JNK signaling pathway. *J. Biochem.* 136:123–126.
23. Patel S, Docampo R. 2009. In with the TRP channels: intracellular functions for TRPM1 and TRPM2. *Sci. Signal.* 2:pe69. doi:10.1126/scisignal.295pe69.
24. Perraud AL, et al. 2001. ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature* 411:595–599.
25. Pyo JO, et al. 2008. Compensatory activation of ERK1/2 in Atg5-deficient mouse embryo fibroblasts suppresses oxidative stress-induced cell death. *Autophagy* 4:315–321.
26. Rabinowitz JD, White E. 2010. Autophagy and metabolism. *Science* 330:1344–1348.
27. Sallmann FR, Vodenicharov MD, Wang ZQ, Poirier GG. 2000. Characterization of sPARP1. An alternative product of PARP1 gene with poly-(ADP-ribose) polymerase activity independent of DNA strand breaks. *J. Biol. Chem.* 275:15504–15511.
28. Sarkar S, Rubinsztein DC. 2006. Inositol and IP3 levels regulate autophagy: biology and therapeutic speculations. *Autophagy* 2:132–134.
29. Sumoza-Toledo A, et al. 2011. Dendritic cell maturation and chemotaxis is regulated by TRPM2-mediated lysosomal Ca^{2+} release. *FASEB J.* 25:3529–3542.
30. Sumoza-Toledo A, Penner R. 2011. TRPM2: a multifunctional ion channel for calcium signalling. *J. Physiol.* 589:1515–1525.
31. Szabadkai G, Duchon MR. 2008. Mitochondria: the hub of cellular Ca^{2+} signaling. *Physiology (Bethesda)* 23:84–94.
32. Szlufcik K, Missiaen L, Parys JB, Callewaert G, De Smedt H. 2006. Uncoupled IP3 receptor can function as a Ca^{2+} -leak channel: cell biological and pathological consequences. *Biol. Cell* 98:1–14.
33. Szumiel I. 2011. Autophagy, reactive oxygen species and the fate of mammalian cells. *Free Radic. Res.* 45:253–265.
34. Tanida I. 2011. Autophagy basics. *Microbiol. Immunol.* 55:1–11.
35. Tóth B, Csanady L. 2010. Identification of direct and indirect effectors of the transient receptor potential melastatin 2 (TRPM2) cation channel. *J. Biol. Chem.* 285:30091–30102.
36. Wei Y, Pattingre S, Sinha S, Bassik M, Levine B. 2008. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Mol. Cell* 30:678–688.
37. Wu GS. 2007. Role of mitogen-activated protein kinase phosphatases (MKPs) in cancer. *Cancer Metastasis Rev.* 26:579–585.
38. Yélamos J, Schreiber V, Dantzer F. 2008. Toward specific functions of poly(ADP-ribose) polymerase-2. *Trends Mol. Med.* 14:169–178.
39. Zhivotovsky B, Orrenius S. 2011. Calcium and cell death mechanisms: a perspective from the cell death community. *Cell Calcium* 50(3):211–221.