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# A novel cardioprotective p38-MAPK/mTOR pathway

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## Abstract

Despite intensive study, the mechanisms regulating activation of mTOR and the consequences of that activation in the ischemic heart remain unclear. This is particularly true for the setting of ischemia/reperfusion (I/R) injury. In a mouse model of I/R injury, we observed robust mTOR activation, and its inhibition by rapamycin increased injury. Consistent with the in-vivo findings, mTOR activation was also protective in isolated cardiomyocytes exposed to two models of I/R. Moreover, we identify a novel oxidant stress-activated pathway regulating mTOR that is critically dependent on p38-MAPK and Akt. This novel p38-regulated pathway signals downstream through REDD1, Tsc2, and 14-3-3 proteins to activate mTOR and is independent of AMPK. The protective role of p38/Akt and mTOR following oxidant stress is a general phenomenon since we observed it in a wide variety of cell types. Thus we have identified a novel protective pathway in the cardiomyocyte involving p38-mediated mTOR activation. Furthermore, the p38-dependent protective pathway might be able to be selectively modulated to enhance cardio-protection while not interfering with the inhibition of the better-known detrimental p38-dependent pathways.

### Keywords

Ischemia/reperfusion; oxidant stress; mTOR; p38 MAPK; cell survival

## INTRODUCTION

Reperfusion is the definitive treatment for acute coronary syndromes including myocardial infarction (MI), but reperfusion injury is, at this point, largely unavoidable [1]. Reactive oxygen species (ROS), which activate a host of signaling pathways including, among others, the stress-activated protein kinases, are key mediators of I/R injury. In an attempt to reduce reperfusion injury, pre-clinical studies have identified a large number of putative targets of ROS, but very few have been validated and much remains to be done to better understand the consequences of modulating their activity in the ischemic heart. The p38 MAPKs are



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clear examples of this. p38s are members of the stress-activated protein kinase family [2] and are activated by various stresses including I/R in the heart [3]. Although several studies report that p38 activation enhances injury in hearts subjected to I/R, other studies suggest that p38 activation may confer protection in some circumstances [4] and reviewed in [5]. There are many reasons for these disparate results. Most notably, different animal models and different protocols have been employed and this likely leads to different magnitudes and time courses of activity. This culminates in different profiles of activation of downstream targets. Importantly, some of the downstream targets of p38 are protective, while others are inducers of cell death, and the overall result of p38 activation may depend on the balance between these [5].

Recently, Downward and co-workers reported the surprising finding that p38 activates mTOR in response to oxidant stress in the A547 cancer cell line [6]. This was surprising on two fronts: 1) mTOR is typically inhibited by stressors [7][8], and 2) p38 had never been implicated in regulating mTOR. Herein we examine whether this novel pathway is active in non-transformed cells, whether it plays an important biological role, and what are the mechanisms regulating activity of the pathway. We chose to focus on cardiomyocytes, since oxidant stress injury plays such a major role in the cell death seen in the setting of ischemia/reperfusion (I/R). We now report that activation of mTOR is protective in the setting of I/R in vivo and H/R in vitro. Furthermore, we delineate an extensive signaling cascade regulated primarily by p38 but also by Akt, that recruits multiple factors that converge on mTOR. We believe that many of these factors could serve as novel targets to limit I/R injury. Our studies significantly advance understanding of I/R injury and the factors regulating it.

## MATERIALS AND METHODS

### Ischemia/reperfusion model

C57/Bl6 mice were utilized in accordance with the *Guide for the Care and Use of Laboratory Animals*. These studies were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. 11 week old male mice, were injected intra-peritoneally with vehicle (DMSO 2%, EtOH 48%, PBS 50%) or rapamycin (2 mg/kg in DMSO 2%, EtOH 48%, PBS 50%) 24 h and 3 h before surgery. They were then anesthetized with 2.0% isofluorane and their heart was exposed using a vertical pericardiotomy. Ischemia was induced by occluding the left anterior descending coronary artery for thirty minutes, followed by release of the occlusion [9]. Twenty-four hours after reperfusion, the animals were anesthetized with 2.0% isofluorane and area at risk (AAR or ischemic region) was determined by injection of Evans Blue solution (SIGMA). Hearts were excised, quickly frozen in dry ice, sliced from apex to base in four 1 mm sections, and incubated in triphenyl tetrazolium chloride (TTC) for 30 minutes at RT to determine the size of the infarct zone (MI). Sections were photographed through a direct light microscope. AAR and MI were quantified using Image J software. AAR was expressed as a percent of total left ventricular area, and the extent of MI was expressed as percent of the AAR. When processed for protein extraction, the animals were sacrificed, the heart was rapidly excised, and the LV was snap-frozen in liquid nitrogen. LV tissue was homogenized in 10 volumes of lysis buffer (50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, with the protease and phosphatase inhibitor cocktail present.

### Cells and reagents

Primary neonatal rat ventricular myocyte (NRVM) cultures were prepared from 1–2-day-old rats using standard methods as previously described [10]. Cells were cultured in growth medium consisting of 10 % Horse Serum (HS), 5 % Fetal Bovine Serum (FBS) and 1 % Penicillin/Streptomycin (PS) in F-10 Nutrient Mixture Media. The experiments were carried

36 h after cells were seeded. HCA2 human fibroblasts, immortalized with telomerase (HCA2-hTERT), were a kind gift from Dr. Gavin Wilkinson (Department of Medicine, University of Wales College of Medicine, Cardiff, UK). Human osteosarcoma (SaOS2) and human embryonic kidney (HEK293) cell lines were purchased from ATCC. Primary mouse embryonic fibroblasts (MEFs) were a gift from Dr. Anxo Vidal (Department of Physiology, University of Santiago de Compostela, Santiago de Compostela, Spain). p38$\alpha^{-/-}$ MEFs were a gift of Dr. Angel Nebreda (CNIO, Spain). SaOS2 culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) with 5 % FBS and 1% PS. Both HCA2 cells and MEFs were cultured in DMEM supplemented with 5% FBS and 1% Glutamine/ Penicillin/Streptomycin (GPS) 1%. Rapamycin was purchased from SIGMA (for experiments with cell lines), or from LC laboratories (*in vivo* experiments). Wortmannin, SB202190, SP600125, and PD98059 were obtained from Calbiochem, SB239063 was obtained from GlaxoSmithKline, VX-702 was obtained from Vertex Pharmaceuticals and Dorsomorphin (Compound C), cobaltum chloride ($CoCl_2$), and LY294002 from SIGMA. For the *in vitro* experiments done with SaOS2, HCA2-htert, and MEFs, all cell culture reagents were acquired from SIGMA; for experiments with NRVMs the reagents used were from GIBCO. All other chemicals were purchased from SIGMA.

## Hypoxia/reoxygenation protocols

NRVM cultures were subject to the following hypoxia/reoxygenation (H/R) protocol: 36 h after being seeded, cells were placed in modified KRH media (NaCl 115.0 mM, $KH_2PO_4$ 1.3 mM, $NaHCO_3$ 25.0 mM, $MgCl_2{\cdot}6H_2O$ 0.5 mM, $CaCl_2{\cdot}7H_2O$ 0.9 mM, sodium lactate 20.0 mM, 2-de-oxi-glucose (2-DG) 2.5 mM, KCl 12.0 mM and sodium dithionite 1.0 mM) adapted from Punn et al. (2000) [11] that had been pre-equilibrated with 5% $CO_2$/95% $N_2$ overnight. Cells were placed in an airtight chamber that was purged at 25 L/min with 5% $CO_2$/95% $N_2$ and were kept at 37 °C for 45 min. Cells were removed from the chamber and placed in KRH media (NaCl 115.0 mM, KCl 3.6 mM, $KH_2PO_4$ 1.3 mM, $NaHCO_3$ 25.0 mM, $MgCl_2{\cdot}6H_2O$ 0.5 mM, $CaCl_2{\cdot}7H_2O$ 0.9 mM and glucose 10.0 mM) that had been pre-equilibrated in air. Cells were then maintained in normoxic conditions at 37 °C, $CO_2$ 5% for the times indicated in the figure legends.

