CHARACTERIZATION OF THE CELLULAR RESPONSE TO HYPOXIA

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Tan Chia Yee
28 December 2013

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Summary

Oxygen is essential to life for all higher organisms. Hypoxia is a condition with low oxygen levels. Under hypoxic conditions there are limited cellular energy resources due to inhibition of oxidative phosphorylation dependent ATP synthesis. Hypoxia activates a variety of complex pathways to enable cells to maintain homeostasis and survive low oxygen conditions. Non-essential processes such as protein synthesis may be inhibited during hypoxia. Furthermore, cells may respond to hypoxic stress by diminishing their proliferative rates through cell cycle arrest.

The mechanistic target of rapamycin complex 1 (mTORC1) is a key regulator of cell growth and proliferation in response to various upstream signals. Hypoxia has been shown to exert a strong inhibitory effect on mTORC1 activity. Various mechanisms involving gene transcription have been proposed to mediate the effect of hypoxia on mTORC1 activity. In this study, I showed that oxygen concentrations regulate mTORC1 activity in a highly dynamic manner. The rapid response of mTORC1 to changes in oxygen concentrations was not mediated by the HIF transcription factor or its transcriptional targets, REDD1 and BNIP3. Interestingly, I observed that the rapid response of mTORC1 activity to changes in oxygen concentrations is independent of transcription and new protein synthesis. This suggests a post-translational regulation mTORC1 activity in hypoxia and reoxygenation. My results also suggest that hypoxia does not regulate mTORC1 via the TSC1/2 or Ragulator pathways but directly at the level of mTORC1. In conclusion, my results suggest that mTORC1 can respond rapidly to changes in oxygen

concentrations via a post-translational mechanism that may involve a heme containing protein.

REDD1 is a negative regulator of mTORC1 that is known to be transcriptionally upregulated in hypoxia. During hypoxic stress, REDD1 has been reported to play an important role as a mediator of mTORC1 inhibition. REDD1 is also subject to highly dynamic transcriptional regulation in response to a variety of other stress signals. In addition, the REDD1 protein is highly unstable. However, it is currently not well understood how REDD1 protein stability is regulated. In this study, I discovered that mTORC1 regulates REDD1 protein stability in a 26S proteasome dependent manner. Inhibition of mTORC1 resulted in reduced REDD1 protein stability and a consequent decrease in REDD1 expression. Conversely, activation of the mTORC1 pathway increases REDD1 protein levels. I show that REDD1 degradation is not regulated by HUWE1, Cul4a or other Cullin E3 ubiquitin ligases. My study shows that mTORC1 increases REDD1 protein stability and reveals a novel mTORC1-REDD1 feedback loop. This feedback mechanism may limit the inhibitory action of REDD1 on mTORC1.

CDC6 is an important component of the pre-replication complex and plays an essential role in the regulation of DNA replication in eukaryotic cells. Deregulation of CDC6 protein levels results in rereplication and genomic instability. CDC6 expression is tightly regulated during the cell cycle. It is known that hypoxia can lead to cell cycle changes. Furthermore, it has been reported that hypoxia affects CDC6 protein levels. Therefore, I hypothesized that altered CDC6 protein stability contributes to hypoxia dependent cell cycle

arrest. However, in my studies I did not observe any significant changes in CDC6 protein levels at low oxygen concentrations. Hence, in my further studies I focused on the post-translational regulation of CDC6 in normoxic conditions. One major mechanism of cell cycle dependent regulation of CDC6 is APC^{Cdh1} mediated protein ubiquitination and degradation during G1 phase. In addition to APC^{Cdh1} dependent degradation, alternative, Cullin RING E3 ubiquitin ligase dependent degradation pathways have been characterized in yeast. In this project, I studied whether Cullin RING E3 ligases also play a role in the turnover of CDC6 protein in mammalian cells. To this end, I used the Nedd8 E1 inhibitor MLN4924, which blocks the activity of all Cullin E3 ligases. I observed that treatment with MLN4924 increased CDC6 protein expression. However, this effect was due to a delay in cell cycle progression from G1 to S phase, resulting in accumulation of cells with high CDC6 protein levels. Therefore, my results indicate that unlike in lower eukaryotes, Cullin E3 ligases are not involved in the basal turnover of CDC6 in mammalian cells.

Interestingly, I also found that the DNA cross-linker mitomycin C induces marked CDC6 protein degradation. Of note, mitomycin C requires bioreduction for activation and has hence been demonstrated to have greater cellular effects under hypoxic conditions. I found that mitomycin C induced CDC6 degradation is not mediated by APC^{Cdh1}, Cullin or HUWE1 E3 ubiquitin ligases. Notably, mitomycin C mediated CDC6 degradation requires the neddylation pathway. My results provide evidence for a novel, cullin independent mechanism of CDC6 posttranslational regulation upon DNA damage that involves the neddylation pathway.

List of Figures

List of publications

1. Tan, CY and Hagen, T. (2013) Post-translational regulation of mTOR complex 1 in hypoxia and reoxygenation. *Cellular Signalling* **25**(5):1235-44.

2. Tan, CY and Hagen, T. (2013) mTORC1 dependent regulation of REDD1 protein stability. *PLoS One* **8**(5): e63970.

3. Tan, CY and Hagen, T. (2013) Destabilization of CDC6 upon DNA damage is dependent on neddylation but independet of Cullin E3 ligases. *International Journal of Biochemistry and Cell Biology* **45**(7):1489-98.

1.0 Characterization of the cellular signaling and response to hypoxia

Hypoxia is a condition with oxygen levels lower than the physiological oxygen concentrations (approximately 7%). Hypoxic stress occurs when there is diminished supply of oxygen to tissues or there is an increase in oxygen demand. To adapt to hypoxia, cells respond by reducing fundamental physiological activities such as protein translation and energy metabolism, increasing protein degradation as well as inducing cell cycle arrest to maintain homeostasis and enable cells to survive low oxygen conditions. The mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway, an important regulator of protein synthesis, is rapidly inhibited upon reduced oxygen availability to conserve energy levels in cells. This is because protein synthesis is a high-energy process and in hypoxia, ATP levels are severely decreased in cells. Hence, inhibition of the mTORC1 signaling pathway in hypoxia and thereby downregulating protein synthesis would allow cells to utilize available energy for more essential survival mechanisms.

The mTORC1 pathway is activated through the inhibition of tuberous sclerosis complex -1 and -2 (TSC1/2) via phosphorylation by different upstream kinases of multiple signaling pathways including PI3K/Akt, MEK/ERK/RSK and MAPK/MK2 (Manning et al., 2002; Inoki et al., 2002; Li et al., 2003; Ma et al., 2005). Inactivation of GTPase activator TSC1/2 leads to the accumulation of the active GTP bound form of Rheb. GTP-Rheb binds to and activates mTORC1 (Figure 1). Recently, it has also been shown that in response to amino acids, mTORC1 is activated by the Rag GTPases and the

Ragulator complex through its translocation to the lysosomal surface where mTORC1 is activated by Rheb (Sancak et al., 2010).

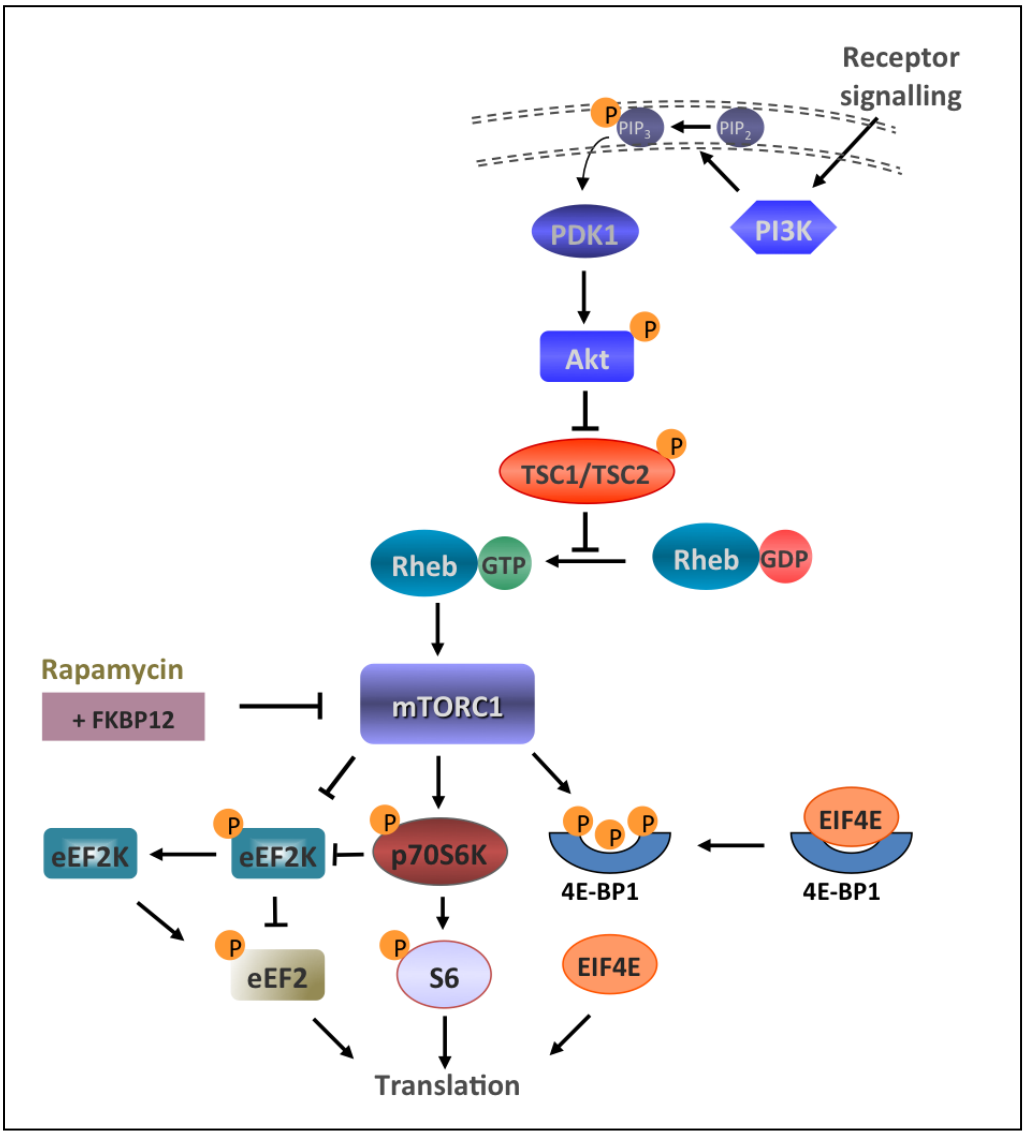


Figure 1. *The mTORC1 pathway*. mTORC1 is a sensor of various stress signals and the mTORC1 pathway regulates translation via phosphorylation of its downstream targets eEF2K, p70S6K and 4E-BP1.

Activated mTORC1 stimulates protein synthesis and cell growth through phosphorylation of its downstream targets: ribosomal S6 kinase 1 (p70S6K), eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and eukaryotic elongation factor 2 kinase (eEF2K) (Browne and Proud, 2004; Fingar et al., 2002) (Figure 1). Activated p70S6K promotes translation

through phosphorylation and activation of its target ribosomal protein S6, a component of the 40S ribosomal subunit. 4E-BP1 is a translational repressor protein that is normally bound to eIF4E to inactivate the binding of eIF4E to the 5' cap of mRNAs to initiate translation (Gingras, Raught and Sonenberg, 1999). Hyperphosphorylation of 4E-BP1 by mTORC1 prevents binding of 4E-BP1 to eIF4E and thereby promotes translation. On the other hand, eEF2K mediates the translocation step of elongation through its phosphorylation and inactivation of eEF2, the protein which controls ribosomal translocation during elongation of the new polypeptide chain (Browne and Proud, 2002).

A further mechanism important in the hypoxic response is protein ubiquitination, which plays a critical role as it allows cells to respond quickly to changes in the environment. The most well studied ubiquitination event in hypoxia is the regulation of the transcription factor, Hypoxia-Inducible Factor 1α (HIF-1α) (Epstein et al., 2001; Bruick, 2001; Bruick and McKnight, 2001). HIF-1 proteins exist in 2 subunits: the oxygen sensitive HIF-1α subunit and the constitutively expressed nuclear subunit, HIF-1β. The expression of the HIF-1α subunit is a highly specific response to hypoxia (Huang et al., 1996). Although HIF-1α mRNA is constitutively expressed in cells, the HIF-1α protein is rapidly degraded in normoxia by the ubiquitin-proteasome pathway via Cullin 2 E3 ubiquitin ligases (Huang et al., 1998; Salceda and Caro, 1997; Maxwell et al., 1999; Cockman et al., 2000; Kamura et al., 2000; Tanimoto et al., 2000). This degradation process is inhibited in hypoxia to allow rapid accumulation of HIF-1α levels in cells followed by nuclear translocation of HIF-1α in response to low oxygen levels (Sutter, Laughner and Semenza,

2000; Kallio et al., 1998) (Figure 2). The HIF-1 transcription factor is known to regulate genes important for survival in hypoxia including genes involved in angiogenesis such as vascular endothelial growth factor (VEGF) (Forsythe et al., 1996) as well as in glycolysis, for instance GLUT1 (Ebert, Firth and Ratcliffe, 1995), signifying its importance in hypoxia.

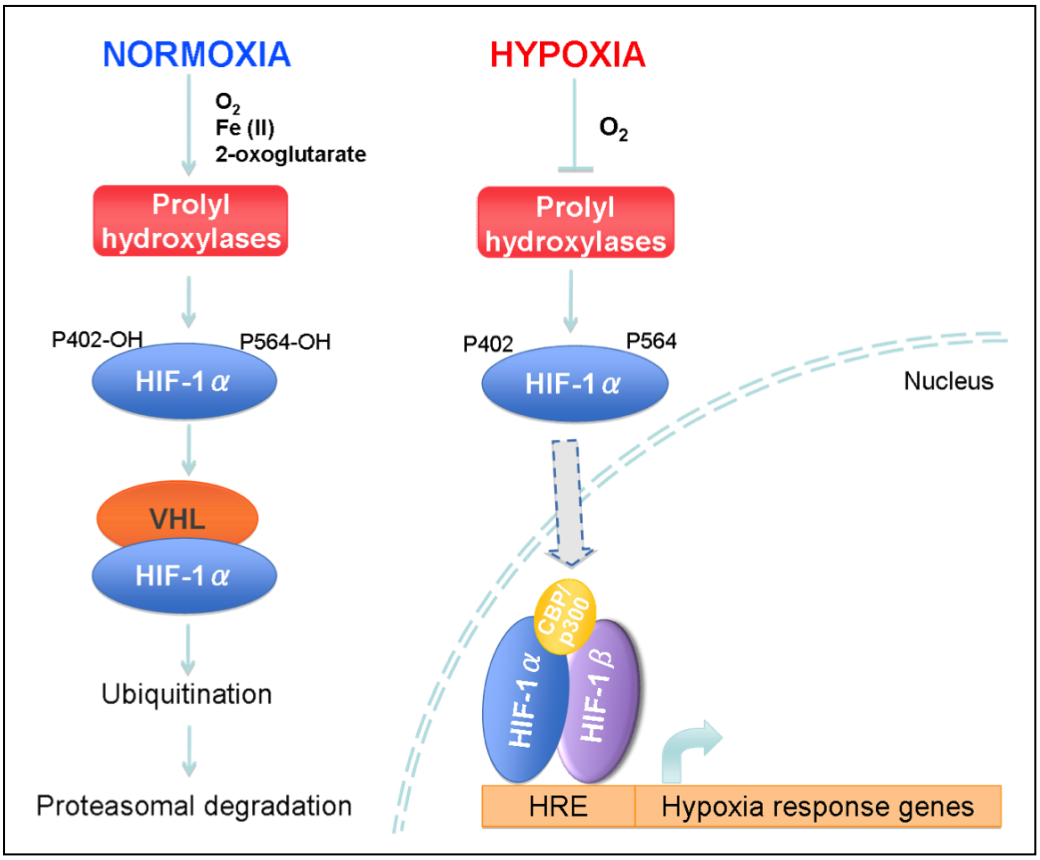


Figure 2. *Regulation of HIF-1α protein stability in normoxia and hypoxia.* In nomoxia, HIF1α is hydroxylated by prolyl hydroxylases and continuously degraded by Cullin 2 VHL E3 Ligase. In hypoxia, prolyl hydroxylases are inactive leading to the stabilization of HIF1α which then translocate into the nucleus to dimerize with HIF1β for the transcription of hypoxia response genes.

Although HIF-1α has always been thought to function as the key regulator in hypoxia, other HIF-1α independent mechanisms also play an important in cellular adaptation to changes in oxygen concentrations. Hypoxic environment occurs during development and also in tumor formation.

In both cases, cells grow rapidly resulting in a hypoxic environment at the inner region of the growing cell mass. It has been reported that the expression of FBXl14 is potently downregulated in hypoxia, thereby leading to increased levels of its substrate, SNAIL1 (Vinas-Castells et al., 2010). SNAIL1 is a transcription factor which plays a fundamental role in initiating epithelial-mesenchymal transition (EMT), a phenotype that is induced in hypoxia.

Also, cell cycle checkpoints are activated in hypoxia to suppress cell proliferation and enable cells to adapt to and survive in hypoxia (Amellem et al., 1998). Diminished oxygen levels lead to the activation of the cell cycle checkpoint at the G1/S phase (Amellem et al., 1998; Schmaltz et al., 1998). An essential step in the transition of the G1/S phase is the phosphorylation of the retinoblastoma protein (Rb) by specific cyclin-dependent kinase (CDK)-cyclin complexes. This leads to the inactivation of the growth suppressive function of Rb (Blagosklonny and Pardee, 2000; Planas-Silva and Weinberg, 1997). However, in hypoxia, CDK2 activity is diminished, resulting in the hypophosphorylation and thereby activation of Rb to induce G1 cell cycle arrest (Amellem et al., 1998; Krtolica, Krucher and Ludlow, 1999).

Various mechanisms are activated in cells in response to hypoxic stress to enable cells to adapt to a changing environment and ensure cell survival. Cellular response to hypoxic stress is complicated as it occurs at many different levels and the different mechanisms be can interdependent in their function in response to cellular stress. The aim of my project is to

characterize different mechanisms activated in hypoxia and my work is divided into three parts. In the first part I studied how oxygen levels regulate the mTORC1 pathway in hypoxia and upon reoxygenation. In the second, I studied how the stability of REDD1 is regulated. The REDD1 protein is a negative regulator of the mTORC1 pathway. REDD1 is normally upregulated in hypoxia and degraded upon reoxygenation. Finally, in the last part, I studied how the protein CDC6, an important protein of the pre-replication complex, is degraded. CDC6 has been reported to be downregulated in hypoxia. I therefore hypothesized that oxygen levels may regulate CDC6 protein stability and consequently, downregulation of CDC6 protein levels may contribute to cell cycle arrest in hypoxia. It is therefore important to understand how this protein is degraded. However, I observed that CDC6 levels in cells exposed to hypoxic conditions were not significantly lower compared to cells in normoxia. Therefore, the focus of the last part of the project was to characterize the hypoxia-independent regulation of CDC6 protein stability.

2.0 Materials and methods

2.1 Cell culture and transfection

Human embryonic kidney (HEK293) (ATCC and Invitrogen), mammary carcinoma (MCF7) (ATCC), Mouse Embryonic Fibroblasts (MEF), Hela cells, renal cell carcinoma (RCC) 786-O cells (Iliopoulos et al., 1995; Lonergan et al., 1998) and $TSC2^{+/+}$-$p53^{-/-}$ and $TSC2^{-/-}$-$p53^{-/-}$ MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Colon carcinoma (HCT116) cells (ATCC) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. Both media were supplemented with 10 % inactivated fetal bovine serum, 2 mM L-glutamine and 1 % penicillin-streptomcin (Invitrogen) and all cell lines were incubated at 37 °C with 5 % CO_2. RCC 786-O VHL *null* and HA-pVHL (WT) reconstituted 786-O cells were kindly provided by Michael Ohh, University of Toronto (Lonergan et al., 1998; Iliopoulos et al., 1995) and $TSC2^{+/+}$-$p53^{-/-}$ and $TSC2^{-/-}$-$p53^{-/-}$ MEFs were kindly provided by D.J. Kwiatkowski (Brigham and Women's Hospital, Harvard Medical School, Boston, MA) (Zhang et al., 2003). For overexpression experiments, sub-confluent cells were transfected using Genejuice (Novagen) according to the manufacturer's instructions. Knockdown experiments using siRNAs (predesigned dsiRNAs, IDT) were performed using Lipofectamine RNAiMax (Invitrogen) according to the instructions by the manufacturer.

2.2 Plasmid constructs

The human BNIP3-V5 pcDNA3 plasmid was constructed by PCR amplification from the cDNA purchased from Mammalian Gene Collection and inserted into the pcDNA3 vector with a C terminal V5 tag using KpnI and XbaI restriction sites with a SacII restriction site inserted between BNIP3 and the V5 tag. HIF-1α P402A/P564A-V5 pcDNA3 plasmid was constructed as described previously (Hagen et al., 2003). The HSPBAP1-V5 plasmid was PCR amplified from HEK293 cDNA and ligated in to the pCDNA3 vector with a C terminal V5 tag using the same restrictions sites as BNIP3-V5 pcDNA3 construct.

For the REDD1-V5 pcDNA3 plasmid, REDD1 gene was first PCR amplified from human brain cDNA and subsequently ligated into the pcDNA3 backbone with a C terminal V5 tag using the same restriction sites as the BNIP3-V5 pcDNA3 construct. REDD2-V5 pcDNA3 was also constructed in the same way. Mutagenesis of REDD1 Threonines 23 and 25 to Alanines or Aspartate was carried out using the Stratagene site-directed mutagenesis kit. Mutation of different combinations of REDD1 lysine residues to alanines was performed using the Stratagene site-directed mutagenesis kit. Construction of REDD1 truncation mutants i.e. C-terminal end truncation mutants (1-132), (1-162), (1-202) and N-terminal end truncation mutant (129-233) were carried out using PCR with the appropriate primers.

The following plasmids were purchased from Addgene: FLAG-TSC2 pcDNA3 (Addgene Plasmid 14129) (Manning et al., 2002), pRK5 DEPTOR-

FLAG (Addgene Plasmid 21334) (Peterson et al., 2009), pBabe GFP Small T Antigen (Addgene Plasmid 10673) (Boehm et al., 2005), pRK5 HA Raptor (Addgene Plasmid 8513) (Kim et al., 2002), FLAG-Rheb pcDNA3 (Addgene Plasmid 19996) (Urano et al., 2007). The FLAG-Rheb S16H pcDNA3 mutant was constructed from the wild type Flag-Rheb pcDNA3 by mutation of Serine to Histidine using Stratagene site-directed mutagenesis kit.

The human IDH1 wt-V5 pcDNA3 plasmid was constructed by PCR amplification from the cDNA Purchased from Mammalian Gene Collection (MGC clone 3889331) and inserted into the pcDNA3 vector with a C terminal V5 tag KpnI and SacII. The IDH1 R132H-HA pcDNA3 plasmid was constructed by mutation of Arginine to Histidine using the Stratagene site-directed mutagenesis kit.

The human PRMT1-V5 pcDNA3 plasmid was constructed by PCR amplification from HEK293 cDNA. The amplified PRMT1 was inserted into the pCDNA3 vector with a C-terminal V5 tag using the same restriction sites as the BNIP3-V5 pcDNA3 construct. The human Siah2-FLAG pcDNA3 was PCR amplified from HEK293 cDNA and inserted into the pCDNA3 vector with a C-terminal FLAG tag.

The human CDC6 pcDNA3 plasmid was constructed by PCR amplification from the cDNA purchased from Mammalian Gene Collection. The amplified CDC6 coding sequence was inserted into the pcDNA3 vector with a C terminal V5 tag using the same restriction sites as BNIP3-V5. The CDC6-V5

ΔD & KEN box mutant was constructed by deleting amino acids 56-83 containing D box and KEN box from full length CDC6-V5 pcDNA3 plasmid.

The S6 kinase plasmids HA-p70S6K1 T389D pcDNA3 and HA-p70S6K1 T389A pcDNA3 were constructed from the pRK7-HA-S6K1-WT (Addgene Plasmid 8984) (Schalm and Blenis, 2002). The HA-p70S6K1 gene was digested from the pRK7-HA-S6K1-WT plasmid using XbaI and EcoRI restriction enzymes and ligated in to the pcDNA3.1 (-). Mutagenesis of p70S6K1 Threonine 289 site to Alanine or aspartate was carried out using the Stratagene site-directed mutagenesis kit. GSK3β and FRAT1 plasmid was previously described (Hagen et al., 2002).

The 3 kb REDD1 promoter pGL-3 basic or 0.6 kb REDD1promoter pGL-3 basic constructs were kindly provided by Leif W. Ellisen (Harvard Medical School) (Ellisen et al., 2002).

The dnCul1-V5 pcDNA3 (amino acids 1-452), dnCul3-V5 pcDNA3 (amino acids 1-427) and dnCul4a-V5 pcDNA3 (amino acids 1-439) plasmids were described previously. The dnCul4b-FLAG pcDNA3 (amino acids 1-594) plasmid was from Addgene (Plasmid 15822) (Jin et al., 2005). The tetracycline-inducible dnUbc12 (C111S), dnCul1-V5 (amino acids 1-452) and dnCul4a (amino acids 1 to 439) cell lines were generated using the T-Rex system (Invitrogen) according to the manufacturer's instructions, as previously described (Chew et al., 2007; Chew and Hagen, 2007).

2.3 Oxygen conditions

Hypoxic condition (1 % O_2, 5 % CO_2 and balanced with N_2) was achieved in a Pro-ox 110 oxygen controller and Pro-ox in vitro chamber (BioSpherix) or an Invivo2 400 hypoxia workstation (Ruskinn Technology). For reoxygenation experiments, cells were first treated with the indicated compounds or transfected before hypoxia incubation, followed by removal from hypoxic chamber and exposure to atmospheric oxygen for reoxygenation.

2.4 Immunoblotting

Whole cell lysates were prepared by rinsing the cells in ice cold 1x PBS followed by cell lysis using triton-X lysis buffer with the following composition: 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 20 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphate, and 0.5 % Triton X-100 containing freshly added protease inhibitor cocktail (Roche Diagnostics) and 0.1 % β-mercaptoethanol. Equal amounts of protein from each sample were separated by SDS–PAGE (10%) and transferred onto nitrocellulose membranes. The blots were probed with a primary antibody followed by a secondary antibody conjugated to horseradish peroxidase. The following primary antibodies were used: rabbit anti-phospho-p70 S6 kinase (Thr389) (9234; Cell Signaling), rabbit anti-p70 S6 kinase (9202; Cell Signaling), rabbit anti-REDD1 (10638-1-AP; Proteintech), mouse anti-HIF-1a (610959; BD Biosciences), mouse anti-a-tubulin (236–10501; Molecular Probes, Invitrogen) and mouse anti-V5 (MCA1360; AbD Serotec), mouse anti-p27 (610241; BD Biosciences), mouse anti-HECTH9 (AX8D1)/HUWE1 (5695; Cell Signaling), mouse anti-GSK3β

(610202; BD Transduction Laboratories), mouse anti-Mcl-1 (sc-12756; Santa Cruz Biotechnology), mouse anti-Cdc6 (sc-9964; Santa Cruz Biotechnology), anti-p21 (F-5) (sc6246; Santa Cruz Biotechnology), mouse anti-Cdh1 (DCS266), mouse anti-SLBP (H00007884-M01; Abnova), mouse anti-MDM2 (Santa Cruz Biotechnology), mouse anti-FLAG M2 (F-3165; Sigma), rat anti-HA (clone 3F10) (Roche Applied Science). Protein levels on the blots were detected using the enhanced chemiluminescence system (GE Healthcare) according to the manufacturer's instructions. Western blots shown are representative of at least two independent experiments.

2.5 Immunoprecipitation

10 µl of anti-FLAG M2 agarose (Sigma) or 1.5 µl of V5 antibody, coupled to 10 µl of protein G-sepharose (Amersham Biosciences) was used for immunoprecipitations. 500 µl pre-cleared lysate from HEK293 cells transfected in 60 mm tissue culture plates was added. The samples were tumbled at 4 °C for 1 h and the agarose or sepharose beads were then washed four times in 1 ml of cold buffer containing 20 mM Tris (pH 7.5), 0.6 M NaCl and 1 mM EGTA and once in buffer containing 50 mM Tris (pH 7.5). The immunoprecipitated proteins were then denatured in SDS-sample buffer and subjected to SDS-PAGE and Western blotting.

2.6 In vitro ubiquitination assay

Pre-cleared lysate from HEK293 cells transfected with 2 µg REDD1-V5 in two 60 mm plates was added to protein G-sepharose beads coupled with V5 antibody. The samples were tumbled at 4 °C for 1 h and sepharose beads were

then washed four times in 1 ml of cold Nonidet P-40 buffer containing 50 mM Tris-HCl pH 7.5, 0.5 % NP-40, 5 % glycerol, 0.5 mM EDTA and 50 mM NaCl. Pre-cleared lysates from untransfected HEK293 cells in 100 mm plates were added to the REDD1-V5 samples bound to sepharose beads. Next, 10 µl of ubiquitination system (Boston Biochem Cat # K-960) containing 25 mM Hepes, 20 nM $MgCl_2$, 10 nM E1 ubiquitin enzyme, 0.1 µM E2 ubiquitin enzyme, 50 µM ubiquitin and 0.5 M ATP was added followed by shaking incubation at 30 °C for 1 h. After that, the samples were washed twice using cold 1 X PBS.

2.7 Luciferase reporter assay

HEK293 cells at approximately 70% confluence were transfected with 0.2 µg firefly luciferase pGL-3 basic reporter plasmids (driven by REDD1 promoters) using GeneJuice according to the manufacturer's instructions. Firefly luciferase activity was measured after 48 hours using the Steady-Glo reporter assay system (Promega).

2.8 iTRAQ analysis

HEK293T cells stably expressing REDD1-FLAG puroMARX or EGFP puroMARX were grown in 100 mm tissue culture plates. MG-132 (20 µM) was added to cells for 6 hours followed by cell lysis with triton-X lysis buffer (described above) or hypotonic lysis buffer (1M Tris-HCl pH 7.5, 0.5 M EDTA, 100 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 20 mM sodium β-glycerophosphate and 10 mM sodium pyrophosphate containing freshly added protease inhibitor cocktail (Roche Diagnostics)). Pre-cleared lysates from the

cells were tumbled at 4 °C for 1 h with 20 µl of anti-FLAG M2 agarose (Sigma) beads and washed four times in 1 ml of cold Nonidet P-40 buffer. Washed samples were eluted from FLAG beads by adding 0.1 M acetic acid and incubated at room temperature by gently shaking for 5 mins. The supernatant was transferred to fresh tubes containing 5 µl neutralizing buffer (1N NaOH). The samples were shipped on dry ice to the UVic Genome BC Proteomics Centre for iTRAQ analysis.

2.9 In vitro phosphorylation of REDD1 and FRAT1

FLAG-immunoprecipates (REDD1-FLAG or FRAT-FLAG) from HEK293 cell lysates were incubated on a shaking platform for 45 minutes at room temperature in 50 mM Tris pH7.5, 25 mM $MgCl_2$ and 2 mM DTT in the presence or absence of 1 mM ATP and/or the recombinant protein GSK3β. Following the reaction, the samples were denatured in SDS-sample buffer and subjected to SDS-PAGE and immunoblotting.

2.10 Cell synchronization and cell cycle analysis

HeLa cells synchronized at G_2/M phase by blocking with thymidine (2 mM) for 20 hours, washed and released in complete medium for 4 hours , followed by incubation in nocodazole (100 ng/ml) for 13 hours. Mitotic cells were plated in 6 well plates (2 X 10^6 cells) in the presence or absence of MLN4924 (1 µM) and collected every 3 hours for 12 hours and at 24 hours. For cell cycle analysis, Hela cells were fixed with 70 % ethanol on ice for at least 2 hours and stained in propidium iodide (20 µg/ml) and RNase A (0.1 µg/ml). Propidium iodide stained cells were analyzed using Epics Altra flow

cytometer (Beckman Coulter) and the data were analyzed using Dako Summit v4.3.

3.0 Post-translational Regulation of mTOR Complex 1 in Hypoxia and Reoxygenation

3.1 Introduction

The mechanistic target of rapamycin complex 1 (mTORC1) functions as a key regulator of cell growth and proliferation by acting as a sensor of various types of stress signals. Under conditions of stress unfavorable for cell growth, the mTORC1 pathway is inhibited. One important negative regulator of the mTORC1 activity is hypoxia (Arsham, Howell and Simon, 2003). Under hypoxic conditions there are limited cellular energy resources due to inhibition of oxidative phosphorylation dependent ATP synthesis. Hence, hypoxia mediated mTORC1 inhibition is of great physiological significance as it downregulates non-essential cellular reactions and pathways in favor of processes that are critical for cell viability.

mTORC1 is a complex consisting of mTOR, a serine/threonine kinase, in association with the regulatory associated protein of mTOR (Raptor) (Hara K et al., 2002), proline-rich Akt substrate 40 (PRAS40) (Wang et al., 2007; Haar et al., 2007) and G-protein β-subunit-like protein/mLST8 (Kim et al., 2003). The mTORC1 kinase stimulates protein synthesis and cell growth through phosphorylation of its downstream targets: ribosomal S6 kinase 1 (p70S6K), eukaryotic initiation factor 4E (eIF4E)-binding protein 4E-BP1 and

eukaryotic elongation factor 2 kinase (eEF2K) (Browne and Proud, 2004; Fingar et al., 2002). mTORC1 is activated via two signaling pathways, depending on its upstream signals. Both pathways activate mTORC1 through binding of the small GTPase Rheb. The first pathway is dependent on the presence of growth factors. Growth factor dependent activation of cellular signaling leads to the inhibition of an important negative upstream regulator of the mTORC1 pathway, the tuberous sclerosis complex -1 and -2 (TSC1/2) complex. This complex normally functions as a GTPase to convert the active GTP-Rheb into the inactive GDP bound form. Under nutrient- and energy-replete conditions different upstream kinases of multiple signaling pathways including PI3K/Akt, MEK/ERK/RSK and MAPK/MK2 (Manning et al., 2002; Inoki et al., 2002; Li et al., 2003; Ma et al., 2005) phosphorylate and inhibit the TSC1/2 complex, thus leading to mTORC1 activation. In the second pathway, presence of amino acids leads to the Rag-GTPases-Ragulator dependent translocation of mTORC1 to the lysosomal surface, where mTORC1 is activated by Rheb (Sancak et al., 2010).

Hypoxia has been reported to inhibit mTORC1 via different mechanisms (Figure 3). For instance, it has been reported that inhibition of mTORC1 in hypoxia is a consequence of activation of AMP-activated protein kinase (AMPK) (Hardie and Hawley, 2001). Low oxygen concentrations block mitochondrial ATP production leading to decreased ATP levels and subsequently, activation of AMPK. AMPK has been reported to activate TSC1/2, leading to inhibition of mTORC1 (Liu et al., 2006). In addition, TSC1/2 is also inhibited in hypoxia through REDD1 (REgulated in

Development and DNA damage responses 1), which is a known target gene of the transcription factor Hypoxia Inducible Factor-1α (HIF-1α) (Reiling and Hafen, 2004; Brugarolas et al., 2004). HIF-1α is stabilized in hypoxia through inhibition of oxygen-dependent prolyl hydroxylases (PHDs) (Bruick, 2001); (Epstein et al., 2001) or activation of ataxia telangieactasia mutated (ATM) (Cam et al., 2010). On the other hand, mTORC1 inhibition in hypoxia has also been shown to be regulated through the inactivation of Rheb by BNIP3 (Bcl2/adnovirus E1B 19 kDA protein-interacting protein 3) (Li et al., 2007). Similar to REDD1, BNIP3 is also transcriptionally induced in hypoxia via the HIF transcription factor. Finally, the promyelocytic leukemia (PML) protein was reported to inhibit Rheb-mTORC1 association and to promote the nuclear accumulation of mTOR, where Rheb is absent, thus preventing mTORC1 activation (Bernardi et al., 2006).

However, studies have shown that mTORC1 inhibition in hypoxia can occur independently of AMPK, HIF-1α and REDD1, suggesting the existence of additional mechanisms (Arsham, Howell and Simon, 2003). Furthermore, the dynamics with which mTORC1 responds to changing oxygen concentrations are currently not well characterized. In this study, I found that the inhibition of mTORC1 is rapidly reversed upon reoxygenation, suggesting a highly dynamic oxygen dependent regulation of mTORC1 activity via post-translational mechanisms. I also found that previously reported mTORC1 inhibitory factors do not play a major role in the rapid mTORC1 regulation in hypoxia and reoxygenation. My results suggest that a heme containing protein regulates this pathway at the level of mTORC1.

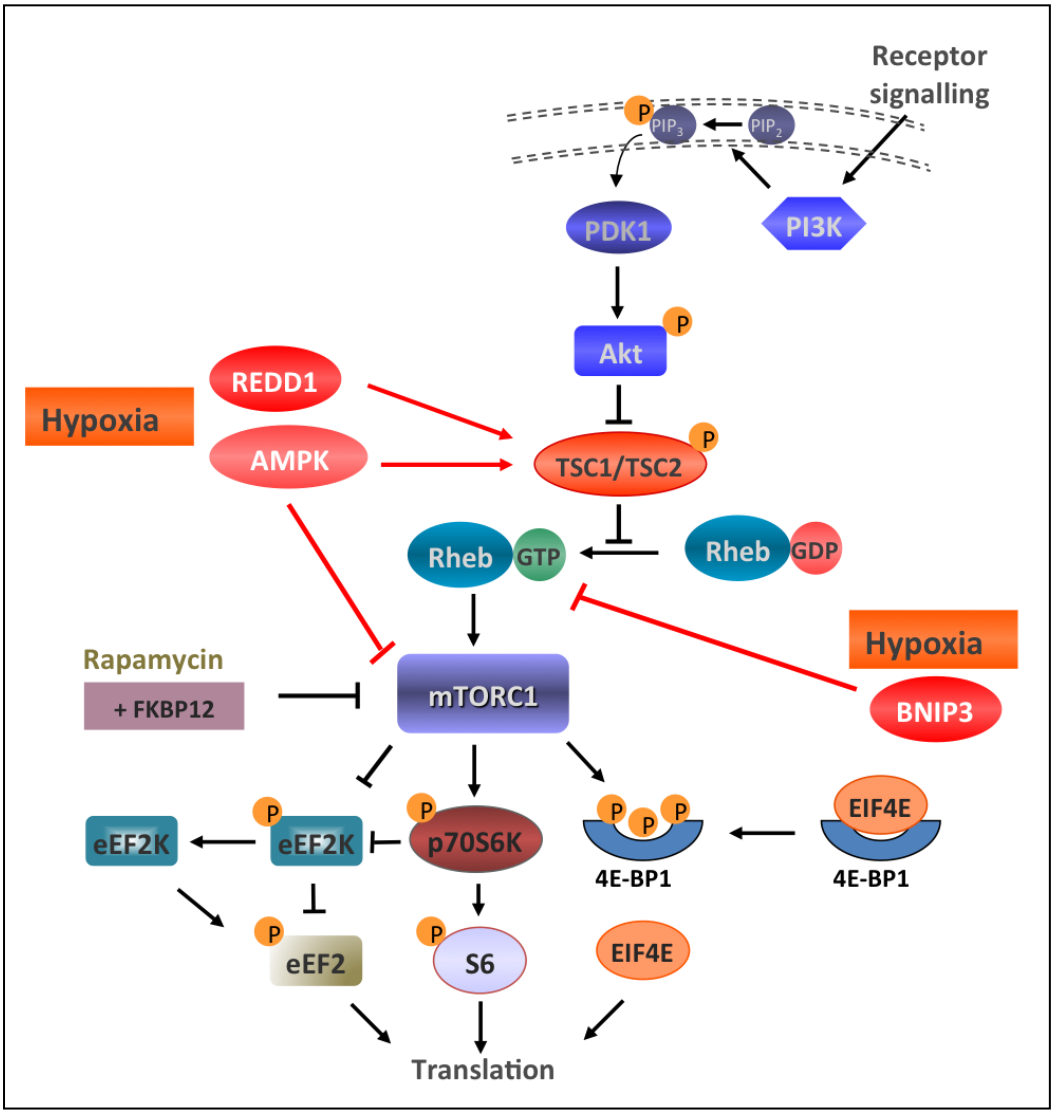


Figure 3. *Regulation of mTORC1 pathway in hypoxia.* The mTORC1 pathway is inhibited in hypoxia by the upregulation of REDD1, BNIP3 and AMPK.

