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# p38α Mediates Cell Survival in Response to Oxidative Stress via Induction of Antioxidant Genes

## *EFFECT ON THE p70S6K PATHWAY*[*] [S]

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**Background:** p38α MAPK is activated by stress stimuli, which can regulate cell death.
**Results:** In response to $H_2O_2$, p38α MAPK increases SOD and catalase levels, impairs ROS accumulation, and leads to cell survival.
**Conclusion:** p38α MAPK signals survival under moderate oxidative stress through up-regulation of antioxidant defenses.
**Significance:** To know how p38α regulates ROS levels is important for cell homeostasis.

**We reveal a novel pro-survival role for mammalian p38α in response to $H_2O_2$, which involves an up-regulation of antioxidant defenses. The presence of p38α increases basal and $H_2O_2$-induced expression of the antioxidant enzymes: superoxide-dismutase 1 (SOD-1), SOD-2, and catalase through different mechanisms, which protects from reactive oxygen species (ROS) accumulation and prevents cell death. p38α was found to regulate (i) $H_2O_2$-induced SOD-2 expression through a direct regulation of transcription mediated by activating transcription factor 2 (ATF-2) and (ii) $H_2O_2$-induced catalase expression through regulation of protein stability and mRNA expression and/or stabilization. As a consequence, SOD and catalase activities are higher in WT MEFs. We also found that this p38α-dependent antioxidant response allows WT cells to maintain an efficient activation of the mTOR/p70S6K pathway. Accordingly, the loss of p38α leads to ROS accumulation in response to $H_2O_2$, which causes cell death and inactivation of mTOR/p70S6K signaling. This can be rescued by either p38α re-expression or treatment with the antioxidants, *N*-acetyl cysteine, or exogenously added catalase. Therefore, our results reveal a novel homeostatic role for p38α in response to oxidative stress, where ROS removal is favored by antioxidant enzymes up-regulation, allowing cell survival and mTOR/p70S6K activation.**

The intracellular redox state is tightly regulated because it is essential for the control of cell fate. High levels of ROS[5] can lead to molecular damage and cell death, whereas low ROS levels can be essential second messengers (1). Pro-oxidant and antioxidant systems are involved in this regulation, preventing an excessive accumulation of ROS.

Different members of the MAPK family, such as ERKs, JNKs, and p38, can be activated by ROS (1). This activation leads to a great variety of biological responses, including cell death or survival. Hence, although the stress MAP kinases can induce apoptosis in response to oxidative stress (2, 3), differences in the duration and magnitude of the oxidative stress might be directly proportional to the state of activation of these kinases, and this might determine cell death or survival.

p38α MAPK plays an important role in the coordination of cellular stress responses to signals such as ROS. In fact, it is well known that p38α MAPK plays an important role in mediating apoptosis (4) and/or senescence induced by different stimuli, including ROS (5, 6). For example, ROS generated by oncogenic H-Ras induces apoptosis through p38α activation, inhibiting tumor initiation (7). In contrast, low levels of oxidative stress can also induce cell cycle arrest (8) or cell survival (9, 10) through p38. Initially, stress signaling mechanisms are pro-survival systems because they tend to repair damage before committing cells to death or senescence. Interestingly, p38α can mediate survival upon activation with $H_2O_2$ (10), and p38α and β can have pro-survival roles (11–13) such as during quiescence of dormant tumor cells (14). However, the precise mechanisms by which p38 signaling achieves cell survival are poorly understood.

We have previously demonstrated that Akt activity is negatively regulated by p38α (15), and recent data from Nogueira *et*

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[5] The abbreviations used are: ROS, reactive oxygen species; SOD, superoxide dismutase; ATF, activating transcription factor; MEF, mouse embryonic fibroblast; DCFH, 2′,7′-dichlorfluorescein-diacetate; NAC, *N*-acetyl cysteine; mTOR, mammalian target of rapamycin.

*al.* (16) showed that Akt activation sensitized cells to oxidative stress through down-regulation of ROS scavengers leading to the accumulation of intracellular ROS and cell death. Therefore, we hypothesized that p38α, through inhibition of Akt, might allow a proper expression of antioxidant genes and cell survival. Thus, we analyzed the precise function of p38α in the regulation of the cell fate using nontransformed WT and p38$\alpha^{-/-}$ MEFs exposed to oxidative stress. Furthermore, we explored the mechanisms involved in the regulation of antioxidant responses, in the context of Akt/mTOR signaling, as well as other pathways linked to ROS level regulation.

## EXPERIMENTAL PROCEDURES

*Cell Lines, Culture Conditions, and Inhibitors*—WT and p38α-deficient MEFs, immortalized either by passages or by LTAg (Large T Antigen) expression, were grown in DMEM supplemented with 10% FBS (Invitrogen) at 37 °C in a humidified atmosphere with 5% $CO_2$. For signaling experiments, confluent cells were stimulated with 0.1–1 mM $H_2O_2$ for 20 min. For cell death analysis, growing cells were treated with 0.1–1 mM $H_2O_2$ for 6–24 h. The mTORC1 inhibitor, rapamycin was used at a concentration of 1–10 μM.

*Treatment with Antioxidants, Actinomycin D, and MG-132*—The cells were treated with the following antioxidants to decrease intracellular levels of ROS and/or to metabolize $H_2O_2$: 50 units/ml of catalase (Sigma; C-1345) and 2.5 mM *N*-acetyl cysteine (Sigma; A-9165). The antioxidants were added 1 h before $H_2O_2$ treatment. To inhibit transcription, the cells were treated with actinomycin D (Sigma; A9415) at 5 μg/ml. To block proteasome-dependent proteins degradation cells were treated with the proteasome inhibitor MG-132 at 1 μM.

*Transfection Assays*—To re-express p38α MAPK in p38$\alpha^{-/-}$ MEFs or to express WT and constitutive active p70S6K, transient transfections were performed using Metafectene-Pro and the following constructs containing: (i) human p38α cDNA cloned into the EcoRI site of the pEFmlink expression vector (4); (ii) wtp70; and (iii) p70Δ29–46 ΔCT104 (deletion of amino acids 29–46 and C-terminal tail, 422–525) active mutant cloned in PMT2 vector containing HA tag (Addgene plasmid 1892) (17). The protocol supplied by the manufacturer was followed using 7 μl/1 μg of DNA/dish (20.000 cells). The cell assays were performed 48 h after transfection.

*ATF-2 RNA Interference*—Transfections of cells with siRNA targeting ATF-2 (Cell Signaling; 6433) or a control scrambled siRNA (Ambion) diluted in medium without serum at a final concentration of 50 nM were performed using siPORT NeoFX transfection reagent (Ambion) following the manufacturer's instructions. Trypsinized cells were resuspended and overlaid onto the transfection complexes. After 24 h under normal cell culture conditions, protein and RNA was isolated, or cell viability was quantified.

*SOD-2 Luciferase Analysis*—To assess SOD-2 promoter activity upon $H_2O_2$ treatment in WT and p38$\alpha^{-/-}$ MEFs, the cells were cotransfected with a construct containing the SOD-2 promoter coupled to luciferase reporter (kindly provided by Daret St. Clair, Kentucky University) and a plasmid-encoding *Renilla* luciferase (Clontech) (100–500 ng). Then cells were treated with $H_2O_2$ 0.5 mM for 4 and 8 h and lysed using the passive lysis buffer from Promega. Luciferase activity was detected with a luminometer (Molecular Devices Spectramax M5E) using a dual luciferase reporter kit from Promega following the manufacturer's instructions. Luciferase activity was normalized to *Renilla* luciferase activity.