## $H_2O_2$ treatment protocols

NRMV cultures were placed in KRH media during 120 min at 37 °C, 5% $CO_2$. After that, $H_2O_2$ 50 μM was added at t = 0, and the cells were kept at 37 °C, 5% $CO_2$ the time needed. When used, inhibitors were added to the media prior to treatment.

SaOS2, HCA2-htert and MEFs cultures were placed in KRH medium during 60 min at 37 °C, 5% $CO_2$. After that, $H_2O_2$ 100 μM was added at t = 0, and the cells were kept at 37 °C, 5% $CO_2$ the time needed. When used, inhibitors were added to the media prior to treatment.

For determination of NRVMs cell death by apoptosis after H/R treatment, cells seeded on 8-well chamber slides (coated with laminin 100 μg/mL) and submitted for 36 hours to the H/R protocol in the presence of DMSO 0,1%, or rapamycin 20 nM. After 14 h of reoxygenation, cells were processed for TUNEL staining using the *ApopTag® Fluorescein In Situ Apoptosis Detection Kit* (Millipore) following manufacturer's instructions. Coverslips were viewed with a Nikon Eclipse E800 microscope and a minimum of five fields were randomly selected and photographed with a Hammamatsu Orca digital camera. Then TUNEL-positive nuclei and total (DAPI-stained) nuclei were counted. TUNEL-positive nuclei were expressed as a percent of total nuclei. For determination of NRVM cell death by necrosis, cells were seeded in 6-well plates and 36 h hypoxia performed in the presence of DMSO 0,1% or rapamycin 20 nM as described above. Samples from cell culture media were

obtained 4 and 8 h after reoxygenation and used to estimate cell viability using the TOXYLIGHT assay (Lonza).

Viability assays in SaOS2 and HCA2-htert cell lines were performed both by trypan blue exclusion, as described by Nogueira et al (2008) [12], and by MTT. In the latter assay at the end of the treatment, cells were incubated in 100 μl of a 0.5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) at 37°C for 4h and lysed in 100 μl of the solubilization solution (0.01M HCl, 10% SDS) at 37°C for overnight. The absorbance of each well was measured at 550 nm in a microplate reader.

### siRNA-mediated knockdown

Pre-designed siRNA targeting rat p38 mRNA and an siRNA control were obtained from Invitrogen (siRNA p38 s135447 and siRNA control AM4611). siRNA transfection was performed using Lipofectamine RNAiMAX according to the manufacturer instructions (Invitrogen) with slight modifications. Briefly, $0.5 \times 10^6$ NRVMs were transfected in 2 ml of F-10 medium containing 500μl of Opti-MEM (Invitrogen), 8 μl of Lipofectamine RNAiMAX and 100 nmol of siRNA.

### Immunoblotting

Cell lysates were prepared as previously described (Nogueira et al (2008) [12], Shao et al. (2006)) [10], resolved by SDS-PAGE and proteins were analyzed by western blot on nitrocellulose membranes. Membranes were incubated for 1 h at room temperature with one of the following antibodies: S6, phospho S6 (S235/236), phospho Akt (S473), phospho Akt (T308), phospho mTOR (S2448), AMPK (T172), phospho GSK3β (S9), 4EBP1, phospho 4EBP1 (S65), phospho p38 (T180/Y182) that were purchased from Cell Signaling Technology; Akt, p38 and phospho p38 (T180/Y182) that were purchased from Santa Cruz Biotechnology; phospho ACC (Ser79) from Millipore; REDD1 (ProteinTech Group); 14.3.3 (Thermo Scientific), α-tubulin (SIGMA); GAPDH (BD biosciences). Antibody binding was detected either with a peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Pierce) followed by a chemiluminescence kit *West Dura* (Pierce) or either using Alexa Fluor® 700 goat anti-mouse, Alexa Fluor® 700 goat anti-rabbit (Invitrogen) followed by *Odyssey Imager* (LI-COR) scanning. All immunoblots shown are representative of at least n = 3 experiments. The bands were quantified by Image J software (http://rsbweb.nih.gov/ij/download.html).

### Immunoprecipitation

HCA2-htert cell extracts were prepared in lysis buffer (HEPES 20 mM pH 7.4, EGTA 2 mM, glycerol 10 %, Triton X-100 1 %, β-glyceraldehyde phosphate, PMSF 2 mM, NaF 1 mM, DTT 20 mM, sodium orthovanadate 1 mM, leupeptin 2.0 g/mL and aprotinin 1.4 mg/L). 1 mg of total protein was pre-cleared with Protein G agarose (Roche). Then Lysates were incubated with 1 μg of 14.3.3 antibody (Thermo Scientific), and collected with Protein G agarose. Beads were washed three times with lysis buffer and proteins were resolved by SDS-PAGE and analyzed by Western blot.

### Statistical analyses

Results are presented as mean ± S.E.M. Statistical significance was evaluated using unpaired Student's t-test. $p < 0.05$ was considered significant.

# RESULTS

## mTOR is activated by I/R and is cardioprotective

The mTOR pathway has been reported to be inhibited by hypoxia and anoxia in cancer cells[13]. We first asked whether mTOR activity was modulated in the heart in the setting of I/R based on the mTOR readout, phosphorylation of the ribosomal S6 protein. We found quite striking activation following reperfusion in vivo that, as expected, was abolished by the mTOR inhibitor, rapamycin (Figure 1A). To further evaluate mTOR activation at the cellular level, neonatal rat ventricular myocytes (NRVMs) were treated with rapamycin vs. vehicle and then were subjected to a period of 45 min of hypoxia followed by re-oxygenation. We found, as expected, that S6 phosphorylation was very low during hypoxia in NRVMs, but, similar to in vivo, phosphorylation increased significantly after reoxygenation (Figure 1B). Consistent with the in vivo observation, the increase in phospho S6 was abolished by rapamycin confirming that S6 phosphorylation during re-oxygenation is dependent on mTOR (Figure 1B).

To determine the consequences of mTOR activation, we subjected C57/BI6 mice to I/R after pre-treatment with rapamycin (2 mg/kg IP), or vehicle. Area at risk (AAR) as a percent of total left ventricular area (LV), was similar in the two groups, but infarct area (IA) as a percent of AAR was significantly increased in the rapamycin-treated mice (Figure 1C). Thus activation of mTOR is cardioprotective in the setting of I/R. This is, to our knowledge, the first demonstration of a cardioprotective role of mTOR following I/R in vivo.

## mTOR inhibition leads to hypoxia/reoxygenation-induced cardiomyocyte cell death

One of the key features of I/R is the burst of ROS following reperfusion [1]. This can lead to cell death by either apoptosis or necrosis [14]. To determine the role of mTOR activation in cell death, NRVMs were treated with rapamycin vs. vehicle and then subjected to a period of 45 min of hypoxia followed by re-oxygenation. At the indicated time points after re-oxygenation (14 hours for apoptosis and 4 or 8 hours for necrosis) the extent of apoptosis and necrosis was determined. H/R did not induce apoptosis to any significant degree (Figure 2A) and rapamycin did not modify this response. In contrast, H/R induced necrotic cell death, and this was significantly increased in cells treated with rapamycin (Figure 2B). Therefore, under these conditions, mTOR inactivation increases cell death, indicating that mTOR serves a pro-survival function in the setting of H/R.

## p38 is required for H/R-induced activation of mTOR

To understand the molecular mechanism of mTOR mediated cardioprotection, we next examined mechanisms responsible for H/R-induced activation of mTOR. We found that p38 MAPK was significantly activated both in vivo (Figure 3A) and in vitro (Figure 3B) in response to I/R and H/R respectively. This activation was not inhibited by rapamycin treatment. In fact, there is a small increase in p38 phosphorylation in rapamycin treated animals. This, together with the results below suggests the existence of feedback loops between p38 and mTOR. Akt was also significantly activated by H/R (Figure 3B).

Importantly, activation of mTOR, as determined by ribosomal S6 protein phosphorylation, was markedly reduced when p38 activity was inhibited by three unrelated small molecule inhibitors, SB202190, SB239063 (a third generation p38 inhibitor) (Figure 3C), and VX-702 (Figure 3D). This same result was obtained when p38 levels were depleted using siRNA stratagies (Figure 3E). To our knowledge, these data demonstrate for the first time that p38 is a central regulator of the mTOR pathway in H/R.