3.2 Results

3.2.1 mTORC1 is inhibited in hypoxia and rapidly reactivated upon reoxygenation

To test for mTORC1 activity in hypoxia and reoxygenation, I incubated cells in a hypoxic chamber at 1 % oxygen for 4 hours followed by reoxygenation at 21 % oxygen and cell lysis at different time points. mTORC1 activity was markedly reduced in all cell types, as detected by the phosphorylation status of the mTORC1 target p70 S6 kinase (p70S6K) (Figure 4), when cells were placed in hypoxia. This is consistent with previous reports that hypoxia inhibits mTORC1 activity (Arsham, Howell and Simon, 2003). Interestingly, upon reoxygenation, there was a rapid reactivation of mTORC1 activity, as shown by the quick accumulation of phosphorylated p70S6K (Figure 4). The effect of reoxygenation on p70S6K phosphorylation is mTORC1 dependent as it is completely prevented in the presence of the specific mTORC1 inhibitor rapamycin (Figure 5). To investigate the mechanism through which hypoxia regulates mTORC1, I initially studied the role of a number of previously reported mediators.

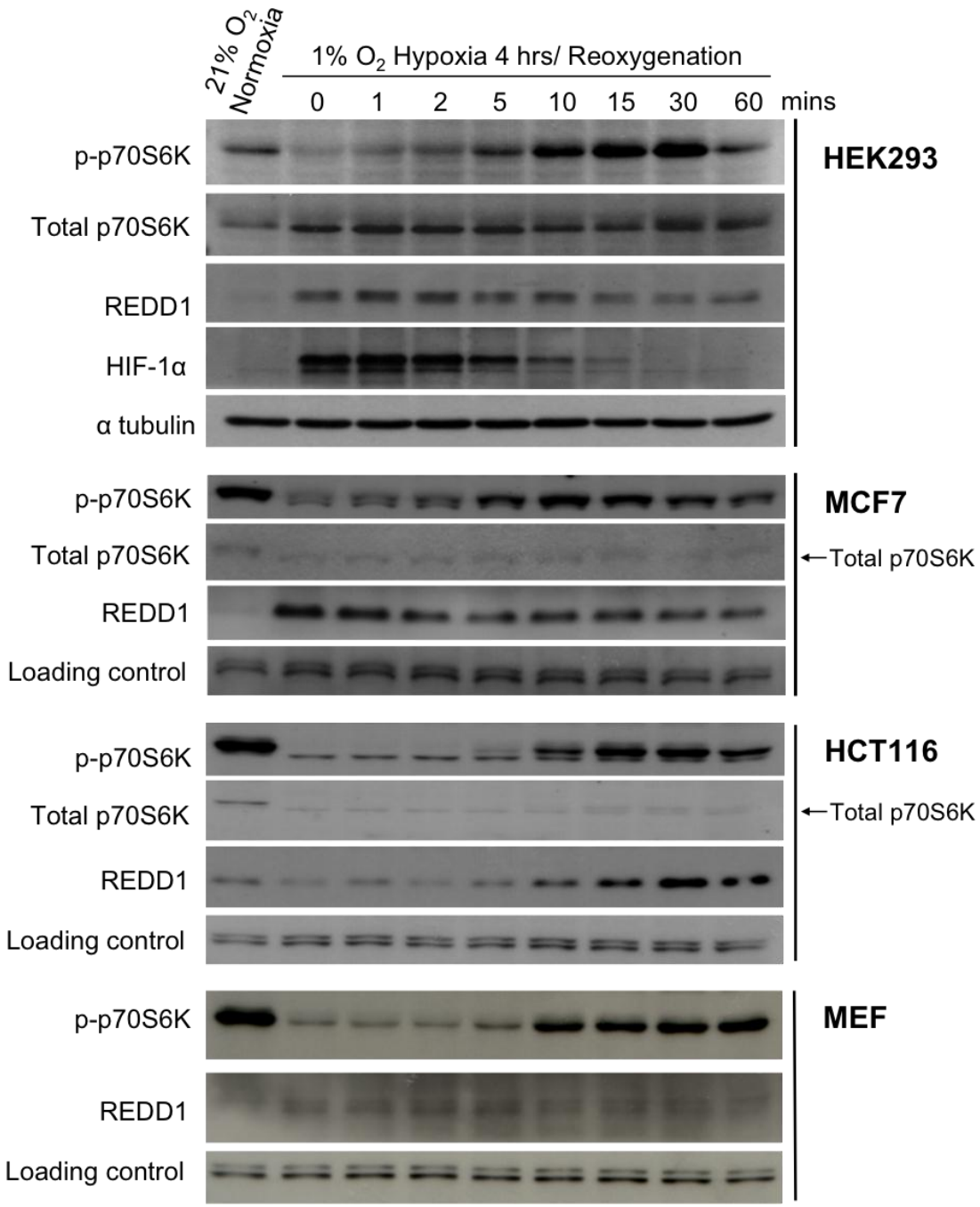


Figure 4. *mTORC1 is inhibited in hypoxia and rapidly reactivated upon reoxygenation.* HEK293, MCF7, HCT116 and MEF cells were incubated at 21 % or 1 % O_2 for 4 hours, followed by reoxygenation in normoxia and cell lysis at the indicated time points. Hypoxia (1% O_2) inhibits mTORC1 activity as indicated by the marked reduction in p70 S6 kinase (p70S6K) T389 phosphorylation compared to normoxia (21% O_2). Upon reoxygenation, mTORC1 activity is rapidly reactivated as shown by the increased p70S6K phosphorylation.

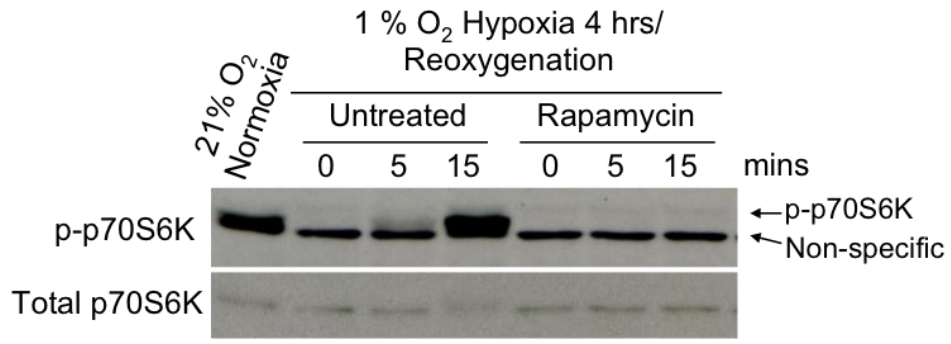


Figure 5. *mTORC1 activity in hypoxia and reoxygenation is mTORC1 dependent.* HEK293 cells were pre-treated with 20 nM rapamycin prior to hypoxic incubation for 4 hours at 1 % O_2, followed by reoxygenation and cell lysis at 0, 5 and 15 min.

3.2.2 BNIP3 and REDD1 are partially responsible for mTORC1 inhibition in hypoxia

BNIP3 is strongly induced in hypoxia and has been shown to mediate mTORC1 inhibition in hypoxia. BNIP3 binds to Rheb and consequently prevents activation of mTORC1 (Li et al., 2007). To determine the effect of BNIP3 on mTORC1 activity, I overexpressed control vector or BNIP3-V5 pcDNA3 in HEK293 cells and determined the phosphorylation status of p70S6K in normoxia and hypoxia. I observed that BNIP3 overexpression did not result in any difference in mTORC1 activity compared to control cells under both nomoxic and hypoxic conditions (Figure 6) indicating that BNIP3 did not induce mTORC1 inhibition. I also used siRNA to silence BNIP3 expression. Efficiency of the siRNAs used to knock down BNIP3 was confirmed in a separate experiment (Figure 7A). I observed that silencing of BNIP3 using siRNAs resulted in only a slight increase in mTORC1 activity in hypoxia compared to controls (Figure 7B). Taken together, my results suggest that BNIP3 does not play a major role in mTORC1 regulation in HEK293 cells.

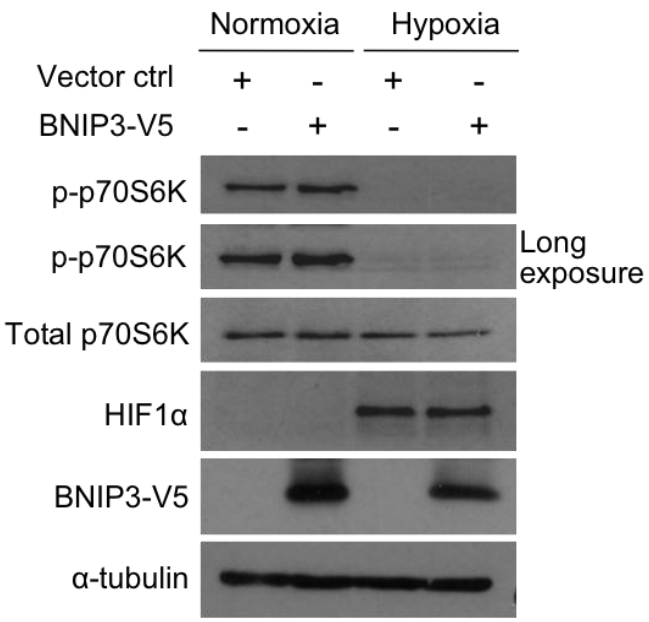


Figure 6. *BNIP3 overexpression has no effect on mTORC1 activity.* HEK293 cells were transfected with 0.4 µg pcDNA3 vector control or BNIP3-V5 pcDNA3 for 3 days followed by hypoxia incubation for 4 hours at 1 % O_2 before lysis.

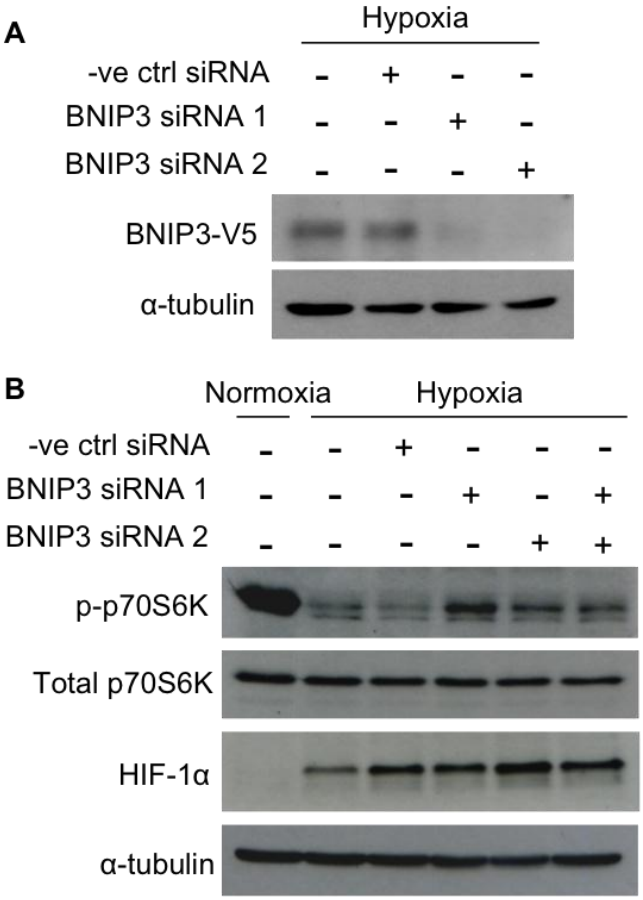


Figure 7. *BNIP3 is partially responsible for mTORC1 inhibition in hypoxia.* (**A**) HEK293 cells were transfected with 20 nM control or BNIP3 siRNAs 16 hours after the cells were transfected with 0.15 µg BNIP3-V5 pcDNA3 to determine siRNA efficiency.

(**B**) HEK293 cells were transfected with 20 nM control or BNIP3 siRNAs for 3 days followed by hypoxia incubation for 4 hours at 1 % O_2 before lysis.

The transcriptional target of HIF-1α, REDD1, has been reported to negatively regulate mTORC1 in a TSC1/2 dependent manner (Reiling and Hafen, 2004; Brugarolas et al., 2004) and may mediate the inhibitory effect of hypoxia on mTORC1 activity. I found that REDD1 overexpression did not result in the inhibition of mTORC1 activity in normoxia as expected but instead caused a slight increase in p70S6K phosphorylation (Figure 8). In hypoxia, REDD1 overexpression also did not result in marked reduction in the phosphorylation of p70S6K compared to control cells (Figure 8). Similarly, overexpression of the REDD1 ortholog, REDD2, did not affect mTORC1 activity in normoxia and hypoxia (Figure 9). This result suggests that increasing the cellular REDD1/REDD2 levels does not result in mTORC1 inhibition. To test whether endogenous levels of REDD1 and REDD2 play a role in regulating mTORC1 activity in hypoxia, I knocked-down both isoforms using siRNAs. Combined knockdown of REDD1 and REDD2 in HEK293 cells only partially reversed hypoxia dependent inhibition of mTORC1 activity (Figure 10).

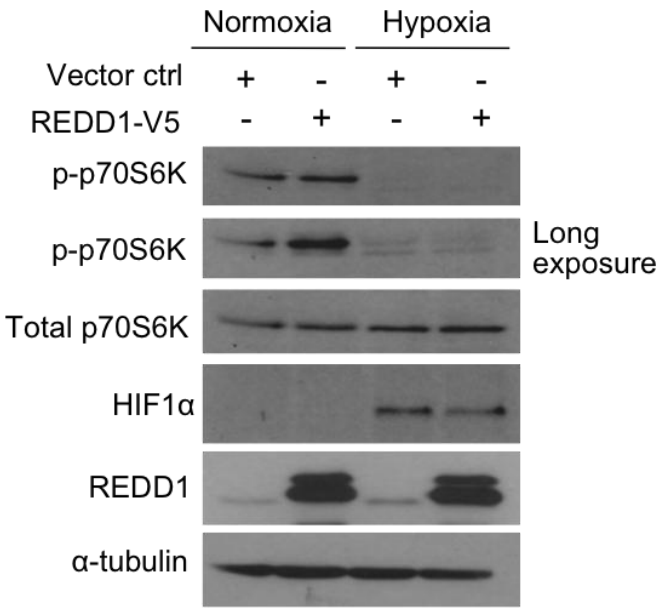


Figure 8. *REDD1 is partially responsible for mTORC1 inhibition in hypoxia.* HEK293 cells were transfected with 0.4 µg pcDNA3 vector control or REDD1-V5 pcDNA3 for 3 days followed by hypoxia incubation for 4 hours at 1 % O_2 before lysis.

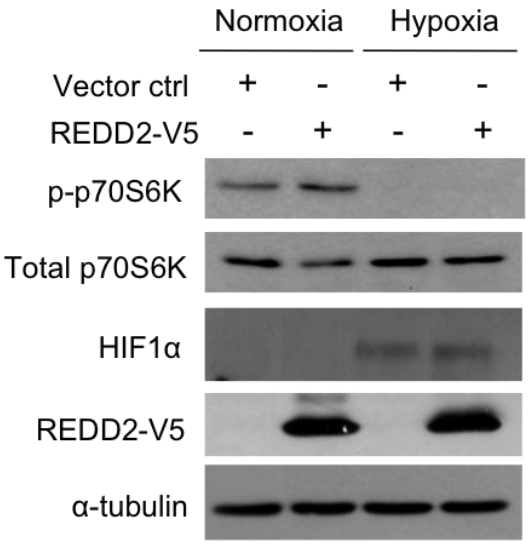


Figure 9. *REDD2 does not regulate mTORC1 activity.* HEK293 cells were transfected with 0.4 µg pcDNA3 vector control or REDD2-V5 pcDNA3 for 3 days followed by hypoxia incubation for 4 hours at 1 % O_2 before lysis.

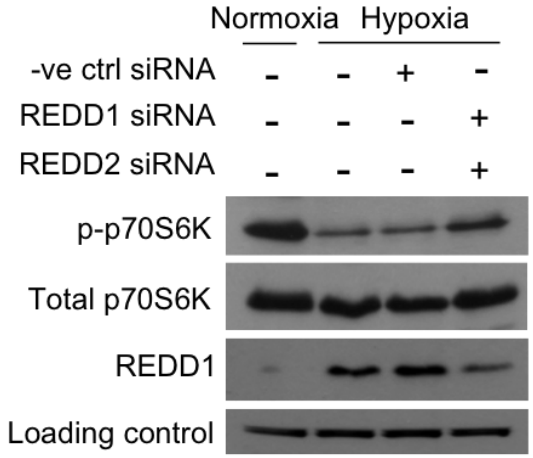


Figure 10. *REDD1 and REDD2 are partially responsible for mTORC1 inhibition in hypoxia.* HEK293 cells were transfected with 20 nM control or REDD1 and REDD2 siRNAs for 3 days and incubated in hypoxia for 4 hours at 1 % O_2 before cell lysis.

To test the possibility that the reactivation of mTORC1 upon reoxygenation is related to REDD1, I determined the hypoxia induced changes in REDD1 protein expression in a number of cell lines. In MCF7, HEK293 and MEF cells, REDD1 expression was upregulated in hypoxia and downregulated upon reoxygenation (Figure 4). However, the downregulation of REDD1 levels occurred at a much slower rate compared to the rapid reactivation of mTORC1 upon reoxygenation (Figure 4). This suggests that the rapid mTORC1 reactivation upon reoxygenation is independent of REDD1. Furthermore, it was observed that in HCT116 cells REDD1 expression in hypoxia and reoxygenation showed the opposite trend compared to MCF7 and HEK293 cells. Thus, REDD1 expression was downregulated in hypoxia and upregulated upon reoxygenation (Figure 4). However, as mentioned above, hypoxia and reoxygenation had comparable effect on mTORC1 activities in all cell lines. Taken together, these results strongly suggest REDD1 does not play a role as the key mediator of the rapid response of mTORC1 to hypoxia and reoxygenation.

3.2.3 HIF-1α is not involved in mTORC1 regulation in hypoxia and reoxygenation

The transcription factor, Hypoxia-Induced Factor 1 (HIF-1) is a key mediator of the cellular response to oxygen deprivation and both BNIP3 and REDD1 are transcriptional targets of HIF-1. I therefore investigated the role of HIF-1α in mTORC1 regulation. HIF-1 comprises of a constitutively expressed HIF-1β subunit and an oxygen regulated HIF-1α subunit (or the HIF-2α or HIF-3α isoforms). Under normoxic conditions, HIF-1α is hydroxylated on conserved proline residues at positions 402 and 564 by oxygen dependent PHDs (Epstein et al., 2001; Bruick, 2001), thus leading to von Hippel-Lindau (VHL) protein dependent ubiquitination and proteasomal degradation (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001). In hypoxia, PHDs are suppressed and the HIF-1α protein is stabilized, resulting in transcription of hypoxia inducible genes (Figure 11).

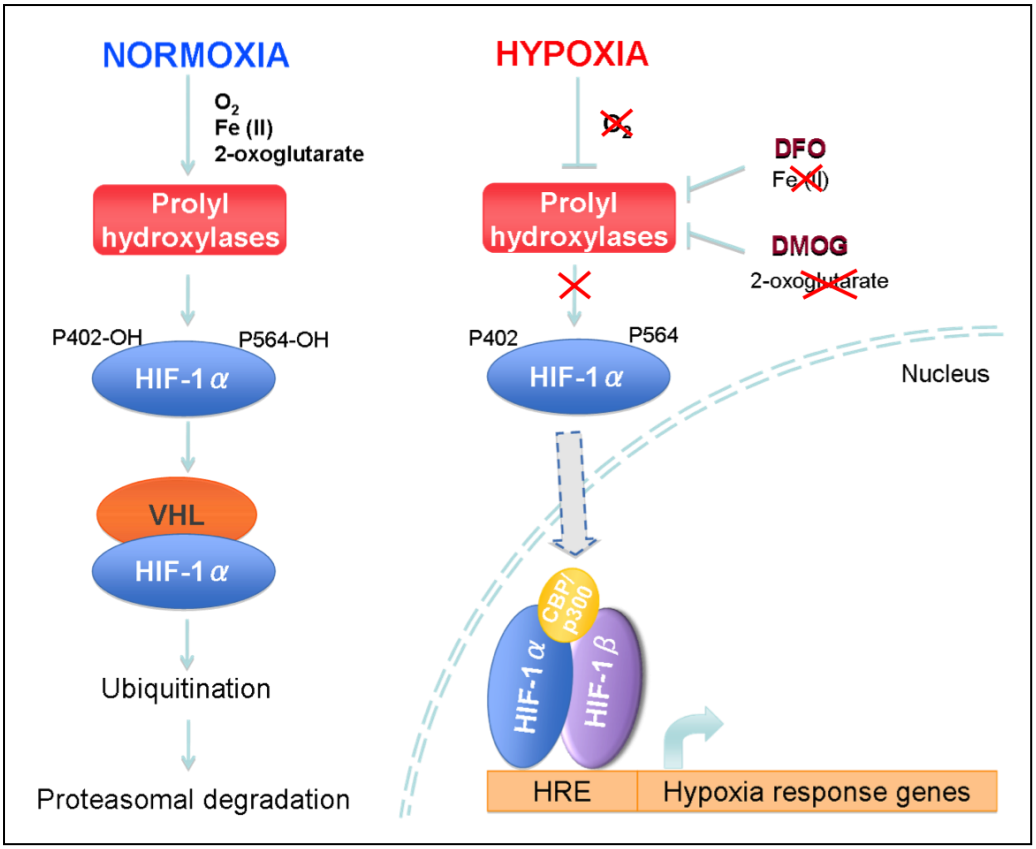


Figure 11. *Regulation of HIF-1α stability in normoxia and hypoxia.* DFO and DMOG act as hypoxia mimics to inhibit prolyl hydroxylases and thereby leading to the stabilization of HIF1α. DFO is an iron chelator and DMOG is a 2-oxoglutarate analog.

To test the involvement of HIF-1α, I used a mutant in which the two proline residues are substituted with alanine resulting in a stable HIF-1α protein. The HIF-1α mutant was transfected into HEK293 cells and the cells were exposed to hypoxia and reoxygenation. The expression level of transfected mutant HIF-1α was higher compared to endogenous HIF-1α expression in hypoxia (Figure 12). If inhibition of mTORC1 is due to HIF-1α, it would be expected that reactivation of mTORC1 is delayed upon reoxygenation. However, overexpression of the stable HIF-1α mutant did not affect mTORC1 activity upon reoxygenation compared to control cells (Figure 12).

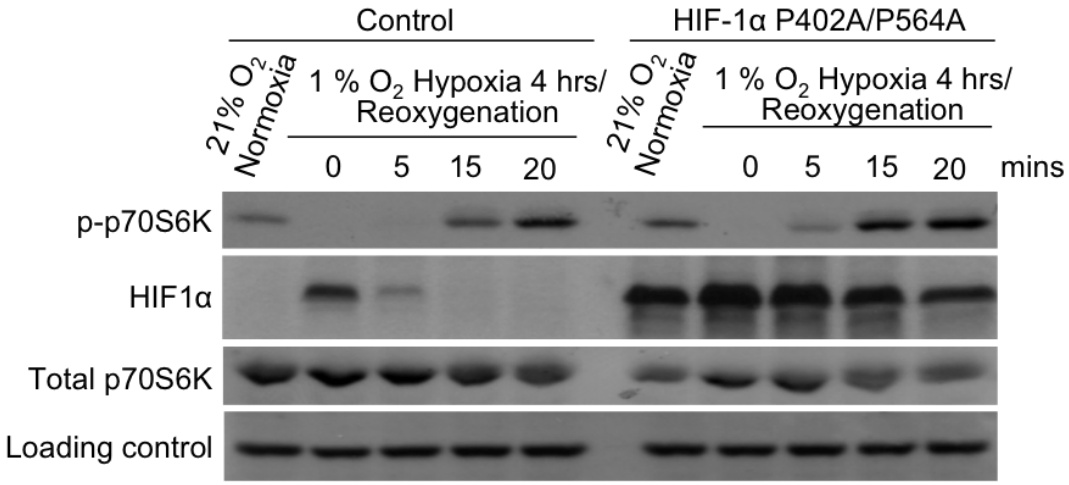


Figure 12. *HIF-1α is not involved in mTORC1 regulation in hypoxia and reoxygenation.* A constitutively active mutant HIF-1α P402A/P564A (0.4 μg) was transfected into HEK293 cells for 3 days and placed in hypoxic condition for 4 hours at 1 % O_2 followed by cell lysis at the indicated time points after reoxygenation.

To further confirm this observation, I used the 786-O VHL *null* cells. These cells do not express HIF-1α and oxygen dependent regulation of HIF-2α protein is prevented due to the absence of pVHL. As shown in Figure 13, hypoxia still caused mTORC1 inhibition in the absence of a functional HIF pathway although to a slightly lesser extent compared to the VHL reconstituted cells. Furthermore, knockdown of HIF-1α in HEK293 cells did not affect mTORC1 activity in hypoxia and upon reoxygenation compared to control siRNA (Figure 14A). To rule out the involvement of the all the 3 HIF-α isoforms, HIF-1β was silenced. This prevents the formation of a functional dimeric HIF transcription factor. HIF-1β knockdown did not prevent mTORC1 reactivation upon reoxygenation (Figure 14B). Taken together, these data suggest that HIF does not play a role in mTORC1 regulation in short-term hypoxia.

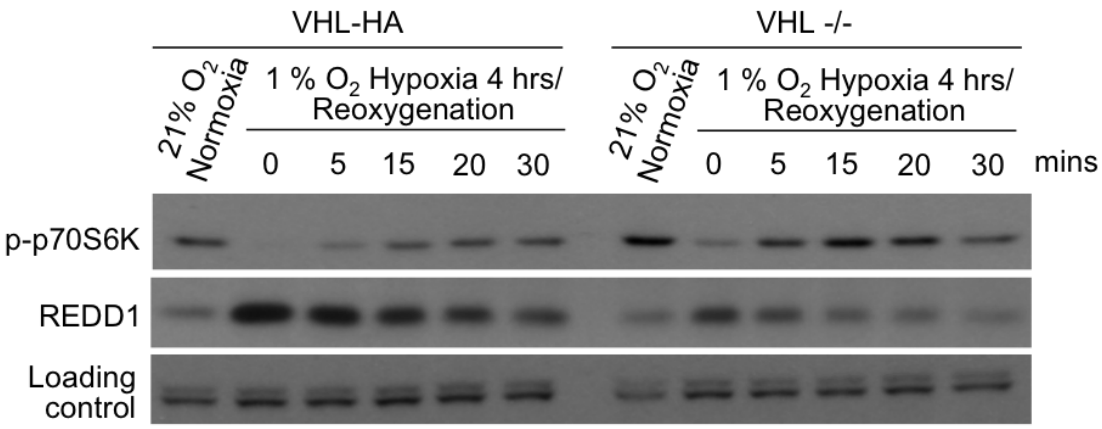


Figure 13. *HIF-1α does not contribute to mTORC1 regulation in hypoxia and reoxygenation.* 786-O cells with VHL *null* or reconstituted VHL-HA were grown to confluency and incubated in hypoxia (1 % O_2) for 4 hours before cell lysis at the indicated time points upon reoxygenation.

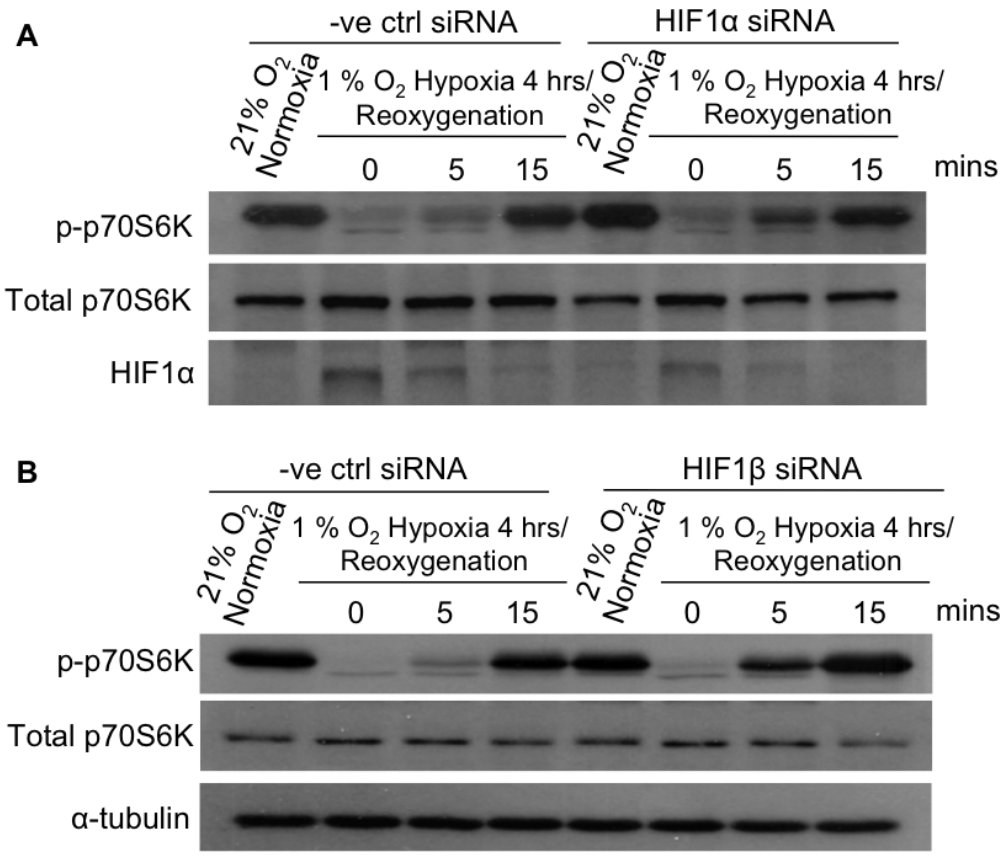


Figure 14. *HIF-1α and HIF-1β are not involved in mTORC1 regulation in hypoxia and reoxygenation.* (**A**,**B**) HEK293 cells were transfected with 20 nM control, HIF-1α (**A**) or HIF-1β (**B**) siRNAs for 3 days and incubated in hypoxia for 4 hours at 1 % O_2 followed by cell lysis at the indicated time points after reoxygenation.

3.2.4 The dynamic regulation of mTORC1 by hypoxia and reoxygenation is mediated via a post-translational mechanism

So far, my results indicated that the dynamic regulation of mTORC1 by cellular oxygen concentrations is independent of previously reported transcriptional mechanisms. Furthermore, reactivation of mTORC1 upon reoxygenation is rapid. These results suggest that mTORC1 regulation is independent of transcription. To confirm this, the transcription and translation inhibitors actinomycin D and cycloheximide, respectively, were added to cells. Notably, the rapid mTORC1 reactivation was still observed in the presence of these inhibitors implying that regulation of mTORC1 is mediated via a posttranslational mechanism (Figure 15).

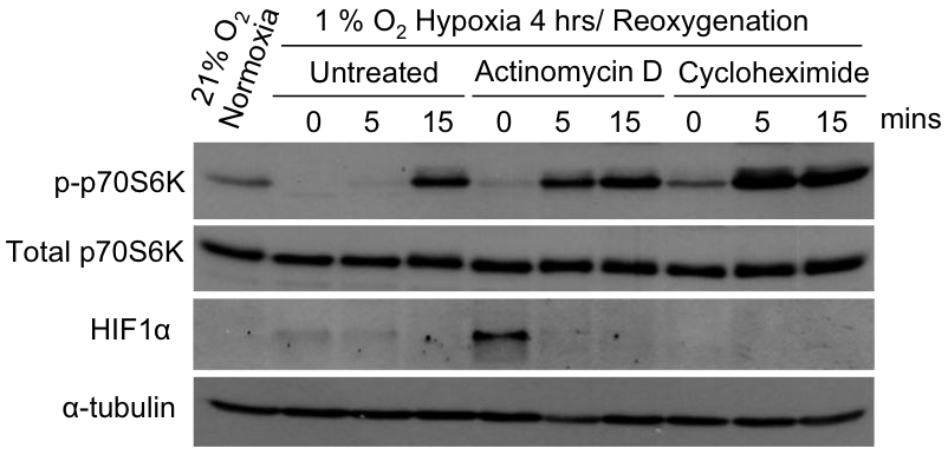


Figure 15. *The dynamic regulation of mTORC1 in hypoxia and reoxygenation is mediated via a post-translational mechanism.* The transcription inhibitor actinomycin D (5 μg/ml) and translation inhibitor cycloheximide (40 μM) were added to HEK293 cells prior to incubation at 1 % O_2 for 4 hours, followed by reoxygenation and cell lysis immediately (0 mins) as well as 5 and 15 min after reoxygenation.

3.2.5 mTORC1 regulation in hypoxia and reoxygenation is independent of protein degradation

mTORC1 activity may be regulated by oxygen dependent changes in the stability of specific proteins (e.g. mTORC1 regulatory proteins). To determine if protein degradation is involved in the decrease in mTORC1 activity in hypoxia as well as its reactivation upon reoxygenation, I used the proteasomal inhibitor MG-132 to block protein degradation. If protein degradation is involved in oxygen dependent mTORC1 regulation, treatment with proteasome inhibitor would prevent inhibition of mTORC1 activity in hypoxia as well as its reactivation upon reoxygenation. However, no change in mTORC1 activity was observed in cells treated with MG-132 compared to controls (Figure 16). This indicates that mTORC1 activity is not regulated by changes in protein stability. Similarly, when the Nedd8 E1 inhibitor MLN4924 which inhibits all cullin E3 ligases was used, no difference was observed in mTORC1 activity between MLN4924 treated and control cells (Figure 16). This suggests that Cullin E3 ligases are not involved in mTORC1 regulation in hypoxia and reoxygenation.

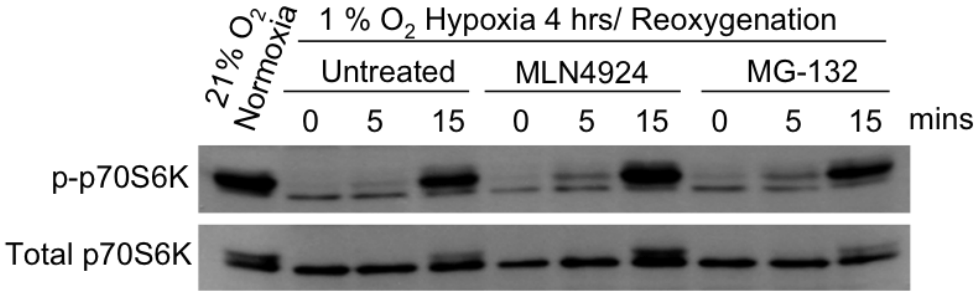


Figure 16. *The dynamic regulation of mTORC1 in hypoxia and reoxygenation is independent of Cullin E3 ubiquitin ligases and protein degradation.* MLN4924 (1 μM), an inhibitor of the Nedd8 activating enzyme (NAE) that blocks the activity of all Cullin E3 ligases, and proteasomal inhibitor MG-132 (20 μM) were added to HEK293 cells prior to incubation at 1 % O_2 for 4 hours, followed by reoxygenation and cell lysis at 0, 5 and 15 minutes.

In addition to proteasomal degradation, proteins are also degraded via the lysosomal pathway. Hence, to test if lysosomal degradation plays a role in mTORC1 regulation in hypoxia and reoxygenation, lysosomal inhibitors ammonium chloride and Pepstatin A + E64 were used. As shown in Figure 17, mTORC1 activity was inhibited in hypoxia and reactivated upon reoxygenation in the presence or absence of lysosomal inhibitors although treatment with ammonium chloride lead to a slight delay in mTORC1 reactivation upon reoxygenation. This indicates that mTORC1 activity is not regulated by lysosomal degradation pathway.

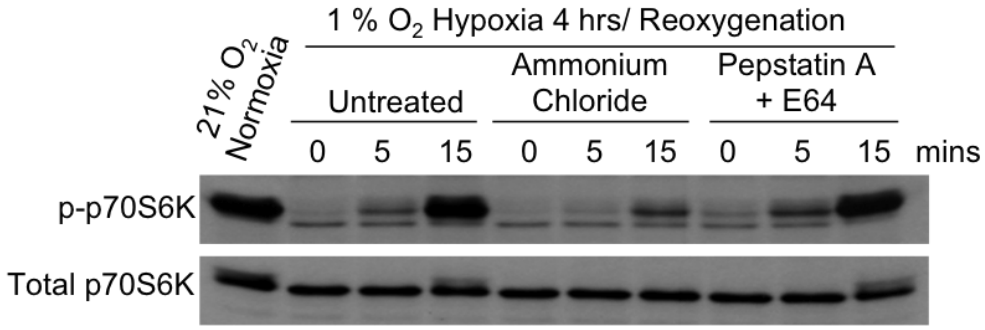


Figure 17. *The dynamic regulation of mTORC1 in hypoxia and reoxygenation is independent of lysosomal degradation.* The lysosomal inhibitors ammonium chloride (20 mM) and pepstatin A (10 μM) plus E64 (25 μM) were added to HEK293 cells prior to incubation at 1 % O_2 for 4 hours, followed by reoxygenation and cell lysis immediately (0 mins) as well as 5 and 15 min after reoxygenation.

3.2.6 mTORC1 regulation in hypoxia and reoxygenation is independent of AMPK, mitochondrial ATP synthesis and reactive oxygen species (ROS)

I next tested a number of potential post-translational mechanisms that may be involved in the regulation of mTORC1 by cellular oxygen concentrations. AMPK is activated under conditions of energy stress such as during nutrient starvation or hypoxia whereby intracellular cellular ATP levels decline and AMP levels increase. AMPK has been shown to inhibit mTORC1 activity via phosphorylation of both TSC2 and the mTORC1 subunit Raptor (Liu et al., 2006; Gwinn et al., 2008). To test for AMPK involvement in hypoxia and reoxygenation dependent mTORC1 regulation, I treated cells with compound C, an inhibitor of AMPK. Compound C did not affect mTORC1 activity in hypoxia and reoxygenation (Figure 18). Furthermore, treatment with an AMPK activator, AICAR, did not prevent mTORC1 reactivation upon reoxygenation (Figure 18).