*Western Blot Analysis*—Western blot analysis was carried out as previously described using total cell extracts (15). Proteins were separated by electrophoresis using Anderson gels (or SDS-PAGE gels) and transferred to nitrocellulose membranes that were probed with the following antibodies: Akt (Cell Signaling; 9272), catalase (Sigma; C-0979), p70S6K (Cell Signaling; 9202), p38α (Santa Cruz; sc-535), phospho-acetyl-CoA-carboxylase (Cell Signaling; 3661), phospho-Akt (Cell Signaling; 9271), phospho-AMPK (Cell Signaling; 2531), phospho-MKK3/6 (Cell Signaling; 9231), SOD-2 (Upstate Biotech; 06-984), TSC-1 (Cell Signaling; 4906), phospho-TSC-2 (Cell Signaling; 3615), phospho-p38 (Cell Signaling; 9211), phospho-p70S6K (Cell Signaling; 4376), anti-HA Clone 16 B12 (Covance; MMS-101P), phospho-ATF-2 (Cell Signaling; 9221), ATF-2 (Cell Signaling; 9226), and α-tubulin (Sigma; T-5168).

*Catalase and SOD Activity Assays*—Catalase activity was measured by quantification of peroxide decomposition in a 50 mM phosphate buffer at pH 7 containing 3 mM $H_2O_2$. This was monitored spectrophotometrically at 240 nm. SOD activity from cell extracts was quantified using a kit (BioVision, reference number K335-100), where WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfo-phenyl)-2H tetrazolium, monosodium salt) is the substrate. WST-1 produces a water-soluble formazan dye upon its reduction with superoxide anion, which can be monitored spectrophotometrically at 450 nm. The rate of the reduction is linearly related to the xanthine oxidase activity and inhibited by SOD, so the $IC_{50}$ of SOD is determined as a measure of SOD activity.

*RT-PCR and RT-Quantitative PCR Analysis*—After the isolation of total RNA with RNeasy Mini kit (Qiagen; 74104), 1–3 μg of RNA was reverse transcribed with SuperScrip III RT kit (Qiagen; 18080) to generate cDNA. Then PCR analysis was performed using specific primers: for SOD-1: forward, 5′GATGAAGAGAGGCATGTTGG-3′, and reverse, 5′-CCAATGATGCAATGGTCTCC-3′ (n141–n160 and n554–n573, respectively; accession number 000082.5); and for SOD-2: forward, 5′TGGGGCTGGCTTGGCTTCAA-3′, and reverse, 5′GCGTGCTCCCACACGTCAAT-3′ (n646–n665 and n751–n770, respectively; accession number 000083.5). The amplified bands were normalized using internal control: GAPDH, forward, 5′-CATCAAGAAGGTGGTGAAGC-3′, and reverse, 5′-CATCGAAGGTGGAAGAGT TGG-3′ in the same PCR. The conditions for the PCR were: 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min for 30 cycles. Quantitative analysis of catalase mRNA levels was performed by real time PCR using SYBR Green (Roche Applied Science) and the following specific primers: forward, 5′-GTCACCGGCACATGAATGGCT-3′ (n738–n759), and reverse, 5′-TGATGCCCTGGTCGGTCTTGT-3′ (n817–n839) using GAPDH primers (referred above) to normalize.

*Chromatin Immunoprecipitation Assay*—ChIP assay was performed essentially as described previously (18). Briefly, the cells ($3 \times 10^6$) were fixed in 1% formaldehyde solution (15 min)

to cross-link DNA with associated proteins. The cross-linking reaction was finished by the addition of 125 mM glycine (5 min), and cells were washed and harvested in PBS containing protease and phosphatase inhibitors. The pelleted cells were lysed on ice in a buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and protease and phosphatase inhibitors. Then cells were sonicated 10 seconds × 6 (at level 4 and 40% of potency). DNA was fragmented in a range of 200–600 bp. Equal amounts of chromatin were diluted in ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and incubated overnight at 4 °C with a P-ATF-2 antibody or a rabbit IgG (negative control), followed by 1 h of incubation with salmon sperm DNA/protein A-agarose beads. 10% of the sample was kept as an input. Then samples were centrifuged, and protein A-agarose beads pellets were sequentially washed with a low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl); a high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl); a LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1); and TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Protein-DNA complexes were eluted in a buffer containing 1% SDS and 0.1 M $NaHCO_3$. Then cross-linking was reversed by incubation in 200 mM NaCl for 4 h at 65 °C followed by incubation in 40 mM Tris-HCl, pH 6.5, 10 mM EDTA, and 20 μg of proteinase K for 1 h at 45 °C to eliminate proteins. DNA was then extracted with phenol/chloroform, precipitated, and analyzed by PCR using primers for AP-1 site (positions 844–853) from mouse SOD-2 promoter: forward, 5′-GCAAGCAGCAGAACTCGCAGC-3′, and reverse, 5′-AGCACTCAGGAGGCAGAGGCA-3′. Inputs were also analyzed by PCR.

*Analysis of Cell Viability and Apoptosis*—Cell viability was assayed through staining of adhered (viable) cells with crystal violet. The cells were washed with PBS, incubated with a crystal violet solution (0.2%, w/v) for 20 min, washed, and dried. Stained cells were lysed in 1% SDS, and absorbance at 560 nm was measured.

Apoptotic cells were quantified by flow cytometric analysis of the cell cycle. The cells were trypsinized, washed with PBS, and fixed with cold ethanol (70% v/v). Then they were washed, resuspended in PBS, and incubated with RNase (25 $\mu g/10^6$ cells) for 30 min at 37 °C. After the addition of 0.05% propidium iodide, the cells were analyzed in the cytometer. The percentage of cells in sub-$G_1$ was determined and considered as apoptotic.

To analyze condensed and fragmented nuclei, characteristic of apoptosis, they were stained with propidium iodide (5 μg/ml in PBS, 0.1% Triton X-100, 0.1 M EDTA supplemented with 5 μg/ml RNase) (19) and visualized by fluorescent microscopy. Apoptotic indices were calculated after counting 500–1,000 cells/treatment in an inverted fluorescence microscope (Eclipse TE300; Nikon).

*Measurement of Intracellular ROS*—The cells were incubated with 5 μM 2′,7′-dichlorfluorescein-diacetate (DCFH) (Sigma; 35845) in PBS for 30–60 min and then treated with $H_2O_2$ (0.1–1 mM) for 15 min. Finally, the cells were washed with PBS and analyzed in an inverted fluorescence microscope (Eclipse TE300; Nikon).

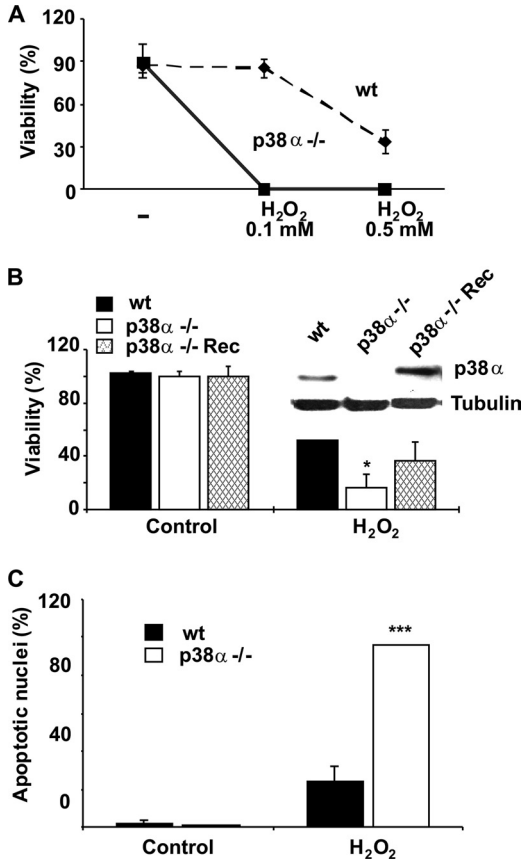


FIGURE 1. **p38α protects from $H_2O_2$-induced cell death.** MEFs (WT and p38$\alpha^{-/-}$) maintained in the presence of serum were treated with $H_2O_2$ when indicated. *A*, p38α expression increases cell viability in cells treated with $H_2O_2$ (0.1 or 0.5 mM) for 24 h. *B*, p38α reconstitution in p38$\alpha^{-/-}$ cells (*Rec*) rescues cells from cell death upon treatment with $H_2O_2$ (1 mM) for 6 h. The data correspond to cell viability expressed as percentages, and p38α expression was determined by Western blot and normalized with tubulin. *C*, loss of p38α increases the number of apoptotic nuclei in cells treated with $H_2O_2$ (0.5 mM) for 3 h. *, $p < 0.05$ and ***, $p < 0.001$, p38$\alpha^{-/-}$ *versus* WT MEFs upon treatment with $H_2O_2$.