During reperfusion or re-oxygenation, the production of reactive oxygen species (ROS) is greatly increased as a sudden supply of oxygen becomes available to the reduced components of the respiratory chain [15]. Therefore we asked if mTOR is also activated by oxidative stress, induced by $H_2O_2$, and if so, whether p38 modulates this activation. To that end, we subjected NRVMs to $H_2O_2$ for 30 min in KRH media. This significantly increased S6 phosphorylation and, as with reoxygenation, Akt and p38 MAPK were also activated (Figure 4A). Also shown for purposes of comparison is the maximal stimulation of mTOR and maximal phosphorylation of S6 with growth media (figure 4A, left panels).

We then asked whether either p38 or Akt regulate mTOR activity after ROS exposure. Both the PI3K/Akt inhibitor, wortmannin, and the p38 inhibitor SB239063, abrogated $H_2O_2$-induced S6 phosphorylation (Figure 4B). However, p38 inhibition affected neither Akt phosphorylation nor phosphorylation of the Akt target GSK3β following $H_2O_2$ (Figure 4C; see right panel for quantification). Of note, inhibition of PI3K/Akt by wortmannin did not affect p38 activation by ROS (Figure 4B, C). Therefore, activation of the mTOR pathway by ROS depends on both PI3K/Akt and p38 MAPK acting in parallel. Taken together, these data demonstrate that p38 is an activator of mTOR in the setting of H/R and oxidant stress, and suggest that ROS may be one of the stressors that activate mTOR in the reperfused heart.

## PI3K/Akt- and p38 MAPK-dependent activation of mTOR by ROS is a general phenomenon that protects cells from dying

We next asked two questions: 1) is the p38-mediated activation of mTOR following oxidant stress a general phenomenon in mammalian cells; and 2) what are the biological consequences of this activation. We exposed a variety of human cell lines as well as mouse embryonic fibroblasts (MEFs) to $H_2O_2$. S6 phosphorylation was increased in all cell types tested, specifically the human osteosarcoma cell line SaOS, the human fibroblast HCA2 cell line, and MEFs (Figure 5A). Consistent with the findings in cardiomycytes, S6 phosphorylation is mTOR dependent as pretreatment with rapamycin prevented it (figure 5B). Furthermore, the time course of activation of the mTOR pathway after oxidative stress in HCA2 cells is concurrent with that of p38, with both being fully activated as early as 30 minutes post treatment (Figure 5C). These data indicate that the concurrent activation of mTOR and p38 by ROS is a general mechanism and is not restricted to cardiomyocytes. Furthermore, mTOR activation after oxidative stress is dependent both on Akt and p38 (Figure 5D), as was the case with cardiomyocytes.

We also wanted to see whether mTOR activity protects from oxidative stress in the various cell lines. In both SaOS and HCA2 cells, pretreatment with rapamycin significantly increased cell death in cells treated with $H_2O_2$ but not in control cells, a result that has been obtained using two different techniques to evaluate cell death: trypan blue exclusion assay (figure 5E) and MTT viability assay (Figure 5F). To further confirm the regulation of mTOR by p38, we treated wild type or p38$\alpha^{-/-}$ MEFs with two different concentrations of $H_2O_2$. Consistent with findings above, deletion of p38 impaired oxidative stress-induced S6 phosphorylation (Figure 5F). Furthermore, p38$\alpha^{-/-}$ cells were more prone to die after oxidative stress than wild-type cells (Figure 5G). Moreover, pretreatment with rapamycin clearly diminished the survival advantage of the wild-type cells (Figure 5G). Thus p38α protects against oxidative stress-induced cell death and this protective effect depends at least in part on mTOR activation. These data confirm that activation of mTOR is protective against oxidative stress and that this is a general phenomenon.

### Oxidant stress-induced activation of mTOR is independent of AMPK

We have shown that the activation of mTOR by stress requires the positive influence of upstream factors (i.e. p38 and PI3K). However, activation of mTOR also requires that inputs from negative regulators are inhibited. The AMP-activated protein kinase (AMPK) is one key negative regulator of mTOR in response to low energy levels [13]. As recent findings suggest that ROS activate AMPK, which should inhibit mTOR [16], we next examined AMPK phosphorylation and its impact on mTOR activation in our system. As expected, AMPK was activated in cells deprived of serum and amino acids (Fig 6A, top panel, lane 2 vs 3), and this activity correlated with a repressed mTOR pathway (middle panel, lanes 2 and 3). However, $H_2O_2$-induced AMPK activation (top panel, lane 4) did not impair the $H_2O_2$-induced activation of mTOR (Figure 6A, middle panel, compare lanes 2 and 4). Moreover, when we treated HCA2 cells with Compound C (an inhibitor of AMPK; Figure 6B) or AICAR (an activator of AMPK; Figure 6C), either in the presence or in the absence of $H_2O_2$, we saw that neither inhibition nor activation of AMPK modified S6 phosphorylation. This lack of effect is specific to oxidative stress since stimulation of AMPK with AICAR inhibited the mTOR activation induced by aminoacids (Figure 6C). These data show that although AMPK is stimulated by ROS, it does not modulate mTOR activity in this setting.

### ROS downregulate REDD1 and promote TSC2/14-3-3 association

Modulation of mTOR activity by various factors converges at the level of the TSC1/2 complex (tuberous sclerosis tumor suppressor proteins). Genetic and biochemical evidence has established that this complex is a negative regulator of mTOR activity [17, 18]. Under hypoxic conditions, enhanced expression of the hypoxia-inducible gene REDD1/RTP801 suppresses mTOR activity by releasing TSC2 from its growth factor-induced association with inhibitory 14-3-3 proteins. To ask whether ROS could modulate REDD1 levels, TSC2/14-3-3 association, and, consequently, mTOR activity, SaOS2 and HCA2 cells were placed in KRH media containing $H_2O_2$ vs. vehicle. REDD1 levels were elevated in KRH media, but declined significantly in cells treated with $H_2O_2$ (Figure 7A). Phosphorylation of mTOR and S6 is low when REDD1 levels are high, and, in contrast, phosphorylation is high when REDD1 levels are low (Figure 7A). We also found that we could recover TSC2 from 14-3-3 immunoprecipitates when cells were treated with $H_2O_2$ and REDD1 levels were low (figure 7B, compare lane 5 vs. 4), and inhibition of p38 MAPK but not PI3K/Akt disrupts this interaction (figure 7C, compare lane 5 vs 7 and 5 vs 6). Moreover, downregulation of REDD1 by $H_2O_2$ was also dependent on p38 (Figure 7D, panel 9).

## DISCUSSION

Herein we show that mTOR is markedly activated in the setting of I/R and that activation is cardioprotective in vivo as well as in various in vitro models of I/R. Furthermore we delineate a novel signaling pathway regulated by the stress activated MAPK, p38, that leads to activation of mTOR. Key to this is p38-mediated inhibition of the mTOR regulator TSC2, which is due, at least in part, to down-regulation of the REDD1 protein. Akt also contributes to mTOR activation by stresses since we find that inhibiting Akt reduces mTOR activation. Our findings are summarized in Figure 8.

Based on the central role of p38 in regulating levels of inflammatory cytokines, p38 inhibitors were developed for use in patients with chronic inflammatory disorders such as rheumatoid arthritis. However liver toxicity was dose-limiting in animal models. Recent pre-clinical studies with SB239063 showed that endothelial dysfunction and atherosclerotic plaque inflammation were reduced. More recently, patients treated with a low dose of p38 inhibitor for 3 months had reduced levels of C-reactive protein (indicative of less

inflammation) and improved vascular reactivity. Another p38 inhibitor, GW856553 has entered into a clinical trial in patients with acute coronary syndromes (http://clinicaltrials.gov/ct2/show/NCT00910962?term=solstice&rank=1). While the evidence is mounting that p38 inhibition might be beneficial in reducing inflammation, it is obviously critical to be certain that p38 inhibition will not exacerbate I/R injury. Unfortunately, there is no clear answer to this question. In a recent review examining our understanding of the role of p38 in ischemic injury, the conclusion was reached that "dissecting the beneficial vs. detrimental aspects of p38 signaling will be a major issue to be addressed in the future."[5] [19]

Herein, we believe we have identified one key beneficial role for p38 in the setting of I/R: activation of mTOR. Recently there has been an increase in interest in the role of mTOR in the heart. Activation of mTOR via over-expression of the wild-type protein was shown to protect against pressure overload induced by thoracic aortic constriction [20]. Deletion of Raptor, a key component of the mTORC1 complex, leads to reduced cardiomyocyte growth and heart failure that is particularly pronounced in the setting of pressure overload. Furthermore, rapamycin was recently proposed as a therapeutic strategy to limit the pathologic hypertrophy seen in LEOPARD syndrome, one of the ras-opathies caused by a mutation in the tyrosine phosphatase SHP2 [21]. Thus mTOR appears to be protective in the setting of excess hypertrophic signaling, possibly irrespective of cause.