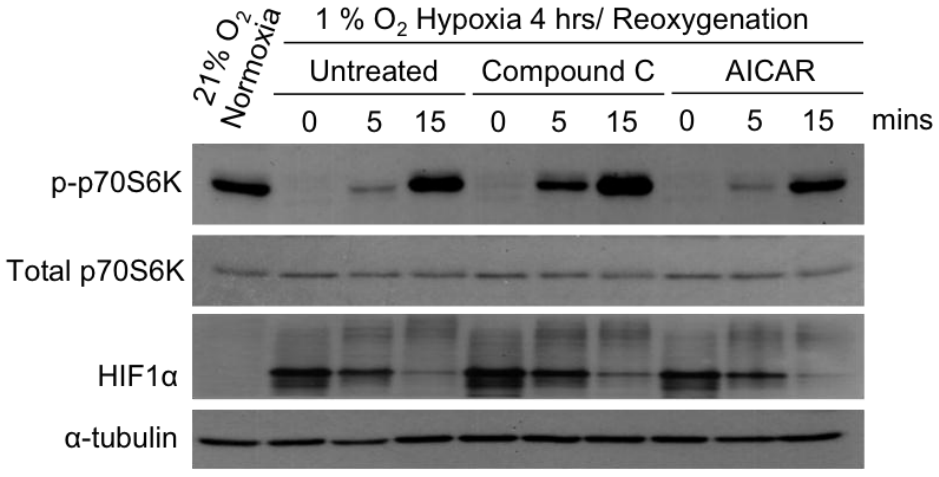


Figure 18. *The dynamic regulation of mTORC1 in hypoxia and reoxygenation is independent of AMPK.* HEK293 cells were incubated for 4 hours at 1 % O_2 followed by reoxygenation and cell lysis at 0, 5 and 15 minutes. Compound C, an inhibitor of AMPK (20 μM) and AICAR (0.5 mM) for AMPK activation were added prior to hypoxia incubation.

I also mimicked the effect of hypoxia on cellular ATP concentrations by treating cells with various electron transport chain (ETC) inhibitors. These inhibitors were used at concentrations that completely blocked oxidative phosphorylation. Thus, the mitochondrial inhibitors would be expected to have a more pronounced effect on mTORC1 compared to hypoxia, which inhibits electron transport chain activity only partially. However, the various mitochondrial inhibitors had a markedly weaker inhibitory effect on mTORC1 activity compared to hypoxia (Figure 19). As the ETC is reactivated upon reoxygenation, it is also possible that the reactivation of mTORC1 pathway is mediated by the increase in ATP levels upon reintroduction of oxygen. Hence, the various mitochondrial inhibitors were added to inhibit ETC activation upon reoxygenation. If mTORC1 activity is regulated by the ETC, mTORC1 pathway would be completely inhibited upon reoxygenation in presence of the ETC inhibitors. However, mTORC1 reactivation upon reoxygenation was observed in the presence of the various inhibitors, although at a slower rate (Figure 20). Taken together, these results suggest that AMPK and mitochondrial ATP synthesis do not play a major role in mTORC1 regulation in hypoxia and reoxygenation.

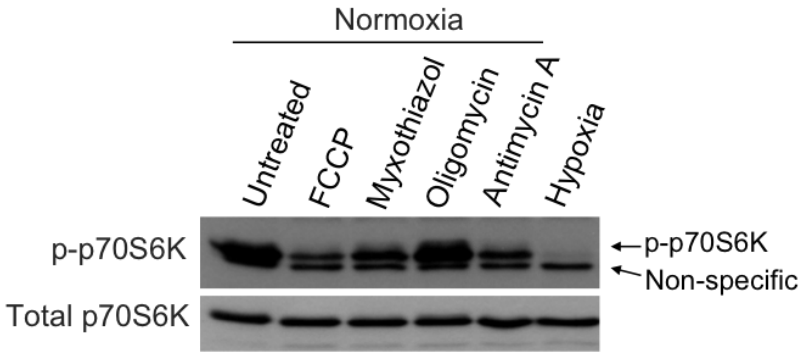


Figure 19. *The dynamic regulation of mTORC1 in hypoxia and reoxygenation is independent of mitochondrial ATP synthesis.* HEK293 cells were treated with mitochondrial inhibitors FCCP (1 μM), myxothiazole (1 μM), oligomycin (10 μM) and antimycin A (1 μg/ml) under normoxic conditions or incubated in hypoxia at 1 % O_2 for 4 hour.

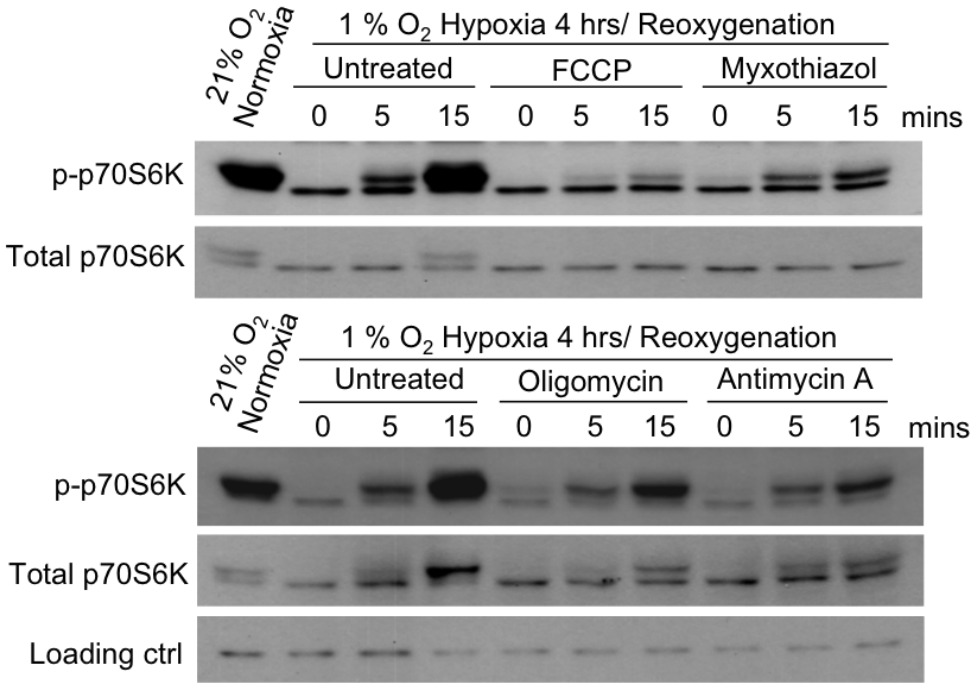


Figure 20. *The dynamic regulation of mTORC1 upon reoxygenation is independent of mitochondrial ATP synthesis.* HEK293 cells were incubated for 4 hours at 1 % O_2 followed by reoxygenation and cell lysis at 0, 5 and 15 minutes. Mitochondrial inhibitors FCCP (1 μM), myxothiazole (1 μM), oligomycin (10 μM) and antimycin A (1 μg/ml) were added prior to hypoxia incubation.

To explain the rapid mTORC1 reactivation upon reoxygenation, I hypothesized that reactive oxygen species (ROS), a factor well known to be associated with reoxygenation, can induce mTORC1 activity. ROS production during reoxygenation is largely a consequence of the highly reduced state of the ETC during hypoxia leading to a burst in ROS production upon reintroduction of oxygen. However, my results show that the antioxidant N-acetylcysteine (NAC), the superoxide dismutase mimetic drug MnTBAP as well as the superoxide dismutase inhibitor Diethylditiolcarbamate (DCC) do not significantly affect mTORC1 reactivation upon reoxygenation (Figure 21). Furthermore, induction of cellular ROS by addition of 2-methoxyestradiol or glucose oxidase did not promote mTORC1 activity (Figure 21). These results suggest that mTORC1 regulation during hypoxia and reoxygenation is independent of ROS.

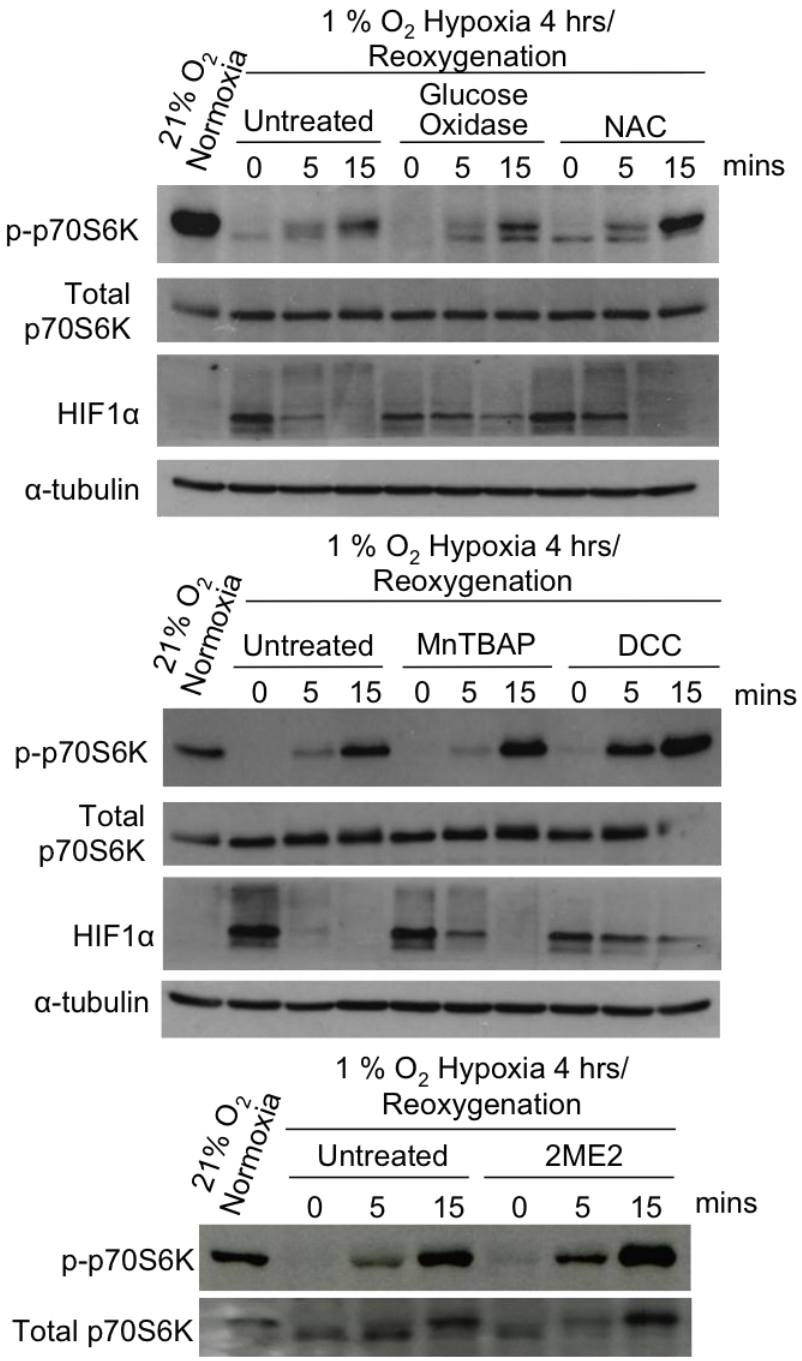


FIGURE 21. *The dynamic regulation of mTORC1 in hypoxia and reoxygenation is independent of reactive oxygen species (ROS).* HEK293 cells were incubated for 4 hours at 1 % O_2 followed by reoxygenation and cell lysis at 0, 5 and 15 minutes. MnTBAP (50 μM), a superoxide dismutase (SOD) mimetic drug to decrease cellular superoxide production; SOD inhibitor Diethyldithiolcarbamate (DCC, 1 mM) to block H_2O_2 production; glucose oxidase (100 mU/ml) to generate H_2O_2, antioxidant N-acetylcysteine (NAC, 10 mM) and 2ME2 (50 μM) to inhibit the ETC Complex I and SOD were added prior to hypoxia incubation.

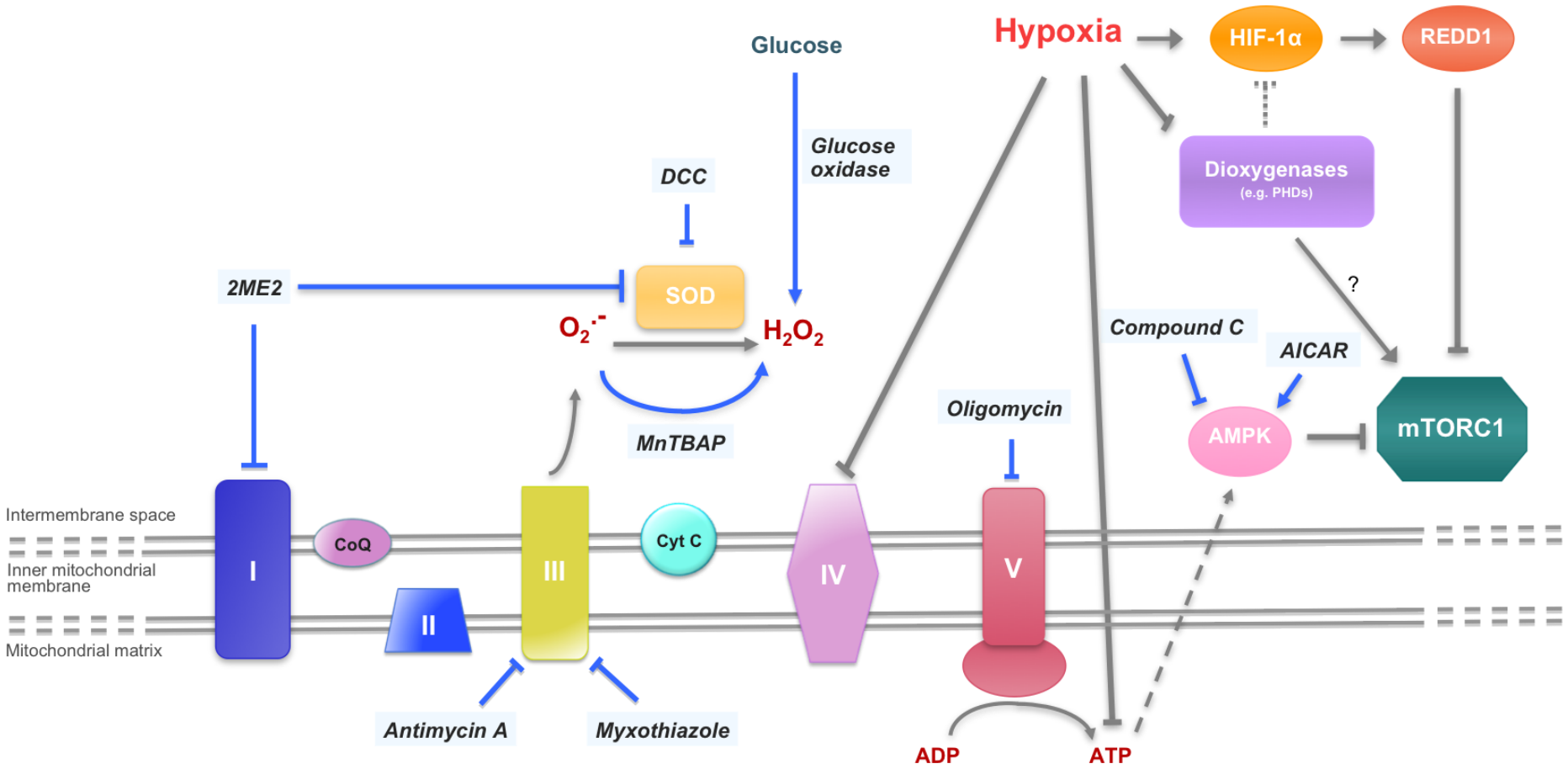


Figure 22. *Summary of the functions of the different compounds used to study mTORC1 regulation in hypoxia and reoxygenation.* Antimycin A, myxothiazole, 2ME2 and oligomycin inhibits mitochondria electron transport chain. MnTBAP decreases cellular superoxide production; SOD inhibitor Diethyldithiolcarbamate (DCC) blocks H_2O_2 production and glucose oxidase generates H_2O_2. Compound C inhibits AMPK whereas AICAR activates AMPK.

3.2.7 mTORC1 activity in hypoxia and reoxygenation is sensitive to the 2-oxoglutarate analog DMOG

PHDs are dioxygenases that functions in an oxygen dependent manner. They are proposed to act as oxygen sensors as they require molecular oxygen for their function to post-translationally regulate the stability of HIF-1α protein as well as a number of other more recently identified proteins including Activating Transcriptional Factor 4 (ATF4) (Koditz et al., 2007) myogenin (Fu et al., 2007), Pax2 (Yan et al., 2011) and β-adrenergic Receptor (Xie et al., 2009). Therefore, it is possible that PHDs also regulate mTORC1 activity in hypoxia and reoxygenation. To test the effect of PHDs in mTORC1 regulation, I used the PHD inhibitors desferrioxamine (DFO), cobalt chloride ($CoCl_2$) and dimethyloxalylglycine (DMOG) (Figure 11). Treatment with the iron chelator DFO and with $CoCl_2$ did not affect p70S6K phosphorylation even though they effectively inhibited PHD activity, as determined by the stabilization of HIF-1α (Figure 23A). Longer incubation and higher concentrations of DFO were also without effect on mTORC1 activity (Figure 23B). However, DMOG, a 2-oxoglutarate (2OG) analog, completely blocked mTORC1 activity under normoxic conditions (Figure 23A).

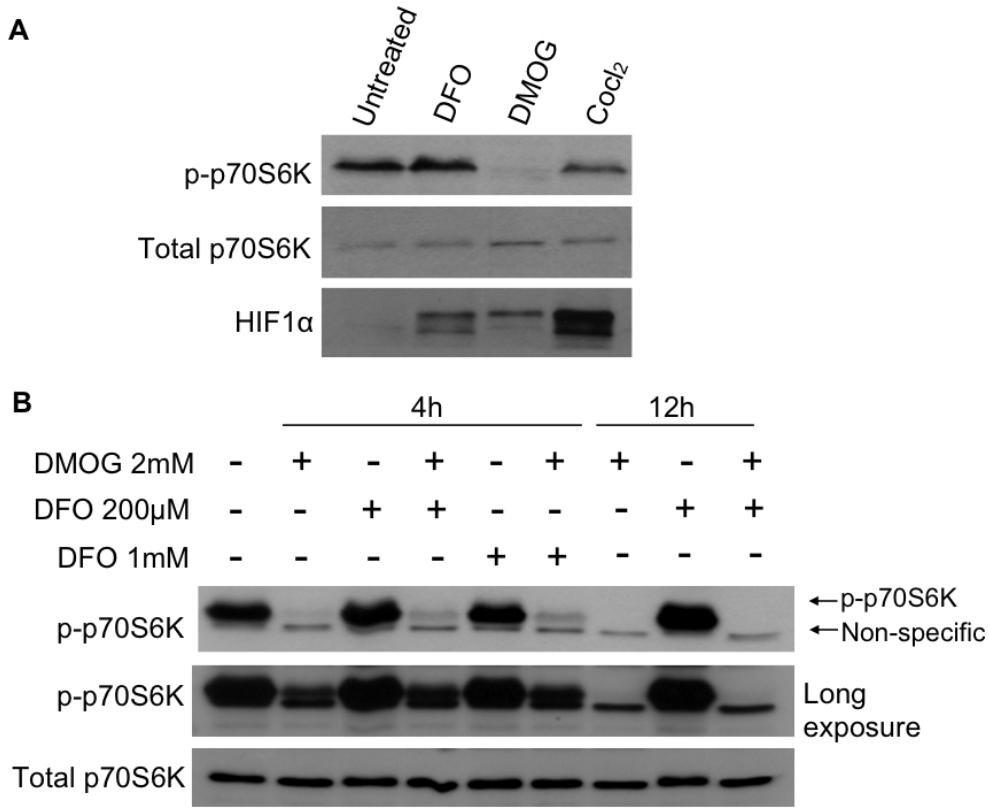


Figure 23. *mTORC1 activity in hypoxia and reoxygenation is independent of HIF-1α but sensitive to the 2-oxoglutarate analog dimethyloxalylglycine (DMOG).* (**A**) HEK293 cells were treated with HIF prolyl hydroxylase (PHD) inhibitors cobalt chloride ($CoCl_2$, 200 μM), desferrioxamine (DFO, 200 μM) and the 2-oxoglutarate analog, dimethyloxalylglycine (DMOG, 2 mM) for 4 hours under normoxic conditions. (**B**) Two different concentrations of DFO (200 μM and 1 mM) were added to HEK293 alone or in combination with DMOG (2 mM) and incubated for either 4 hours or 12 hours in normoxia before cell lysis.

Furthermore, the inhibitory effect of DMOG on mTORC1 was comparable to hypoxia (Figure 24). Importantly, DMOG prevented reactivation of mTORC1 upon reoxygenation (Figure 24) but not $CoCl_2$ or the HIF-1α inhibitor chetomin (Figure 25). The inhibitory effect is also independent of protein degradation as treatment with proteasomal inhibitor MG-132 and the Nedd8 E1 inhibitor MLN4924 did not prevent mTORC1 inhibition by DMOG (Figure 26). Moreover, similar to oxygen regulation of mTORC1 pathway, inhibition of mTORC1 by DMOG was reversible. It can

be observed that mTORC1 activity is reactivated upon the removal of DMOG from cells (Figure 27).

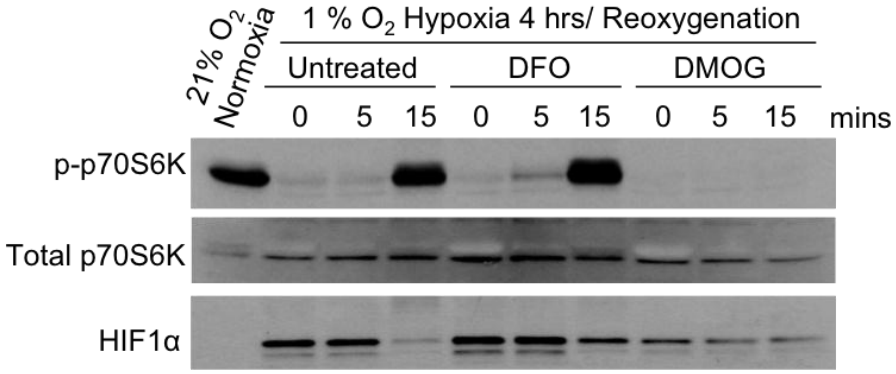


Figure 24. *mTORC1 activity in hypoxia and reoxygenation is independent of PHDs but sensitive to the 2-oxoglutarate analog dimethyloxalylglycine (DMOG).* DFO (200 μM) and DMOG (2 mM) were added to HEK293 cells prior to hypoxic incubation at 1 % O_2 for 4 hours and cells were lysed at the indicated time points after reoxygenation.

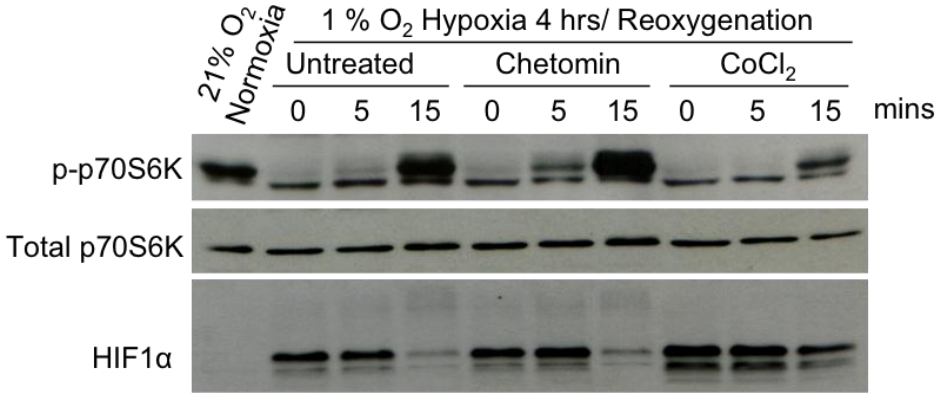


Figure 25. *mTORC1 activity in hypoxia and reoxygenation is independent of PHDs and HIF-1α.* Chetomin (50 nM) and $CoCl_2$ (200 μM) were added to HEK293 cells prior to hypoxic incubation at 1 % O_2 for 4 hours and cells were lysed at the indicated time points after reoxygenation.

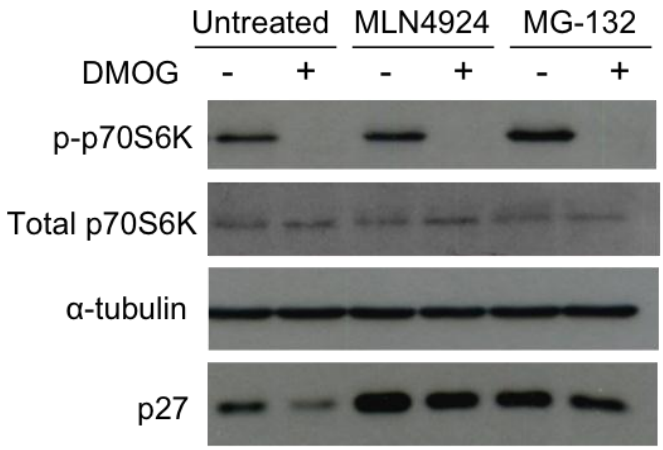


Figure 26. *mTORC1 inhibition by DMOG is independent of Cullin E3 ubiquitin ligases and protein degradation.* HEK293 cells were treated with DMOG (2 mM), the Nedd1 E1 inhibitor MLN4924 (1 μM) which inhibits all Cullin E3 ligases and the proteasome inhibitor MG-132 (20 μM) for 4 hours followed by cell lysis.

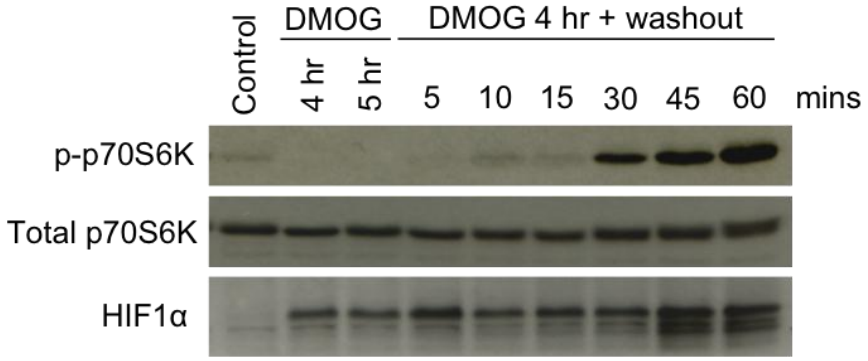


Figure 27. *DMOG washout reactivated mTORC1 activity.* HEK293 cells were treated with 2mM DMOG for 4 hours and removed by washing twice with 1X PBS followed by cell lysis at the indicated time points after washout. DMOG treated cells were lysed at 4 and 5 hours after DMOG treatment to serve as control for 0 and 60 mins after washing out.

To characterize how DMOG regulates mTORC1 activity, I looked at different proteins in the mTORC1 pathway to better understand the mechanism of action of DMOG on mTORC1 regulation. It can be observed that DMOG reduced mTOR phosphorylation at both serine 2448 and 2481 residues (Figure 28A). This indicates that the effect of DMOG is at the level of mTOR or upstream of mTOR. No change was observed in the expression levels of GβL, Raptor (unique to mTOR complex1) as well as Rictor (specific to mTOR complex 2) (Figures 28A and B). This shows that these proteins are not targets of DMOG.

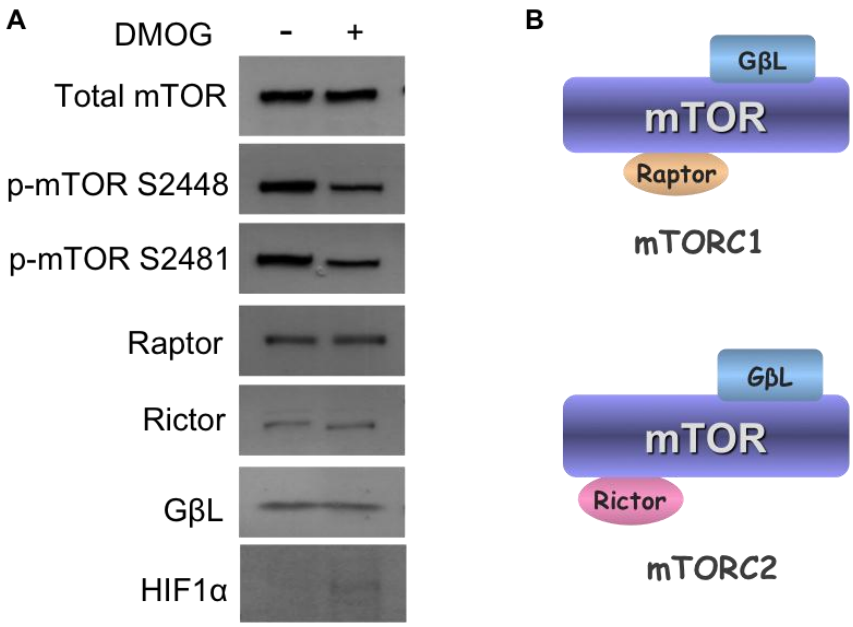


Figure 28. *DMOG treatment decreased mTOR phosphorylation.* (**A**) HEK293 cells were treated with 2 mM DMOG for 4 hours followed by cell lysis. (**B**) Components of mTOR complex 1 and 2.

However, a slight mobility shift of Rictor was observed with treatment of DMOG (Figure 28). Therefore, it is possible that DMOG also regulates mTORC2. If DMOG regulates both mTORC1 and mTORC2 pathways, it is highly likely to be via a common protein present in both pathways. Akt is an

activator of the mTORC1 pathway located upstream of TSC1/2 that is phosphorylated at threonine 308 and serine 473 when it is active (Figure 1). It is a downstream target of mTORC2 that is phosphorylated at serine 473 when mTORC2 is active. It is plausible that if DMOG inhibits mTORC2, Akt phosphorylation and activation would in turn be reduced and this would lead to the inhibition of mTORC1. Hence, I looked at the effect of DMOG treatment on Akt phosphorylation. As shown in Figure 29, DMOG treatment led to a slight reduction of Akt phosphorylation at S473. However, no change was observed at Akt T308 phosphorylation site (Figure 29). This result indicates that the effect of DMOG on mTORC1 is independent of mTORC2 and Akt.

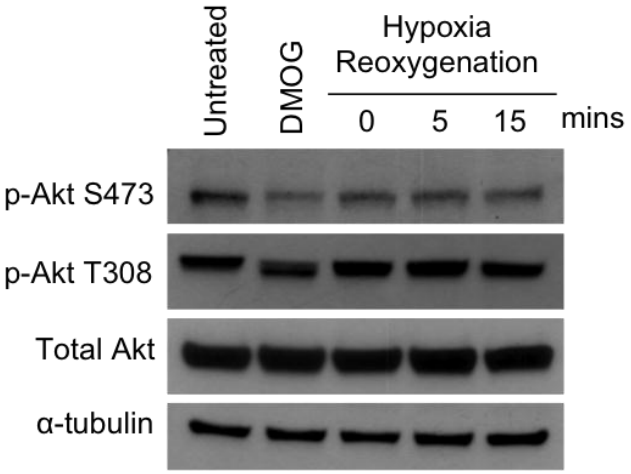


Figure 29. *Regulation of mTORC1 activity is independent of Akt.* HEK293 cells were treated with 2mM DMOG or incubated at 1 % O_2 for 4 hours followed by cell lysis at the indicated time points.

So far, my results showed that the effect of DMOG on mTORC1 inhibition is downstream of Akt and upstream of or at the level mTOR. Hence, I tested if the effect of DMOG is upstream of mTOR using a constitutively active Rheb mutant. Mutation of residue serine 16 on Rheb to histidine (S16H) results in a mutant Rheb that exhibits gain-of-function properties (Yan et al., 2006). As expected, overexpression of Rheb S16H led to increased p70S6K

phosphorylation (Figure 30). However, Rheb S16H overexpression did not prevent mTORC1 inhibition upon treatment of DMOG (Figure 30). This indicates that the effect of DMOG may be at the level of Rheb or downstream of Rheb.

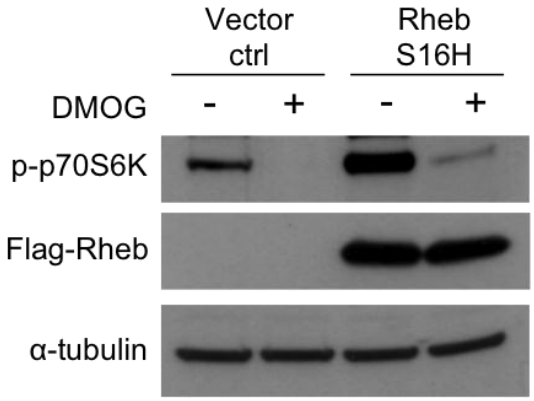
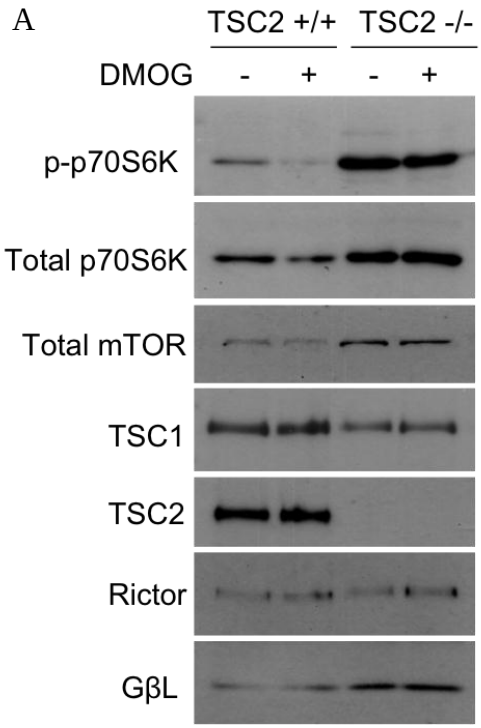


Figure 30. *mTORC1 regulation by DMOG is not at the level of Rheb.* HEK293 cells were transfected with 0.4 μg FLAG-Rheb S16H pcDNA3 for 3 days and treated with DMOG for 4 hours followed by cell lysis.

However, it has been reported that the constitutively active Q64L Rheb mutant is sensitive to TSC2 overexpression (Li, Inoki and Guan, 2004). Although this was not shown for the S16H mutant I used, I also tested if TSC2 is involved in mTORC1 regulation. It has previously been shown that AMPK regulates mTORC1 pathway by phosphorylating TSC2 and enhancing its inhibitory activity (Inoki, Zhu and Guan, 2003). Therefore, I looked at the involvement of TSC1/2 in mTORC1 regulation by DMOG and also in hypoxia and reoxygenation. $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were treated with DMOG to determine if the absence of TSC2 affects the effect of DMOG on mTORC1 activity. As expected, in $TSC2^{+/+}$ cells, DMOG inhibited mTORC1 activity as shown by the reduced p70S6K phosphorylation (Figures 31A and B). However, interestingly, treatment of DMOG did not affect mTORC1 activity in $TSC2^{-/-}$ cells (Figures 31A and B). This suggests that DMOG regulation of mTORC1 requires TSC2.



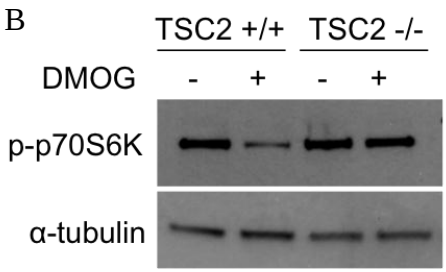


Figure 31. *The effect of DMOG on mTORC1 activity may be regulated via TSC2.* (**A & B**) MEF $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were treated with 2 mM DMOG for 4 hours followed by cell lysis. The amount of cell lysates loaded in (**B**) was adjusted to show equal phosphorylation levels for both $TSC2^{+/+}$ and $TSC2^{-/-}$ cell lines in the control samples.

TSC2 forms a functional complex with TSC1 and the interaction between TSC1 and TSC2 is required to prevent TSC2 ubiquitination (Benvenuto et al., 2000). Hence, to test if DMOG affects the formation of TSC1/2 complex, I performed immunoprecipitation to pull down TSC2-FLAG in the presence or absence of DMOG. As shown in Figure 32, treatment of DMOG did not affect TSC1 binding to TSC2. This indicates that the effect of DMOG on mTORC1 inhibition is independent of TSC1/2 complex formation.

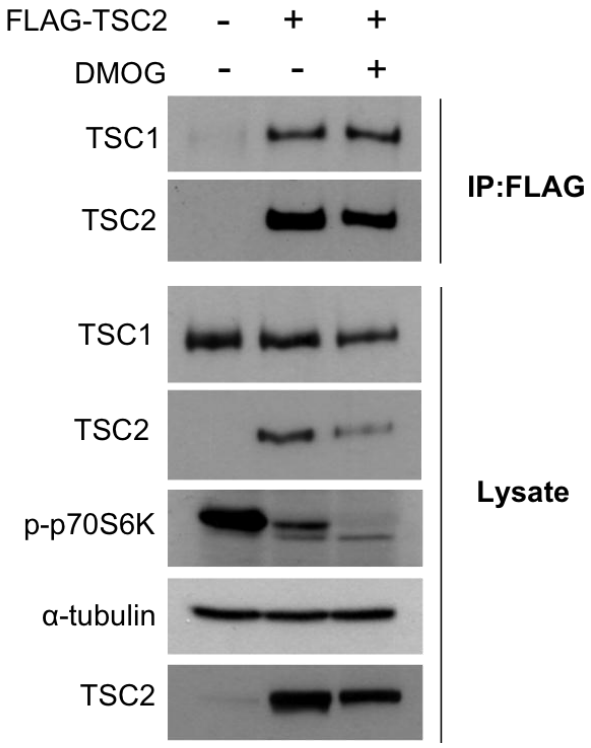


Figure 32. *The effect of DMOG on mTORC1 inhibition is independent of TSC1/2 complex formation.* HEK293 cells were transfected with 1.5 μg FLAG-TSC2 pcDNA3 in 60 mm tissue culture plates for 3 days followed by cell lysis and immunoprecipitation with FLAG antibody as described in Materials and Methods.

Next, I tested if regulation of mTORC1 activity by hypoxia and reoxygenation is also mediated by TSC2. Surprisingly, unlike DMOG, the effect of hypoxia on mTORC1 activity in $TSC2^{+/+}$ MEFs is weak (Figures 33 and 34) although mTORC1 is reactivated upon reoxygenation (Figure 34). These results suggest that the effect of DMOG and hypoxia on mTORC1 inhibition may work via different mechanisms.