*Statistical Analysis*—The data are represented as the means ± S.E. The comparisons were made between two experimental groups. An unpaired Student's *t* test was used, and alternatively, an analysis of variance test was carried out for comparisons of more than two experimental groups.

## RESULTS

*Loss of p38α Sensitizes Cells to $H_2O_2$-induced Cell Death*—We first tested viability of WT and p38α Knock-out MEFs in response to a level of oxidative stress able to generate damage but not massive toxicity. As shown in Fig. 1*A*, WT MEFs exhibited a higher resistance to $H_2O_2$ (0.1–0.5 mM) than p38α-deficient MEFs. This suggests that p38α allows cells to survive under mild levels of oxidative stress. This was confirmed by p38α reconstitution in p38$\alpha^{-/-}$ cells, which increased cell viability in response to $H_2O_2$ up to the levels observed in WT cells (Fig. 1*B*). The morphological analysis of nuclei showed a significantly higher number of condensed and/or fragmented nuclei in p38α-deficient MEFs than in WT cells upon $H_2O_2$ treatment (Fig. 1*C*), which suggests that cells would be dying by apoptosis. These data demonstrate that p38α protects from low levels of $H_2O_2$-induced cell death.

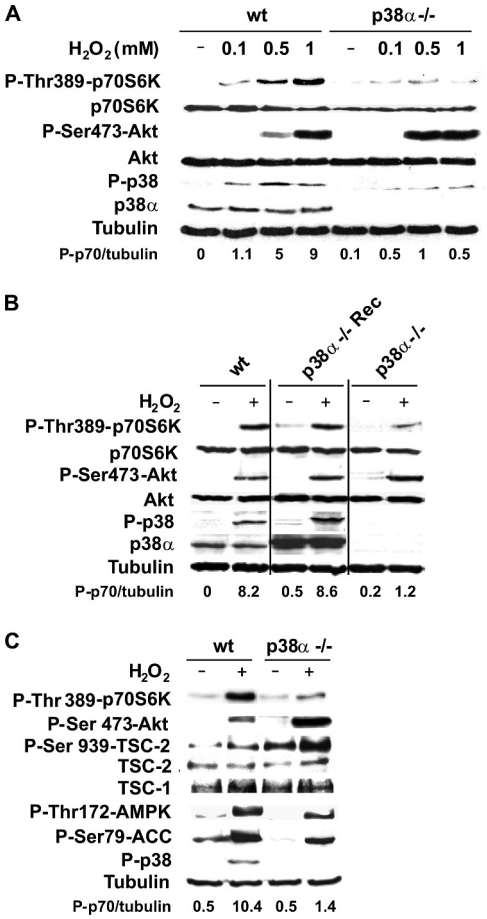


FIGURE 2. **p38α MAPK positively regulates p70S6K through an Akt independent mechanism.** MEFs (WT and p38$\alpha^{-/-}$) maintained in the presence of serum were treated with $H_2O_2$ (0.1–1 mM in *A*, 1 mM in *B* and *C*) for 20 min when indicated. Western blot analysis of the levels of Thr(P)-389-p70S6K (*P-Thr389-p70S6K*), Ser(P)-939-TSC-2 (*P-Ser939-TSC-2*), Ser(P)-473-Akt (*P-Ser473-Akt*), Thr(P)-172-AMPK (*P-Thr172-AMPK*), Ser(P)-79-acetyl-CoA-carboxylase (*P-Ser79-ACC*), Thr/Tyr(P)-180/182-p38 MAPK (P-Thr/Tyr-180/182-p38 MAPK) (*P-p38*) Ser (P)-189/207-MKK3/MKK6 (P-Ser-189/207-MKK3/MKK6) (*P-MKK3/6*), as well as total levels of p70S6K, Akt, TSC-1, and p38α normalized with tubulin are shown. *A* and *B*, effect of p38α expression on the activation of Akt and p70S6K in response to $H_2O_2$. p38α-deficient cells show a decrease activation of p70S6K as compared with WT or p38$\alpha^{-/-}$ with reconstitution of p38α (*Rec*). *C*, analysis of the activation of the p70S6K pathway by p38α showing p70S6K phosphorylation by mTOR and the activation of different upstream regulators (positive and negative). *P-p70/tubulin* represents the relative value resulting from the densitometric analysis of Thr(P)-389-p70S6K *versus* tubulin levels multiplied by 10.

*Loss of p38α MAPK Impairs mTOR/p70S6K Activation in Response to $H_2O_2$ through Akt-independent Mechanisms*—Akt is involved in the activation of mTORC1 through the phosphorylation and inactivation of TSC-2, which inhibits mTOR through inhibition of Rheb (20, 21). We have previously shown that Akt activity is negatively regulated by p38α (15), so that Akt is hyperactivated in p38$\alpha^{-/-}$ cells, leading to increased survival in response to serum deprivation (15). However, whether this has any effect on ROS sensitivity remains unknown. Recently, Nogueira *et al.* (16) showed that Akt activation sensitized cells to oxidative stress through down-regulation of ROS scavengers, which increased intracellular ROS. This led us to hypothesize that p38α, through inhibition of Akt, might allow a proper expression of antioxidant genes. Thus, we next tested the activation of the Akt/mTOR/p70S6K pathway in response to $H_2O_2$ in WT and p38α-deficient MEFs. As shown in Fig. 2*A*, Akt phosphorylation in response to $H_2O_2$ (0.1–1 mM) was higher (particularly, at 0.5 mM) in p38$\alpha^{-/-}$ than in WT MEFs. Surprisingly, this activation was uncoupled from mTOR-mediated p70S6K phosphorylation in Thr-389, because phosphorylation at this site was markedly reduced in p38α-deficient cells. Under these conditions, p38α was activated by $H_2O_2$ in WT cells, whereas in p38$\alpha^{-/-}$ MEFs another p38 isoform with a higher mobility was slightly activated (Fig. 2*A*). Therefore, these data suggest that in the absence of p38α, mTOR/p70S6K pathway activation becomes uncoupled from that of Akt and is impaired in response to $H_2O_2$ treatment. This was confirmed by p38α reconstitution in p38$\alpha^{-/-}$ cells, which rescued the levels of p70S6K activation (see Thr(P)-389-p70S6K levels; Fig. 2*B*), reaching a level comparable with WT cells treated with $H_2O_2$.

To gain further insight into the mechanisms involved in the regulation of mTOR/p70S6K pathway by p38α, we analyzed whether the regulators of mTORC1 were differentially modulated in the absence of p38α. As shown in Fig. 2*C*, the high level of Akt phosphorylation in p38α-deficient cells was correlated with Akt-mediated TSC-2 phosphorylation in Ser-939 (an inhibitory site) in response to $H_2O_2$, whereas TSC-1 and TSC-2 levels remained unchanged in all cases. Nevertheless, this did not result in a high activation of mTOR/p70S6K pathway. Hence, we measured activation of AMPK, an inhibitor of mTORC1 pathway. However, phosphorylation of AMPK (in Thr-172) and its substrate, acetyl-CoA-carboxylase in response to $H_2O_2$ was enhanced in WT MEFs (Fig. 2*C*). As a consequence, these changes in AMPK activity in WT and p38α-deficient cells did not explain the higher activation of mTOR/p706SK in WT cells exposed to oxidative stress. Therefore, neither TSC-1/2 nor AMPK appear to mediate the inhibition of mTOR signaling observed in p38$\alpha^{-/-}$ cells, which indicates that other mechanisms might be involved. However, it is clear that Akt and AMPK signaling is uncoupled from mTOR/p70S6K activation under these conditions.