The role of mTOR in regulating ischemic injury is less clear in that it has been examined in a number of settings with what appear to be disparate conclusions. [22, 23], [24–26]. Several studies have examined the role of mTOR in insulin cardioprotection and have shown that mTOR inhibition with rapamycin or its analogs negate the beneficial effects of insulin infusion. Rapamycin also reduces the protection provided by ischemic pre-conditioning (IPC) [25]. More recently, Buss et al. examined the role of mTOR in a chronic MI model and reported that inhibition of mTOR with everolimus reduced infarct size. In contrast, Lajoie et al.[27] used a similar model to that of Buss et al. [26]and concluded that rapamycin increased infarct size in female rats.

The only other report of which we are aware that addressed the role of mTOR in "standard" I/R (i.e. no IPC, no insulin infusion, etc.) stated that rapamycin decreased infarct size in a Langendorf model. Our findings clearly differ from those of Khan et al. [24]since we demonstrate that rapamycin increased I/R injury. The disparate conclusions underline the confusion in this area and, we believe, in order to understand the true roles of mTOR in the heart, it is key to identify molecular mechanisms regulating mTOR in the stressed heart.

Therefore, we set out to identify the signaling pathways that regulate mTOR activity in the heart exposed to I/R. Our central finding is that the mTOR pathway is positively regulated by p38 MAPK and inability to activate mTOR via this mechanism increases cell death in models of I/R injury. We have also delineated the pathway upstream of mTOR activation in response to H/R and oxidant stress, defining p38 and Akt as two essential activators of the mTOR pathway in these situations. Importantly, we were able to reproduce this p38-dependent mTOR activation by ROS in different cell types, confirming that this is not a cell-type-specific phenomenon.

The fact that stress activates mTOR is a relatively new concept, and in fact the vast majority of work has suggested that stress inactivates mTOR. This inactivation was reported to depend on AMPK-mediated TSC2 phosphorylation in the setting of energy stress and hypoxia, and by HIF-1 inducible REDD1-mediated displacement of inhibitory 14-3-3 from TSC2 in the setting of hypoxic stress ([28][13][29][30] and reviewed in [31]. In contrast, we found that with oxidative stress, despite AMPK being activated, AMPK was unable to

inactivate mTOR or to block its activation. Regulation of TSC2 by AMPK is known to be a complex event that requires phosphorylation of TSC2 at several residues, in which a priming phosphorylation by AMPK is followed by additional phosphorylation events by GSK3β. We speculate that inactivation of GSK3β by Akt in cells exposed to ROS (Figure 4C) may impair the GSK3β-dependent phosphorylation of TSC2. Experimental confirmation of this hypothesis will depend on the availability of antibodies specific to GSK3β-dependent phosphorylation sites of TSC2.

We also find that REDD-1 is down-regulated by reactive oxygen species and this likely accounts for the inactivation of TSC2 and activation of mTOR in this setting. p38 MAPK, but not Akt, is necessary for this downregulation. Overall, this shows that p38 stimulates the mTOR pathway in a specific and an unexpected manner.

To our knowledge, there have been only two reports prior to this showing that p38 can activate mTOR. Li et al [32], have proposed a model whereby the p38 substrate MK2 phosphorylates TSC2 at Ser1210, thereby inducing its binding to 14-3-3.We however were unable to detect phosphoSer1210 TSC2 after $H_2O_2$ treatment using commercially available antibodies (data not shown). However, we do see a clear downregulation of REDD1 after $H_2O_2$ treatment that is dependent on p38. Since TSC2 is inactive in hypoxia mainly due to high REDD1 levels, the mechanism we describe here is likely to be generally important in ischemic syndromes. The second publication was from Cully and coworkers who recently identified p38 as an upstream regulator of TORC1 activity, at least in Drosophila melanogaster cells and in a transformed human cell line, but the functional significance of this remains unclear. The effects of p38 described by Cully et al. are independent of TSC2, at least in Drosophila, and, as such, the mechanism differs significantly from our findings [6]. The existence of several possible routes by which p38 can modulate the mTOR pathway suggests that this may be a central mechanism of mTOR regulation.

In summary, mTOR activation protects cardiomyocytes from I/R injury and cell death. Mechanistically, ROS-induced activation of mTOR is mediated by p38-driven TSC2 inactivation which occurs despite activation of AMPK. These data underline the fact that regulation of downstream targets by p38 is complex as it is regulating both pro-survival and pro-death pathways, sometimes simultaneously. Our findings raises some concerns over the use of small molecule inhibitors of p38, some of which are advancing through clinical trials. The findings also suggest that the development of effective treatments based on inhibition of the p38 pathway might be better-targeted at factors downstream of p38 itself.

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## NON-STANDARD ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **I/R** | ischemia/ reperfusion injury |
| **MI** | myocardial infarction |
| **H/R** | hypoxia/reoxigenation |
| **ROS** | reactive oxygen species |
| **AAR** | area at risk |

| | |
|---|---|
| **IA** | infarct area |
| **NRVM** | neonatal rat ventricular myocytes |

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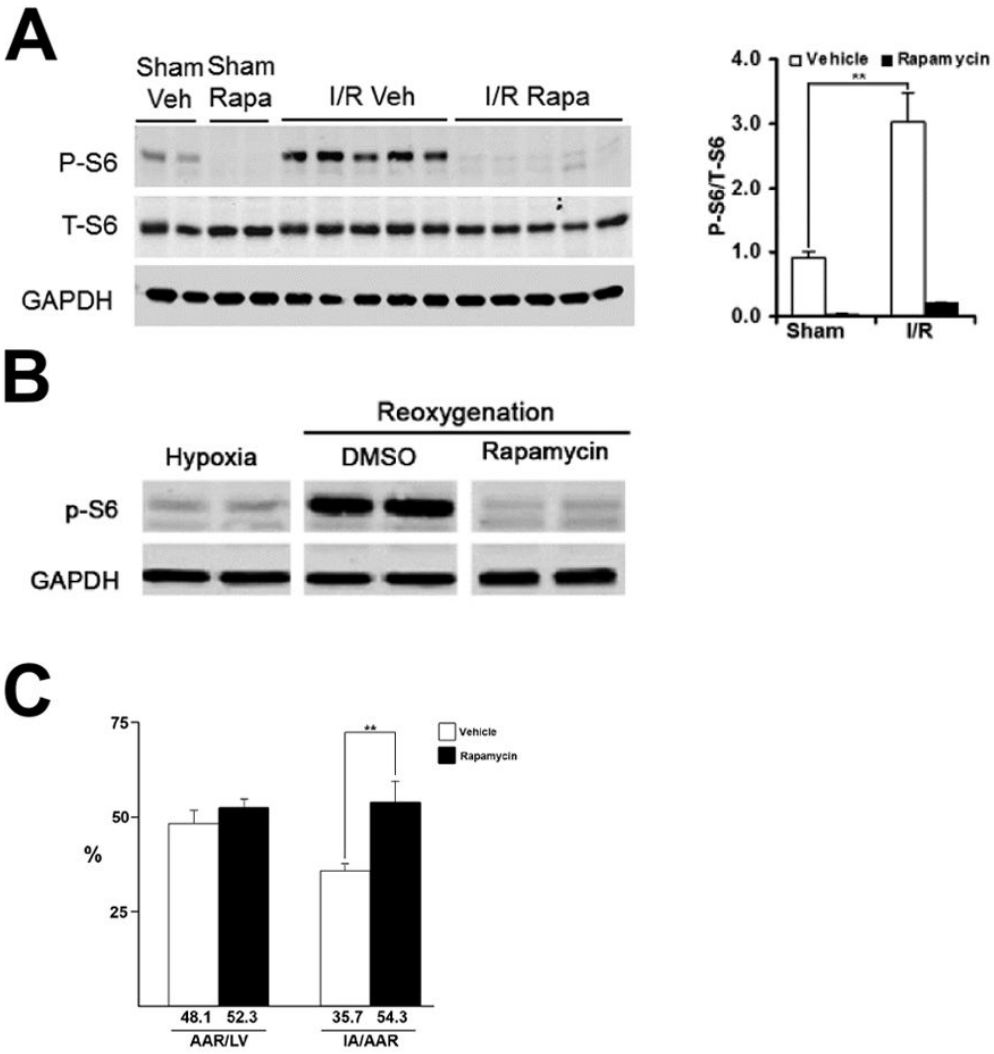