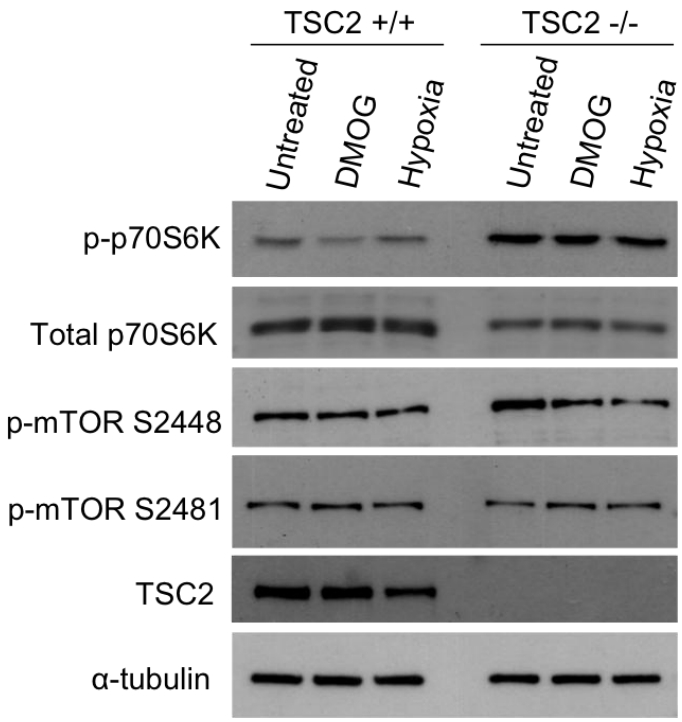


Figure 33. *Hypoxia and DMOG may work via different mechanisms to regulate mTORC1 activity.* MEF TSC2$^{+/+}$ and TSC2$^{-/-}$ cells were treated with 2 mM DMOG or incubated at 1 % O_2 for 4 hours followed by cell lysis.

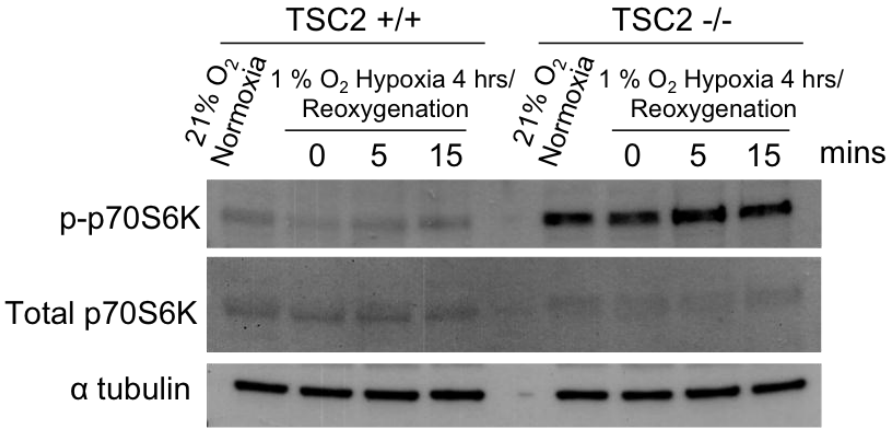


Figure 34. *Hypoxia and reoxygenation had a weak effect on mTORC1 activity in MEF TSC2$^{+/+}$ cells.* MEF TSC2$^{+/+}$ and TSC2$^{-/-}$ cells were treated with 2 mM DMOG or incubated at 1 % O_2 for 4 hours followed by cell lysis at the indicated time points after reoxygenation.

It is interesting that even though hypoxia did not have a marked effect on mTORC1 activity in $TSC2^{+/+}$ cells, changes in oxygen levels had no effect on mTORC1 activity in $TSC2^{-/-}$ cells (Figures 33 and 34). Hence, to determine if TSC2 plays a role in mTORC1 regulation in response to hypoxia and reoxygenation, I overexpressed TSC2 in the $TSC2^{-/-}$ cells. If TSC2 plays a role in the regulation of mTORC1 activity, reintroduction of TSC2 would enable the $TSC2^{-/-}$ cells to sense changes in oxygen levels. Overexpression of TSC2 to the $TSC2^{-/-}$ MEFs resulted increased sensitivity to DMOG but not hypoxia (Figure 35). This suggests that it is unlikely that the effect of hypoxia on mTORC1 is mediated by TSC2.

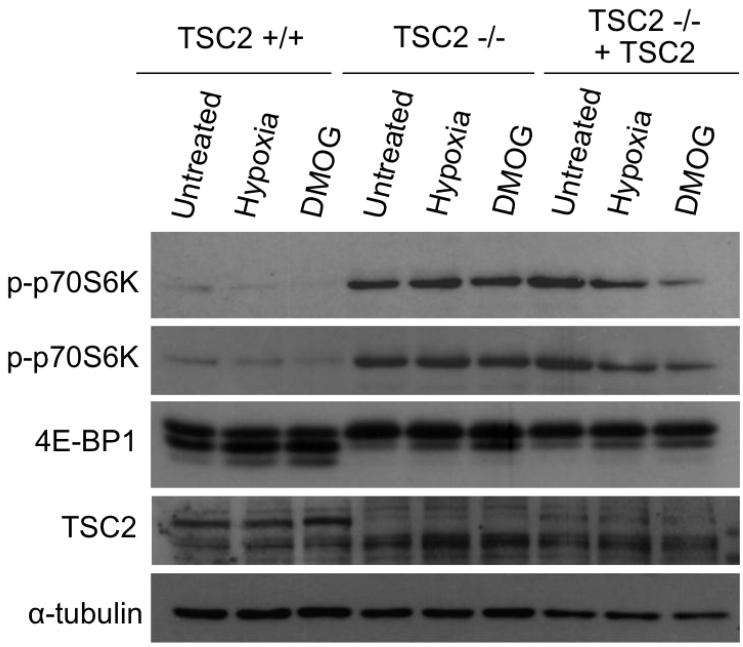


Figure 35. *Overexpression of TSC2 in $TSC2^{-/-}$ cells did not lead to hypoxia induced mTORC1 inhibition.* MEF $TSC2^{-/-}$ cells were transfected with 1 μg FLAG-TSC2 pcDNA3 for 3 days. All cells were treated with 2 mM DMOG or incubated at 1 % O_2 for 4 hours followed by cell lysis.

As the mTORC1 pathway in $TSC2^{+/+}$ MEF cells was not inhibited in response to hypoxia treatment, it is therefore difficult to use the $TSC2^{+/+}$ and $TSC2^{-/-}$ MEF cells to determine if mTORC1 regulation in hypoxia is regulated by TSC2. Hence, I used HEK293 cells to perform overexpression and knockdown studies. Overexpression of TSC2 did not affect mTORC1 activity in hypoxia and reoxygenation compared to controls (Figure 36). Knockdown of TSC2 in HEK293 cells partially blocked the inhibitory effect of DMOG on mTORC1 (Figure 37) but did not affect the regulation of mTORC1 by hypoxia and reoxygenation (Figure 38). Taken together, these results indicate that the effect of hypoxia and reoxygenation on mTORC1 is independent on TSC2.

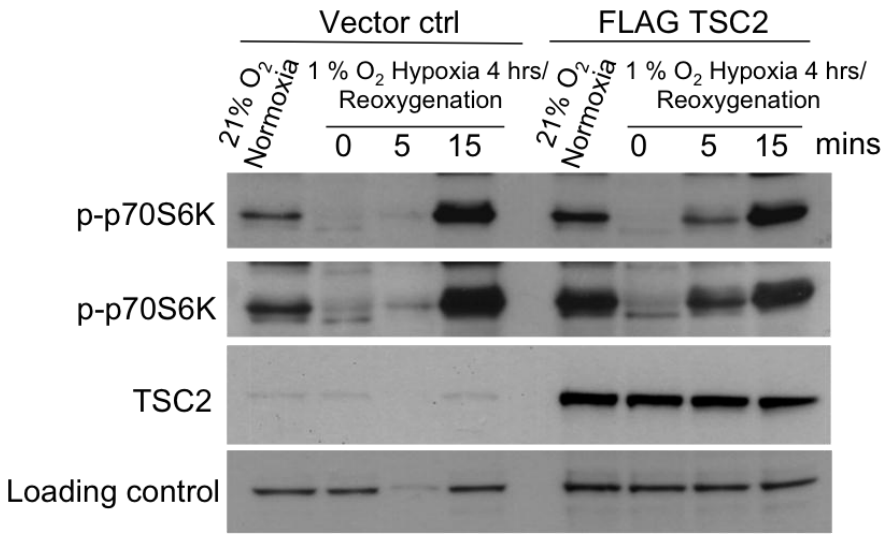


Figure 36. *TSC2 is not involved in mTORC1 regulation in hypoxia and reoxygenation.* HEK293 cells were transfected with 0.4 μg FLAG-TSC2 pcDNA3 and incubated at 1 % O_2 for 4 hours followed by cell lysis at the indicated time points upon reoxygenation.

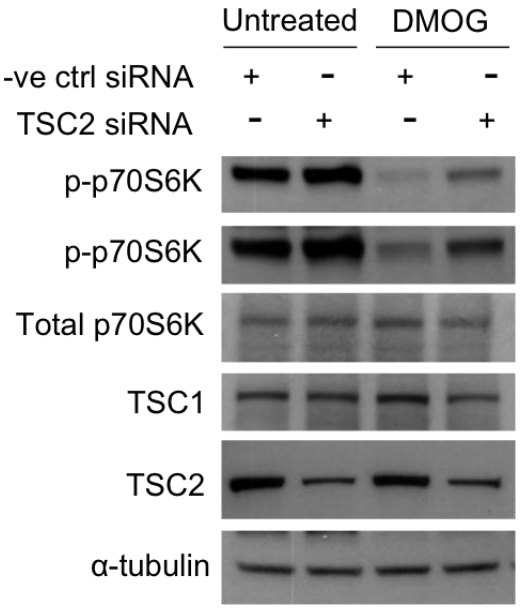


Figure 37. *TSC2 knockdown partially reverses the effect of DMOG on mTORC1.* HEK293 cells were transfected with 20 nM control or TSC2 siRNA for 3 days followed by treatment with 2mM DMOG and cell lysis.

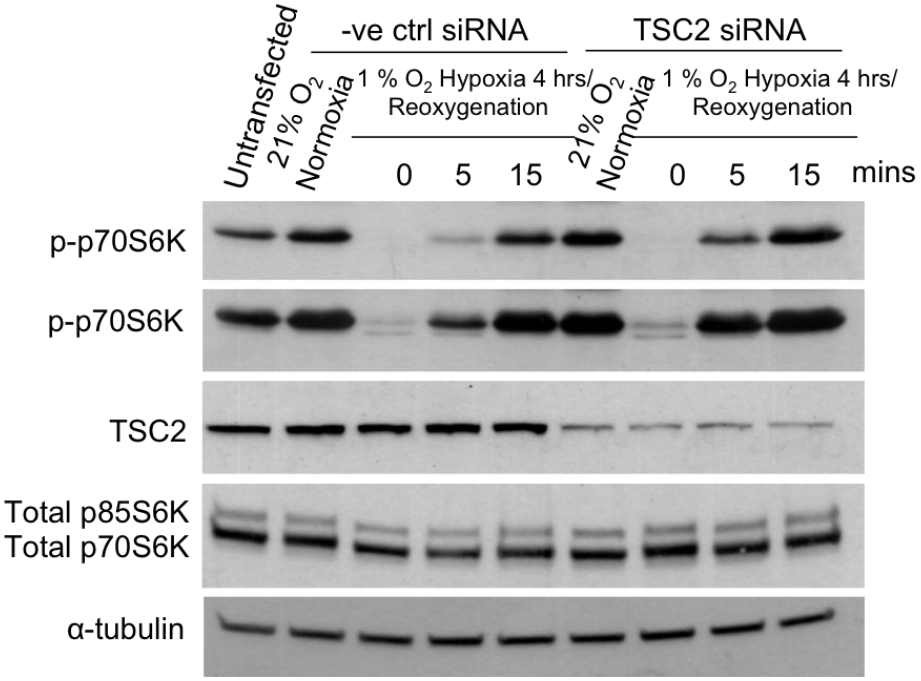


Figure 38. *Regulation of mTORC1 activity in hypoxia and reoxygenation is independent of TSC2.* HEK293 cells were transfected with 20 nM control or TSC2 siRNA for 3 days and incubated at 1 % O_2 for 4 hours followed by cell lysis at the indicated time points upon reoxygenation.

The lack of effect of the known PHD inhibitors, DFO and $CoCl_2$, suggests that the DMOG dependent mTORC1 regulation is independent of PHD enzymes. Alternatively, it is also possible that DFO and $CoCl_2$ mediated mTORC1 inhibition is masked by a simultaneous, PHD independent activation of mTORC1 by these inhibitors. To test this possibility, I treated HEK293 cells with DMOG in the presence of either DFO or $CoCl_2$. If DFO and $CoCl_2$ activate mTORC1 in a PHD independent manner, it would be expected that the two inhibitors reverse the effect of DMOG. However, DMOG mediated inhibition of mTORC1 was reversed by DFO to a small degree but not affected by $CoCl_2$ (Figure 39) suggesting that these iron chelators do not activate mTORC1 via a PHD independent pathway. Therefore, I conclude that PHD enzymes are unlikely to be involved in the oxygen dependent regulation of mTORC1.

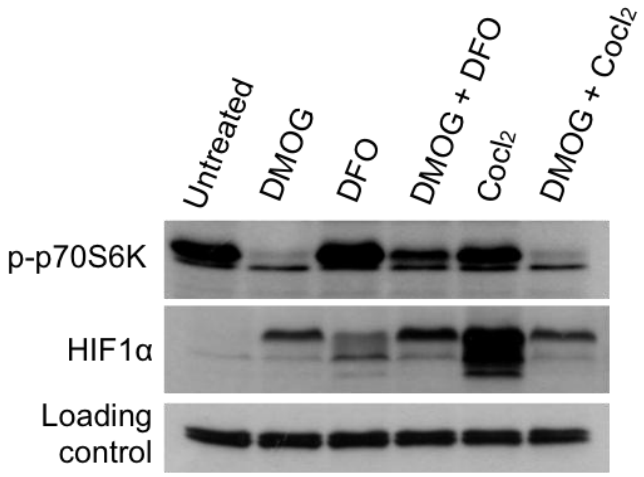


Figure 39. *mTORC1 activity in hypoxia and reoxygenation is independent of HIF-1α but sensitive to the 2-oxoglutarate analog dimethyloxalylglycine (DMOG).* HEK293 cells were treated with PHD inhbitors DFO (200 µM), $CoCl_2$ (200 µM) and DMOG (2 mM) alone or in the indicated combinations for 4 hours.

In support of these results, knockdown of the three HIF PHDs (PHD1-3) and the 2OG dependent Factor Inhibiting HIF (FIH) in HEK293 cells did not reveal differences in p70S6K phosphorylation levels compared to control (Figure 40). Furthermore, I also confirmed that the effect of DMOG is HIF independent by treating 786-O VHL *null* cells with DMOG. As shown in Figure 41, DMOG inhibited mTORC1 activity in VHL *null* cells indicating that the inhibitory effect of DMOG on mTORC1 is independent of HIF. Collectively, these data suggest that a DMOG sensitive 2-OG dependent dioxygenase enzyme might be responsible for the regulation of mTORC1 activity.

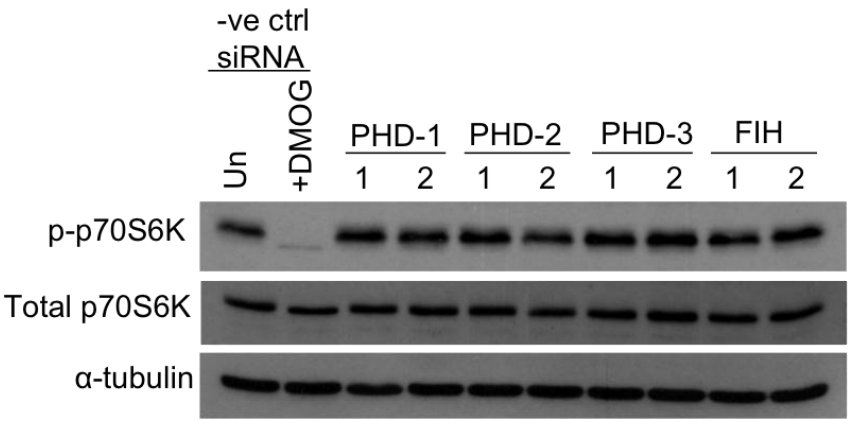


Figure 40. *mTORC1 activity is independent of the three HIF PHDs (PHD1-3) and the 2OG dependent Factor Inhibiting HIF (FIH).* HEK293 cells were transfected with 20 nM control, HIF PHDs (PHD1-3) and FIH siRNAs for 3 days followed by cell lysis.

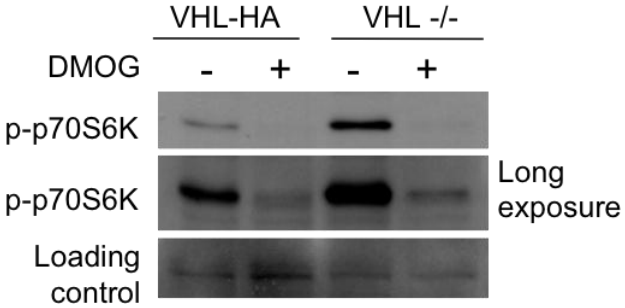


Figure 41. *mTORC1 activity is independent of HIF-1α but sensitive to the 2-oxoglutarate analog dimethyloxalylglycine (DMOG).* 786-O VHL *null* and reconstituted VHL-HA cells were treated with 2mM DMOG for 4 hours.

To determine the dioxygenase that regulates mTORC1 activity, 11 possible candidate dioxygenases were selected for siRNA screening using two different siRNAs each. These candidates included dioxygenases with known cytoplasmic subcellular localization, enzymes with known metabolic function as well as family members that are not well characterized functionally. I identified phosphatidylserine receptor (PTDSR) and heat shock 27kDa associated protein 1 (HSPBAP1) as possible candidates from the screening (Figure 42).

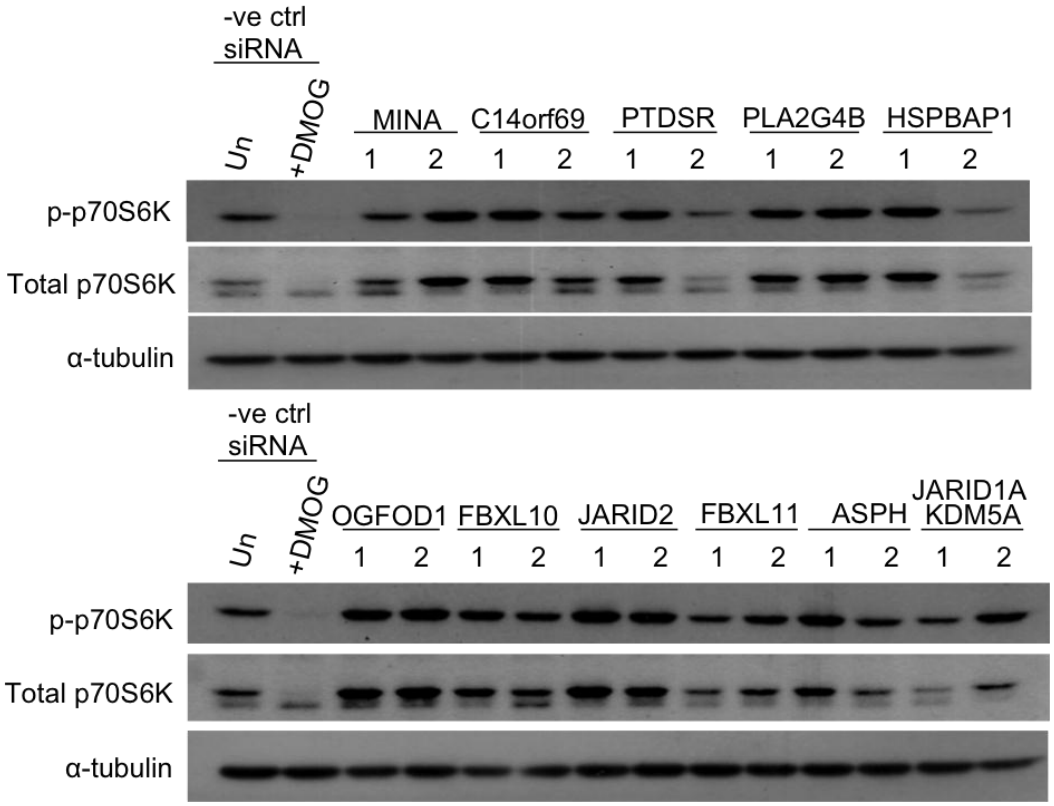


Figure 42. *mTORC1 activity is not regulated by oxygen and 2-oxoglutarate dependent dioxygenases.* HEK293 cells were transfected with 20 nM control siRNAs or two different siRNAs for each of the candidate dioxygenase for 3 days before cell lysis. DMOG (2mM) was added for 4 hours.

However, hypoxia and reoxygenation experiments upon siRNA mediated silencing of the enzymes showed that only HSPBAP1 knockdown delayed mTORC1 reactivation upon reoxygenation (Figure 43B) whereas PTDSR knockdown did not have any effect on mTORC1 activity (Figure 43A). I therefore hypothesized that inhibition of mTORC1 upon hypoxia or DMOG treatment is due to HSPBAP1 inhibition. Hence, it can be predicted that overexpression of HSPBAP1 would reverse the effect of hypoxia and DMOG.

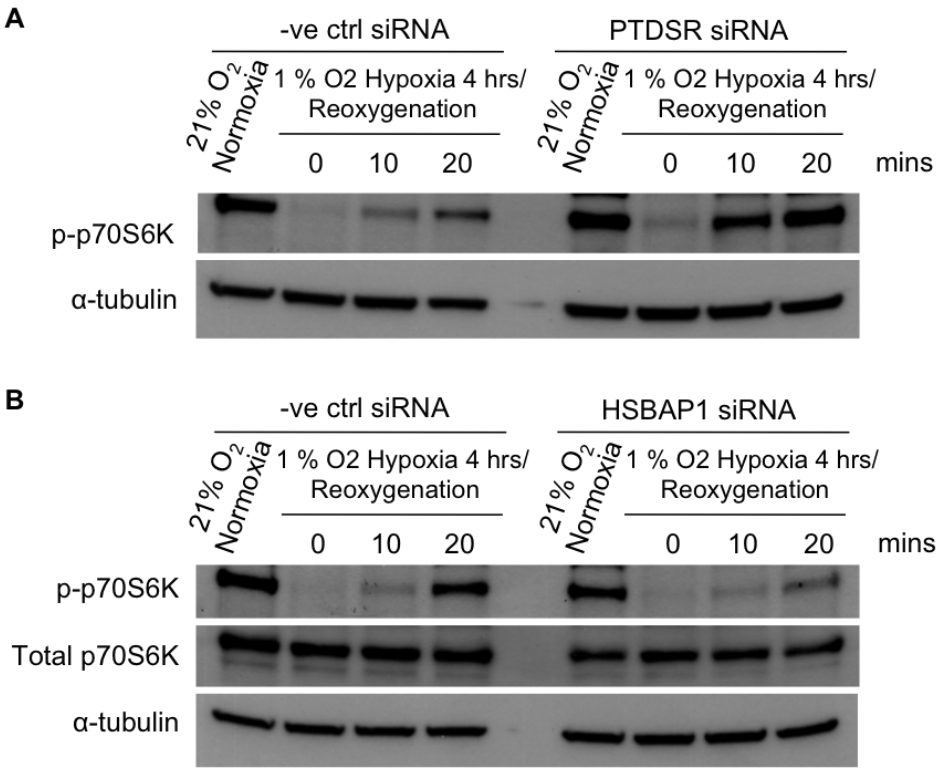


Figure 43. *mTORC1 activity is not regulated by the oxygen and 2-oxoglutarate dependent dioxygenases, PTDSR and HSPBAP1.* (**A**, **B**) The siRNA for the two candidate dioxygenases, PTDSR (**A**) and HSPBAP1 (**B**) with positive mTORC1 inhibition were transfected (20 nM) to HEK293 cells for 3 days and incubated in hypoxia for 4 hours at 1 % O_2 followed by cell lysis at the indicated time points after reoxygenation.

However, contrary to my hypothesis, it was observed that HSPBAP1 overexpression was without effect on hypoxia and DMOG mediated mTORC1 inhibition (Figure 44). This suggests that HSPBAP1 does not regulate mTORC1 activity. To rule out the possibility that the DMOG and oxygen concentrations used inhibited HSPBAP1 completely, even when overexpressed, I used lower concentrations of DMOG (Figure 45) or a less severe hypoxia treatment (Figure 46). However, HSPBAP1 overexpression did not have an effect on mTORC1 activity compared to vector control under these conditions indicating that HSPBAP1 is not a regulator of mTORC1.

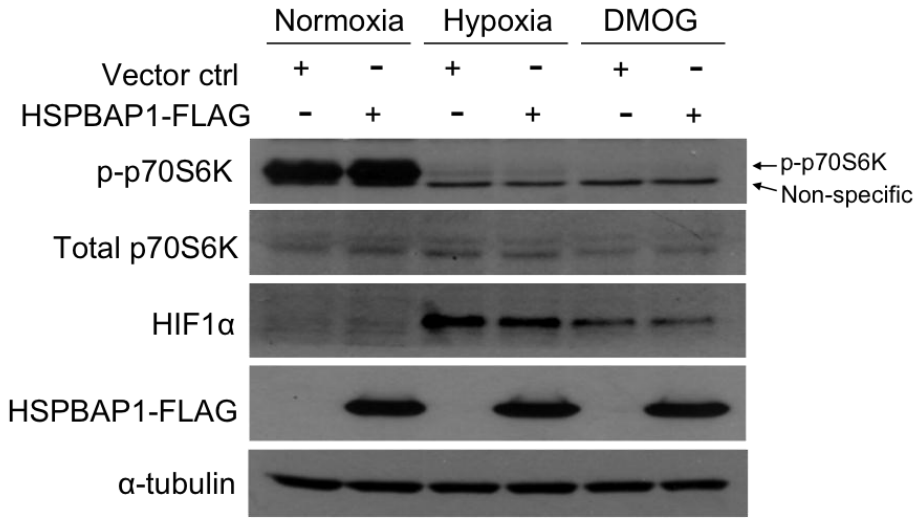


Figure 44. *mTORC1 activity is not regulated by the oxygen and 2-oxoglutarate dependent dioxygenase, HSPBAP1 in hypoxia and with DMOG treatment.* HSPBAP1-FLAG or empty pcDNA3 vectors (0.4 μg) were transfected into HEK293 cells for 3 days before DMOG treatment (2 mM) or hypoxic incubation (1 % O_2) for 4 hours followed by cell lysis.

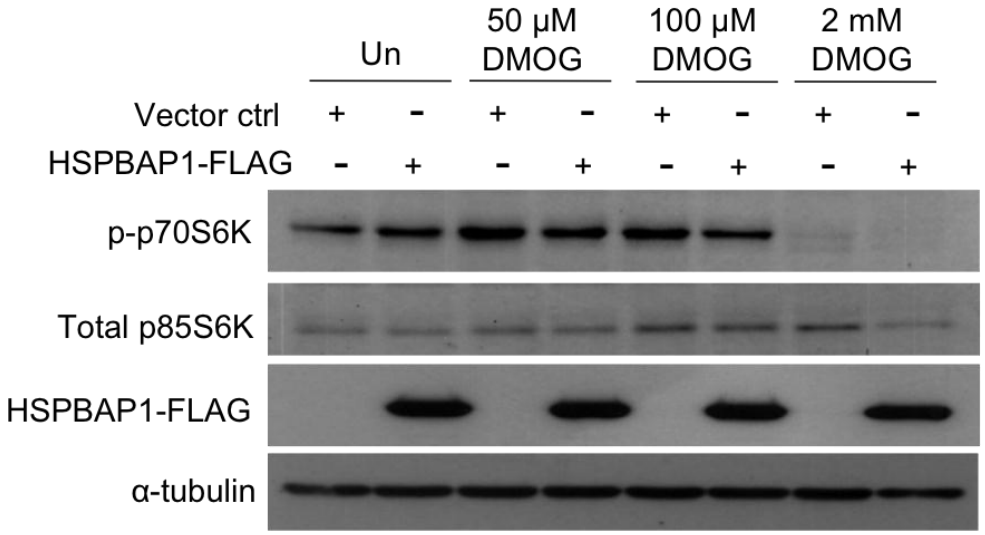


Figure 45. *mTORC1 activity is not regulated by the oxygen and 2-oxoglutarate dependent dioxygenase, HSPBAP1 with different concentrations of DMOG.* HEK293 cells were transfected with empty pcDNA3 or HSPBAP1-FLAG (0.4 μg) for 3 days followed by treatment with the indicated concentration of DMOG for 4 hours.

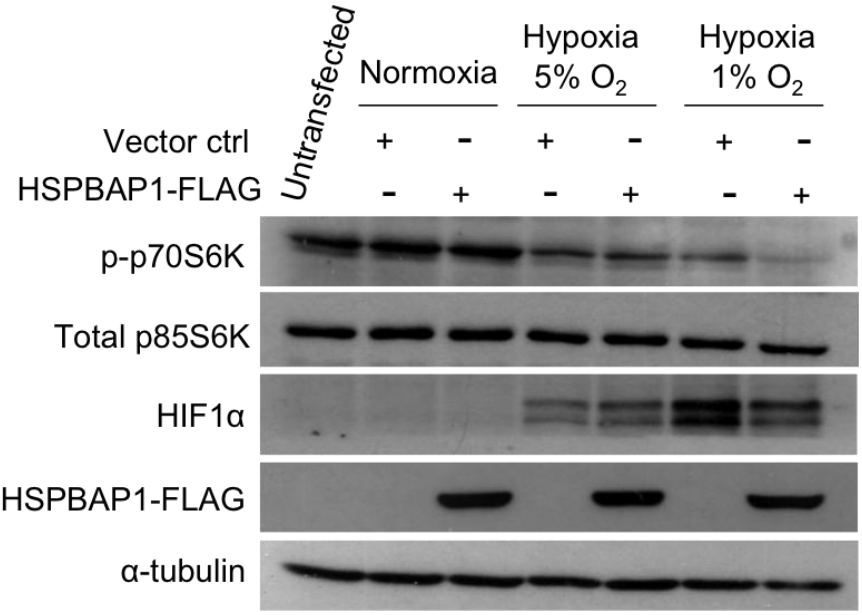


Figure 46. *mTORC1 activity is not regulated by the oxygen and 2-oxoglutarate dependent dioxygenase, HSPBAP1 under different hypoxic conditions.* HEK293 cells were transfected with empty pcDNA3 or HSPBAP1-FLAG (0.4 μg) for 3 days followed by incubation in hypoxic condition with 1 % or 5 % O_2 for 4 hours.

My results indicated that 2-oxoglutarate dioxygenases are not involved in mTORC1 regulation although DMOG completely inhibited mTORC1 activity in hypoxia and reoxygenation. Therefore, to further verify the inhibition of mTORC1 activity by DMOG, I tested if other inhibitors of 2-oxoglutarate dioxygenases affect mTORC1 activity. N-(2-Mercaptopropionyl) glycine (NMPG) is a 2-oxoglutarate analog which functions similarly as DMOG to act as a competitive inhibitor to 2-oxoglurate dependent dioxygenases (Wang et al., 2002) (Figure 47). NMPG inhibits the function of PHDs, hence, as expected, HIF-1α protein is not degraded when oxygen is reintroduced in the presence of NMPG (Figure 48). The effect of NMPG on HIF-1α stability upon reoxygenation is markedly stronger than DMOG indicating that NMPG may be a stronger inhibitor. However, treatment of NMPG did not affect mTORC1 activity in hypoxia and reoxygenation when compared to controls. In contrast to DMOG treatment, NMPG did not inhibit mTORC1 reactivation when oxygen is reintroduced (Figure 48).

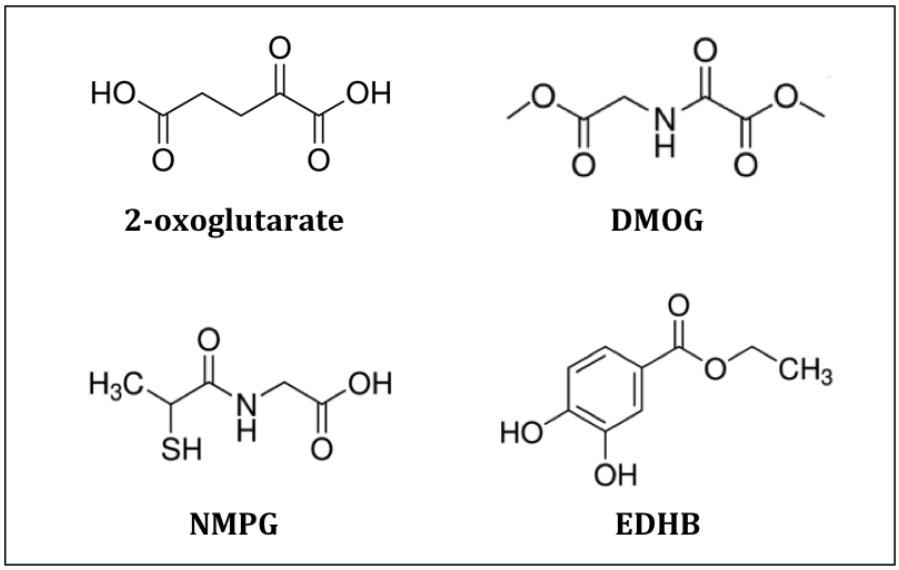


Figure 47. *2-oxoglutarate and its analogs dimethyloxalylglycine (DMOG), N-(2-Mercaptopropionyl) glycine (NMPG) and Ethyl 3,4-dihydroxybenzoate (EDHB).*

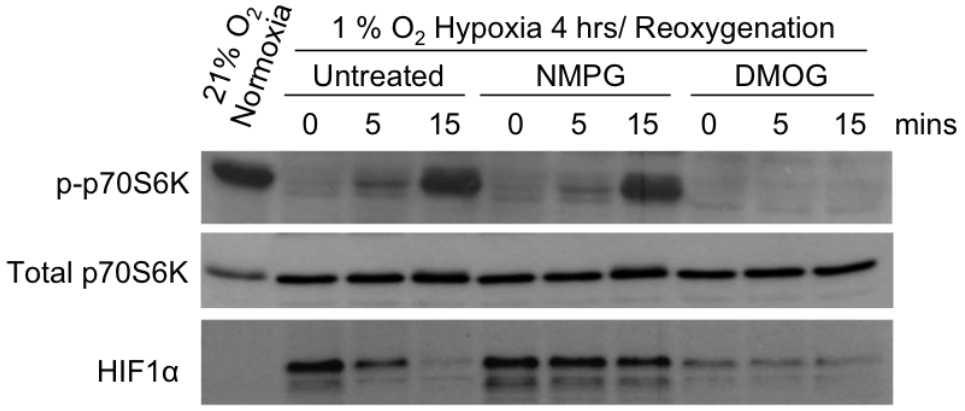


Figure 48. *mTORC1 activity in hypoxia and reoxygenation is sensitive to DMOG but not N-(2-Mercaptopropionyl) glycine (NMPG).* NMPG (5 mM) and DMOG (2 mM) were added to HEK293 cells prior to hypoxic incubation at 1 % O_2 for 4 hours and cells were lysed at the indicated time points after reoxygenation.

Ethyl 3,4-dihydroxybenzoate (EDHB) is also a 2-oxoglutarate analog that has been shown to inhibit 2-oxoglutarate dependent dioxygenases including PHDs (Warnecke et al., 2003) and collagen prolyl 4-hydroxylases (Majamaa, Sasaki and Uitto, 1987; Sasaki, Majamaa and Uitto, 1987) (Figure 47). It would be expected that EDHB treatment would prevent mTORC1 reactivation upon reoxygenation if it functions similarly as DMOG. However, mTORC1 activity was not inhibited in the presence of EDHB when oxygen was reintroduced (Figure 49). Taken together, these results indicate that 2-oxoglutarate dependent dioxygenases are not regulators of mTORC1 activity in hypoxia and reoxygenation. The inhibition of mTORC1 by DMOG may be due to an off target effect.

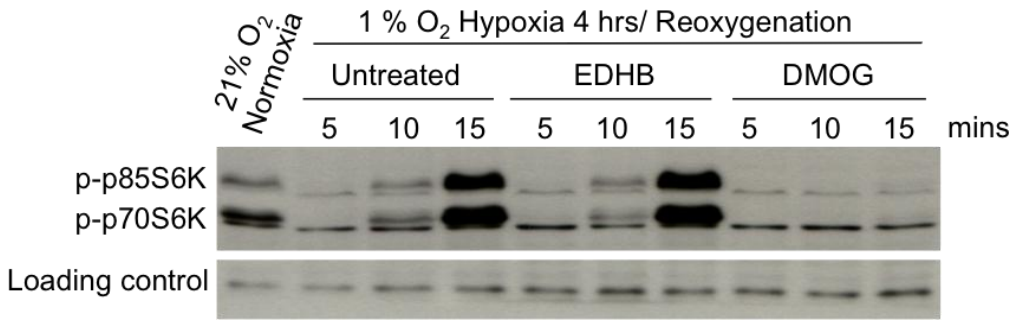


Figure 49. *mTORC1 activity in hypoxia and reoxygenation is sensitive to DMOG but not Ethyl 3,4-dihydroxybenzoate (EDHB).* EDHB (400 µM) and DMOG (2 mM) were added to HEK293 cells prior to hypoxic incubation at 1 % O_2 for 4 hours and cells were lysed at the indicated time points after reoxygenation.

As a further confirmation of the 2-oxoglutarate analogs results to show that mTORC1 regulation is independent of 2-oxoglutarate dependent dioxygenases, I performed overexpression of Isocitrate dehydrogenase 1 (IDH1). IDH1 are enzymes that catalyze the conversion of isocitrate to 2-oxoglutarate in the Krebs Cycle (Figure 50). It has been reported that a mutation at arginine 132 of IDH1 to histidine (R132H) found in gliolastomas resulted in the conversion of 2-oxoglutarate to 2-hydroxyglutarate (2HG) (Dang et al., 2009). Consequently, this IDH1 R132H mutation leads to the reduction of 2-oxoglutarate and accumulation the dioxygenase competitive inhibitor 2HG. Therefore, if mTORC1 is regulated by 2-oxoglutarate dependent dioxygenases, I would expect mTORC1 activity to be inhibited when IDH1 R132H mutant is overexpressed in cells due to the increased levels of 2HG. However, no change was observed in mTORC1 activity with the overexpression of wild type as well as R132H mutant IDH1 (Figure 51). This indicates that mTORC1 is not regulated by 2-oxoglutrate dependent dioxygenases.