We considered the possibility that the higher mTOR/p70S6K activation in cells expressing p38α could be responsible for the increased survival in response to $H_2O_2$. Thus, we inhibited this pathway with rapamycin to evaluate it. As shown in Fig. 3*A*, treatment with rapamycin completely abolished p70S6K phosphorylation by mTOR in response to $H_2O_2$. However, rapamycin did not sensitize WT cells to ROS-induced cell death (Fig. 3*B*). Thus, the higher activation of mTOR/p70S6K appears not to be responsible for the p38α-mediated increased survival. Nevertheless, rapamycin was able to decrease cell size in WT cells but not in those deficient in p38α (data not shown), suggesting a potential function for the p38α-mTOR pathway promoting and/or maintaining cell size and homeostasis under oxidative stress conditions. This is in agreement with recent published results (22). Moreover, transfection of either an active p70S6K (p70Δ29–46 ΔCT104 mutant) or a WT p70S6K construct did not increase the cell viability of p38α-deficient MEFs treated with $H_2O_2$ (Fig. 3*C*). In contrast, basal viability was slightly reduced upon expression of these p70S6K constructs.

On the other hand, Akt inhibition with the specific chemical inhibitor, A443354 (23) had no effect on $H_2O_2$-induced cell

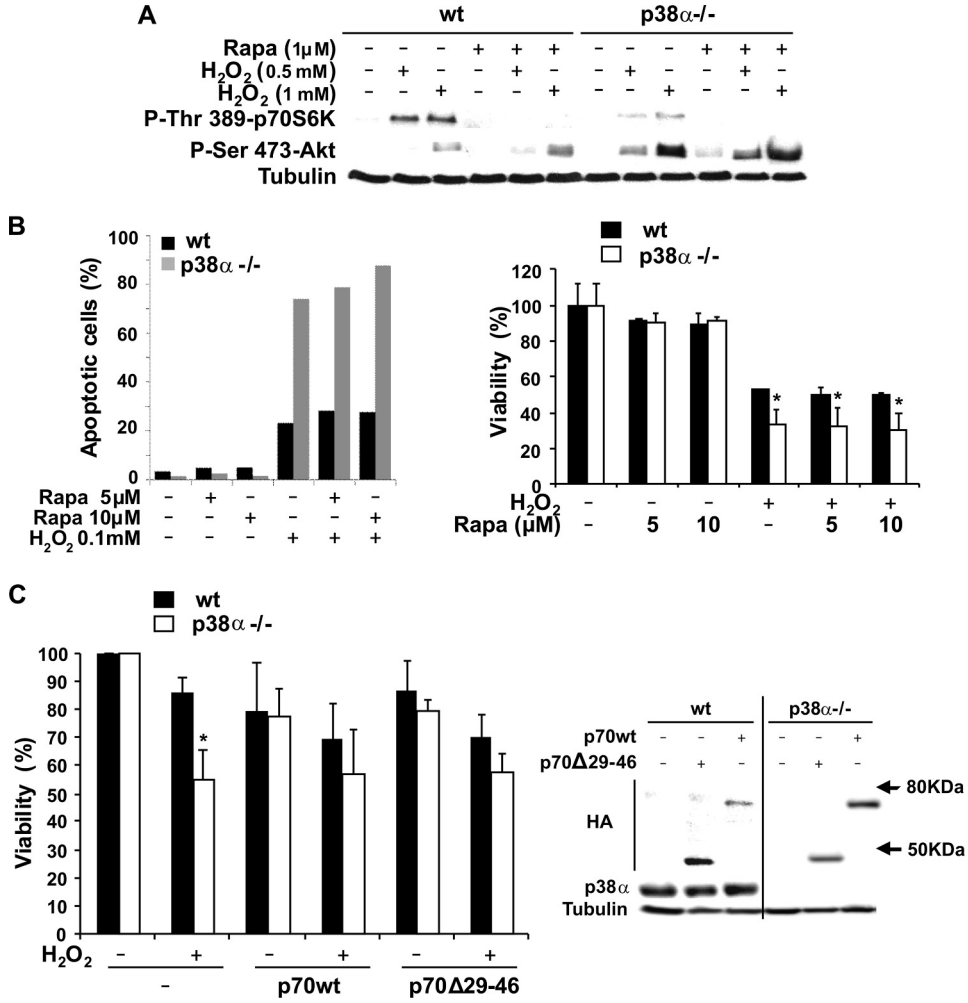


FIGURE 3. **mTORC1/p70S6K does not mediate p38α-dependent survival in response to $H_2O_2$.** MEFs (WT and p38$\alpha^{-/-}$) maintained in the presence of serum were treated with $H_2O_2$ (0.1, 0.5, or 1 mM) when indicated. *A*, effect of mTORC1 inhibition by rapamycin (*Rapa*) on $H_2O_2$-induced activation p70S6K and Akt. Western blot analysis of the levels of Thr(P)-389-p70S6K (*P-Thr389-p70S6K*) and Ser(P)-473-Akt (*P-Ser473-Akt*) normalized with tubulin upon treatment with $H_2O_2$ for 20 min. *B*, effect of rapamycin on $H_2O_2$-induced apoptosis (*left panel*) and cell viability (*right panel*) after 24 h. The results show the percentage of hypodiploid cells (apoptotic cells) analyzed by flow cytometry and the percentage of viable cells determined by crystal violet staining. *, $p < 0.05$, p38$\alpha^{-/-}$ *versus* WT MEFs treated with 0.1 mM $H_2O_2$. No significant differences were found in the presence of rapamycin. *C*, effect of the expression of transfected WT p70S6K and p70Δ29–46ΔCT104 active mutant on cell viability. *, $p < 0.05$, p38$\alpha^{-/-}$ *versus* WT MEFs treated with $H_2O_2$. No significant differences were found upon expression of WT p70S6K and p70Δ29–46ΔCT104. Western blot analysis shows HA expression in cells transfected with WT p70S6K and p70Δ29–46ΔCT104 active mutant. *Arrows* indicate the migration of 80- and 50-kDa molecular weight markers.

death, either in WT or p38$\alpha^{-/-}$ MEFs (supplemental Fig. S1), which suggests that the Akt pathway does not play a major role in p38α-mediated survival. We also tested other pathways that could be potentially involved in cell death, such as JNKs. We found a higher JNK activation in p38$\alpha^{-/-}$ MEFs in response to $H_2O_2$ and under basal conditions (supplemental Fig. S2). However, JNK inhibition with SP600125 did not decrease cell death, which indicates that JNK is not responsible for the enhanced cell death observed in p38α-deficient cells.

*Activation of p38α Prevents the Accumulation of ROS upon $H_2O_2$ Treatment via Induction of Antioxidant Enzymes*—We next explored alternative mechanisms by which p38α might protect cells from ROS damage, allowing cell survival. Although p38α MAPK is known to mediate cell death in response to oxidative stress (5–7), p38 has been shown to mediate the expression of antioxidant enzymes (24, 25). This led us to hypothesize that p38α through the regulation of the antioxidant response might maintain low ROS intracellular levels, leading to cell survival.

As shown in Fig. 4, the percentage of cells with detectable levels of ROS (positive for DCFH) was slightly higher in untreated p38α-deficient cells and was highly increased upon treatment with $H_2O_2$ in a dose-dependent manner. This suggests that upon treatment with $H_2O_2$, p38$\alpha^{-/-}$ cells are unable to scavenge ROS, leading to a high and progressive accumulation of ROS (Fig. 4*B*).