**Figure 1. Activation of mTOR in reperfused hearts is cardioprotective**

A. Anesthetized C57/BI6 mice were subject to left coronary artery ischemia (30 min) followed by reperfusion for 24 h. Mice were either previously pretreated with rapamycin (2 mg/kg, 24h and again at 3h before ischemia, n= 20), or left untreated (vehicle, DMSO 2%, EtOH 48%, PBS 50%, n= 17). Hearts were processed for immunoblotting as described in Methods. Immunoblot shows S6 phosphorylation (a readout of mTOR activity) in hearts subjected to sham I/R or I/R, either following rapamycin treatment or vehicle treatment (left panel with quantification shown in right panel). B. Reoxygenation following hypoxia activates mTOR as determined by p-S6. NRVMs in modified KRH medium were subjected to hypoxia (45 min) followed by reoxygenation for 30 min, in the presence of 0,1% DMSO or 20 nM rapamycin. Immunoblot shows levels of phosphorylated S6 (p-S6). C, mTOR inhibition increases infarct size. Anesthetized C57/BI6 mice were treated as in A. Tissue was processed as described in Methods. Quantification of area at risk of infarction (AAR) as a percentage of total left ventricular area (LV; AAR/LV)), and infarct area (IA) as percentage of the AAR (IA/AAR) are shown. ** p< 0,01

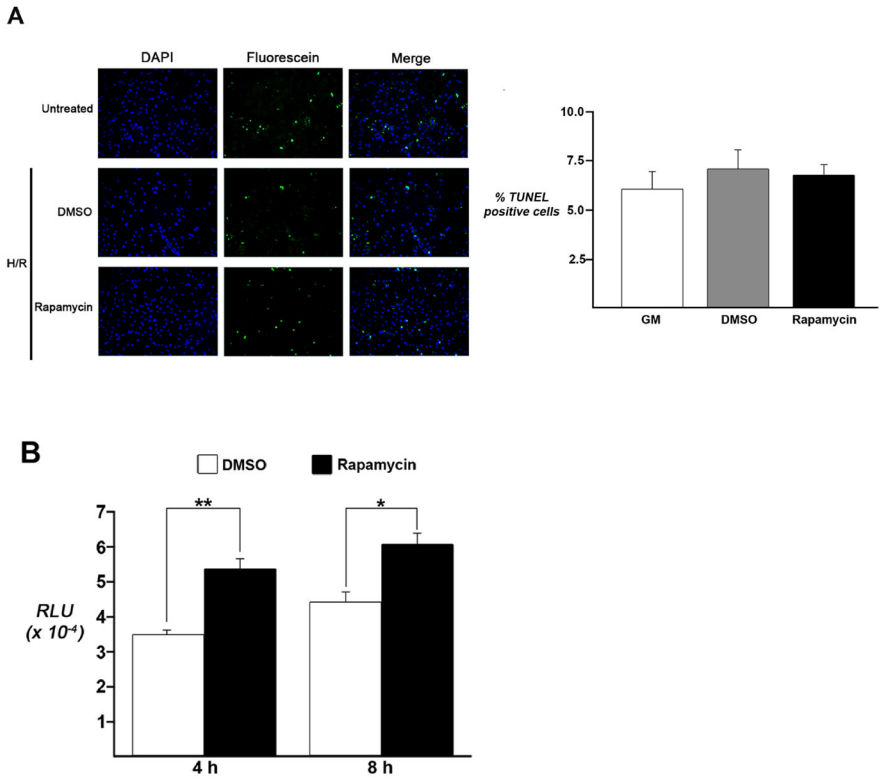


**Figure 2. mTOR inhibition enhances H/R-induced necrotic cell death in cardiomyocytes**

**A**. H/R does not induce apoptotic cell death in cardiomyocytes. NRVMs in modified KRH medium were either left untreated or were subjected to hypoxia (45 min) followed by re-oxygenation for 14 hours (H/R), in the presence of 0.1 % DMSO or 20 nM rapamycin in DMSO. Representative images of TUNEL assays are shown in panel A. Apoptosis was quantified by counting the number of TUNEL positive nuclei and plotted as percentage of total nuclei stained with DAPI (panel A). Shown is the mean ± SEM of three independent experiments. n= total number of nuclei counted with at least 200 cells being examined in each experiment). **B**. Rapamycin enhances cardiomyocyte necrotic cell death induced by H/R. NRVMs were either left untreated or were subjected to hypoxia for 45 min in modified KRH media followed by reoxygenation for 4 or 8 hours in KRH media in the presence of vehicle (0,1 % DMSO) or 20 nM rapamycin. Necrosis was quantified measuring adenylate kinase phosphotransferase activity in the culture supernatant and expressed as relative luciferase units (RLUs). Shown is the mean ± SEM of three independent experiments. ** p< 0,01; * p< 0,05.