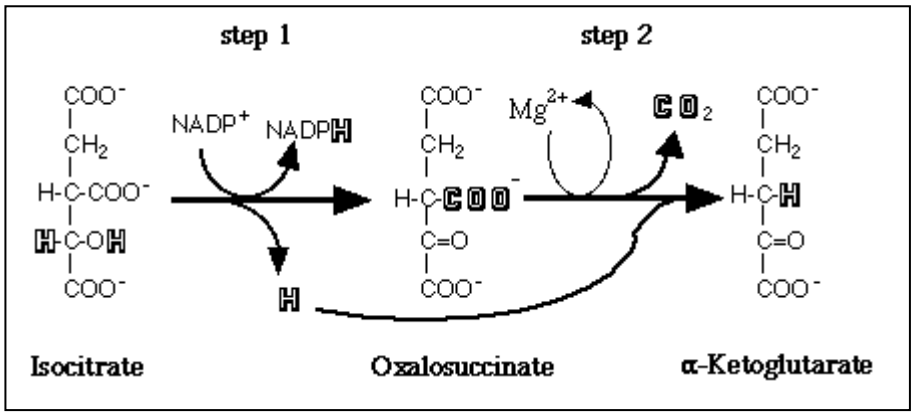


Figure 50. Conversion of isocitrate to 2-oxoglutarate (α-ketoglutarate).

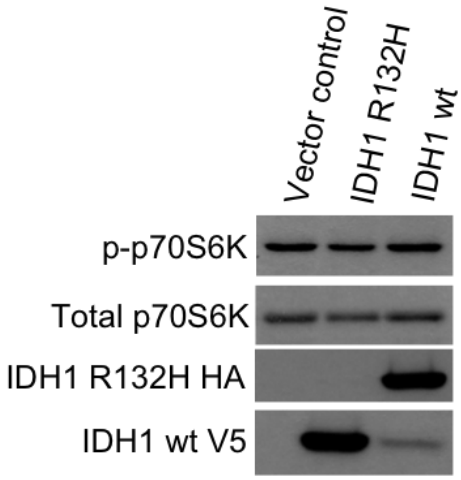


Figure 51. *Regulation of mTORC1 activity is independent of 2-oxoglutarate.* (**A**) HEK293 cells were transfected with 0.4 μg pcDNA3 vector control, IDH1 R132H mutant or IDH1 wt pcDNA3 for 3 days followed by cell lysis.

3.2.8 mTORC1 activity is not regulated by DEPTOR, PRMT1, Siah2 and SV40 T Antigen

To identify proteins that regulate mTORC1 activity in hypoxia, I tested several candidate proteins to determine if they affect mTORC1 activity. These proteins were selected based on several different criteria, including their function as inhibitors of the mTORC1 pathway or their upregulation in hypoxia, hence possibly affecting mTORC1 activity in hypoxia.

3.2.8.1 DEPTOR

DEPTOR is an inhibitor of mTORC1 and mTORC2. The DEPTOR protein interacts via its PDZ domain with the FAT domain of mTOR (Peterson et al., 2009). DEPTOR (also known as DEPDC6) is named in reference to its DEP (Dishevelled, Egl-10, Pleckstrin) domains and its specific interaction with mTOR. The expression level of DEPTOR is low in most cancers, consistent with the fact that many human cancers have activated mTORC1 and mTORC2 pathways (Peterson et al., 2009). However, no studies have looked at the activity and expression of DEPTOR in hypoxia. Hence, to test if DEPTOR might regulate mTORC1 activity in hypoxia, I performed overexpression of DEPTOR. No changes in mTORC1 activity were observed with the overexpression of DEPTOR compared to controls in both normoxia and hypoxia (Figure 52A). This indicates that regulation of mTORC1 activity is independent of DEPTOR in hypoxia. However, it is also possible that

DEPTOR overexpression is not sufficient to inhibit mTORC1 activity as no decrease in p70S6K phosphorylation was observed (Figure 52A).

Interestingly, a mobility shift was observed in hypoxia when lower amount of DEPTOR was overexpressed, suggested a change in DEPTOR posttranslational modification (Figure 52B). The significance of this finding is currently not clear. Of note, it has been reported that the phosphorylation of DEPTOR facilitates binding and ubiquitylation by the F box protein βTrCP thereby leading to its proteasomal degradation (Dang et al., 2009; Gao et al., 2011; Zhao, Xiong and Sun, 2011). It is therefore possible that oxygen concentrations affect the stability of endogenous DEPTOR protein.

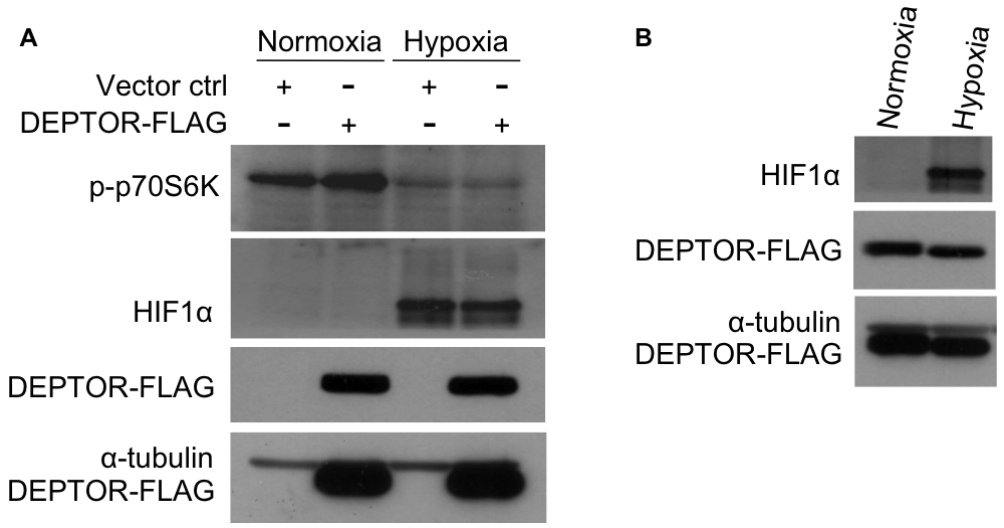


Figure 52. *mTORC1 activity is not regulated by DEPTOR.* **(A)** HEK293 cells were transfected with 0.4 μg pcDNA3 vector control or DEPTOR-FLAG pcDNA3 for 3 days followed by hypoxia incubation for 4 hours at 1 % O_2 before lysis. **(B)** HEK293 cells were transfected with 0.15 μg DEPTOR-FLAG pcDNA3 for 3 days followed by hypoxia incubation for 4 hours at 1 % O_2 before lysis.

3.2.8.2 PRMT1

Protein arginine methyl-transfecrases (PRMTs) are a family of enzymes that catalyze protein arginine methylation. PRMTs methylate arginine residues within an Akt consensus phosphorylation motif (RxRxxS/T) in their target proteins. This methylation blocks the phosphorylation of Akt on these substrates (Sakamaki et al., 2011; Yamagata et al., 2008). As Akt is an important upstream activator of the mTORC1 pathway (Figure 1), it is possible that PRMTs regulate the mTORC1 pathway by inhibiting the phosphorylation of Akt target proteins. Therefore, to test this, I overexpressed PRMT1 in cells. No difference was observed in cells with PRMT1 overexpression compared to controls (Figure 53). Similarly, mTORC1 activity was not affected by PRMT1 in hypoxia or with DMOG treatment (Figure 53). This result indicates that PRMT1 is unlikey to be involved in mTORC1 regulation.

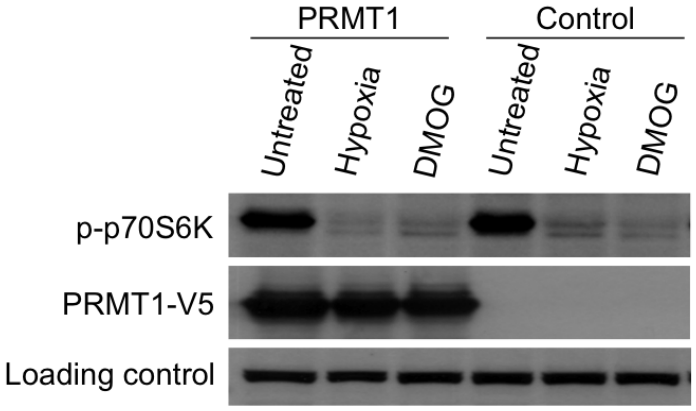


Figure 53. *PRMT1 is not involved in mTORC1 regulation.* HEK293 cells were transfected with 0.4 μg pcDNA3 vector control or PRMT1-V5 pcDNA3 for 3 days followed by hypoxia incubation at 1 % O_2 or DMOG (2mM) treatment for 4 hours before lysis.

3.2.8.3 Siah2

The ubiquitin ligase Siah 2 is upregulated in hypoxia and functions to degrade PHD1 and 3 (Nakayama et al., 2004; Nakayama, Qi and Ronai, 2009). Although PHD2 is reported to be the most important PHD under normoxia (Berra et al., 2003), PHD3 appears to retain its activity in mediating HIF-1α hydroxylation in hypoxia (Nakayama et al, 2004). Hence, degradation of PHD3 facilitates HIF-1α stabilization in hypoxia, leading to the transcription of its downstream targets.

Recently, it has been discovered that Siah2 protein is itself regulated and stabilized by a member of the tumor necrosis factor receptor superfamily, $p75^{NTR}$. In hypoxia, Siah2 is bound to the soluble domain of the adaptor protein $p75^{NTR}$, which functions to stabilize Siah2 and prevent its autoubiquitination (Le Moan et al., 2011). Therefore, as Siah2 is upregulated in hypoxia, it is possible that Siah2 regulates the mTORC1 pathway in hypoxia. However, I found that overexpression of Siah2 in normoxia to mimic the increased Siah2 levels in hypoxia did not affect mTORC1 activity (Figure 54). This result suggests that mTORC1 regulation in hypoxia is independent of Siah2.

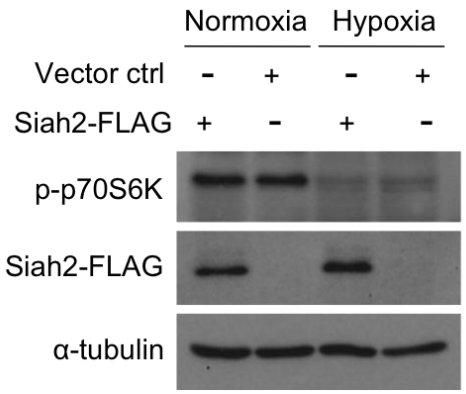


Figure 54. *Siah 2 is not involved in mTORC1 regulation.* HEK293 cells were transfected with 0.4 μg pcDNA3 vector control or Siah2-FLAG pcDNA3 for 3 days followed by hypoxia incubation for 4 hours at 1 % O_2 before lysis.

3.2.8.4 SV40 T Antigen

In addition to the cell lines used in Figure 4, I also used HEK293T cells to study mTORC1 activity in hypoxia and reoxygenation. As expected, mTORC1 activity is inhibited in hypoxia, as shown by the decrease in p70S6K phosphorylation (Figure 55). However, interestingly, mTORC1 activity remained inhibited upon reoxygenation and a delay in mTORC1 reactivation was observed (Figure 55). This is surprising as the HEK293T cells are a variant of the original HEK293 cells with an addition of the SV40 Large T antigen. Hence, it is possible that the presence of the SV40 Large T antigen in HEK293 cells affects mTORC1 reactivation upon reoxygenation.

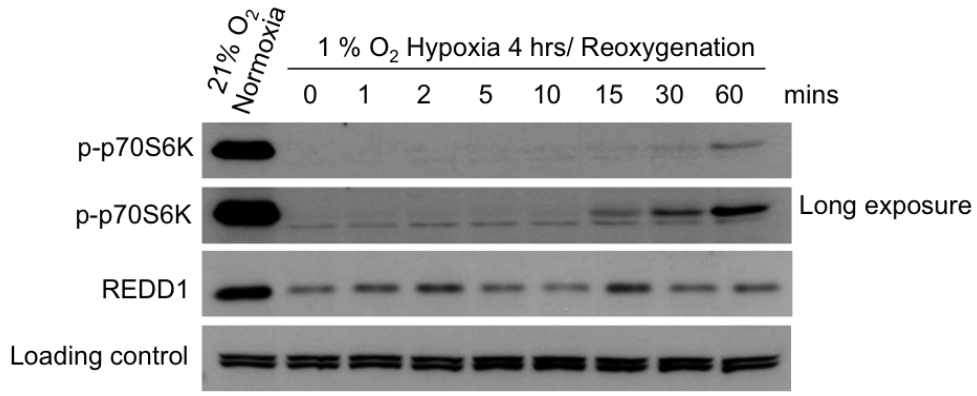


Figure 55. *mTORC1 reactivation upon reoxygenation is delayed in HEK293T cells.* HEK293T cells were incubated at 21 % or 1 % O_2 for 4 hours, followed by reoxygenation in normoxia and cell lysis at the indicated time points.

To test this, knockdown of SV40 Large T antigen was carried out in HEK293T cells. If the SV40 Large T antigen were involved in mTORC1 inhibition upon reoxygenation, knockdown of SV40 Large T-antigen would result in a rapid reactivation of mTORC1 activity similar to the rate observed in HEK293 cells. However, there was no difference in mTORC1 activity in

control and SV40 Large T antigen knockdown cells (Figure 56). This indicates that SV40 Large T antigen is not involved in mTORC1 regulation.

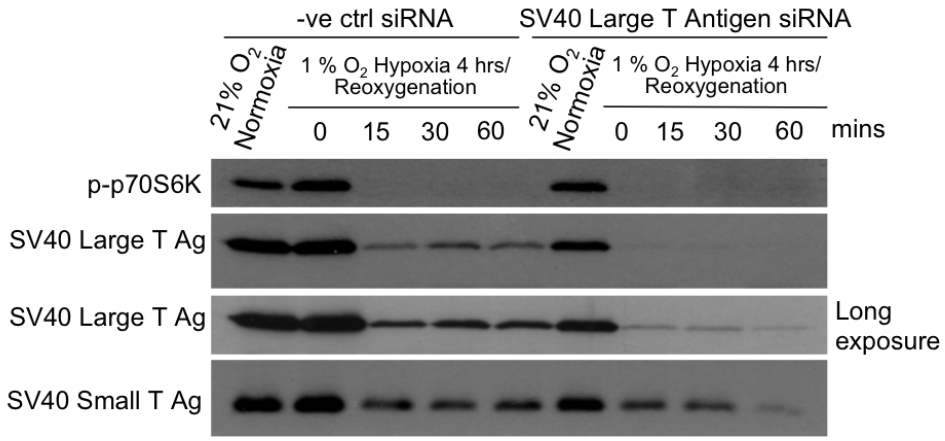


Figure 56. *mTORC1 activity in hypoxia and reoxygenation is not regulated by SV40 large T Antigen.* HEK293T cells were transfected with 20 nM control or SV40 Large T antigen siRNA for 3 days and incubated in hypoxia for 4 hours at 1 % O_2 followed by cell lysis at the indicated time points after reoxygenation.

To confirm the result, I overexpressed SV40 Large T antigen in HEK293 cells to mimic the presence of SV40 Large T antigen in HEK293T cells. Overexpression of SV40 Large T antigen did not affect mTORC1 reactivation upon reoxygenation (Figures 57A and B). Taken together, these results show that SV40 Large T antigen is not involved in the regulation of mTORC1 activity.

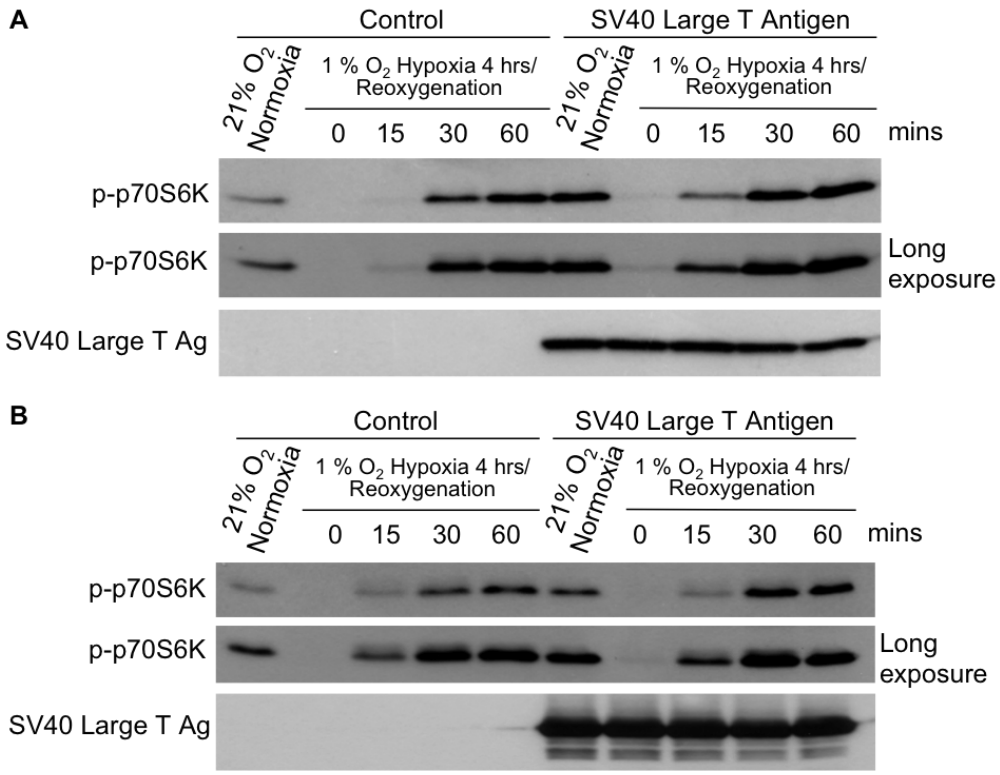


Figure 57. *SV40 Large T Antigen is not involved in mTORC1 regulation in hypoxia and reoxygenation.* (**A** and **B**) HEK293 cells were transfected with 0.4 μg pcDNA3 vector control, SV40 Large T Antigen pcDNA3 (**A**) or SV40 Large T Antigen pcDNA4/TO (**B**) for 3 days with 1 μg/ml tetracycline induction for (**B**) at the last 24 hours prior to cell lysis. Cells were incubated for 4 hours at 1 % O_2 followed by cell lysis at the indicated time points after reoxygenation.

In addition to the Large T antigen, the SV40 vector also encodes an alternatively spliced gene known as the Small T antigen. Hence, to test if the SV40 Small T antigen is involved in mTORC1 regulation, overexpression of the SV40 Small T antigen alone or in combination with the SV40 Large T antigen was carried out. No difference in mTORC1 reactivation was observed upon reoxygenation in the presence of the SV40 Small T antigen (Figure 58) or both SV40 Large and Small T antigens (Figure 59). Taken together, these results showed that SV40 Large and Small T antigens are not involved in mTORC1 regulation. The delayed reactivation of mTORC1 observed in

HEK293T cells upon reoxygenation may be due to other secondary changes in these cells independent of the SV40 T antigens.

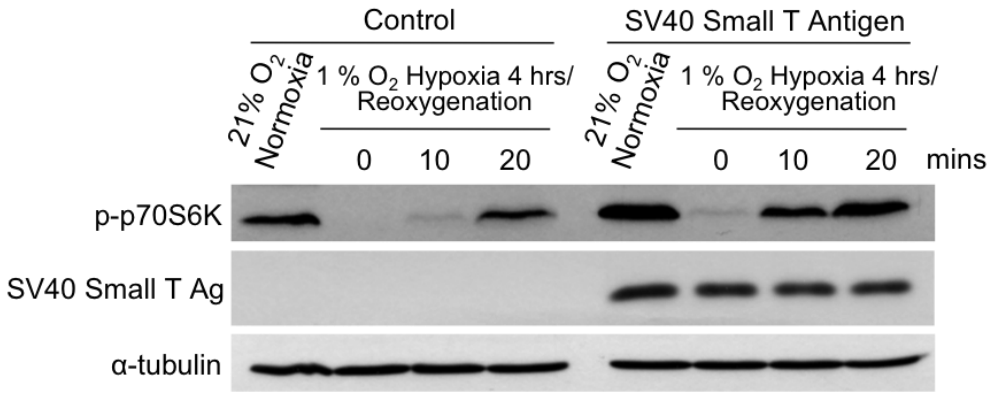


Figure 58. *mTORC1 activity is not regulated by Small T antigen.* HEK293 cells were transfected with 0.4 μg pcDNA3 vector control or SV40 Small T Antigen pcDNA3 for 3 days and incubated for 4 hours at 1 % O_2 followed by cell lysis at the indicated time points after reoxygenation.

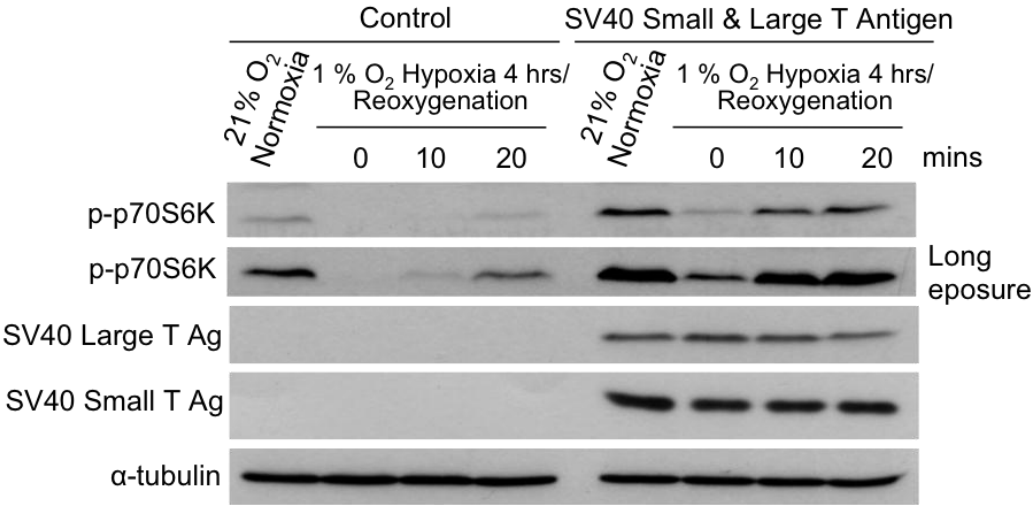


Figure 59. *mTORC1 activity is not regulated by SV40 Large and Small T antigen.* HEK293 cells were transfected with 0.4 μg pcDNA3 vector control, SV40 Large T Antigen pcDNA3 or SV40 Small T Antigen pcDNA3 for 3 days and incubated for 4 hours at 1 % O_2 followed by cell lysis at the indicated time points after reoxygenation.

3.2.9 mTORC1 activity in hypoxia and reoxygenation is regulated at the level of the mTORC1 complex directly

mTORC1 is activated by two independent signaling pathways in response to different upstream signals. Growth factors activate mTORC1 through the inactivation of the TSC1/2 complex whereas amino acids availability activates mTORC1 via the Ragulator-Rag GTPase complex resulting in its translocation to the lysosomes. To determine if hypoxia exerts its inhibitory effect via the TSC1/2 pathway, I deprived cells of amino acids. Under these conditions, only the serum dependent TSC1/2 pathway will be active. In the absence of amino acids, mTORC1 activity in normoxia was low but detectable (Lane 1 in Figure 60). In the absence of amino acids, hypoxia completely inhibited mTORC1 activity and the effect was rapidly reversed upon reoxygenation (Figure 60). This suggests that hypoxia can inhibit mTORC1 activity via the serum dependent TSC1/2 pathway. On the other hand, to test if hypoxia also acts on the amino acids dependent Ragulator-Rag GTPase pathway, serum deprivation was carried out to inhibit mTORC1 activation via the TSC1/2 pathway. Thus only the Ragulator-Rag GTPase would be responsible for mTORC1 activity (Figure 60). mTORC1 was completely inhibited in hypoxia and this inhibition was rapidly reversed upon reintroduction of oxygen. This suggests that hypoxia could also work via the Ragulator-GTPase pathway to regulate mTORC1 activity. One potential interpretation of my results is that hypoxia exerts its effect on two independent pathways to affect mTORC1 activity. However, it is highly unlikely that hypoxia exerts such a marked inhibitory effect on two completely independent

signaling pathways. It is therefore much more probable that hypoxia inhibits at a common downstream target of both pathways. Because hypoxia inhibits the phosphorylation of all known mTORC1 targets, the target of oxygen dependent regulation of the mTORC1 pathway is likely the mTORC1 complex itself.

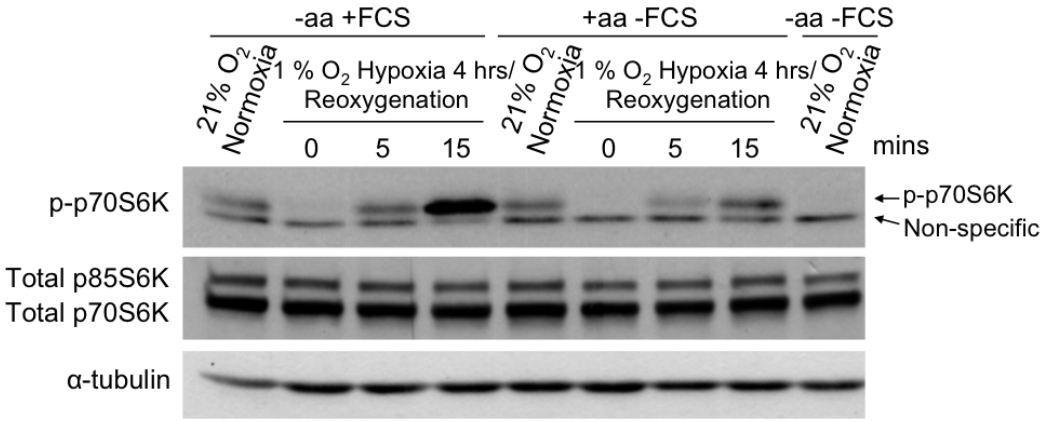


Figure 60. *mTORC1 activity in hypoxia and reoxygenation is regulated at the level of mTORC1.* HEK293 cells were pre-treated with serum and amino acids free Krebs buffer with 25 mM glucose for 1 hour, followed by addition of 10% serum or amino acids to the Krebs buffer. Cells were then incubated for 4 hours at 1 % O_2 followed by reoxygenation and cell lysis at the indicated time points.

3.2.10 mTORC1 may be regulated by heme binding proteins in hypoxia and reoxygenation

My experiments suggest that hypoxia inhibits mTORC1 via a post-translational mechanism by directly regulating the mTORC1 complex. As I have ruled out the involvement of the 2-oxoglutarate and oxygen dependent dioxygenases in mTORC1 regulation, I hypothesized that oxygen dependent heme containing proteins mediate mTORC1 regulation. To test the hypothesis, I used a number of general inhibitors of heme containing proteins. Interestingly, under basal conditions, inhibition of heme binding proteins with treatment of sodium azide and the NO donor, GSNO, both mimicked the effect of hypoxia by inhibiting mTORC1 activity (Figure 61). Furthermore, upon reoxygenation, sodium azide treatment prevented mTORC1 reactivation (Figure 62). These results suggest that heme binding proteins may be involved in mTORC1 regulation in hypoxia and reoxygenation. As some of the mitochondria ETC proteins including cytochrome c oxidase also belong to the heme binding protein family, it is possible that the inhibitors of heme binding proteins regulate mTORC1 activity via inhibition of mitochondrial ETC proteins. However, the cytochrome c oxidase inhibitor potassium cyanide (KCN) did not have any effect on mTORC1 activity (Figure 61). Furthermore, treatment with other mitochondrial inhibitors did not result in marked inhibition of mTORC1 activity (Figure 19). This shows that the inhibitory effect of the heme binding protein inhibitors is not mediated through the mitochondrial ETC. These results suggest that the regulation of

mTORC1 in hypoxia is mediated via an oxygen dependent, heme containing protein that is not part of the mitochondrial ETC.

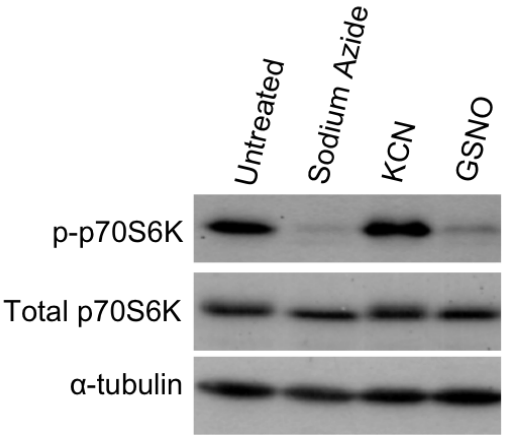


Figure 61. *Regulation of mTORC1 activity in hypoxia and reoxygenation may involve heme binding proteins.* HEK293 cells were treated with inhibitors of heme binding proteins: sodium azide (1 mM), potassium cyanide (KCN, 1 mM) and S-Nitrosoglutathione (GSNO, 1 mM) for 4 hours.

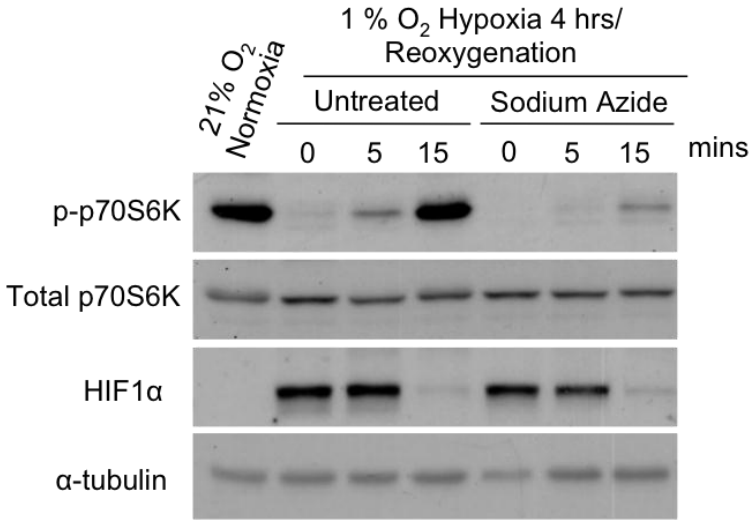


Figure 62. *Regulation of mTORC1 activity in hypoxia and reoxygenation may involve heme binding proteins.* HEK293 cells were pre-incubated at 1 % O_2 in the hypoxia workstation for 4 hours and sodium azide (1 mM) was added to cells 15 mins before cell lysis at the indicated time points.

3.3 Discussion

Hypoxia is one of the major signals that regulate mTORC1 activity. However, the involved mechanisms are currently not well understood. In this study, I show that hypoxia dependent inhibition of mTORC1 is highly dynamic and rapidly reversed (within 15 minutes) upon reintroduction of oxygen. This suggests that regulation of mTORC1 by changes in oxygen concentrations is primarily mediated via post-translational mechanisms. Indeed, my results indicate that oxygen dependent mTORC1 regulation is intact in the absence of ongoing transcription and translation. Furthermore, consistent with previous reports (Arsham, Howell and Simon, 2003; Liu et al., 2006), my results show that HIF, the major transcription factor induced under hypoxia, is not involved in mTORC1 regulation.

In further experiments, I tested a number of potential mediators that may play a role in the regulation of mTORC1 signaling in hypoxia and reoxygenation. REDD1, a known transcriptional target of HIF, has been reported to inhibit mTORC1 in a TSC1/2 dependent manner although the exact mechanism is currently unknown. My results showed that REDD1 is not involved in the rapid regulation of mTORC1. This is based on a number of findings, including the lack of significant effect of REDD1 overexpression as well as silencing of both REDD1 and 2 paralogs on mTORC1 activity in normoxia and hypoxia. Furthermore, the rapid reactivation of mTORC1 during reoxygenation was not accompanied by a concomitant downregulation of REDD1. Finally, when comparing different cell lines, I observed that

mTORC1 was strongly inhibited in the tested cell lines. However, REDD1 induction by hypoxia was cell line dependent. The observed difference in REDD1 expression levels under hypoxia in the different cell lines tested is in line with the findings of another study which showed that REDD1 regulation of mTORC1 in hypoxia is cell type specific (Wolff et al., 2011). It should also be noted that REDD1 has been reported to mediate mTORC1 inhibition under hypoxia as a result of its HIF dependent transcriptional upregulation (Brugarolas et al., 2004). However, given that HIF was not required for the dynamic oxygen dependent regulation of mTORC1 in my experiments, REDD1 is likely not a major mediator of oxygen dependent regulation of mTORC1 under these conditions.

Another important upstream regulator that may mediate the dynamic regulation of mTORC1 in hypoxia is AMPK. Low oxygen concentrations inhibit mitochondrial oxidative phosphorylation, thereby leading to reduced ATP production and subsequently, activation of AMPK. AMPK is known to phosphorylate TSC2 directly to regulate mTORC1 activity in hypoxia (Liu et al., 2006). In addition, AMPK has been shown recently to regulate mTORC1 independent of TSC1/2 by phosphorylating the mTORC1 subunit Raptor directly to induce 14-3-3 binding (Gwinn et al., 2008). Although these reports showed that AMPK plays an important role in mTORC1 regulation, other studies concluded that AMPK is not involved in mTORC1 regulation in hypoxia (Arsham, Howell and Simon, 2003; Brugarolas et al., 2004). The results of my study suggest that AMPK is not a major regulator of mTORC1 during the rapid response to hypoxia/reoxygenation. This is based on

experiments using small molecule inhibitor and activator of AMPK. Furthermore, complete blocking of mitochondrial ATP production using various inhibitors exerted smaller inhibitory effects on mTORC1 compared to incubation of cells at 1 % oxygen where oxidative phosphorylation is still partially functional (Chua et al., 2010). This indicates that hypoxia functions at least partially via oxidative phosphorylation and AMPK independent mechanisms.

mTORC1 is activated via two different pathways, the growth factor dependent TSC1/2 pathway or the amino acids dependent Ragulator-Rag complex pathway. My results show that hypoxia inhibits both growth factor and amino acids dependent mTORC1 activation. Although I cannot rule out the possibility that hypoxia inhibits mTORC1 by directly affecting both pathways independently, it is more likely that hypoxia inhibits a common downstream target of both pathways. This strongly suggests that hypoxia exerts its regulatory effect directly at the level of mTORC1.

In summary, hypoxia inhibits the mTORC1 pathway post-translationally at the level of mTORC1 directly. This inhibition is likely independent of known oxygen sensors which includes dioxygenases such as PHDs. Therefore, I tested the possibility that a different family of oxygen dependent enzymes is involved in mTORC1 regulation and found evidence that heme containing proteins might be novel regulators of mTORC1. This family of proteins includes respiratory cytochromes, gas sensors, P450 enzymes (CYPs), catalases, peroxidases, nitric oxide synthases (NOS), guanyl

cyclases and even transcription factors. Using general inhibitors, I obtained evidence that mTORC1 activity is inhibited in the absence of active heme containing proteins. This inhibition is likely not via oxidative phosphorylation because treatment with KCN, an inhibitor of cytochrome c oxidase, did not inhibit mTORC1 activity, in contrast to sodium azide and GSNO treatment. Also, mTORC1 activity was not significantly affected by the treatment of cells with mitochondrial ETC inhibitors. This is in further support that mTORC1 regulation is independent of oxidative phosphorylation. Identification of the specific mediator involved in the post-translational regulation of mTORC1 in hypoxia in future studies might reveal a novel drug target through which mTOR could be inhibited in a mTORC1-specific manner.

4.0 mTORC1 dependent regulation of REDD1 protein stability

4.1 Introduction

REDD1 (Regulated in Development and DNA Damage responses 1) is a negative regulator of mTORC1 in hypoxia and functions in a TSC2 dependent manner (Brugarolas et al., 2004; Reiling and Hafen, 2004). REDD1 was first identified to be upregulated in response to hypoxia and DNA damage (Shoshani et al., 2002; Ellisen et al., 2002). The *REDD1* gene (also known as RTP801/Dig1/DDIT1) belongs to a family of genes that includes its paralog *REDD2* (TRP801L/Dig2/DDIT4L) (Ellisen et al., 2002) and the Drosophila orthologs *Scylla* and *Charybdis* (Reiling and Hafen, 2004). REDD1 is ubiquitously expressed and is found in most adult tissues, however the expression of REDD2 is highly restricted (Reiling and Hafen, 2004; Ellisen et al., 2002; Shoshani et al., 2002). *REDD1* is upregulated through transcriptional mechanisms in response to different stress stimuli such as DNA damage, ER stress, hypoxia, serum deprivation, glucocorticoid-, hydrogen peroxide-, dexamethasone-treatment (Ellisen et al., 2002; Shoshani et al., 2002; Lee et al., 2004; Whitney, Jefferson and Kimball, 2009; Wang et al., 2003). Moreover, the induction of *REDD1* is mediated by different transcription factors including p53, p63, ATF4, Sp1 and HIF1, indicating that REDD1 is an important regulator in response to diverse stress conditions (Ellisen et al., 2002; Shoshani et al., 2002; Lee et al., 2004; Whitney, Jefferson and Kimball, 2009).

REDD1 is a highly unstable protein. The REDD1 protein half-life has been reported to be between 5-7 mins (Kimball et al., 2008; Katiyar et al., 2009). This indicates that REDD1 is also subject to stringent post-translational control. However, as there are no known structural domains or functional motifs present in REDD1 and not much is known about the regulation of REDD1 stability. It has been reported that REDD1 is degraded by the Cul4a (Cullin 4a)-DDB1 (DNA damage-binding protein 1)-ROC1 (regulator of cullins 1)-β-TRCP ubiquitin E3 ligase complex through a phosphorylation dependent mechanism mediated by glycogen synthase kinase 3β (GSK3β) (Katiyar et al., 2009). However, it is not known how the stability of REDD1 is regulated in response to physiological signals.

In this study, I identified a novel mTORC1-REDD1 feedback loop whereby mTORC1 regulates REDD1 protein stability. Furthermore, I observed that REDD1 stability is not regulated by GSK3β dependent phosphorylation and that REDD1 is not ubiquitinated by Cul4a or other Cullin RING E3 ubiquitin ligases.

4.2 Results

4.2.1 mTORC1 regulates cellular REDD1 protein levels

I initially observed that overexpression of REDD1-V5 in HEK293 cells led to reduced levels of endogenous REDD1 protein when compared to REDD1 protein in untransfected cells (Figure 63). High levels of REDD1 are known to inhibit the mTORC1 pathway. This suggested that inhibition of mTORC1 activity may be responsible for the downregulation of the REDD1 protein levels. I therefore tested if inhibition of the mTORC1 pathway with rapamycin causes a similar downregulation of the REDD1 protein. Indeed, treatment of cells with rapamycin resulted in a marked reduction in REDD1 protein abundance (Figure 64). This effect was also observed in a different cell line, HepG2 (Figure 65). Importantly, when cells were treated with a different mTORC1 inhibitor PP242, REDD1 protein levels were also markedly reduced (Figure 65). This indicates that the effect of mTORC1 inhibition on REDD1 is specific.

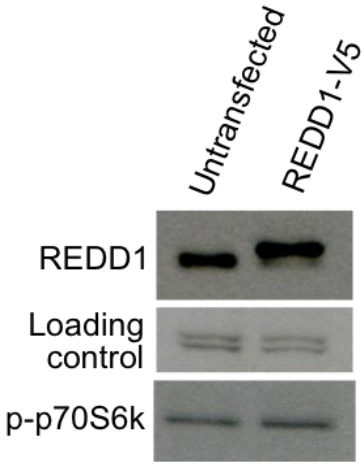


Figure 63. *Overexpression of REDD1-V5 led to reduced levels of endogenous REDD1 protein.* REDD1-V5 pcDNA3 (0.4 μg) was transfected in HEK293 cells for 3 days followed by cell lysis and detection of endogenous REDD1 proteins by Western blotting.