This high ROS accumulation in p38α-deficient cells could be a consequence of a deficiency in the activation of antioxidant mechanisms. Thus, we analyzed the expression of relevant antioxidant enzymes such as SOD or catalase. As shown in Fig. 5*A*, p38$\alpha^{-/-}$ cells expressed lower protein levels of SOD-2 and catalase than WT cells under basal conditions. Moreover, upon treatment with $H_2O_2$, these cells were either unable to efficiently induce the expression of these enzymes, as observed for

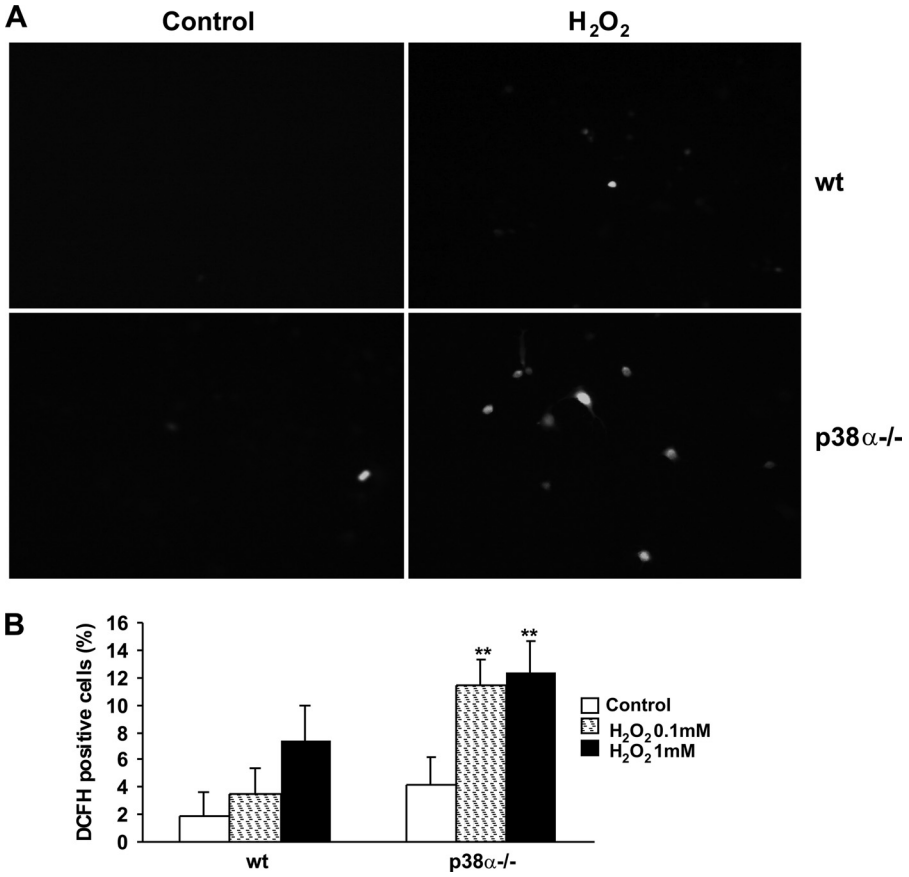


FIGURE 4. **ROS accumulation upon $H_2O_2$ treatment is higher in cells lacking p38α.** MEFs (WT and p38$\alpha^{-/-}$) were incubated with DCFH and treated with $H_2O_2$ (1 mM for *A*, and 0.1 and 1 mM for *B*) for 15 min. *A*, analysis of DCFH-positive cells using an inverted fluorescence microscope. *B*, percentage of DCFH-positive cells. **, $p < 0.01$, p38$\alpha^{-/-}$ *versus* WT MEFs treated with the same dose of $H_2O_2$.

SOD-2, or had a delay and a reduced induction, as happens for catalase. These results suggest that impaired or delayed induction of antioxidant enzymes in p38$\alpha^{-/-}$ MEFs could be responsible for its higher sensitivity to $H_2O_2$-induced cell death. This was supported by the fact that p38α reintroduction in p38α-deficient cells led to an increase in catalase protein levels after 2 h of treatment with $H_2O_2$ (Fig. 5*B*). In addition, basal and $H_2O_2$-induced catalase and SOD activities were significantly higher in WT than in p38$\alpha^{-/-}$ MEFs (Fig. 5, *C* and *D*, respectively). Therefore, all of these data indicate that the presence of p38α highly increases the antioxidant activity of cells.

To get further insight into the role of p38α as a regulator of antioxidant enzyme expression, we measured SOD-1 and SOD-2 mRNAs levels by RT-PCR. The levels were significantly lower in p38$\alpha^{-/-}$ cells under basal conditions, and $H_2O_2$ only induced an increase in SOD-1 mRNA after 20–60 min, whereas SOD-2 mRNA remained unchanged (Fig. 5*E*).

To better understand the regulation of SOD-2 expression by p38α in response to $H_2O_2$, we analyzed SOD-2 promoter activity using luciferase as a reporter. As shown in Fig. 5*F*, $H_2O_2$ treatment induced a significant increase in SOD-2 reporter activity in WT cells after 8 h, but not in p38$\alpha^{-/-}$ MEFs. This lack of activation of SOD-2 promoter in the absence of p38α could be a consequence of the lower activation of the transcription factor ATF-2 (Fig. 6*A*). To address this issue, ATF-2 knockdown experiments were performed using an ATF-2 siRNA (which markedly reduced ATF-2 protein levels, Fig. 6*B*), and results showed a high decrease in SOD-2 mRNA levels in WT cells, either untreated or treated with $H_2O_2$ (Fig. 6*B*). Moreover, ChIP assays revealed a significant binding of P-ATF-2 to SOD-2 promoter in WT MEFs treated with $H_2O_2$, whereas in p38$\alpha^{-/-}$ cells, there was no detectable binding (Fig. 6*C*). In addition, ATF-2 activation was required for p38α-mediated cell survival in the presence of $H_2O_2$, so its knockdown induced death of WT cells (Fig. 6*D*). Therefore, these results indicate that p38α through ATF-2 regulation induces SOD-2 expression and resistance to $H_2O_2$ treatment.

We next explored the mechanisms involved in the regulation of catalase expression. Catalase mRNA levels were increased by $H_2O_2$ progressively at 2 and 4 h in both WT and p38α-deficient cells, but to a higher extend in WT cells at 4 h (Fig. 7*A*). However, after 8 h of treatment, catalase mRNA highly decreased to the level of control in p38$\alpha^{-/-}$ cells, whereas in WT cells, these levels remained above control values. Inhibition of transcription by actinomycin D abolished the increase in catalase mRNA levels observed at 2 and 8 h, regardless of the presence of p38α. However, at 4 h it was just a partial decrease in catalase mRNA upon actinomycin D treatment, specially, in WT cells. This would suggest that increases in the catalase mRNA level are only partially dependent on transcription, and p38α might stabilize catalase mRNA at 4–8 h of treatment. In addition, the fact that the proteasome inhibitor MG-132 increased catalase