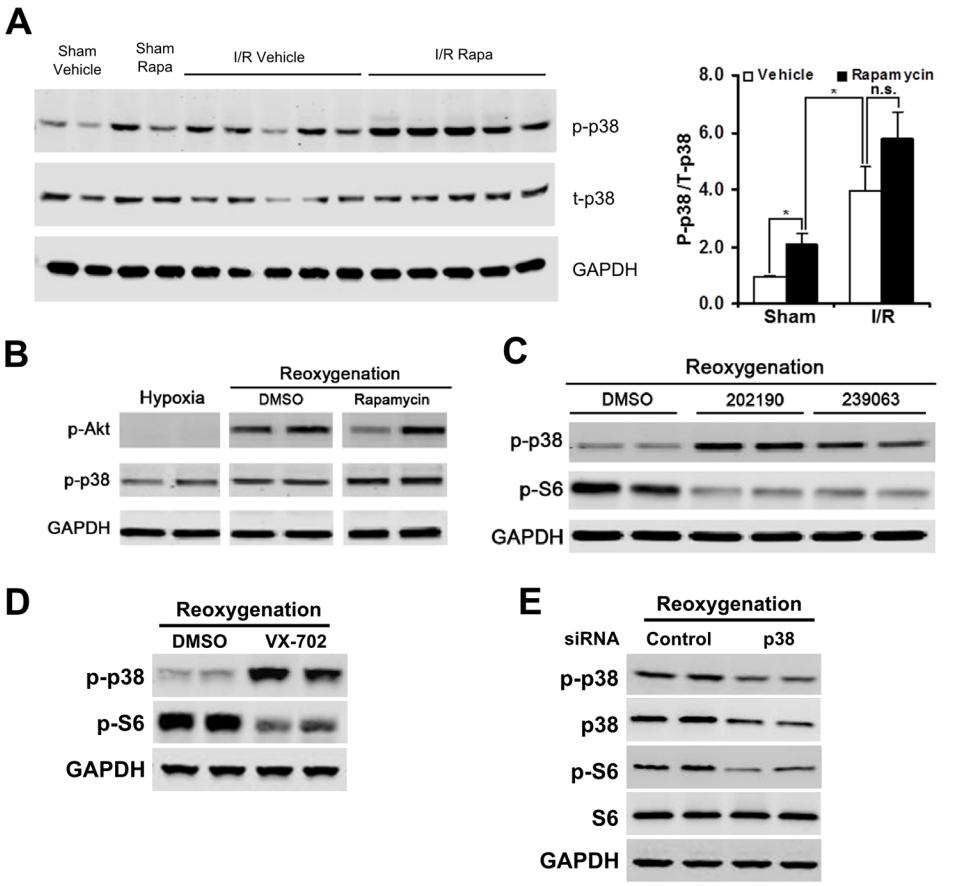


**Figure 3. p38 is required for H/R-induced activation of mTOR**

**A**. Activation of p38 by I/R in vivo. Mice were subjected to ischemia 30 min followed by reperfusion for 3 h. Hearts were processed for immunoblotting as described in Methods. Quantification of phospho-p38 (p-p38) normalized to total p38 (t-p38) is shown in the graph. **B**. NRVMs were subjected to hypoxia in modified KRH media (45 min) followed by reoxygenation for 30 min. Levels of phosphorylated Akt (Ser473) and p38 MAPK (Thr180/Tyr182) are shown (representative of n=3 experiments). GAPDH is shown as a loading control. **C and D**. S6 phosphorylation after H/R is dependent on p38 activity. NRVMs undergoing the identical H/R protocol as in B were treated with 10 μM of the p38 inhibitors, SB 202190, SB236090 and 7,5 μM VX-702. Levels of phosphorylated S6 (Ser235/236) and phosphorylated p38 MAPK (Thr180/Tyr182) were determined by immunoblotting. The images shown are representative of n=3 experiments. Please note, ATP-competitive small molecule inhibitors typically lead to increased phosphorylation of the target kinase but the kinase activity toward downstream substrates remains fully inhibited. GAPDH is shown as a loading control. **E**. p38 mediated phosphorylation of S6 is compromised in siRNA p38 depleted cells. NRVM were transiently transfected with control or p38 siRNA. Levels of phosphorylated S6 and phosphorylated p38 were determined by immunoblotting. Shown are also total p38 levels as demostration of siRNA efficiency, and S6 and GAPDH as loading controls.

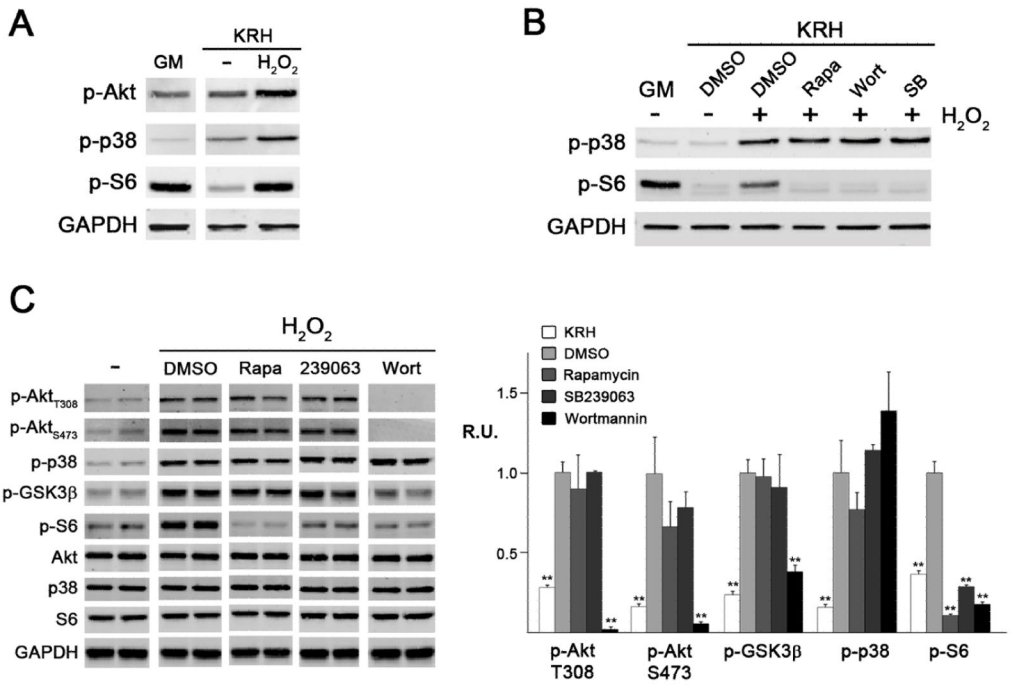


**Figure 4. Oxidant stress activates the mTOR pathway in cardiomyocytes**

**A**. Oxidant stress activates AKT, p38, and the mTOR pathway in cardiomyocytes. NRVM were either cultured in normal growth medium (GM), or placed in KRH solution for 1 h to down-regulate mTOR activity (as determined by p-S6). Cells in KRH were then incubated for 30 minutes in the presence of 50 μM $H_2O_2$ or vehicle. Levels of phosphorylated Akt (Ser473), p38 MAPK (Thr180/Tyr182), and S6 (Ser235/236) were determined by immunoblotting. The immunoblot is representative of n=3 experiments. **B**. Activation of the mTOR pathway by ROS is dependent on PI3K/AKT and p38. NRVM were either incubated in normal growth medium (GM), or placed in KRH solution for 1 hour in the presence of either 20 nM rapamycin (Rapa), 500 nM Wortmannin (Wort), 10 μM SB239063 (SB), or vehicle (0,1% DMSO). Cells in KRH were then incubated for 30 minutes in the presence of 50 μM $H_2O_2$ or vehicle. Note that $H_2O_2$-induced activation of mTOR (p-S6) is abolished by all three inhibitors. **C**. PI3K/AKT and p38 act in parallel to activate the mTOR pathway after oxidative stress. Left panel. NRVMs were placed in KRH solution for 1 h in the presence of either 20 nM rapamycin (Rapa), 500 nM Wortmannin (Wort), 10 μM SB239063 (SB), or vehicle (0,1% DMSO). Cells were then incubated for 30 minutes with 50 μM $H_2O_2$ or vehicle. Levels of phosphorylated Akt (Thr308 and Ser473), p38 (Thr180/Tyr182), GSK3β (Ser9) and S6 (Ser235/236) were determined by immunoblotting. Total S6 and GAPDH were used as a loading control. Note the marked reduction in pS6 after treatment with either the p38 inhibitor SB239063 or the PI3K/Akt inhibitor wortmannin. Phosphoprotein levels normalized to loading controls are plotted in right panel as mean ± SEM. n = 3 experiments. RU (Relative units). ** $p< 0,01$ relative to $H_2O_2$ treatment.