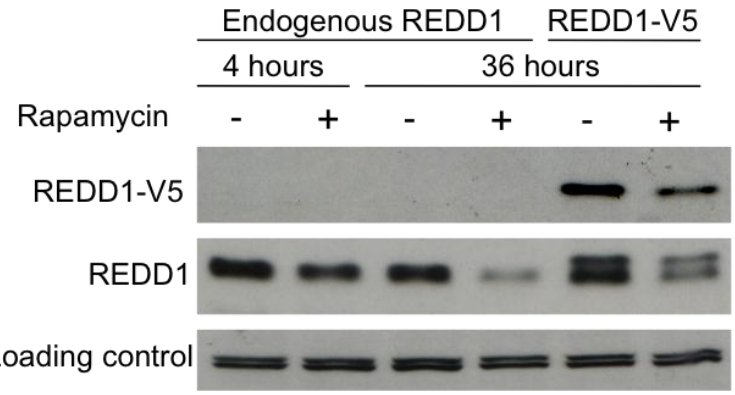


Figure 64. *Rapamycin treatment resulted in marked reduction in REDD1 protein abundance.* HEK293 cells with and without the transfection of REDD1-V5 pcDNA3 (0.15 μg) were treated with 20 nM rapamycin for 4 or 36 hours followed by cell lysis.

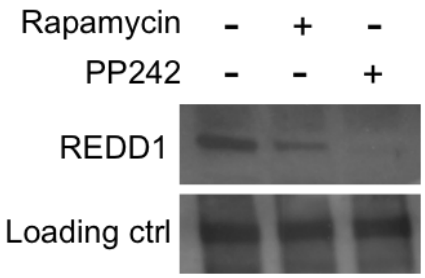


Figure 65. *Rapamycin and PP242 treatment led to marked reduction in REDD1 protein levels.* HepG2 cells were treated with 20 mM rapamycin or 2 μM PP242 for 24 hours followed by cells lysis.

To test if the mTORC1 regulation of REDD1 is due to a decrease in transcription, I also determined the effect of mTORC1 inhibition on transfected REDD1 levels as the expression of REDD1-V5 pcDNA3 plasmid is driven by a constitutively active CMV promoter. Transfected REDD1 levels also decreased with rapamycin treatment (Figure 64). This indicates that the mTORC1 regulation of REDD1 is independent of transcription and is likely a result of altered REDD1 degradation.

One of the major downstream targets of mTORC1 is p70 S6 kinase (p70S6K). To determine the involvement of S6K1 in the feedback regulation of REDD1, I overexpressed the inactive form of p70S6K (with threonine 389

residue mutated to alanine) in HEK293 cells. Interestingly, this led to reduced REDD1 expression levels and hence mimicked the effect of rapamycin (Figure 66). On the other hand, mutation of the threonine 389 residue to aspartate to mimic phosphorylation resulted in an increase in the co-transfected REDD1 levels (Figure 67). This indicates that active mTORC1 increases REDD1 levels via p70S6K.

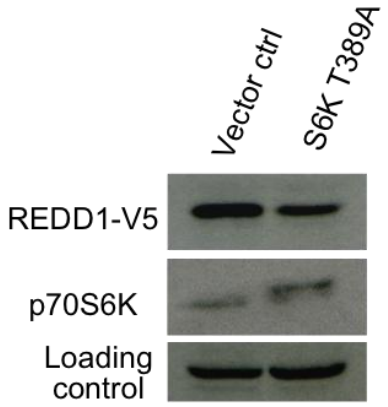


Figure 66. *Inactive p70S6K mutant led to reduced REDD1 protein abundance.* REDD1-V5 pcDNA3 (0.1 μg) was co-transfected with 0.2 μg empty pcDNA3 or p70S6K T389A for 3 days followed by cell lysis.

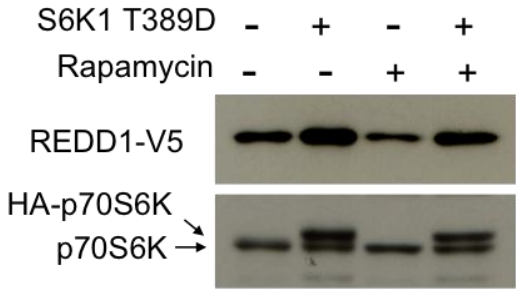


Figure 67. *Active p70S6K mutant increases REDD1 protein abundance.* HEK293 cells were transfected with REDD1-V5 pcDNA3 (0.15 μg) and/or of 0.35 μg p70S6K T389D in the indicated combinations for 3 days and treated with 20 nM rapamycin for 36 hours before cell lysis.

To further confirm the effect of mTORC1 activity on REDD1 protein concentrations, I used $TSC2^{+/+}$ and $TSC2^{-/-}$ mouse embryonic fibroblasts (MEFs). These cells have differential mTORC1 activities due to the presence or absence of the mTORC1 upstream negative regulator TSC2. In the $TSC2^{-/-}$ MEFs where mTORC1 is constitutively active, REDD1 levels are much higher compared to $TSC2^{+/+}$ cells (Figure 68). Thus, consistent with my hypothesis, increased mTORC1 activity led to higher REDD1 protein

abundance. As expected from the results in HEK293 cells, treatment with the mTORC1 inhibitors rapamycin or PP242 reduced REDD1 expression markedly in both $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs (Figure 68). Taken together, these results indicate that REDD1 protein levels are regulated by mTORC1 activity.

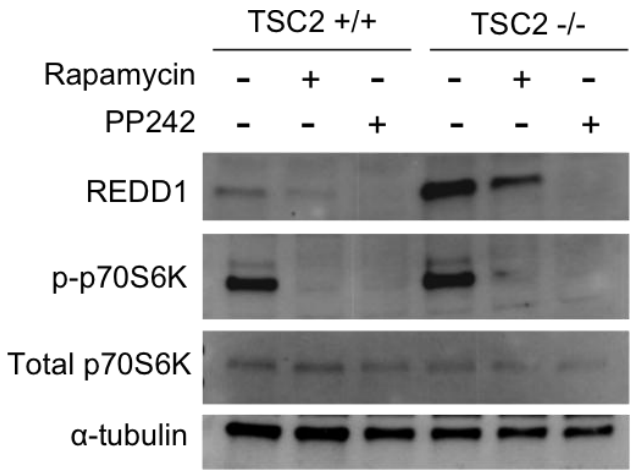


Figure 68. *REDD1 protein is more abundant in TSC2 -/- cells.* MEF $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were treated with rapamycin (40 nM) or PP242 (2 μM) for 24 hours before cell lysis.

4.2.2 mTORC1 regulates REDD1 protein stability

I next determined whether the downregulation of REDD1 upon mTORC1 inhibition is due to increased protein turnover. To this end, I treated cells with the protein synthesis inhibitor cycloheximide in the presence or absence of rapamycin and determined protein degradation rates. REDD1 half-life was shorter when mTORC1 was inhibited with rapamycin (Figure 69). In control cells, REDD1 protein decreased by 27% after 15 mins of cycloheximide treatment. In contrast, in the presence of rapamycin, the decrease in REDD1 protein during the same time period was much more dramatic (70%). Similarly, inhibition of mTORC1 with treatment of PP242 also decreased REDD1 half-life (Figure 70).

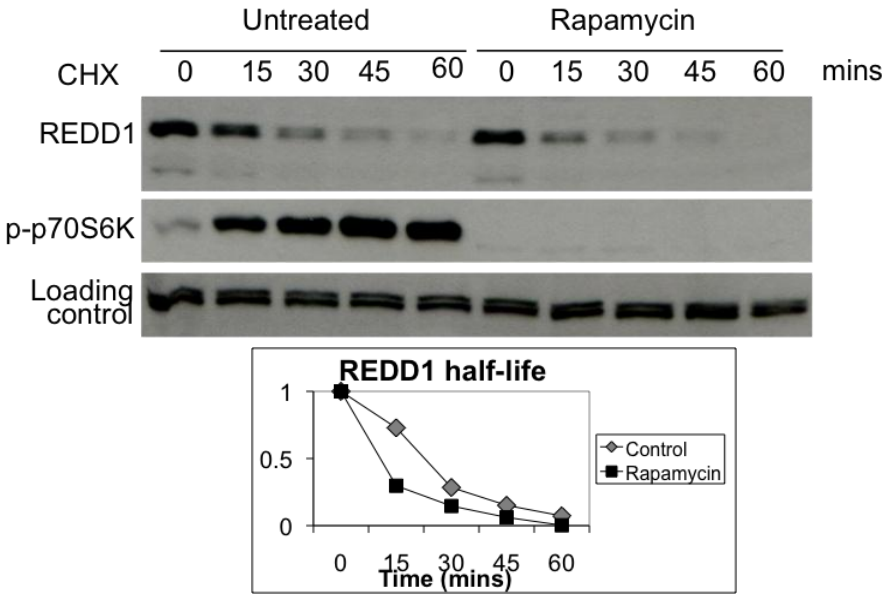


Figure 69. *mTORC1 inhibition with rapamycin treatment increases REDD1 degradation.* HEK293 cells were pretreated with 20 nM rapamycin for 1 hour followed by treatment with cycloheximide (40 μM) and cell lysis at the indicated time points.

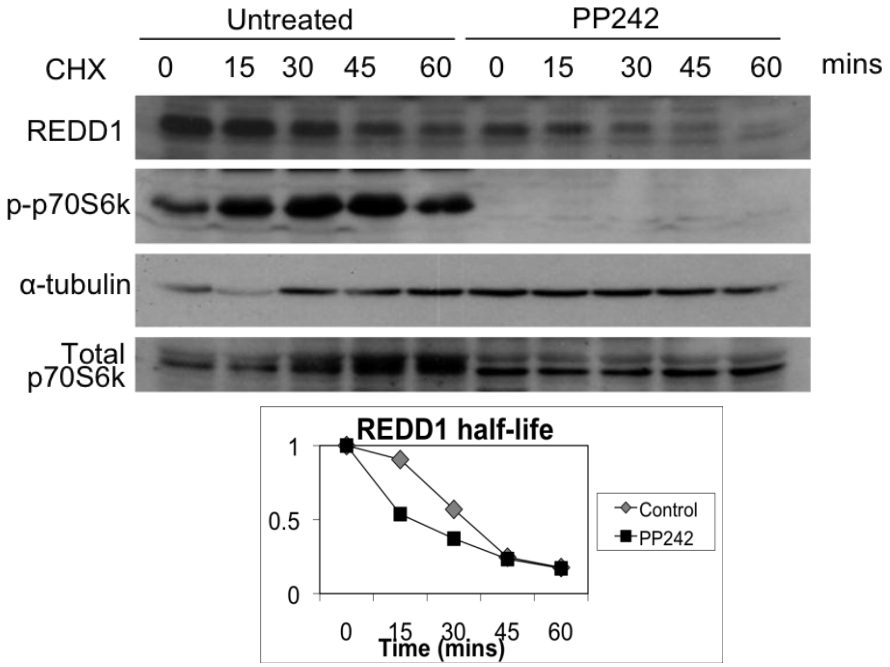


Figure 70. *mTORC1 inhibition with PP242 treatment increases REDD1 degradation.* HEK392 cells cells were pretreated with 2 μM PP242 for 4 hour followed by treatment with cycloheximide (40 μM) and cell lysis at the indicated time points.

The decrease in REDD1 levels upon treatment with mTORC1 inhibitors rapamycin and PP242 treatment was reversed with addition of proteasome inhibitor, MG-132 (Figure 71). This indicates that upon mTORC1 inhibition REDD1 is degraded in a 26S proteasome dependent manner.

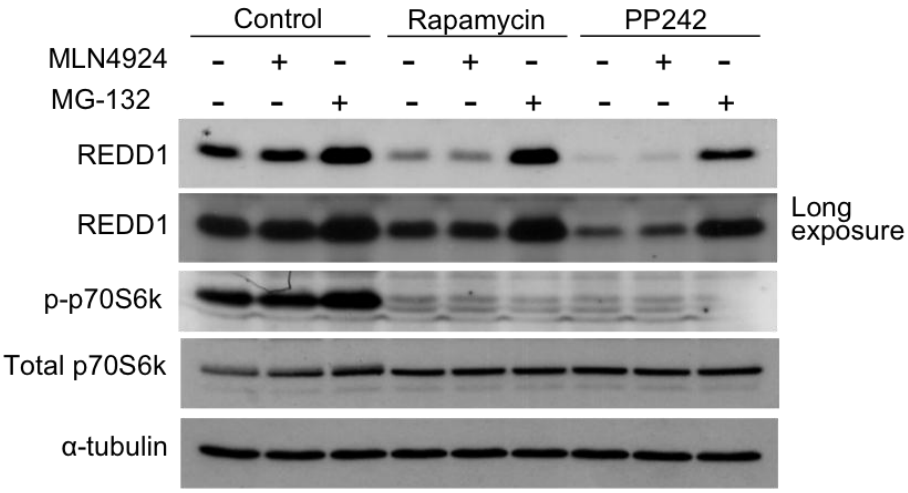


Figure 71. *REDD1 degradation induced by mTORC1 inhibition was reversed with proteasome inhibitor MG-132 treatment.* Co-treatment of rapamycin (20 nM) or PP242 (2 μM) with MLN4924 (2 μM) or MG-132 (20 μM) were performed in HEK293 cells as indicated for 4 hours followed by cell lysis.

When comparing the effects of rapamycin and PP242, it was noted that the decrease in REDD1 protein levels upon PP242 treatment was much stronger compared to rapamycin treatment for both 4 and 24 hours (Figure 72). Yet, both PP242 and rapamycin inhibited mTORC1 activity markedly, as shown by the absence of p70S6K phosphorylation. Furthermore, it was found that a pronounced effect of PP242 treatment on REDD1 protein stability could only be observed with endogenous, but not transfected REDD1 (Figure 72). These results suggested that PP242 exerts additional effects on REDD1. The differential effect of PP242 on endogenous and transfected REDD1 could be explained by the fact that their transcription is driven from different promoters (endogenous REDD1 promoter versus CMV promoter). We therefore hypothesized that PP242 may regulate REDD1 transcription, in addition to regulating REDD1 protein stability.

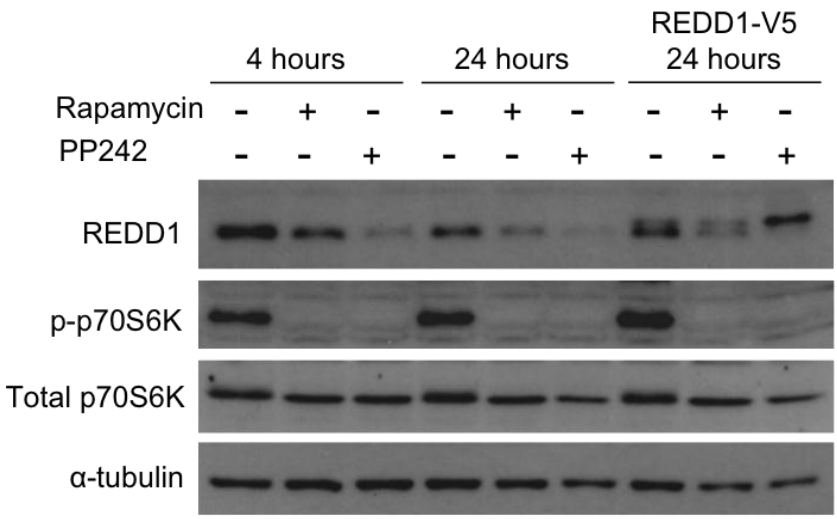


Figure 72. *mTORC1 inhibition with rapamycin and PP242 treatment increases REDD1 degradation.* HEK392 cells were transfected with 0.4 μg REDD1-V5 pcDNA3 followed by treatment with 20 nM rapamycin or 2 μM PP242 for the indicated time before cell lysis.

To test the effect of PP242 on REDD1 transcription, I performed reporter assays using two different REDD1 promoter constructs containing two fragments of the REDD1 promoter, a 0.6 kb fragment immediately upstream of exon 1 and a 3 kb fragment further upstream (Figure 73A). If PP242 treatment affects REDD1 expression at the promoter region, we would expect to see a reduction in luciferase signal intensity. When testing the effect of rapamycin, it was observed that both REDD1 promoter constructs showed reduced promoter activity (Figure 73B). This suggests that rapamycin may regulate the entire 3.6 kb REDD1 promoter region. However, a more likely interpretation would be that mTORC1 inhibition with rapamycin exerts a non-specific effect on REDD1 as it may be unlikely that rapamycin regulates REDD1 expression at two different promoter regions. On the other hand, similar to rapamycin treatment, treatment of PP242 led to a decrease in promoter activity for the 0.6 kb REDD1 promoter construct (Figure 73B). However, PP242 treatment resulted in a much more dramatic decrease in REDD1 promoter activity in the 3 kb REDD1 promoter construct (Figure 73B). This result suggests that PP242 has a specific inhibitory effect on REDD1 transcription mediated through the 3 kb promoter region upstream of exon 1. Hence, this may be the reason why PP242 treatment led only to a small decrease in the CMV promoter driven REDD1-V5 pcDNA3 expression as the effect of PP242 requires REDD1 promoter region. Thus, in addition to mTORC1 inhibition, PP242 may have additional effect on REDD1 promoter activity. These results suggest that PP242 affects REDD1 expression in two ways, ie by decreasing REDD1 half-life and regulating REDD1 promoter region.

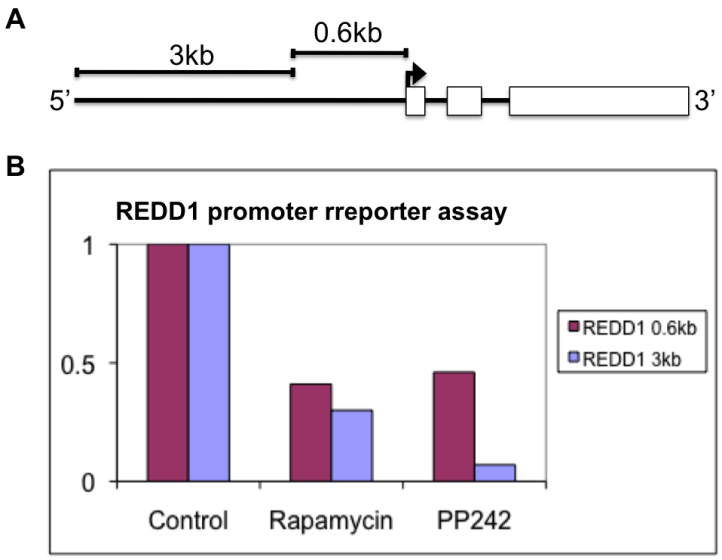


Figure 73. *PP242 affects REDD1 promoter*. (**A**) Schematic diagram to show the location of the 3 kb and 0.6 kb REDD1 promoter in the luciferase reporter constructs used. The three white boxes are the three exons encoding REDD1. This diagram was adapted from Ellisen et al., 2002. (**B**) HEK293 cells were transfected with 3 kb REDD1 promoter pGL-3 basic (0.25 μg) or 0.6 kb REDD1 promoter pGL-3 basic (0.25 μg) constructs for 30 hours. Six hours after transfection, cells were treated with rapamycin (40 nM) or PP242 (2 μM) treatment for 24 hours followed by reporter assay analysis using Steady-Glo luciferase reporter assay system.

4.2.3 REDD1 is ubiquitinated

To characterize how REDD1 is degraded, I first tested if REDD1 is ubiquitinated *in vitro*. Polyubiquitination is an important post-translational modification that targets proteins for degradation through the proteasomal pathway. It can be observed that REDD1 is polyubiquitinated *in vitro* as shown by the high molecular weight smear in lane 4 (Figure 74). This suggests that REDD1 degradation is regulated by the ubiquitin-dependent proteasomal degradation pathway.

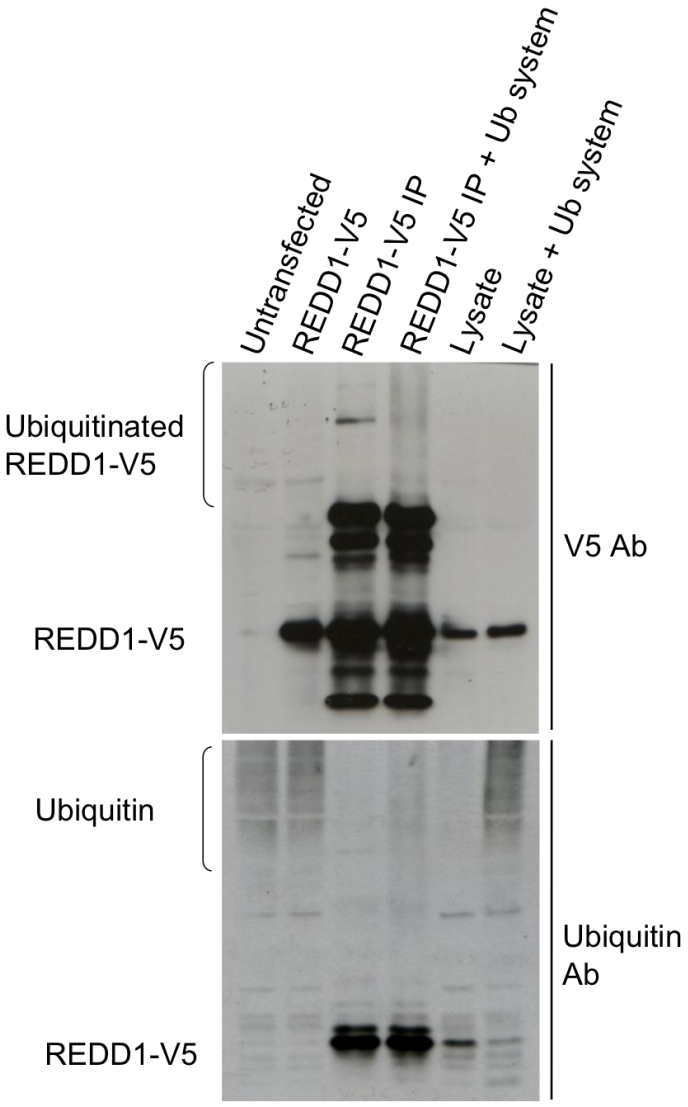


Figure 74. *REDD1 is ubiquitinated.* HEK293 cells were transfected with 1.5 μg REDD1-V5 pcDNA3 in 60 mm tissue culture plates for 3 days, followed by cell lysis and immunoprecipitation with V5 antibody. In vitro ubiquitination assay was performed as described in Materials and Methods.

4.2.4 Lysine residues are not involved in REDD1 ubiquitination

Ubiquitination of protein occurs with the attachment of ubiquitin to lysine residues on substrate proteins. Hence, to understand the ubiquitination and degradation of REDD1, I mutated lysine residues on REDD1 to alanine. There are a total of 6 lysine residues on REDD1 (K129, K155, K188, K218, K219 and K220) (Figure 75A). Mutation of different combinations of lysine residues to alanine did not stabilize REDD1 as treatment with MG-132 resulted in a similar accumulation of wild type REDD1 and REDD1 mutant proteins (Figure 75B). Importantly, when all 6 lysine residues were mutated to alanines in the K(0) mutant, REDD1 protein remained unstable (Figure 75B). Treatment with MG-132 led to an increase in REDD1 K(0) protein levels (Figure 75B) and increased in the half-life of REDD1 K(0) mutants (Figure 76). These results showed that the REDD1 K(0) mutant is degraded by the 26S proteasome even in the absence of lysine residues. Furthermore, the half-life of the K(0) mutant was not significantly longer compared to controls (Figure 77). Taken together, these results suggest that REDD1 ubiquitination is independent of lysine residues. Several proteins including p21, MyoD and Id2 proteins have been reported to be degraded via N-terminal ubiquitination whereby the first ubiquitin is attached to the α-NH_2 group of the N-terminal residue (Bloom et al., 2003; Breitschopf et al., 1998; Fajerman, Schwartz and Ciechanover, 2004). It would be interesting to test if REDD1 is ubiquitinated in the same manner.

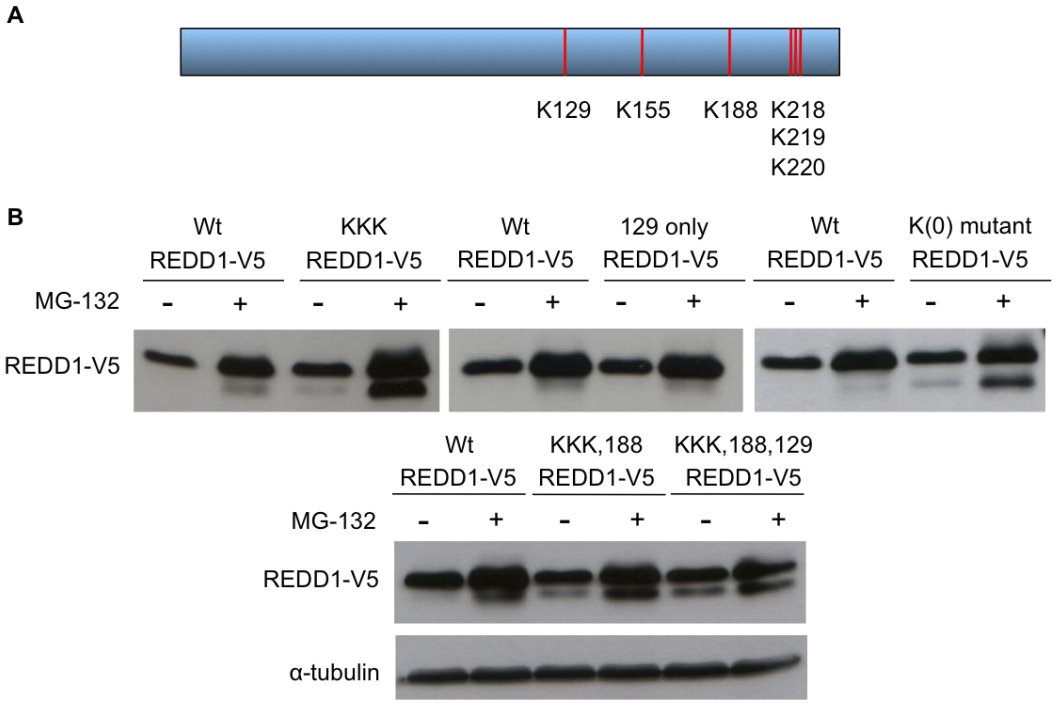


Figure 75. *REDD1 lysine mutants are degraded by 26S proteasome*. (**A**) Schematic diagram to show the lysine residues on REDD1. (**B**) HEK293 cells were transfected with 0.4 μg REDD1 wild-type (wt) or REDD1 lysine mutants (with lysine residues mutated to alanine) for 3 days followed by MG-132 (20 μM) treatment for 6 hours and cell lysis. KKK is a combination of K218, K219 and K220. K(0) is a lysine-less mutant with all 6 lysine residues mutated to alanine.

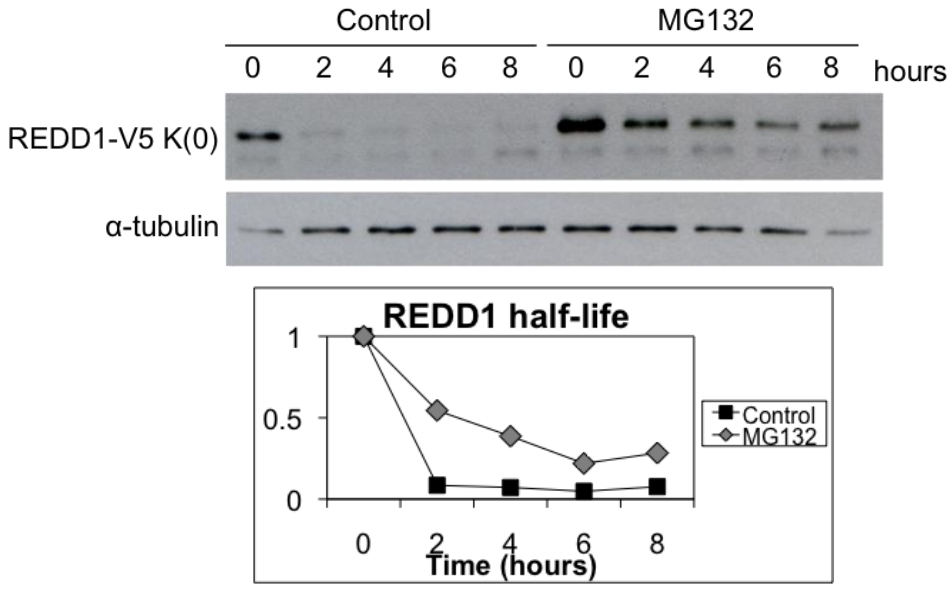


Figure 76. *REDD1 protein stability is independent of its lysine residues.* HEK293 cells were transfected with 0.4 μg REDD1 K(0) mutant pcDNA3 for 3 days followed by MG-132 (20 μM) treatment for 4 hours. After that, cycloheximide (40 μM) treatment was performed and cells were lysed at indicated time points.

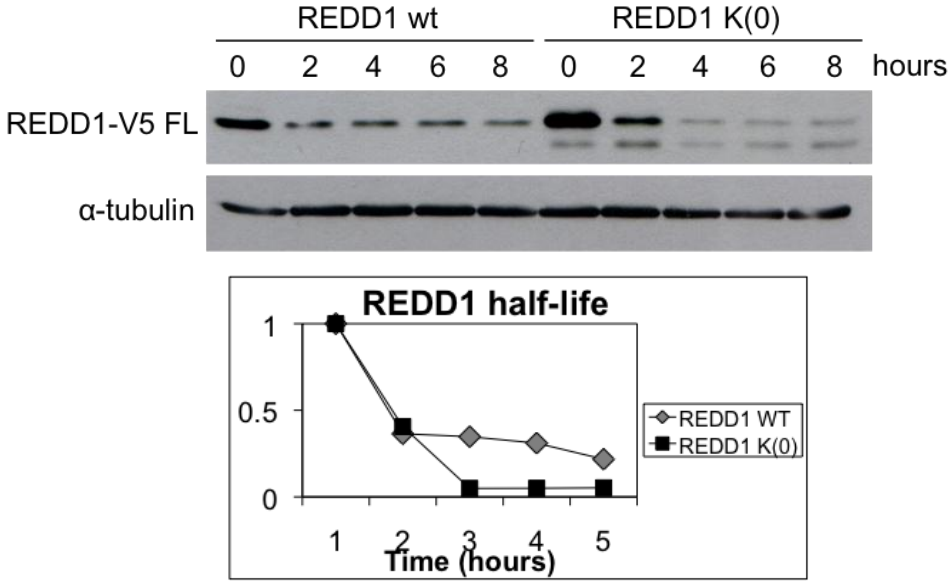


Figure 77. *REDD1 protein stability is independent of its lysine residues.* HEK293 cells were transfected with 0.4 μg REDD1 wild type or REDD1 K(0) mutant pcDNA3 for 3 days followed by cycloheximide (40 μM) treatment and cell lysis at indicated time points.

4.2.5 REDD1 truncation mutants do not reveal any degradation motifs or sequences

Next, to characterize important degradation motifs or sequences in REDD1, I performed deletion analyses. First I aligned the REDD1 protein sequence with that of its ortholog, REDD2 (Figure 78). REDD1 and REDD2 are both inhibitors of mTORC1 and share about 50% sequence identity to each other but show little homology to other known proteins (Brugarolas et al., 2004; Corradetti et al., 2004). As REDD1 and REDD2 may be regulated in a similar manner, I truncated the REDD1 protein based on regions with the highest similarities with REDD2. Truncations of the C-terminal ends of REDD1 did not stabilize REDD1 (Figure 79). Similarly, when the N-terminal end was truncated, REDD1 protein remained unstable as indicated by the accumulation of truncated REDD1 with MG-132 treatment (Figure 80). These results suggest that REDD1 degradation motifs could be located at both the C- and N-terminal ends.

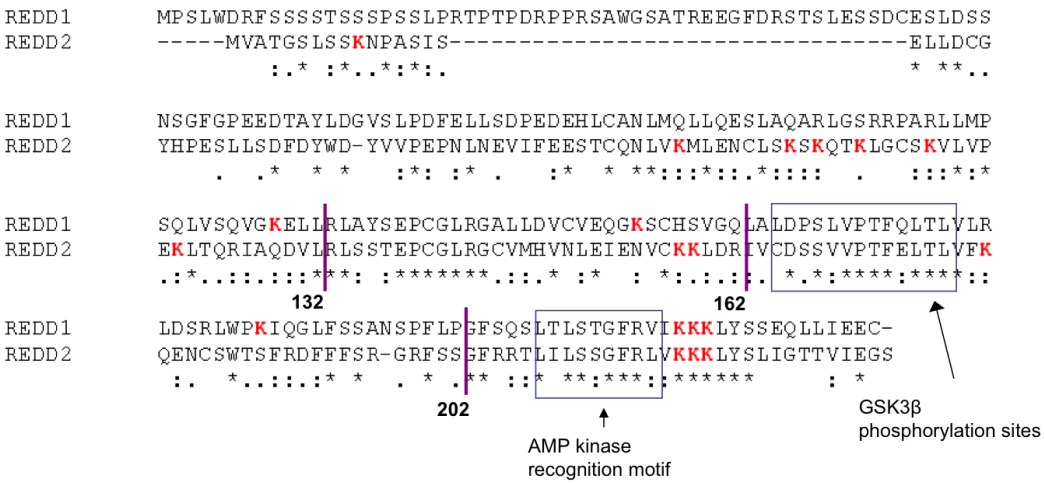


Figure 78. *Alignment of REDD1 and REDD2 protein sequences.* Boxed sequences indicate GSK3β phosphorylation sites and AMP kinase recognition motif. Vertical lines indicate truncation sites.

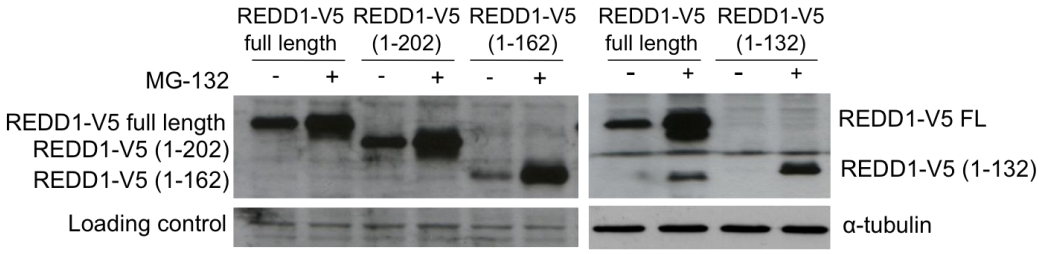


Figure 79. *REDD1 C-terminal end truncations did not stabilize REDD1 protein.* HEK293 cells were transfected with 0.4 μg REDD1 (full length or truncated mutants) for 3 days followed by MG-132 (20 μM) treatment for 6 hours and cell lysis. REDD1-V5 (1-202) mutant has its AMPK motif truncated whereas both AMPK motif and GSK3β sites are truncated in the REDD1-V5 (1-162) mutant. REDD1-V5 (1-132) is the shortest mutant with only one lysine residue in the protein.

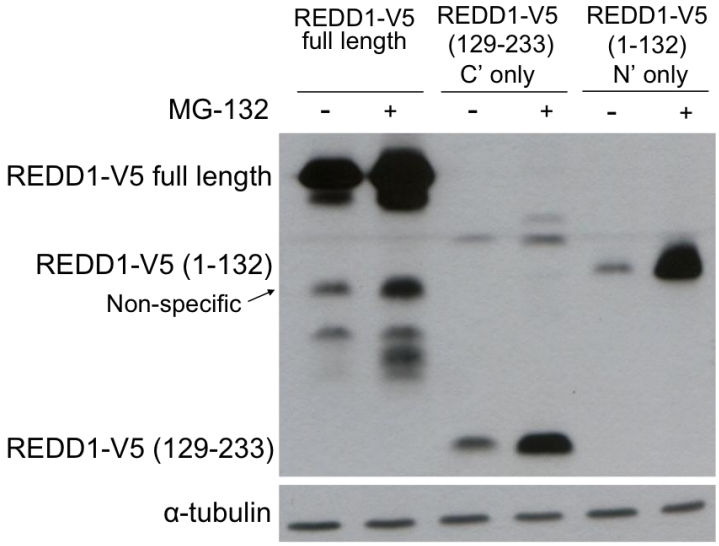


Figure 80. *REDD1 N- and C-terminal end truncations did not stabilize REDD1 protein.* HEK293 cells were transfected with 0.4 μg REDD1 (full length or truncated mutants) for 3 days followed by MG-132 (20 μM) treatment for 6 hours and cell lysis. REDD1-V5 (129-233) mutant is the C-terminal section that was truncated from the REDD1-V5 (1-132) mutant.

4.2.6 iTRAQ analysis of REDD1 did not reveal potential binding proteins that could mediate REDD1 ubiquitination and degradation

iTRAQ (isobaric tags for relative and absolute quantitation) is a mass spectrometry based approach for relative quantification of proteins. This technique allows quantitation of differences in protein amount between different samples by tagging peptides with iTRAQ isotope followed by peptide separation with 2D-liquid chromatography. In my project, a 4-plex analysis was used and 4 different iTRAQ reagents were used to label the 4 samples. Identical peptides obtained from the different samples can be identified based on the iTRAQ isotopes labeling and quantitation of the amount is analyzed from the signal intensity ratios of labels. In the experiment, I used lysate from HEK293 cells transfected with FLAG-REDD1 and untransfected HEK293 cells as control. The FLAG-REDD1 protein was immunoprecipitated using FLAG agarose. After that, FLAG-REDD1 protein complexes were eluted with the addition of acetic acid followed by neutralizing buffer for iTRAQ analysis (see Materials and Methods for details). The samples were then sent for iTRAQ analysis by a commercial lab at UVic Genome BC Proteomics Centre.

The iTRAQ analysis revealed a significant number of potential REDD1 binding proteins that were enriched in the FLAG-REDD1 transfected compared to the untransfected sample. The potential REDD1 interacting proteins included two proteins that are known to be involved in the ubiquitin-proteasome pathway, namely Cullin 2 and Cullin-associated and neddylation-

dissociated 1 (CAND1) proteins. Cullin 2 forms an E3 ligase that utilizes Elongin B and C as adaptor proteins to recruit substrate receptors. Hence, to test if Cullin 2 plays a role in the regulation of REDD1 protein stability, siRNA mediated silencing of the Cullin 2 adaptor protein, Elongin C, was performed. HIF1α, a substrate of Cullin 2, is stabilizes with knockdown of Elongin C (Figure 81). However, no significant increase in REDD1 protein level was observed (Figure 81). This result indicates that Cullin 2 is not involved in the regulation of REDD1 protein stability.

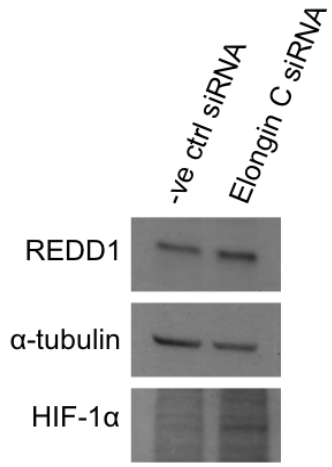


Figure 81. *REDD1 stability is independent of Elongin C and Cul2 E3 Ligase.* HEK293 cells were transfected with 20 nM control or Elongin C siRNAs for 3 days before cell lysis.