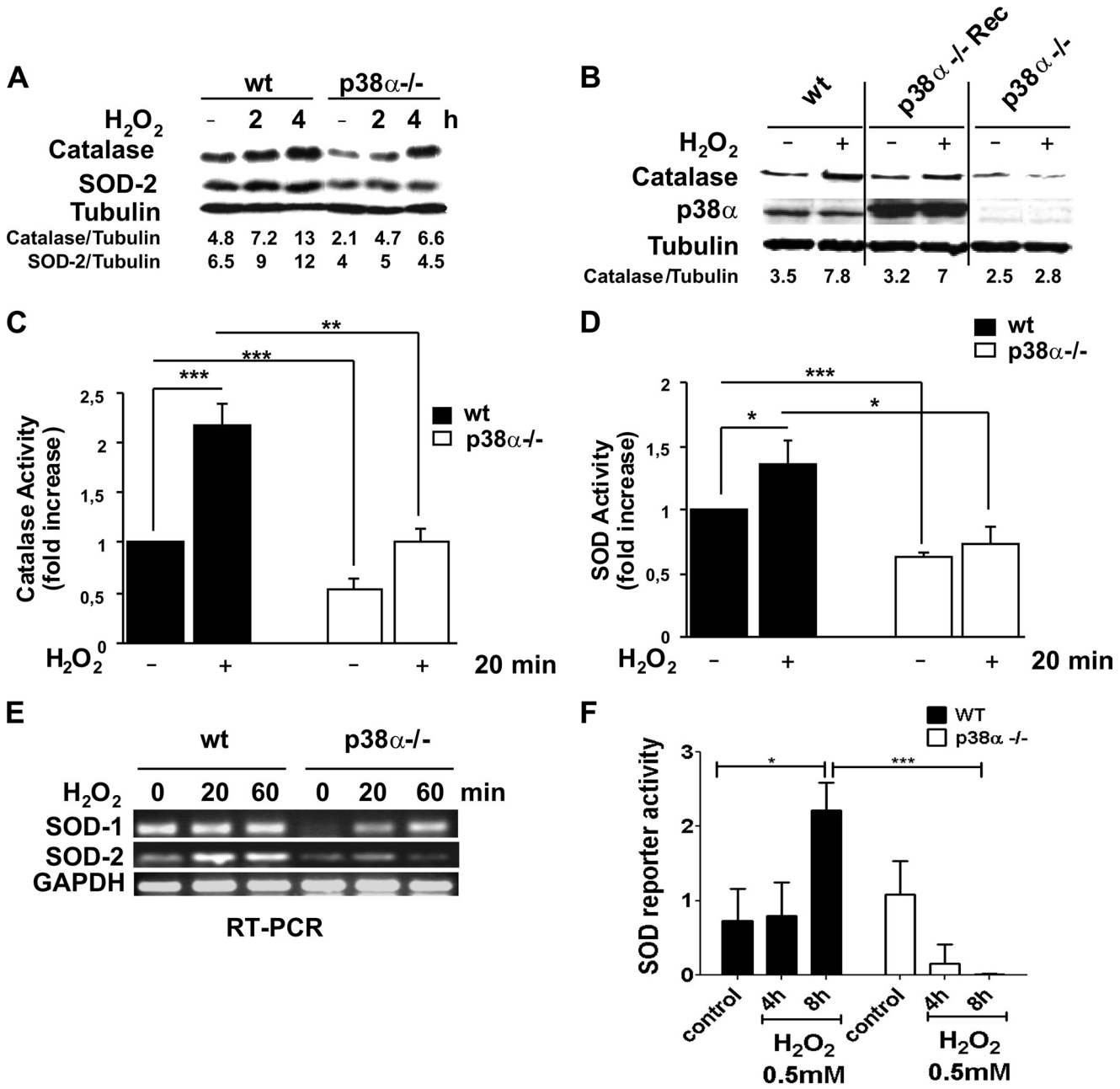


FIGURE 5. **Loss of p38α reduces the expression and activity of antioxidant enzymes.** MEFs (WT and p38$\alpha^{-/-}$) maintained in the presence of serum were treated with $H_2O_2$ (0.5 mM) for the indicated time periods. *A*, catalase and SOD-2 protein levels determined by Western blot analysis and normalized with tubulin. *B*, rescue of catalase protein expression by p38α reconstitution in p38$\alpha^{-/-}$ cells (*Rec*) treated with $H_2O_2$ for 2 h. Western blot analysis of catalase and p38α normalized with tubulin. *A* and *B*, catalase/tubulin and SOD-2/tubulin represents the relative value resulting from the densitometric analysis of catalase or SOD-2, respectively, *versus* tubulin levels multiplied by 10. *C* and *D*, catalase and SOD activities, respectively, are shown as a fold increase of that of WT untreated cells (9.35 milliunits/mg protein for catalase and 7.32 milliunits/mg protein for SOD). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *E*, RT-PCR analysis of the expression of SOD-1 and SOD-2 mRNAs. *F*, SOD-2 reporter activity quantification using luciferase as reported. The results are expressed using arbitrary units. *, $p < 0.05$; ***, $p < 0.001$.

protein levels only in p38α-deficient cells (Fig. 7*B*) strongly suggests that catalase protein would be also stabilized by p38α. Hence, based on these results, p38α might be a positive regulator of catalase through protein stabilization and mRNA expression and/or stabilization.

*Treatment with Either the Antioxidant N-Acetyl Cysteine or Catalase Protects from ROS-induced Cell Death: Effect on mTOR/p70S6K Pathway*—Our data indicate that p38α activation in MEFs can protect from $H_2O_2$-induced cell death through a mechanism that reduces ROS accumulation via induction of antioxidant enzymes. Thus, we next studied the effect of the antioxidant *N*-acetyl cysteine (NAC) on $H_2O_2$-induced cell death.

As shown in Fig. 8*A*, NAC significantly decreased the number of apoptotic nuclei induced by $H_2O_2$ in p38$\alpha^{-/-}$ cells. This correlates with a decrease in Ser(P)-18-p53 levels (supplemental Fig. S3), so p53 could be an important mediator of this process of cell death. Moreover, NAC not only protected from cell

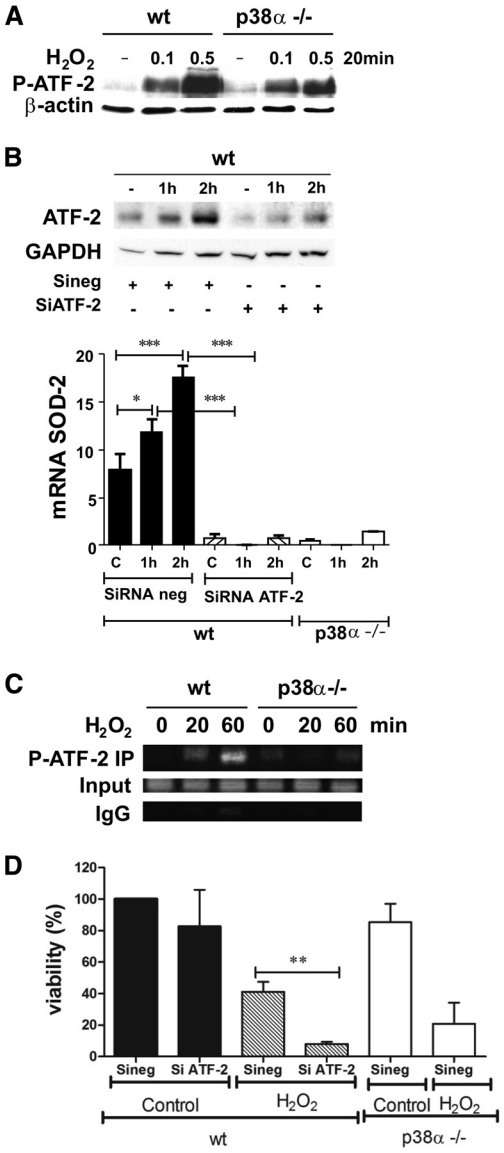


FIGURE 6. **ATF-2 is an important mediator of p38α in the induction of SOD-2 expression and cell viability upon $H_2O_2$ treatment.** MEFs (WT and p38$\alpha^{-/-}$) maintained in the presence of serum were treated with $H_2O_2$ (0.1 and 0.5 mM in *A* or 0.5 mM in *B–D*) for different time periods, as indicated. *A*, Western blot analysis of the levels of P-ATF-2 normalized with β-actin. *B*, effect of ATF-2 siRNA on SOD-2 mRNA levels. *Top panel*, Western blot analysis of ATF-2 levels normalized with GAPDH; *lower panel*, histograms showing SOD-2 mRNA levels. *, $p < 0.05$; ***, $p < 0.001$. *C*, ChIP analysis of P-ATF-2 binding to SOD-2 promoter. PCR analysis of DNA immunoprecipitated by a P-ATF-2 antibody and of input DNA. *D*, effect of ATF-2 siRNA decreasing cell viability of WT MEFs treated with $H_2O_2$ for 24 h. **, $p < 0.01$.