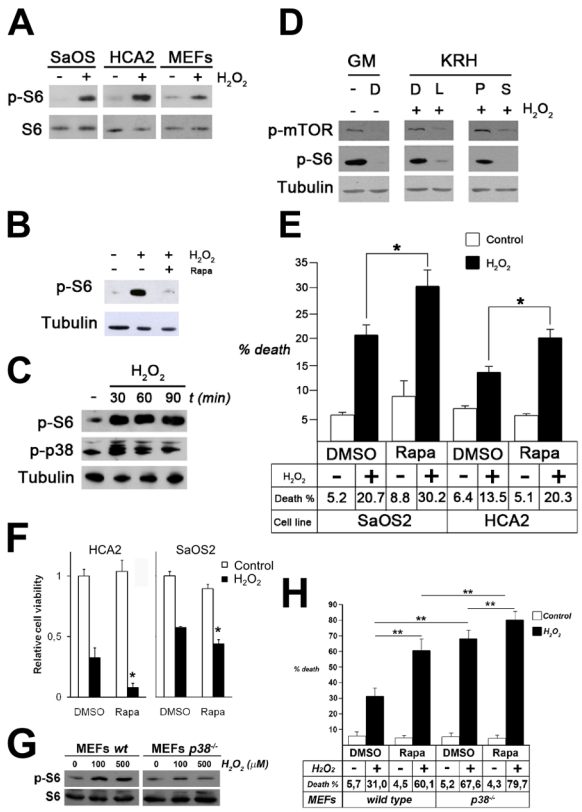


**Figure 5. p38 MAPK-mediated activation of mTOR by ROS is a general phenomenon that reduces cell death**

**A**. ROS-induced activation of the mTOR pathway in various cell types. SaOS2, HCA2 and MEFs were placed in KRH for 1 hour to downregulate mTOR activity. They were then incubated for 30 minutes in the presence of 100 μM $H_2O_2$ or vehicle. Levels of phosphorylated S6 (Ser235/236) were determined by western blotting. Total S6 protein was used as a loading control. Immunoblot is representative of n = 3 experiments. **B**. Rapamycin blocks $H_2O_2$-induced activation of mTOR. HCA2 cells were placed in KRH for 1 hour in the presence of 20 nM rapamycin or vehicle (0,1% DMSO). They were then incubated for 30 minutes in the presence of 100 μM $H_2O_2$ or no $H_2O_2$. Levels of phosphorylated S6 were determined by immunoblotting. Tubulin was used as a loading control. Immunoblot is representative of three independent experiments. **C**. The time course of activation of the mTOR pathway after oxidative stress is concurrent with that of p38. HCA2 cells were placed in KRH for 1 hour and then were treated with 100 μM $H_2O_2$ or vehicle for the indicated times. Levels of phosphorylated p38 MAPK /Thr180/Tyr182) and S6 (Ser235/236) were determined by western blotting. D. mTOR activation after oxidative stress in human fibrobasts is dependent on PI3K/AKT and p38. HCA2 cells were cultured in growth medium (GM) or placed in KRH for 1 hour, and then were treated with 0.1% DMSO (D) as control, 10 μM LY294002 (L) a PI3K inhibitor, 50 μM PD98059 (P) a MEK/ERK pathway inhibitor, or 10 μM SB202190 (S) a p38 inhibitor. Cells were then incubated for 30 minutes with 100 μM $H_2O_2$ or vehicle. Levels of phosphorylated mTOR (Ser2448) and S6 (Ser235/236) were assessed by western blot, using tubulin as a loading control. Note that both L and S, but not P, prevented H2O2-induced activation of mTOR. **E and F.** mTOR protects human fibroblasts and osteosarcoma cells from oxidative stress. SaOS2 and HCA2 cells were incubated for 1 hour in KRH in the presence of 20 nM rapamycin or vehicle (0,1% DMSO). 100 μM $H_2O_2$ vs. vehicle $H_2O_2$ (−) was then added to the cells for 4 hours.

Medium was then changed to media lacking $H_2O_2$ with 20 nM rapamycin or vehicle (0,1% DMSO), and 24 hours later the percentage of non viable cells was determined by trypan blue exclusion (E, shown is the mean ± SEM of three independent experiments. *, p< 0,05) or by MTT assay expressed as the percentage of cell survival (F, values are the mean + SEM of four samples. *, p< 0,05). **G.** Activation of the mTOR pathway afer oxidative stress is abrogated in p38$\alpha^{-/-}$ cells. WT and p38 $\alpha^{-/-}$ MEFs were placed in KRH for 1 hour. Cells were then treated with vehicle $H_2O_2$ (−), 100 μM or 500 μM $H_2O_2$ for 60 min. Phosphorylated S6 (Ser235/236) was determined by western blotting. Also shown are total S6 levels.H. p38α deficiency and mTOR inhibition increase susceptibility of cells to oxidative stress. WT and p38$\alpha^{-/-}$ MEFs were placed in KRH for 1 hour in the presence of 20 nM rapamycin or vehicle (0,1% DMSO). 100 μM $H_2O_2$ or vehicle was then added to the media for 4 hours. The medium was then changed to growth medium with 20 nM rapamycin or vehicle (0,1% DMSO), and 24 hours later cells were trypsinized and the percentage of non viable cells was determined by trypan blue exclusion. Shown is the mean ± SEM of three independent experiments. ** p< 0,01.

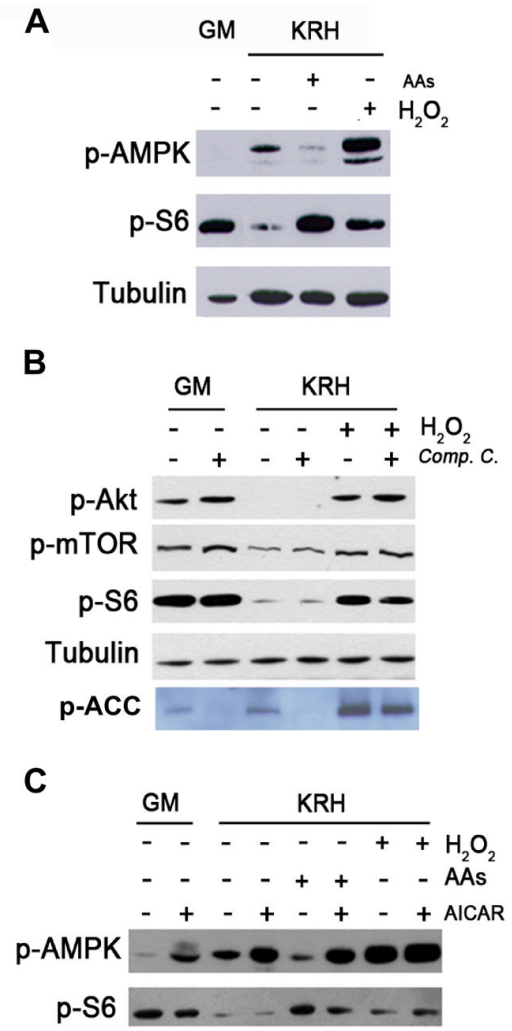


**Figure 6. p38 and PI3K/AKT signal upstream of mTOR upon ROS exposure, and AMPK does not prevent mTOR activation**

**A**. Both mTOR and AMPK pathways are activated after oxidative stress. HCA2 cells were placed in growth medium (GM, lane 1) or in KRH (lanes 2–4). Cells in KRH were then incubated for 30 minutes in amino acid-rich solution (AAs +, lane 3), 100 μM $H_20_2$ (lane 4), or no additive (lane 2). Phosphorylated AMPK (Ser172) and S6 (Ser235/236) were determined by western blotting. Tubulin was used as a loading control. Figure is representative of n = 3 experiments **B**. Inhibition of AMPK does not further activate the mTOR pathway after oxidative stress. HCA2 cells were incubated for 1 hour in normal growth medium (GM), or in KRH, in the presence of 5 μM compound C or vehicle (0.1% DMSO). Cells were then incubated for another hour with 100 μM $H_2O_2$ ($H_2O_2$+) or vehicle. Levels of phosphorylated mTOR and S6 were determined by western blotting. Tubulin was used as a loading control. pACC is shown as a readout of AMPK activity. **C**. Activation of AMPK does not prevent activation of the mTOR pathway by oxidant stress. HCA2 cells were incubated for 1 hour in normal growth medium (GM), or in KRH, in the presence of 1 mM AICAR or vehicle (0.1% DMSO). Cells were then incubated for another hour with 100 μM $H_2O_2$ ($H_2O_2$), aminoacid rich solution (AAs), or kept in their previous medium. Cells were harvested and levels phosphorylated AMPK (Thr172) and S6 (Ser235/236) were determined by western blotting.