CAND1 is a regulatory protein that interferes with the assembly of the SKP1-Cul1-F-box (SCF) and other Cullin E3 ubiquitin ligase complexes and hence regulates the activity of Cullin E3 ligases. Therefore, we were interested to find out if REDD1 is regulated by CAND1. Knockdown of CAND1 led to a decrease in REDD1 protein levels (Figure 82). However, the decrease in REDD1 protein levels was not correlated with CAND1 expression as the first siRNA, which was more effective in silencing, did not decrease REDD1 protein levels as much as the second CAND1 siRNA (Figure 82).

This result suggests that the regulation of REDD1 protein stability is independent of CAND1.

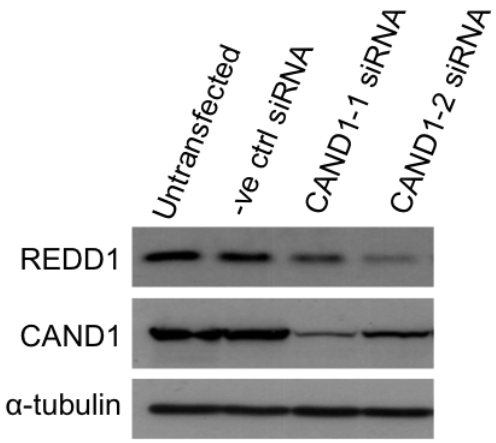


Figure 82. *REDD1 protein stability is independent of CAND1.* HEK293 cells were transfected with 20 nM control or CAND1 siRNAs for 3 days before cell lysis.

4.2.7 Regulation of REDD1 by the HUWE1 E3 ubiquitin ligase

To identify the E3 ligase involved in the REDD1 degradation upon mTORC1 inhibition, I tested a number of candidates. Interestingly, while performing experiments to study the regulation of CDC6 protein stability (see 5.2.5), I observed that the knockdown of the E3 ubiquitin ligase HUWE1 caused an increase in REDD1 steady state levels (Figure 83 and 84). This effect was not due to changes in mTORC1 activity, as indicated by the unaltered levels of p70S6K phosphorylation (Figure 84). An increase in the steady state level of the known HUWE1 substrate Mcl-1 served as a positive control (Figure 84). I also observed that when mTORC1 activity was inhibited with rapamycin or PP242, REDD1 protein levels were higher in cells transfected with HUWE1 siRNA compared to control cells (Figure 84). However, when HUWE1 was immunoprecipitated, REDD1 protein was not detected indicating that the two proteins do not interact (Figure 85). As the enrichment of HUWE1 in the immunoprecipitate was very weak, a reciprocal IP was performed whereby REDD1 was immunoprecipitated. Again, no interaction was observed between HUWE1 and REDD1 (Figure 86). Furthermore, the increase in REDD1 protein levels upon HUWE1 knockdown was similar in mTOR inhibiter treated and untreated cells (Figure 84). Based on these results, I concluded that HUWE1 is not involved in the degradation of REDD1 upon inhibition of mTORC1.

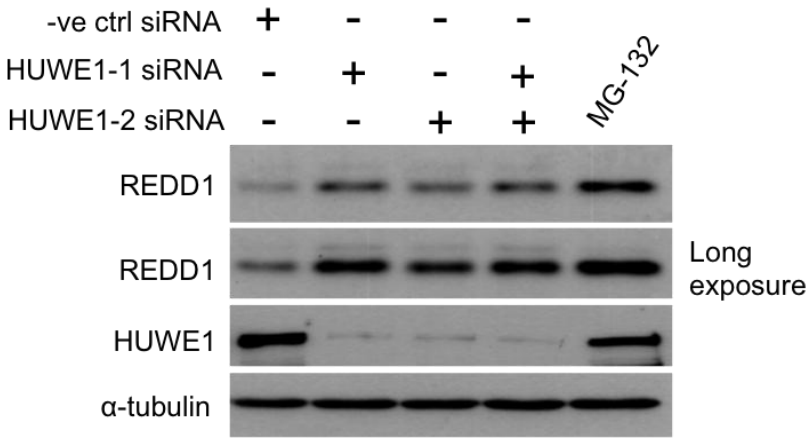


Figure 83. *REDD1 protein stability is regulated by HUWE1 ubiquitin ligase.* HEK293 cells were transfected with 20 nM negative control siRNA or HUWE1-1 or -2 siRNAs in the indicated combinations for 3 days and MG-132 (20 μM) was added to cells for 4 hours followed by cell lysis.

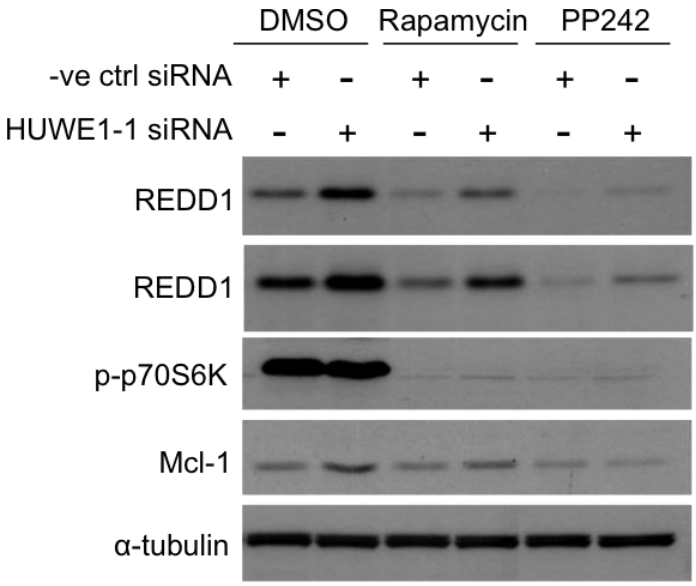


Figure 84. *REDD1 protein stability is regulated by HUWE1 ubiquitin ligase.* HEK293 cells were transfected with 20 nM control or HUWE1 siRNAs for 3 days and rapamycin (40 nM) or PP242 (2 μM) were added to cells for 8 hours followed by cell lysis.

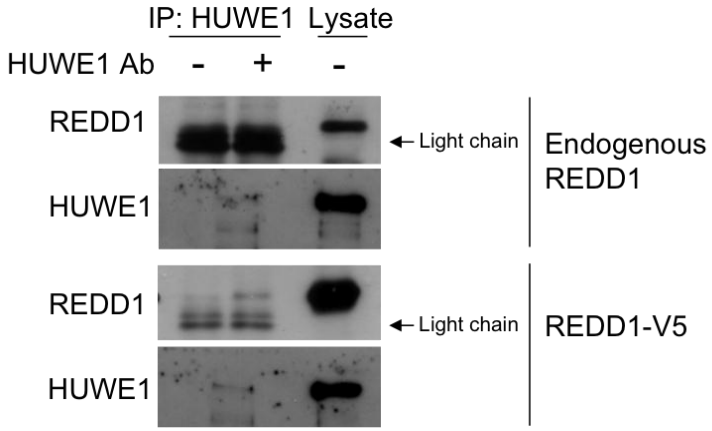


Figure 85. *REDD1 does not interact with HUWE1.* HEK293 cells were grown in 60 mm tissue culture plates for 4 days followed by cell lysis and immunoprecipitation with HUWE1 antibody as described in Materials and Methods.

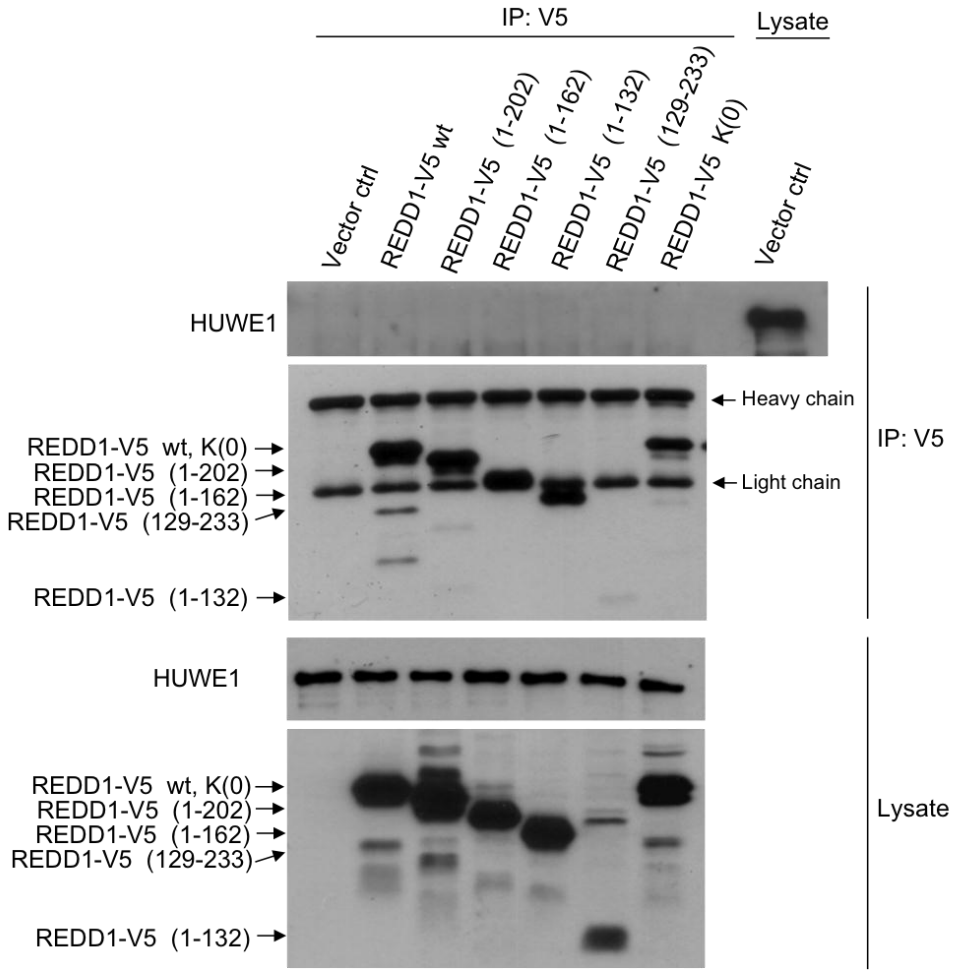


Figure 86. *HUWE1 does not interact with REDD1.* HEK293 cells were transfected with the full-length and truncation mutants of REDD1 pcDNA3 (1.5 μg for all REDD1-V5 pcDNA3 except for REDD1-V5 (129-233) pcDNA3 at 2 μg) in 60 mm tissue culture plates for 3 days followed by cell lysis and immunoprecipitation with V5 antibody as described in Materials and Methods.

Given that HUWE1 knockdown caused an increase in the steady state levels of the REDD1 protein, I next tested whether the HUWE1 is involved in the basal turnover of REDD1. I measured the protein half-life using cycloheximide chase with two different HUWE1 siRNAs. However, with both siRNAs there was no significant change in the half-life of REDD1 in cells with HUWE1 knockdown compared to controls (Figures 87 A and B). This suggests that HUWE1 regulates REDD1 via a mechanism that is independent of protein degradation.

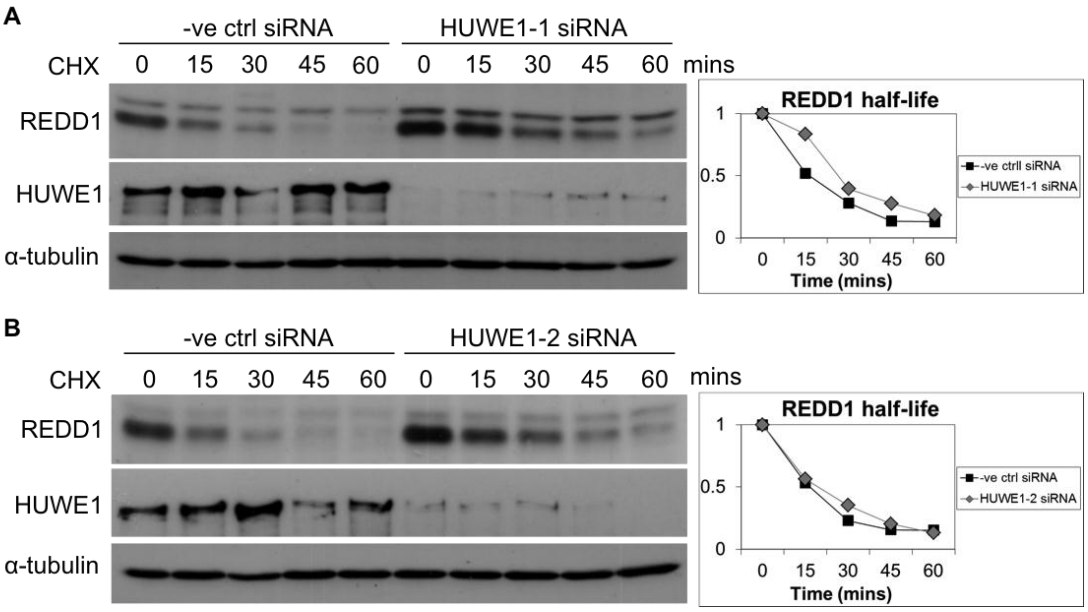


Figure 87. *REDD1 protein stability is not regulated by HUWE1 ubiquitin ligase.* (**A**,**B**) HEK293 cells were transfected with 20 nM negative control or HUWE1-1 (**A**) or -2 (**B**) siRNAs for 3 days followed by cycloheximide (40 μM) treatment and cell lysis at indicated time points.

4.2.8 REDD1 protein stability is not regulated by Cullin E3 ubiquitin ligases

Cullin E3 ubiquitin ligases constitute approximately half of the cellular E3 ligases. Furthermore, Cullin 4 E3 ligase has previously been implicated in regulating the basal turnover of REDD1. I therefore determined whether the family of Cullin E3 ligases is involved in REDD1 degradation upon mTORC1 inhibition. This was tested using MLN4924, an inhibitor of Nedd8 E1 activating enzyme which inhibits all cullin E3 ligases (Soucy et al., 2009). I observed that MLN4924 did not reverse the degradation of REDD1 upon treatment of cells with the mTORC1 inhibitors rapamycin or PP242 (Figure 71). Furthermore, unlike the proteasome inhibitor MG-132, MLN4924 also had no effect on the basal protein levels of REDD1 (Figure 71). This result was surprising given the previous report of Cullin 4 dependent regulation of REDD1 protein stability [11]. Hence, in further studies, I characterized the involvement of Cullin E3 ligases in the regulation of REDD1 stability in detail.

As an alternative approach to using MLN4924, I utilized a genetic approach by expressing the dominant negative C111S mutant of the Ubc12 Nedd8 E2 conjugating enzyme. A HEK293 cell line in which dominant-negative Ubc12 (dnUbc12) was stably transfected and expressed under control of a tetracycline-inducible promoter was used (Chew et al., 2007). The dnUbc12 cells have a defective neddylation pathway and this leads to the inactivation of the Cullin RING E3 ubiquitin ligases. Similar to MLN4924 treatment, induction of dnUbc12 did not have any significant effect on both

endogenous and transfected REDD1 proteins (Figures 88 and 89). Overexpression of dnUbc12 caused a dramatic increase in the known Cullin E3 ligase substrate p27 which was even more pronounced compared to the proteasome inhibitor MG-132 (Figure 89). This shows that REDD1 steady state level is not regulated by Cullin ubiquitin ligases. I subsequently performed experiments to determine the effect of pharmacological and genetic inhibition of Cullin E3 ligases on the REDD1 protein half-life. I observed no difference in the half-life of both endogenous and transfected REDD1 when HEK293 cells were treated with MLN4924 (Figures 90 A and B) or when dnUbc12 expression was induced with tetracycline (Figure 91). In contrast, as expected, p27 was stabilized with both inhibitors. These results indicate that REDD1 stability is not regulated by Cullin RING E3 ubiquitin ligases.

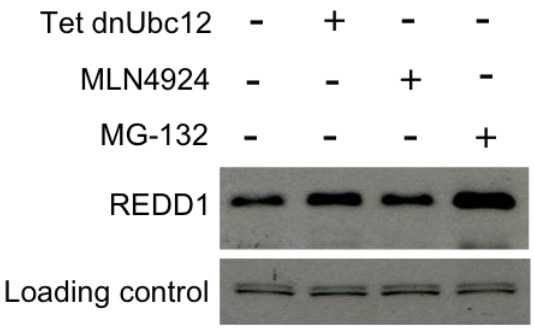


Figure 88. *REDD1 is not regulated by Cullin E3 Ubiquitin ligases.* HEK293 cells stably expressing tetracycline inducible dnUbc12-HA were induced with 1 μg/ml tetracycline for 24 hours or treated with 3 μM MLN4924 or 20 μM MG-132 for 8 hours followed by cell lysis.

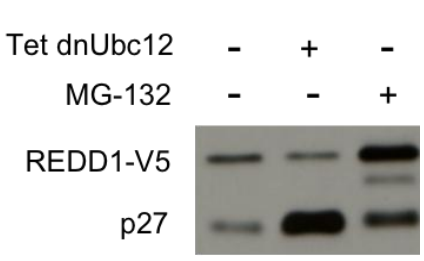


Figure 89. *REDD1 is not regulated by Cullin E3 Ubiquitin ligases.* HEK293 cells stably expressing tetracycline inducible dnUbc12-HA were transfected with REDD1-V5 pcDNA3 (0.3 μg) and induced with 1 μg/ml tetracycline for 24 hours or treated with 20 μM MG-132 for 8 hours followed by cell lysis

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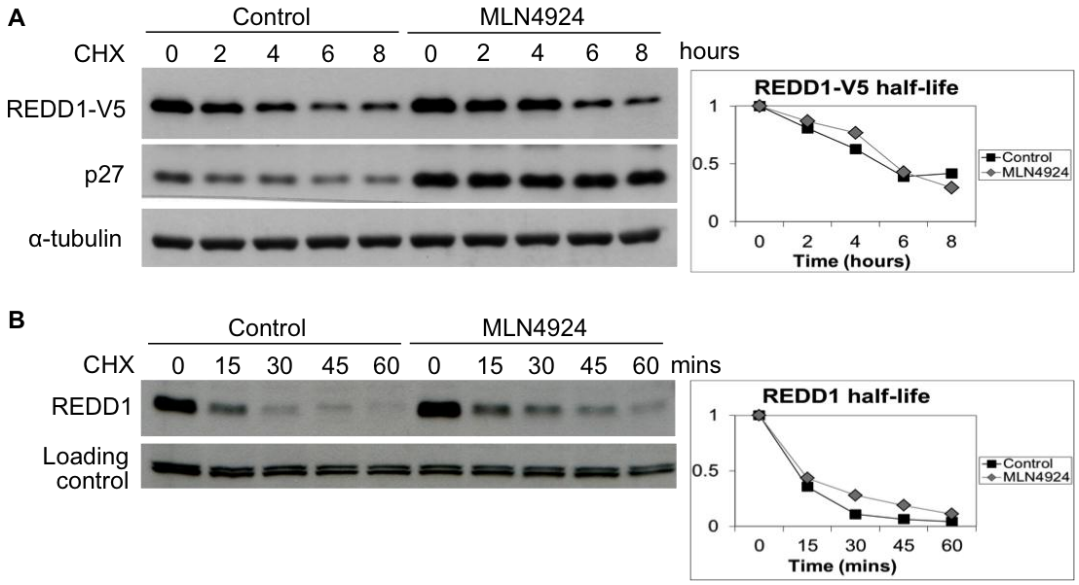


Figure 90. *REDD1 is not regulated by Cullin E3 Ubiquitin ligases.* (**A**,**B**) Untransfected HEK293 (**A**) or HEK293 transfected with REDD1-V5 pcDNA3 (0.3 μg) (**B**) were pre-treated with 3 μM MLN4924 followed by cycloheximide (40 μM) treatment and cell lysis at the indicated time points.

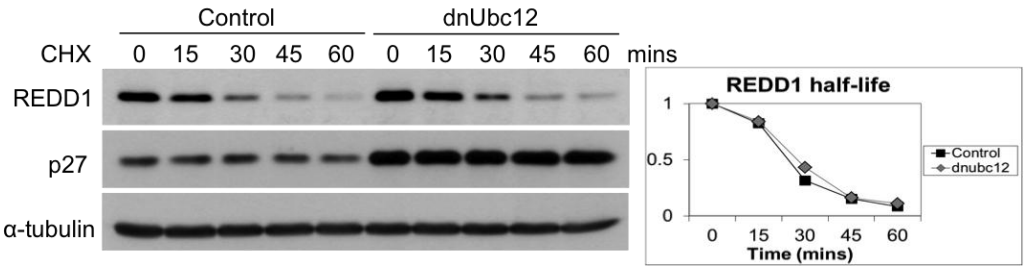


Figure 91. *REDD1 is not regulated by Cullin E3 Ubiquitin ligases.* HEK293 cells stably expressing tetracycline inducible dnUbc12-HA were induced with 1 μg/ml tetracycline for 24 hours followed by cycloheximide (40 μM) treatment and cell lysis at the indicated time points.

4.2.9 Both Cul4a and phosphorylation of REDD1 by GSK3β are not involved in basal REDD1 protein turnover

It has been reported that REDD1 degradation is mediated by the Cul4a E3 ubiquitin ligase complex and that this in turn is dependent on REDD1 phosphorylation at Thr23 and Thr25 sites by GSK3β. Therefore, to confirm this, I overexpressed a tetracycline inducible dominant negative Cul4a-V5 plasmid (dnCul4a, amino acids 1–439) in HEK293 cells (Chew and Hagen, 2007). dnCul4a lacks the C-terminal half of the Cul4a protein. Hence, dnCul4a is able to interact with substrate proteins, but unable to recruit the ubiquitin-charged E2 ubiquitin conjugating enzyme. As a result, dnCul4a sequesters substrates in inactive complexes and inhibits their ubiquitination and degradation. If REDD1 is a substrate of Cul4a, an increase in REDD1 protein expression would be expected upon induction of dnCul4a. However, it was observed that REDD1 levels were not affected by dnCul4a induction (Figure 92). To confirm that dnCul4a overexpression and induction was able to inhibit Cul4a function, I demonstrated that dnCul4a markedly reduced the neddylation levels of coexpressed full length Cul4a (Figure 92). Nedd8 modification (neddylation) of cullin proteins is required for the function of all cullin E3 ligases. Cullin neddylation is dependent on the binding of substrate proteins (Chew and Hagen, 2007). Hence, the markedly reduced neddylation of full length Cul4a indicates that substrate binding is inhibited. The dnCul4a experiment suggests that REDD1 protein stability is not regulated by Cul4a.

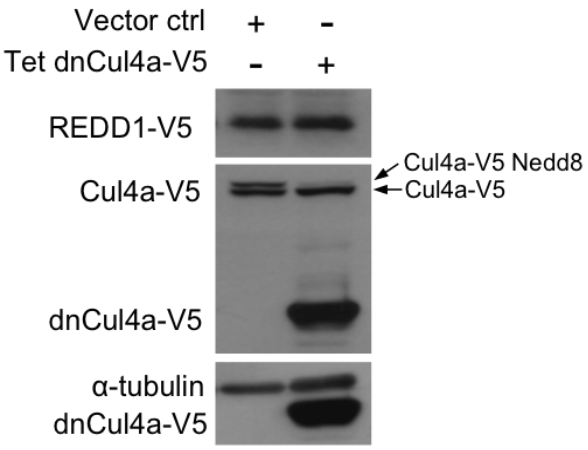


Figure 92. *REDD1 is not degraded by Cul4a E3 Ubiquitin Ligase.* HEK293 cells were transfected with empty vector or tetracycline inducible dnCul4a-V5 pcDNA4/TO (1 μg) and Cul4A-V5 pcDNA3 (0.03 μg) followed by tetracycline (1 μg/ml) induction for 24 hours and cell lysis.

I also used siRNA mediated silencing of Cul4a to confirm the results obtained with the dnCul4a cell line. I used two different siRNA oligonucleotides and Cul4a siRNA-2 proved more effective (Figure 93). I observed that knockdown of Cul4a did not affect REDD1 expression (Figure 94). As expected, the level of the Cul4a-DDB1-DDB2 substrate p27 level was markedly increased with Cul4a siRNA-2. In conclusion, Cul4a is unlikely to be involved in the regulation of REDD1 protein stability.

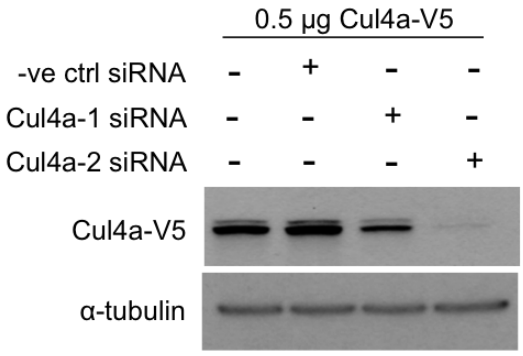


Figure 93. *Cul4a siRNA decreased Cul4a expression.* HEK293 cells were transfected with 0.5 μg Cul4a-V5 pcDNA3 for 15 hours followed by transfection of 20 nM control or Cul4a siRNAs to determine siRNAs efficiency.

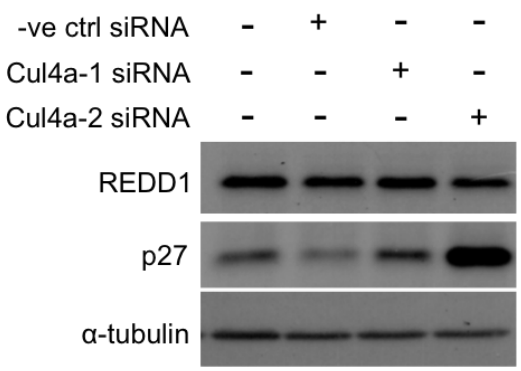


Figure 94. *REDD1 is not degraded by Cul4a E3 Ubiquitin Ligase.* HEK293 cells were transfected with 20 nM control or Cul4a siRNAs for 3 days followed by cell lysis.

It has been reported that GSK3β phosphorylates REDD1 at residues Thr23 and Thr25, resulting in REDD1 recruitment to the Cullin 4a-β-Trcp E3 ligase complex. To confirm involvement of these threonine residues in the regulation of REDD1 protein stability, I mutated both Thr23 and Thr25 to alanines. These mutations would be expected to stabilize REDD1 protein. However, I did not observe any significant difference in the stability of REDD1 mutant compared to controls (Figure 95). Addition of proteasome inhibitor caused a similar increase in wild type and T23A/T25A mutant REDD1 protein levels, suggesting that the phosphorylation sites are not important for the regulation of REDD1 protein stability. Furthermore, mutation of the threonine 23 and 25 residues to aspartate to mimic phosphorylation also did not have any effect on REDD1 stability (Figure 95).

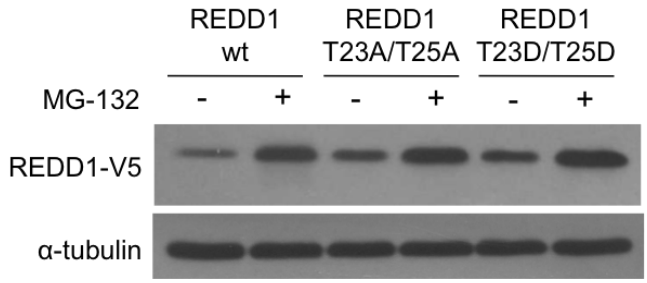


Figure 95. *REDD1 is not degraded via phosphorylation by GSK3β at Thr23 and Thr25.* REDD1-V5 pcDNA3 wild type, T23A T25A or T23D T25D plasmids (0.4 μg) were transfected in HEK293 cells for 3 days and treated with 20 μM MG-132 for 6 hours followed by cell lysis.

To further confirm that GSK3β does not regulate REDD1 protein levels, two different inhibitors of GSK3β were added to HEK293 cells, lithium chloride and GSK3 inhibitor IX. Both inhibitors blocked the activity of GSK3β, as indicated by the marked decrease in the phosphorylation of the GSK3β substrate β-catenin (Figure 96). Inhibition of GSK3β is expected to

increase REDD1 stability. However, I did not observe any significant difference in REDD1 expression levels upon addition of the GSK3β inhibitors (Figure 96). Moreover, overexpression of GSK3β did not result in decreased REDD1 stability despite a dramatic increase in GSK3β expression compared to untransfected cells (Figure 97). I also tested whether incubation of REDD1 with recombinant GSK3 causes a band shift indicative of phosphorylation. Of note, Katiyar *et al*. reported that GSK3β dependent phosphorylation of REDD1 causes a faster migration in SDS gels (Katiyar et al., 2009). However, no change in electrophoretic mobility was detected, whereas the reported GSK3β substrate FRAT1 displayed the expected band shift (Figure 98). Taken together, my results suggest that both basal and mTORC1 regulated REDD1 degradation is mediated via a novel mechanism that does not involve Cullin E3 ligases and GSK3β dependent phosphorylation.

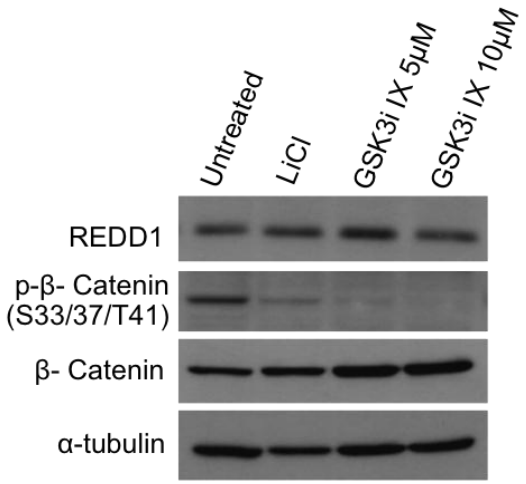


Figure 96. *REDD1 degradation is independent of GSK3β.* HEK293 cells were treated with 30 mM LiCl or GSK3 inhibitor IX (5 μM or 10 μM) for 20 hours followed by cell lysis.

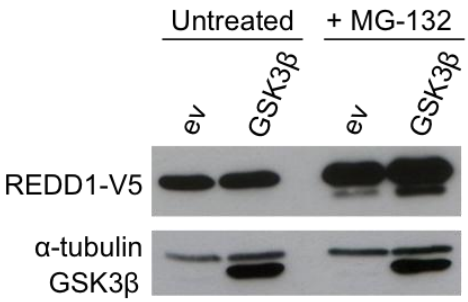


Figure 97. *REDD1 degradation is independent of GSK3β.* HEK293 cells were co-transfected with REDD1-V5 pcDNA3 (0.2 μg) and GSK3β pcDNA3 (0.3 μg) or empty pcDNA3 (0.3 μg) for 3 days followed by MG-132 (20 μM) treatment for 6 hours followed by cell lysis.

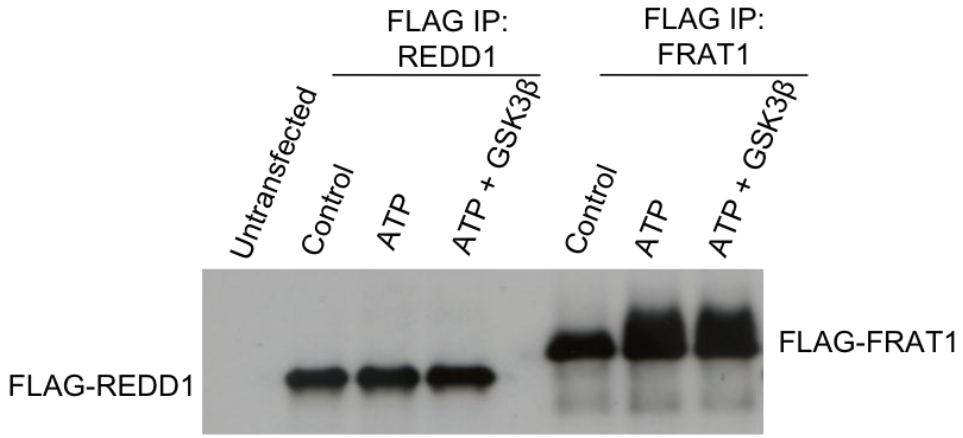


Figure 98. *REDD1 degradation is independent of GSK3β.* HEK293 cells were transfected with 3 μg FLAG-REDD1 or FLAG-FRAT1 for 3 days followed by cell lysis and FLAG immunoprecipitation. In vitro phosphorylation of REDD1 and FRAT1 was carried out as described in Materials and Methods.

4.3 Discussion

REDD1 is an important negative regulator of mTORC1 in response to stress. In this study, I have shown that mTORC1 in turn also regulates REDD1. mTORC1 dependent regulation of REDD1 is at the level of the REDD1 protein stability. Inhibition of mTORC1 using the small molecules rapamycin and PP242 or by overexpressing REDD1 led to reduced REDD1 protein stability and a consequent decrease in REDD1 expression. This mTORC1-REDD1 feedback loop would limit the inhibitory action of REDD1 on mTORC1 (Figure 99). The physiological significance of the mTORC1-REDD1 feedback mechanism is currently not clear and requires further study. However, my finding highlights that in addition to the extensive transcriptional control of REDD1, the REDD1 protein is also subject to posttranslational regulatory mechanisms.

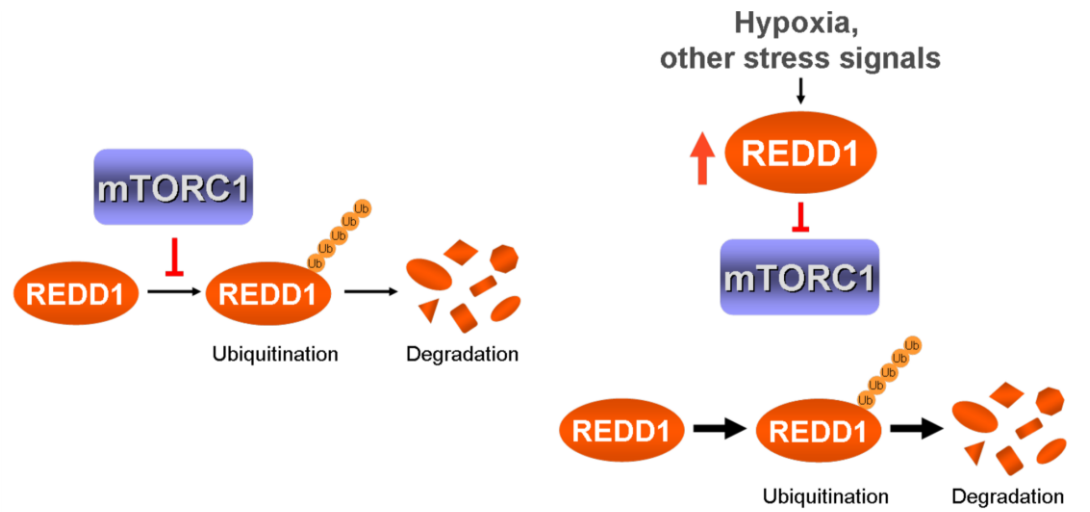


Figure 99. *The mTORC1-REDD1 limits the inhibitory action of REDD1 on mTORC1.* When mTORC1 is active, ubiquitination and proteasome dependent degradation of REDD1 is inhibited (left panel). Under conditions of hypoxia and other stress stimuli, REDD1 is transcriptionally induced, leading to mTORC1 inhibition. As a consequence, REDD1 ubiquitination and degradation is no longer restricted by mTORC1 (right panel). This mTORC1-REDD1 feedback mechanism limits the inhibitory action of REDD1.

In further experiments, I studied the mechanism through which REDD1 stability is regulated. I found that REDD1 degradation in response to mTORC1 inhibition is proteasome dependent. However, contrary to a previous report (Katiyar et al., 2009), I observed that mTORC1 protein stability is not controlled by the Cul4a E3 ubiquitin ligase. The discrepancy between the reported Cul4a dependent regulation of REDD1 and my results is unlikely to be due to differences in cell type as both studies used HEK293 cells. Furthermore, I was unable to confirm the role of GSK3β in targeting REDD1 for ubiquitination. Thus, I found that mutation of the reported GSK3β phosphorylation sites in REDD1 to alanine or aspartate did not affect REDD1 protein levels. Similarly, inhibition of GSK3β with two different inhibitors and overexpression of GSK3β were without effect on REDD1 protein expression. Furthermore, no band shift was observed upon incubation of REDD1 with GSK3β in vitro. It should be noted that mutation of serine or threonine residues to aspartate does not always have phospho-mimetic effects and phosphorylation events do not cause a lower mobility in every instance. Despite these limitations, when taking all of my findings together, there is strong evidence against a role of GSK3β in the regulation of REDD1 protein stability.

My results suggest that an alternative E3 ligase is responsible for both basal REDD1 ubiquitination and ubiquitination that is induced upon mTORC1 inhibition. Using pharmacological and genetic inhibitory approaches, I have ruled out any role for members of the Cullin RING E3 ligase family, which constitutes about half of all cellular E3 ubiquitin ligases. Although

knockdown of the HUWE1 E3 ubiquitin ligase resulted in increased REDD1 protein steady state levels, my further studies indicated that this effect is not due to an effect of HUWE1 on REDD1 protein stability. Hence, the identity of the E3 ligase that mediates basal REDD1 ubiquitination and ubiquitination upon mTORC1 inhibition is currently unknown. Identification of this E3 ligase is important as this ligase may be a unique drug target for specific inhibition of mTORC1.

5.0 Destabilization of CDC6 upon DNA damage is dependent on neddylation but independent of Cullin E3 ligases

5.1 Introduction

Cell division cycle 6 (CDC6) is an essential regulator of DNA replication in eukaryotic cells. CDC6 functions as an important component of the pre-replication complex (preRC). In addition to CDC6, the preRC is composed of the origin recognition complex (ORC) as well as Cdt1. The preRC is responsible for the stable loading of the minichromosome maintenance complex (MCM) onto origins of replication (Liang and Stillman, 1997; Donovan et al., 1997; Tanaka, Knapp and Nasmyth, 1997). The preRC is formed during the initiation step of DNA replication. The assembly of the preRC is one of the most highly regulated events during DNA replication. Tight regulation of preRC assembly is important to ensure that sufficient preRCs are formed during G1 to promote replication. Furthermore, it is also crucial that new preRCs are not assembled after cells enter S phase to prevent rereplication (Archambault et al., 2005; Vaziri et al., 2003; Melixetian et al., 2004; Zhu, Chen and Dutta, 2004). One important mechanism of preRC regulation is the cell cycle dependent control of CDC6 protein stability. Furthermore, DNA replication is inhibited in hypoxia and the downregulation of CDC6 as a result of ATR activation in hypoxia contributes to DNA replication checkpoint in hypoxic cells (Martin et al., 2012). Protein ubiquitination plays a critical role in the hypoxia response as it is one of the mechanisms that allow cells to respond quickly to changes in the environment.

Mammalian CDC6 is ubiquitinated during early G1 phase by APC^{Cdh1} in a manner dependent on both the D box and KEN box in the CDC6 protein (Petersen et al., 2000). The APC^{Cdh1} mediated CDC6 ubiquitination and degradation is important to ensure a timely licensing of replication origins during G1 phase and to prevent rereplication. There is also evidence for APC independent ubiquitination and degradation of the CDC6 protein. Of particular significance are reports of Cullin RING E3 ligase dependent CDC6 ubiquitination in budding yeast, fission yeast and possibly in mammalian cells (Chew et al., 2007; Lin et al., 2010; Soucy et al., 2009).