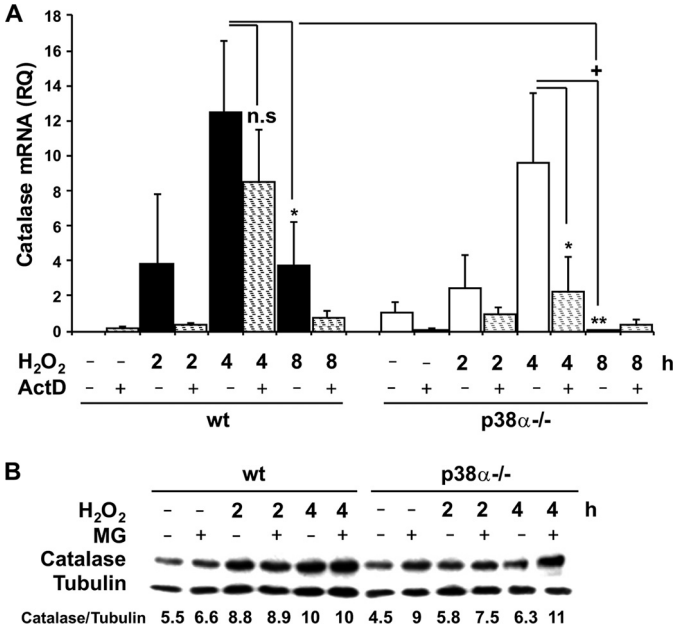


FIGURE 7. **The presence of p38α stabilizes catalase protein.** MEFs (WT and p38$\alpha^{-/-}$) maintained in the presence of serum were treated with $H_2O_2$ (0.5 mM) in the presence or absence of actinomycin D (*ActD*) or MG-132 for the indicated time periods. *A*, analysis of catalase mRNA levels by RT-quantitative PCR in the presence or absence of actinomycin D. Catalase mRNA expression was normalized using GAPDH (catalase $C_t$ − GAPDH $C_t$ = $\Delta C_t$) and then referred to WT control values to calculate the RQ value ($2^{-\Delta\Delta Ct}$). The histograms show the mean values ± S.E. *, $p < 0.05$; +, $p < 0.05$; **, $p < 0.01$; $n = 4$. *B*, effect of the proteasome inhibitor MG-132 on catalase protein expression. Western blot analysis of catalase normalized with tubulin. Catalase/tubulin represents the relative value resulting from the densitometric analysis of catalase *versus* tubulin levels multiplied by 10.

death but also induced an increase in Thr(P)-389-p70S6K levels, which was particularly strong in p38α-deficient cells, reaching a similar level to that found in WT cells (Fig. 8*B*). In contrast, Akt and p38α phosphorylation decreased in NAC-treated cells. All of these results suggest that $H_2O_2$-induced cell death would be a consequence of the damage induced by ROS accumulation, which is higher in p38$\alpha^{-/-}$MEFs. Hence, because catalase levels were lower in these cells, we added exogenous catalase to mimic WT cells situation. As shown in Fig. 8*A*, catalase highly decreased the number of apoptotic nuclei in p38α-deficient MEFs treated with $H_2O_2$. In addition, a parallel increase in P-Thr389-p70S6K levels was observed (Fig. 8*C*). In contrast, pretreatment with the cell-permeable SOD mimetic, Mn-TBAP, which is a superoxide scavenger, neither protected p38$\alpha^{-/-}$ MEFs from $H_2O_2$-induced cell death nor protected from mTOR/p70S6K inactivation (supplemental Fig. S4). Therefore, only the selective $H_2O_2$ scavenger, catalase, which is down-regulated in p38α-deficient cells, allows p70S6K activation in the presence of $H_2O_2$. Moreover, in agreement with the idea that a high accumulation of ROS impairs p70S6K activation, we observed that treatment of WT MEFs with a very high dose of $H_2O_2$ (5 mM) impaired p70S6K activation (supplemental Fig. S5), which indicates that a very high accumulation of ROS either prevents activation or inactivates the mTOR/p70S6K pathway.

## DISCUSSION

p38α plays an important role as a mediator of apoptosis in response to different stress stimuli (4, 15, 26), including oxidative stress (5, 7). For example, p38α functions as a tumor suppressor in the transformation induced by oncogenic H-Ras (7), because of its ability to induce apoptosis upon generation of ROS. However, we have recently found a survival effect of p38α in response to $H_2O_2$ (10), which agrees with some data from other groups (8, 9, 27). Therefore, we have further evaluated the mechanisms by which p38α might protect cells from oxidative stress.

Here, we reveal a pro-survival function of p38α, which is dependent on the regulation of the antioxidant response. In addition, we found that although this response is associated with reduced Akt activity and enhanced mTOR/p70S6K signal-

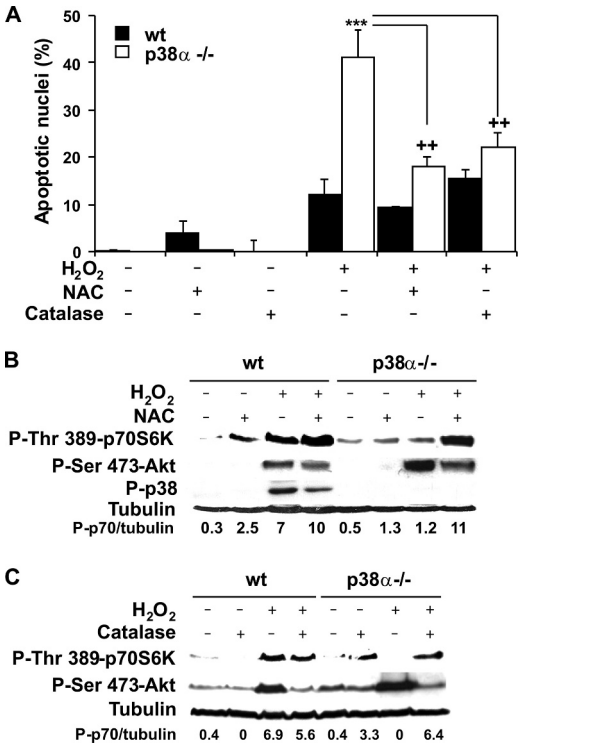


FIGURE 8. **Treatment with the antioxidant, *N*-acetyl cysteine, or catalase protects from ROS-induced apoptosis and allows activation of p70S6K by mTOR.** MEFs (WT and p38$\alpha^{-/-}$) maintained in the presence of serum were treated with $H_2O_2$ (1 mM) for 20 min (*B*) or 3 h (*A*). When indicated, the cells were pretreated for 1 h with NAC (2.5 mM) or catalase. *A*, effect of NAC and exogenous catalase on $H_2O_2$-induced apoptosis. The results show the percentage of apoptotic nuclei. ***, $p < 0.001$, p38$\alpha^{-/-}$ *versus* WT MEFs upon treatment with $H_2O_2$; and ++, $p < 0.01$, as compared with p38$\alpha^{-/-}$ MEFs treated with $H_2O_2$ plus NAC or catalase. *B*, effect of NAC on $H_2O_2$-induced p70S6K activation. *C*, effect of catalase on $H_2O_2$-induced p70S6K activation. *B* and *C*, Western blot analysis of the levels of Thr(P)-389-p70S6K (*P-Thr 389-p70S6K*) and Ser(P)-473-Akt (*P-Ser 473-Akt*) normalized with tubulin. *P-p70/tubulin* represents the relative value resulting from the densitometric analysis of Thr(P)-389-p70S6K *versus* tubulin levels multiplied by 10.

ing, these cascades are not immediately responsible for the p38α-mediated survival effect, but instead their modulation is a consequence of the ROS content in the cells. In contrast, the enhanced mTOR/p70S6K signaling caused by p38 activation appears to favor cell size maintenance during stress, a function recently identified in *Drosophila melanogaster* cells (22). Hence, the loss of p38α induces ROS accumulation, which leads to cell death and inactivation of the mTOR/p70S6K pathway. Although we did not measure this process, autophagic cell death could contribute to the loss of cells observed in p38α-deficient cells treated with $H_2O_2$.