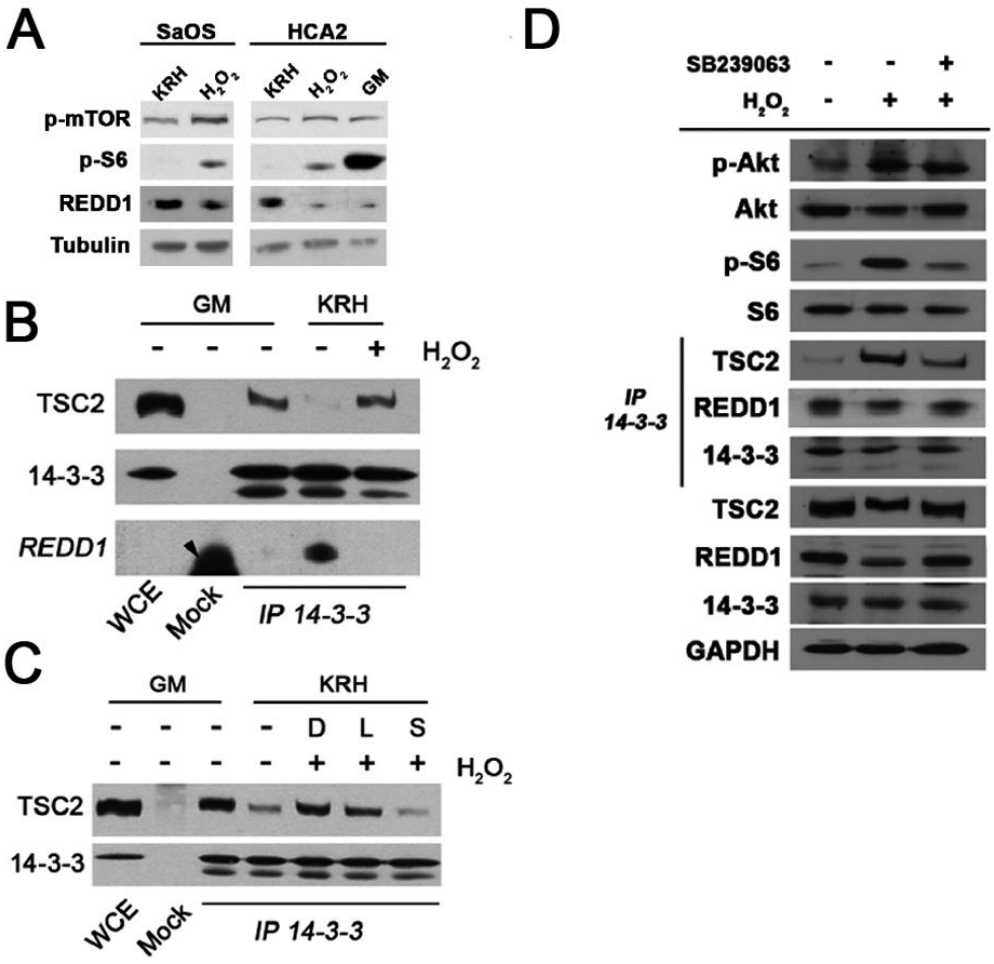


**Figure 7. Regulation of mTOR by ROS, REDD1 downregulation and TSC2/14-3-3 association**

**A**. Oxidative stress downregulates REDD1 protein. SaOS2 and HCA2 cells were placed in KRH for 1 h. They were then incubated for 30 minutes in the presence 100 μM $H_2O_2$ or vehicle. For HCA2 cells, the third lane is from cells incubated in normal growth medium (GM) and is shown for comparison. Levels of phosphorylated mTOR and S6 and total REDD1 were determined by western blotting. Tubulin was used as a loading control. Immunoblot is representative of n = 3 experiments. **B**. Oxidative stress induces dissociation of 14-3-3 from REDD1 and association with TSC2. HCA2 cells were either incubated in growth medium (GM), or placed in KRH for 1 h. Cells in KRH were then incubated for 30 minutes in the presence of 100 μM $H_2O_2$ where indicated. Cell extracts were either run directly, or immunoprecipitated with anti-14-3-3 or an non-specific antibody (Mock). TSC2, 14-3-3 and REDD1 levels were determined by western blot. The arrowhead points to a non-specific band seen only in the extracts immunoprecipitated with the non-specific antibody. **C**. Association of TSC2 with 14-3-3 after oxidative stress is dependent on p38 but not on PI3K/AKT. HCA2 cells were either incubated in growth medium (GM), or placed in KRH for 1 h, in the presence or absence of 10 μM LY294002 (L), 10 μM SB202190 (S), or vehicle (DMSO, D). They were then incubated for 30 minutes in the presence 100 μM of $H_2O_2$ where indicated. Cell extracts were either run directly, or immunoprecipitated with anti-14-3-3 or a non-specific antibody (Mock). TSC2 and 14-3-3 levels in the immunoprecipitate were determined by western blotting. Note the markedly lower amount

of TSC2 associating with 14-3-3 in the setting of p38 inhibition with SB202190 (lane 7). **D.** REDD1 downregulation after oxidative stress is dependent on p38. HCA2 cells were incubated for 1 hour in KRH in the presence of 20 μM SB239063 or vehicle. They were then incubated for 30 minutes in the presence 100 μM of $H_2O_2$ or vehicle. Cell extracts were either run directly, or immunoprecipitated with anti-14-3-3 antibody. Shown are TSC2, REDD1 and 14-3-3 in the inmunoprecipitate, and phosphorylated Akt (Ser473), phosphorylated S6 (Ser235/236), total Akt, S6, TSC2, REDD1 and 14-3-3 in whole cell extracts. GAPDH was used as a loading control. Note the reduction in the amount of TSC2 in the 14-3-3 immunoprecipitate when the p38 inhibitor SB239063 is present ($5^{th}$ panel, compare lanes 2 and 3).

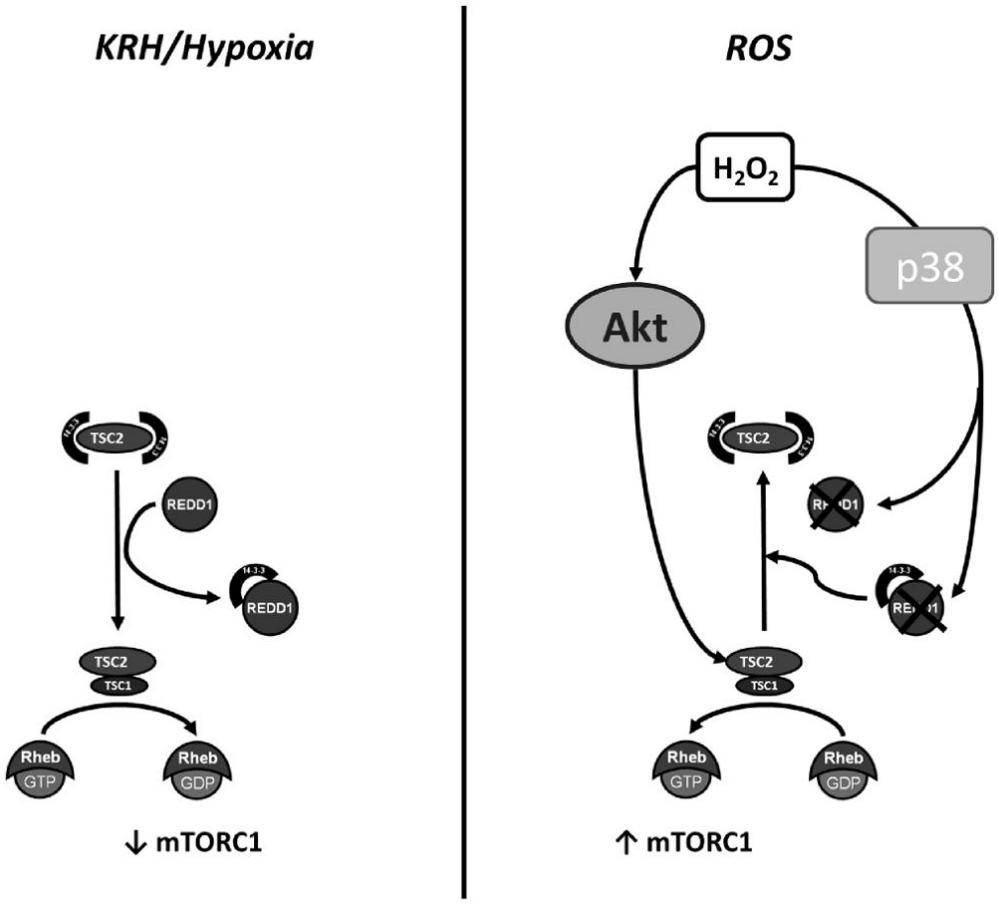


**Figure 8. Proposed model for p38 regulation of ROS mediated mTOR activation and cell survival**

A. mTOR activity is negatively regulated by Tsc1 and Tsc2 in the setting of low concentrations of amino acids, when energy or nutrient stores are low in the cell (as with KRH media), or in the setting of hypoxia. With hypoxia, REDD1 levels rise, and REDD1 sequesters 14-3-3 proteins, allowing the Tsc1/Tsc2 complex to form inhibiting mTOR. B. In the setting of I/R and ROS, mTOR is activated and this activation is cardioprotective both in vitro and in vivo. Activation is dependent on both p38 and Akt. Mechanistically, ROS reduce levels of REDD1, and this is dependent on p38 since it is blocked by SB compounds. The decline in REDD1 leads to the dissociation of the mTOR inhibitory complex of Tsc1/Tsc2 via enhanced association of Tsc2 with 14-3-3 family members. p38 is necessary for the Tsc2/14-3-3 interaction, and in the setting of p38 inhibition (but not PI3K/Akt inhibition), this interaction breaks down, preventing activation of mTOR and increasing cell death.