In agreement with the proposed role for p38α as a positive regulator of antioxidant enzymes expression, it was previously reported that p38 MAPK up-regulated catalase (24) and heme oxygenase-1 (25) mRNAs in response to $H_2O_2$. We have now characterized the precise role of p38α in the regulation of the expression and activity of different antioxidant enzymes, as well as the mechanisms involved. Our data reveal a novel function for p38α controlling (i) $H_2O_2$-induced SOD-2 expression through direct regulation of transcription via ATF-2 activation and (ii) basal and $H_2O_2$-induced catalase expression through regulation of mRNA expression and/or stability and protein stability (Fig. 9).

It is worth highlighting that we describe for the first time a p38α-ATF-2-dependent transcriptional regulation of SOD-2 in response to $H_2O_2$. Previously, it had been shown that low levels of $H_2O_2$ induced ATF-2 expression (8) or activation through p38α MAPK (29), leading to growth arrest. A role for JNKs-ATF-2 and p38 MAPK in the regulation of heme oxygenase-1 in response to oxidative stress was also demonstrated (25). However, we now show that the p38α MAPK-ATF-2 cascade also mediates SOD-2 up-regulation and cell survival in response to low levels of oxidative stress.

In contrast to our results demonstrating that mTOR/p70S6K cascade is not responsible for the p38α-mediated survival effect in response to $H_2O_2$, there are a number of data in the literature proposing that p38 MAPK activation and/or mTOR inhibition are required for $H_2O_2$-induced cell death in other cell types (1, 5, 30–32). For example, in keratinocytes activation of p38 by AMPK contributes to $H_2O_2$-induced apoptosis, as well as to mTOR activity down-regulation (30). In PC12 cells, AMPK-mediated mTOR inhibition was shown to be partially responsible for the apoptotic cell death induced by $H_2O_2$ (32). Our findings are not incompatible with these results. They simply highlight the complexity of the mechanisms and that the function of these pathways may depend on the cell type, $H_2O_2$ dose, and duration of the stress signal.

Based on our results, it is unclear that Akt has any potential role in the response to $H_2O_2$ mediated by p38α in MEFs. The expression of SOD and catalase is higher in WT than in p38$\alpha^{-/-}$ cells under basal conditions, when Akt activation is quite similar in both cell lines, and the Akt inhibitor does not affect survival. Hence, these findings do not support the involvement of Akt antagonizing the antioxidant response in these cells, as found in other systems (16).

Regarding the regulation of mTOR/p70S6K by $H_2O_2$, there are also conflicting data in the literature. Some data indicate that mTOR can be inhibited by $H_2O_2$ treatment through different mechanisms such as up-regulation of REDD1 (regulated in development and DNA damage response 1) (33) or inhibition of PDK1 and Akt, accompanied by AMPK activation (32). In addition, AMPK through p38-dependent and -independent mechanisms can decrease mTOR activation by $H_2O_2$ (30), mediating cell death (30, 32). In contrast, our results indicate that p38α do not act as a negative regulator of mTOR/p70S6K as previously suggested (26). This would be in agreement with previous data demonstrating a pro-survival role of p38α and a p38α-dependent activation of mTOR/p70S6K signaling in response to different types of stresses (22, 28). Hence, in agreement with the results of Cully *et al.* (22), we also found that in cells treated with $H_2O_2$ at a low dose, mTORC1 activation is dependent on p38α. We additionally found that p38α regulates mTORC1/p70S6K in an indirect way, which is dependent on ROS accumulation. Therefore, in cells lacking p38α, a high accumulation of ROS is produced, which impairs activation of this pathway. However, in the presence of either catalase or *N*-acetyl cysteine, which prevents a high ROS accumulation, mTOR/p70S6K can be activated in p38α-deficient cells treated with $H_2O_2$.

However, the mechanisms controlling mTOR by p38 can be different depending on the context. Hence, in quiescent tumor cells, activation of the transcription factor ATF6 via p38α up-

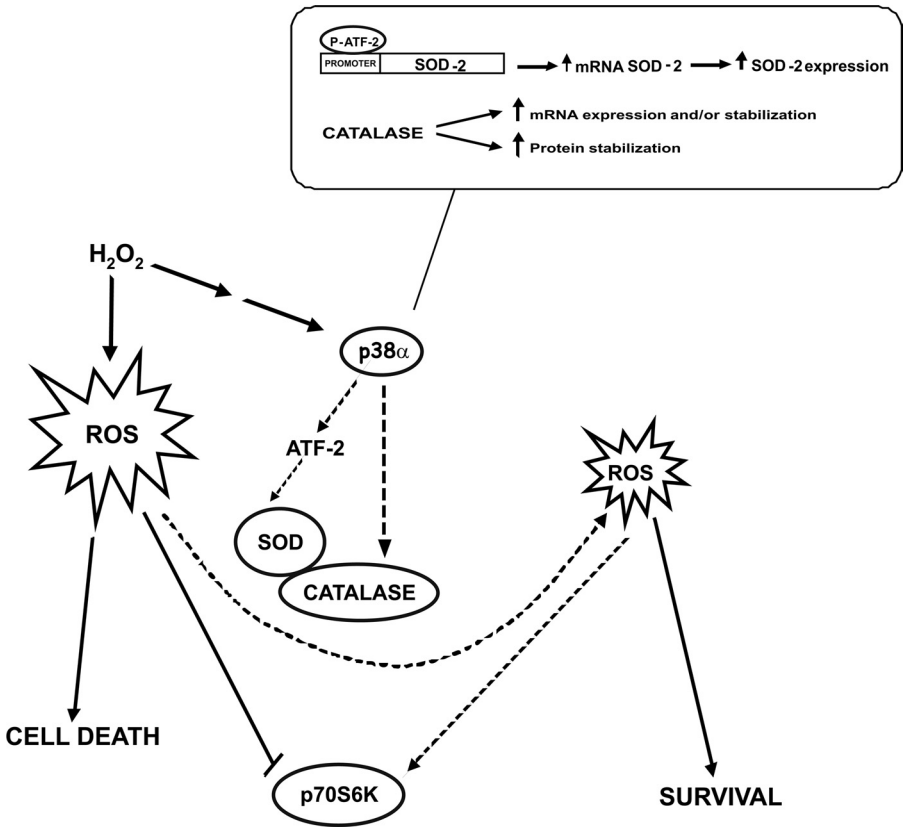


FIGURE 9. **The up-regulation of antioxidant genes by p38α MAPK promotes cell survival and allows p70S6K activation.** Model showing the effect of p38α MAPK increasing the levels of the antioxidant enzymes SOD and catalase in response to $H_2O_2$ through different mechanisms, which leads to the removal of ROS. As a consequence, ROS levels decrease, which allows cell survival and mTOR/p70S6K activation.

regulates Rheb, which in turn activates mTOR and survival through an Akt-independent mechanism (28).

We conclude that p38α has a pro-survival function because of its ability to up-regulate antioxidant genes expression, preventing from a high accumulation of ROS upon exposure to low or moderate doses of $H_2O_2$ (Fig. 9). In this way, cell damage can be overcome, allowing cell survival and mTOR/p70S6K pathway activation. In contrast, in the absence of p38α the antioxidant defense is not properly activated, leading to ROS accumulation and high cell damage. As a consequence, the mTOR/p70S6K pathway is inactivated, which might avoid protein synthesis and cell growth of damaged cells. In agreement with other studies (21, 33), we also found that mTOR/p70S6K activation is dependent on p38α activation in an oxidative microenvironment. We believe this could be critical for other cellular responses such as autophagy that can also control cell size and to maintain cellular homeostasis and function.

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