Cullin RING E3 ligases comprise the largest family of ubiquitin ligases. They consist of several subunits, including a cullin scaffold protein, substrate receptor proteins that bind to the cullin N-terminus and the RING domain protein Rbx1, which binds to the cullin C-terminus. Cullin RING E3 ligases function by recruiting the substrate via a specific substrate receptor and the ubiquitin charged E2 ubiquitin conjugating enzyme via Rbx1. The close proximity of the substrate protein and the ubiquitin charged E2 enzyme facilitates the transfer of ubiquitin molecules onto the substrate. Ubiquitinated substrates are then targeted for degradation by the 26S proteasome. To be active, Cullin E3 ubiquitin ligases require the modification of a conserved lysine residue in the cullin protein with the ubiquitin-like protein Nedd8. Neddylation of cullins is mediated via a cascade that involves the Nedd8 Activating Enzyme (NAE) and the Nedd8 conjugating enzyme Ubc12.

In *Saccharomyces cerevisiae*, CDC6 is ubiquitinated in S phase by the SKP1-Cul1-F-box (SCF) Cullin E3 ligase whereby CDC4 functions as cullin substrate receptor (Drury, Perkins and Diffley, 1997). In *Schizosaccharomyces pombe*, CDC18 (the CDC6 homolog) ubiquitination in S phase is mediated by the Cullin E3 ligase substrate receptor Pop1, which belongs to a WD-repeat family with highest homology to CDC4 (Kominami and Toda, 1997). It has been hypothesized that CDC6 may also be regulated by Cullin E3 ligases in mammalian cells (Lin et al., 2010).

In addition to being regulated during the cell cycle, CDC6 has also been reported to be degraded in response to DNA damage in mammalian cells (Duursma and Agami, 2005; Blanchard et al., 2002). CDC6 downregulation during DNA damage helps to prevent replication and allow DNA repair to take place. Regulation of CDC6 stability in response to DNA damage is mediated by the HUWE1 E3 ubiquitin ligase (Hall et al., 2007).

In this study, I determined the role of Cullin RING E3 ligases in the regulation of CDC6 protein stability in mammalian cells. I found that Cullin E3 ligases are not involved in CDC6 degradation. Stabilization of CDC6 upon inhibition of Cullin E3 ligases is a secondary consequence of a delay in cell cycle progression. In addition, I found that the DNA damage inducing agent mitomycin C causes CDC6 protein degradation. Mitomycin C induced CDC6 degradation is not mediated by the known regulators of CDC6 protein stability, HUWE1 or APC, and is also independent of Cullin E3 ligases. Interestingly, my results indicate that the neddylation pathway is required for mitomycin C induced CDC6 degradation.

5.2 Results

5.2.1 CDC6 protein is not markedly downregulated in hypoxia

The initial aim of this part of my project was to characterize the regulation of CDC6 stability in hypoxia as it has been reported that CDC6 is downreguated in hypoxia (Martin et al., 2012). However, my results showed that CDC6 protein level is not markedly downregulated in hypoxia (Figure 100A & B). This may be due to the different cell lines used in the study. Hence, the focus of my study was to determine how CDC6 stability is regulated.

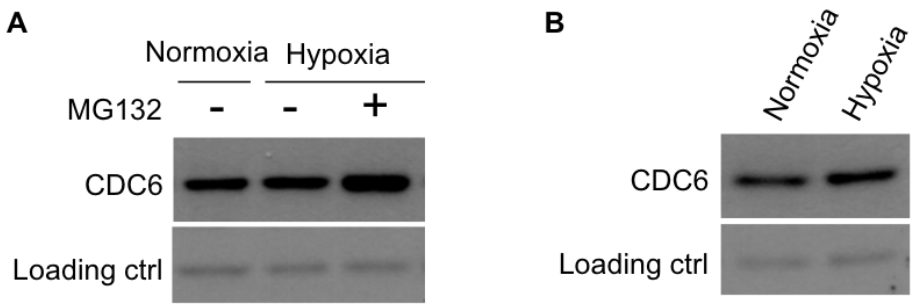


Figure 100. *CDC6 protein is not downregulated in hypoxia.* HEK293 (**A**) or HepG2 (**B**) cells were incubated in 1 % O_2 or treated with MG-132 (20 μM) for 4 hours followed by cell lysis.

5.2.2 CDC6 stability in mammalian cells is not regulated by Cul1 and Cul4 E3 Ligases

It has been shown that treatment with MLN4924 (an investigational inhibitor of Nedd8 activating enzyme (NAE) that inhibits the activity of all Cullin E3 ligases) increases CDC6 protein expression in mammalian cells (Soucy et al., 2009; Lin et al., 2010). Furthermore, inactivation of Cullin E3 ligases by the induction of dominant negative ubc12 (dnUbc12) in HEK293 cells stabilizes CDC6 protein (Chew et al., 2007). To confirm these results, I treated various human cell lines including HEK293, Hela, HCT116 and MCF7 cells with the proteasome inhibitor (MG-132) and the NAE inhibitor MLN4924. CDC6 expression was markedly increased when cells were treated with the proteasome inhibitor MG-132 (Figure 101). This indicates that CDC6 protein is degraded by the proteasome. To test if Cullin E3 ligases are involved, I used MLN4924. I observed that MLN4924 treatment markedly stabilized the known Cullin E3 ligase substrate p27, which served as a positive control (Figure 101). Similarly, the CDC6 protein expression level increased with MLN4924 treatment suggesting that CDC6 stability may be regulated by Cullin E3 ligases (Figure 101). However, the increase in CDC6 expression levels upon treatment with MLN4924 was only observed in HEK293, HCT116 and MCF7 cells but not in Hela cells (Figure 101).

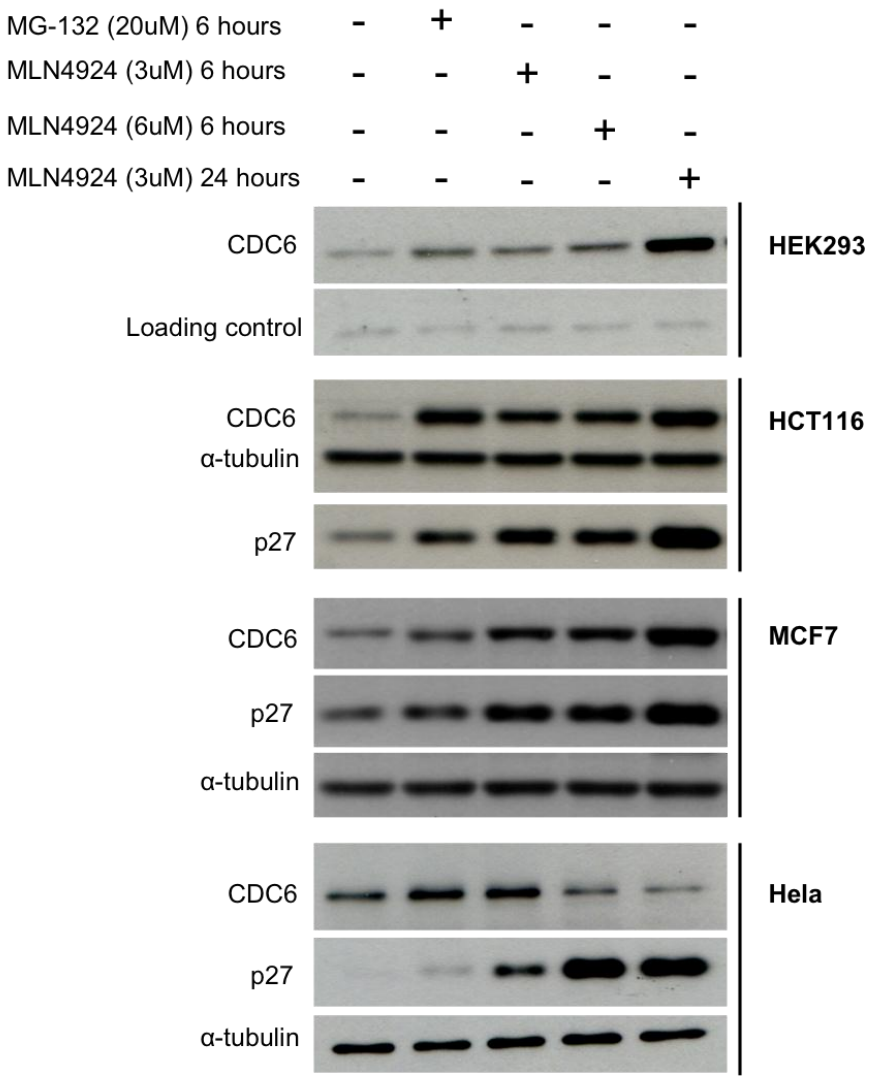


Figure 101. *MLN4924 treatment stabilizes CDC6 in multiple cell lines.* HEK293, Hela, HCT116 and MCF7 cells were treated with 3 μM or 6 μM MLN4924 and 20 μM MG-132 for the indicated duration followed by cell lysis.

In fission and budding yeast, Cul1 has been implicated in the degradation of CDC6. Furthermore, in mammalian cells it has been shown that Cdt1, a member of the preRC, can be ubiquitinated by Cul1 and Cul4 E3 ligases. I therefore tested whether the effect of MLN4924 is a consequence of the inhibition of Cul1 or Cul4 based E3 ligases. To this end, I utilized HEK293 cell lines stably expressing tetracycline inducible dominant negative mutants of Cullin E3 ligases (Chew et al., 2007; Chew and Hagen, 2007).

Induction of dominant negative cullin 1 (dnCul1-V5, amino acids 1-452) strongly stabilized the Cul1 E3 ligase substrate p27 (Figure 102). The increase in the p27 protein concentration is similar to that observed upon MLN4924 treatment. There was also a slight increase in both endogenous and transfected CDC6 expression levels (Figure 102). However, this increase was much less compared to the increase with MLN4924. Therefore, it is unlikely that CDC6 is a substrate of Cul1.

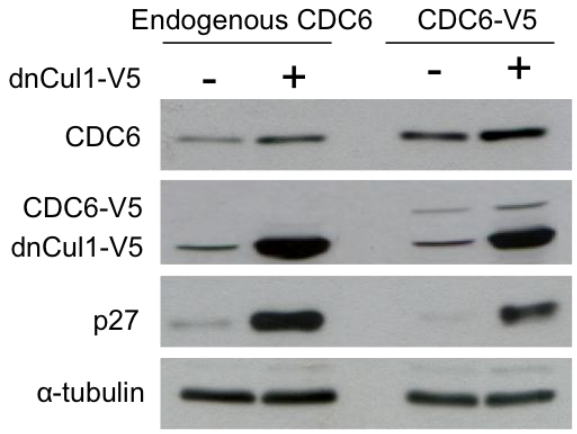


Figure 102. *CDC6 protein stability is not regulated by Cul1 E3 Ligases.* HEK293 cells stably expressing dnCul1-V5, with or without CDC6 (1 μg) transfection for 3 days, were induced with 1 μg/μl tetracycline for 24 hours before cell lysis.

Similarly, induction of dominant negative cullin 4a (dnCul4a-V5, amino acids 1-439) led to an increase in the expression levels of its substrates p27 and p21 (Figure 103). However, no change in CDC6 levels was observed, thus indicating that CDC6 protein is unlikely to be a substrate of Cul4a (Figure 103). In conclusion, my results show that CDC6 protein is not a substrate of Cul1 and Cul4 E3 ligases.

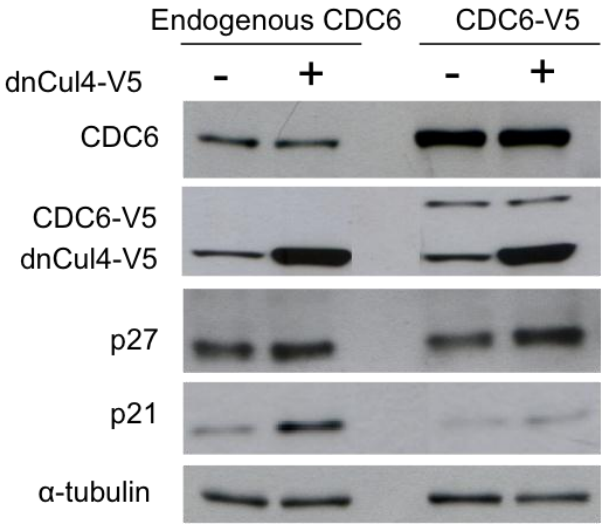


Figure 103. *CDC6 protein stability is not regulated by Cul4 E3 Ligases.* HEK293 cells stably expressing dnCul4a-V5, with or without transfection of 1 μg CDC6 for 3 days, were induced with 1 μg/μl tetracycline for 24 hours followed by cell lysis.

5.2.3 CDC6 stabilization by MLN4924 is due to a delay in cell cycle progression

MLN4924 has been shown to lead to accumulation of cells in S phase (Soucy et al., 2009). Given that APC ubiquitinates CDC6 during early G1 phase (Petersen et al., 2000), I therefore hypothesized that changes in the cell cycle profile might be responsible for the regulation of CDC6 stability. To test this hypothesis, I synchronized Hela cells at the G2/M phase using nocodazole treatment followed by release from the nocodazole block. Consistent with previous reports, CDC6 protein is stable in G2/M phase as illustrated by high expression at t=0h in both MLN4924 treated and control cells (Figure 104). Upon release, CDC6 levels decreased in control cells initially and accumulated at t=6h and 9h but decreased again when cells entered S phase at t=12h (Figure 104). In cells treated with MLN4924, CDC6 was also rapidly degraded during early G1 phase. However, the subsequent increase in CDC6 level was markedly delayed whereby accumulation of CDC6 to high levels was only observed at 24 hours (Figure 104). Cell cycle analyses indicated that the delayed CDC6 stabilization correlates with the delayed transition from G1 to S phase in MLN4924 treated cells (Figure 105). This result suggests that CDC6 stabilization by MLN4924 is a consequent of checkpoint activation at late G1.

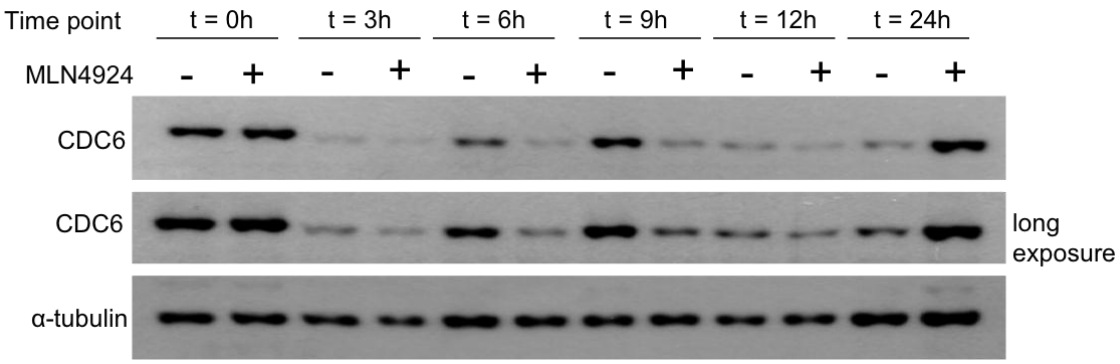


Figure 104. *MLN4924 treatment arrests cells at G1 phase.* Hela cells were synchronized in G2/M phase with thymidine (2 mM) treatment for 20 hours, followed by nocodazole (100 ng/ml) treatment for 13 hours before cell seeding in the presence or absence of MLN4924 (1 μM) and cell lysis at the indicated time points as described in Materials and Methods.

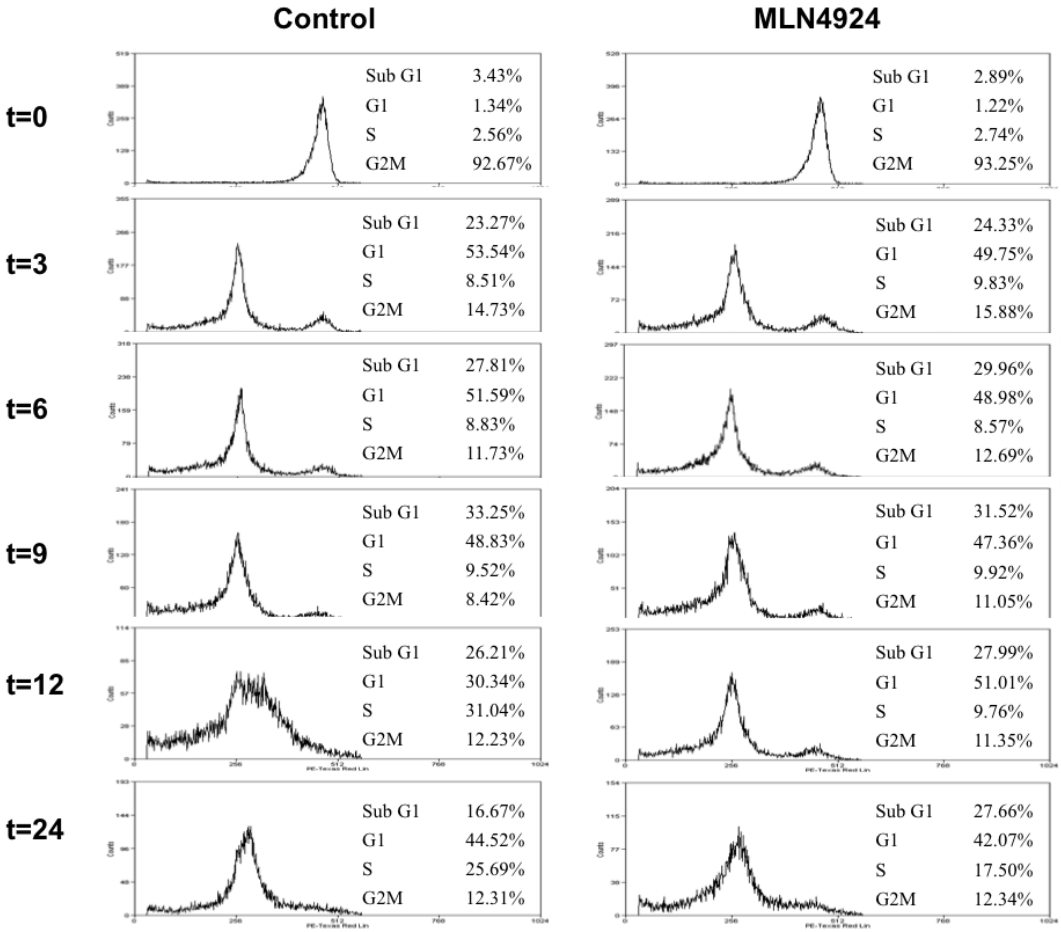


Figure 105. *MLN4924 treatment arrests cells at G1 phase.* Hela were synchronized at G2/M phase as described, seeded in the presence of absence of MLN4924 (1 μM) and collected for flow cytometry analysis at the indicated time points.

I next confirmed that CDC6 accumulation with MLN4924 treatment at t=24h is due to delayed cell cycle progression and not a direct consequence of inhibition of Cullin E3 ligases. Hela cells were harvested 24 hours after G2/M release but MLN4924 treatment was carried out either throughout the 24 hours or only for 4 hours before cell lysis. If CDC6 accumulation is directly due to the inhibition of Cullin E3 ligase mediated CDC6 ubiquitination, 4 hour MLN4924 treatment would be sufficient to observe an increase in CDC6 expression. However, CDC6 protein accumulation was only observed upon prolonged treatment of MLN4924 for 24 hours while the levels of CDC6 proteins remained low with only 4 hours MLN4924 treatment (Figure 106). These results strongly suggest that that the MLN4924 induced CDC6 stabilization is a secondary consequence of a delay in G1 progression and accumulation of cells in G1 and S phase. Taken together, my results indicate that CDC6 stability is not directly regulated by Cullin E3 ligases.

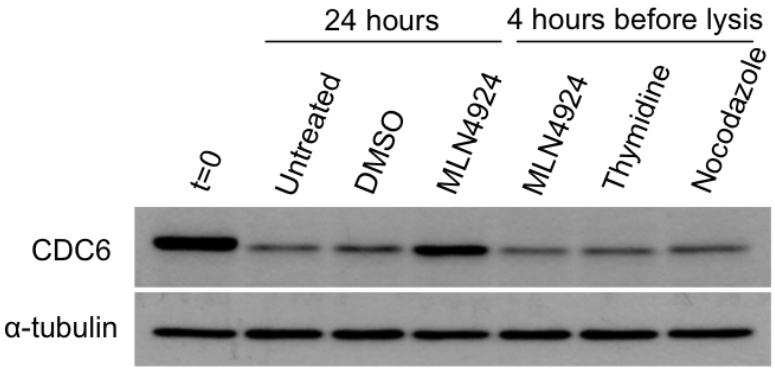
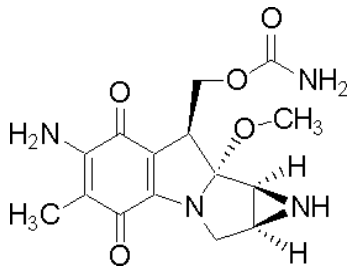


Figure 106. *MLN4924 treatment arrests cells at G1 phase.* Hela cells were synchronized in G2/M as described and cells were lysed at t=24 hours after release from G2/M with treatment of MLN4924 (1 μM) for 24 hours or MLN4924 (1 μM), thymidine (2 mM) or nocodazole (100 ng/ml) for 4 hours before cell lysis.

5.2.4 Mitomycin C treatment induces CDC6 protein degradation

In addition to the CDC6 protein degradation during normal cell cycle progression, it has been reported that CDC6 is also degraded in response to DNA damage. This likely serves to prevent rereplication and promote checkpoint functions to block cell cycle progression when DNA damage occurs. DNA damage induced CDC6 ubiquitination and degradation has been reported to be mediated by APC^{Cdh1} and HUWE1 E3 ligases (Hall et al., 2007; Blanchard et al., 2002; Duursma and Agami, 2005). I observed that the DNA damaging agent mitomycin C, which causes DNA cross-linking, markedly reduces CDC6 stability in various cell types tested (Figure 107A and B). Treatment of cells with mitomycin C resulted in a shorter half-life of the CDC6 protein compared to no treatment (Figure 108). In control cells, the half-life of the CDC6 protein is approximately 3 hours. In contrast, treatment of mitomycin C resulted in a markedly shorter half-life of CDC6 protein levels (approximately 1 hour) (Figure 108). Subsequently, I tested if inhibition of the proteasome can stabilize CDC6. As expected, CDC6 is stabilized in the presence of proteasome inhibitors MG-132 and lactacystin under basal conditions. Mitomycin C treatment caused a dramatic decrease in CDC6 protein expression (Figure 109). The decrease in CDC6 stability upon mitomycin C treatment was prevented by proteasome inhibition with MG-132 and CDC6 was restored to the level in cells treated with proteasome inhibitor alone (Figure 109). This indicates that the mitomycin C induced decrease in CDC6 is mediated by the proteasome.

A



B

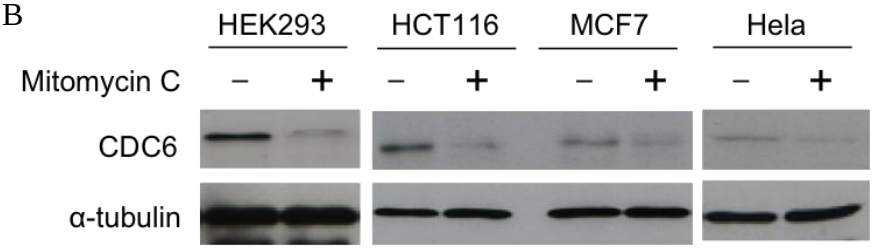


Figure 107. *Mitomycin C treatment induces CDC6 protein degradation.* (**A**) Structure of mitomycin C. (**B**) HEK293, HCT116, MCF7 and Hela cells were treated with mitomycin C (10 μg/μl) for 10 hours followed by cell lysis.

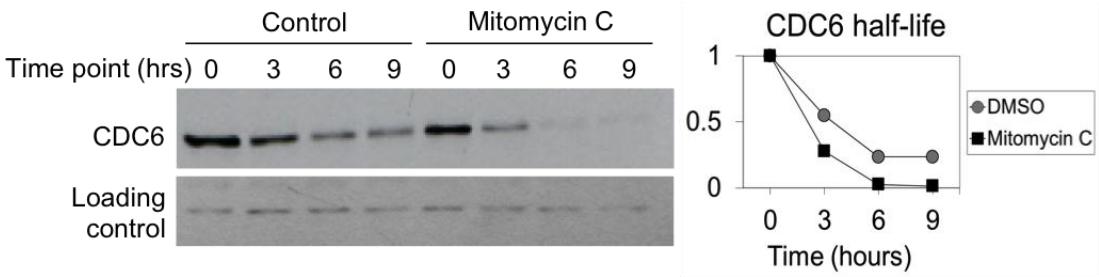


Figure 108. *Mitomycin C treatment induces CDC6 protein degradation.* HEK293 cells were pre-treated with mitomycin C (10 μg/μl) or DMSO as control for 4 hours followed by addition of cycloheximide (40 μM) and cell lysis at the indicated time points.

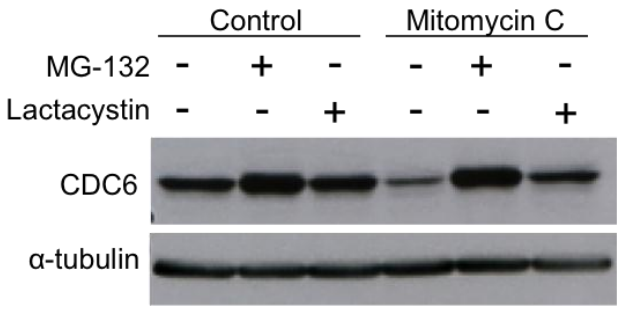


Figure 109. *Mitomycin C treatment induces CDC6 protein degradation.* HEK293 cells were treated with MG-132 (25 μM) or lactacystin (2.5 μM) in the presence of absence of 10 μg/μl mitomycin C for 6 hours before cell lysis.

5.2.5 CDC6 degradation upon mitomycin C treatment is independent of HUWE1 or APC^{Cdh1}

To identify the E3 ligase that regulated CDC6 protein stability with mitomycin C treatment, I tested different possible candidates. Firstly, to test the involvement of HUWE1 E3 ligase, siRNA mediated silencing of HUWE1 was carried out. Cells were treated with mitomycin C or methyl methane sulfonate (MMS), which has been reported to induce HUWE1 mediated CDC6 degradation. CDC6 protein levels were only slightly higher in cells transfected with HUWE1 siRNAs compared to controls, despite highly efficient knockdown of HUWE1 (Figure 110). Furthermore, the increase in CDC6 protein levels upon HUWE1 knockdown was similar in untreated and mitomycin C treated cells (Figure 110), indicating that HUWE1 is not specifically involved in the regulation of CDC6 protein stability upon mitomycin C treatment. Similarly, my results suggest that MMS dependent CDC6 protein ubiquitination and degradation is also independent of HUWE1.

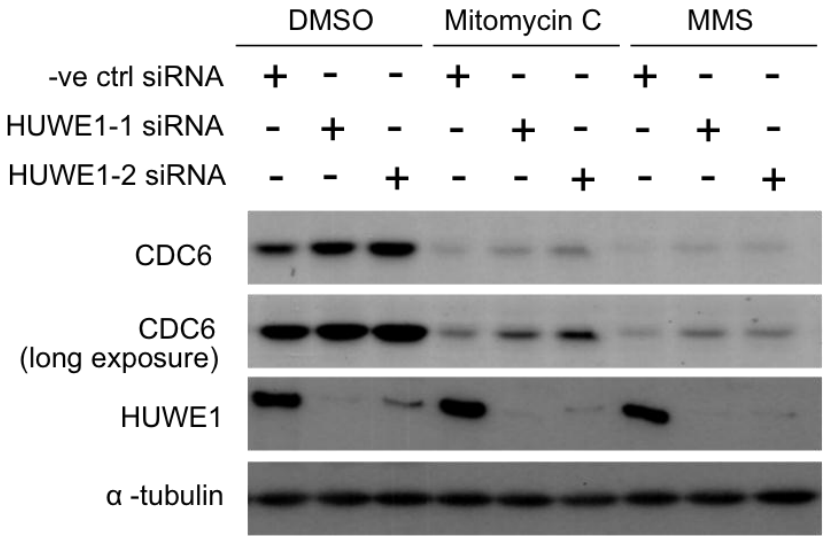


Figure 110. *CDC6 degradation upon mitomycin C treatment is not mediated by HUWE1.* HEK293 were transfected with 20 nM control siRNA of HUWE1-1 or -2 siRNAs for 3 days followed by treatment of mitomycin C (10 μg/μl) or MMS (1 mM) for 8 hours before cell lysis.

Next, I tested if APC^{Cdh1} is involved in CDC6 downregulation upon mitomycin C treatment. When Cdh1 was silenced with Cdh1 siRNA, an increase in CDC6 levels was observed (Figure 111). However, knockdown of Cdh1 did not affect CDC6 degradation in mitomycin C treated cells (Figure 111). This indicated that APC^{Cdh1} is not the E3 ligase that mediates mitomycin C induced CDC6 degradation. To further confirm this, I deleted the D box and KEN box of CDC6 which are important for the recognition by Cdh1 (Petersen et al., 2000) (Figure 112). As expected, the CDC6 deletion mutant showed increased protein stability (Figure 113). However, mitomycin C treatment markedly decreased the expression levels of the CDC6 deletion mutant (Figure 113). This result further confirms that the effect of mitomycin C on CDC6 stability is independent of APC^{Cdh1}.

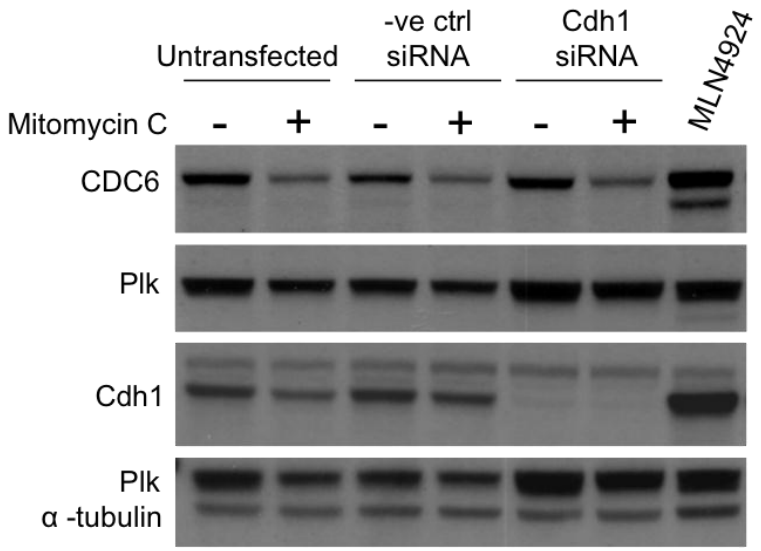


Figure 111. *CDC6 degradation upon mitomycin C treatment is not mediated by APC^{Cdh1}.* HEK293 cells were transfected with 20 nM control siRNA or Cdh1 siRNA for 3 days followed by mitomycin C (10 μg/μl) or MLN4924 (1 μM) treatment for 8 hours before cell lysis.

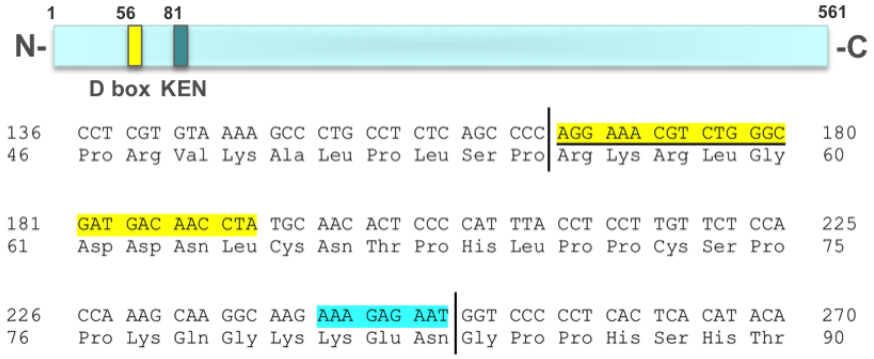


Figure 112. *Location of D box and KEN box in CDC6 protein.* D box (**RXXL**XXXXN) is highlighted in yellow and KEN box (KEN) is highlighted in turquoise. Vertical line indicates area that is removed in the CDC6-V5 ΔD & KEN box deletion mutant.

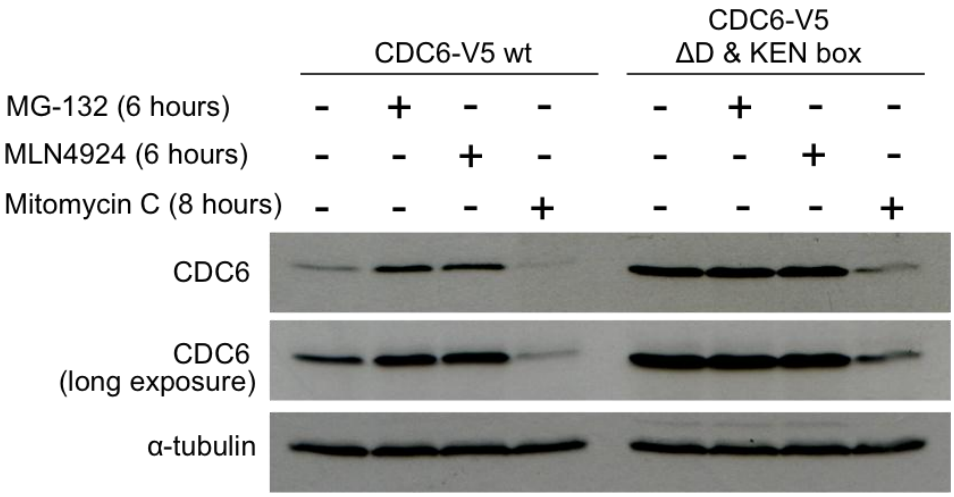


Figure 113. *CDC6 degradation upon mitomycin C treatment is not mediated by APC*^{Cdh1}. HEK293 cells were transfected with wild type CDC6-V5 (0.4 μg) or CDC6-V5 D and KEN box deletion mutant (0.4 μg) for 3 days followed by treatment with MG-132 (20 μM), MLN4924 (3 μM) or mitomycin C (10 μg/μl) for the indicated duration before cell lysis.

5.2.6 CDC6 degradation upon mitomycin C treatment is not mediated by a Cullin RING E3 Ligase but is dependent on the neddylation pathway

To test the involvement of Cullin RING E3 ligases in the mitomycin C dependent CDC6 degradation, I used MLN4924 to inhibit the activity of all Cullin E3 ligases. Interestingly, MLN4924 treatment prevented CDC6 protein degradation upon mitomycin C treatment (Figures 114). These results suggested that Cullin E3 ligases may play a role in mediating the effect of mitomycin C on CDC6 stability. To further confirm the effect of MLN4924 on CDC6 stability upon mitomycin C treatment, I used a tetracycline inducible form of the dominant negative (C111S mutant) Ubc12 (Chew et al., 2007). The dnUbc12 cells have a defective neddylation system and therefore inactive Cullin E3 ligases. Induction of dnub12 expression also prevented the degradation of the CDC6 protein upon treatment with mitomycin C (Figure 115).

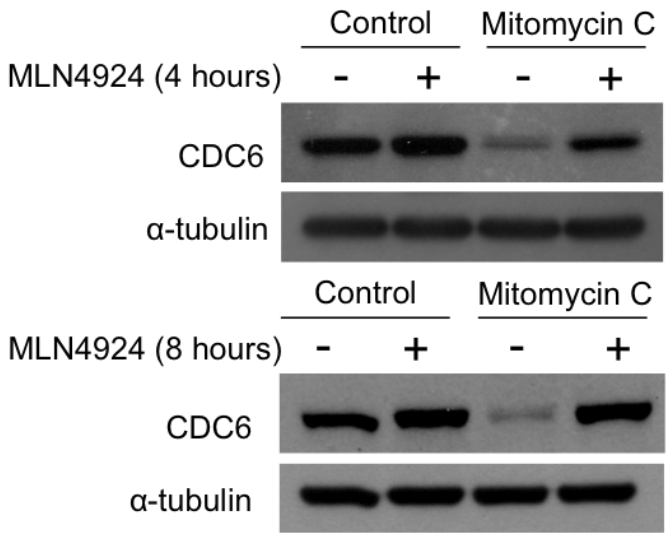


Figure 114. *CDC6 degradation upon mitomycin C treatment is dependent on the Nedd8 pathway.* HEK293 cells were treated with mitomycin C (10 μg/μl) in the presence or absence of MLN4924 (3 μM) followed by cell lysis.

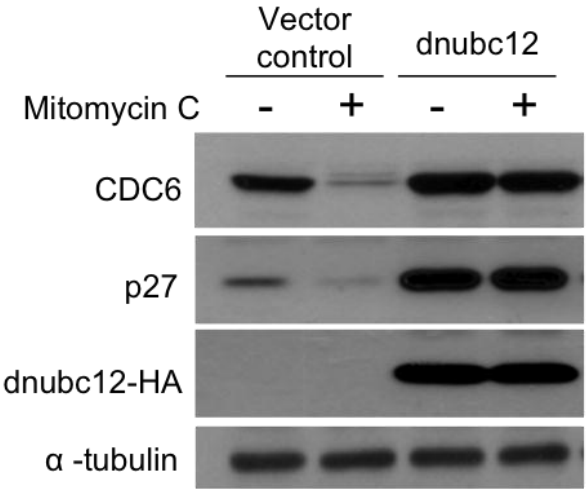


Figure 115. *CDC6 degradation upon mitomycin C treatment is dependent on the Nedd8 pathway.* HEK293 cells were transfected with 0.4 μg dnUbc12-HA pcDNA3 or pcDNA3 vector control for 3 days followed by addition of mitomycin C (10 μg/μl) for 8 hours and cell lysis.

Next, I wanted to identify which member of the Cullin E3 ligase family is involved in the mitomycin C mediated CDC6 degradation. To this end, dominant negative mutant forms of the different members of the Cullin E3 ligase family were used. Dominant-negative cullin proteins contain only the N-terminal portion of the respective cullin proteins. They are able to bind to substrate receptor subunits and substrate, but are unable to interact with the RING domain protein Rbx1. Dominant-negative cullins therefore lack E3 ubiquitin ligase activity. They are widely used to block the function of specific Cullin E3 ubiquitin ligases and to validate substrate proteins. I validated dnCul1 by confirming increased expression levels of the known Cul1 substrate p27 (Figure 116). In contrast, no increase in CDC6 protein levels was observed upon induction of dnCul1 (Figure 116). This indicates that Cul1 E3 ligases are not responsible for the mitomycin C mediated CDC6 ubiquitination and degradation. Similarly, induction of dominant negative Cul3 (dnCul3-V5, amino acids 1-427) in a HEK293 cell line stably expressing inducible dnCul3 had no effect on CDC6 stability upon mitomycin C

treatment (Figure 117). This shows that Cul3 does not mediate mitomycin C induced CDC6 degradation.

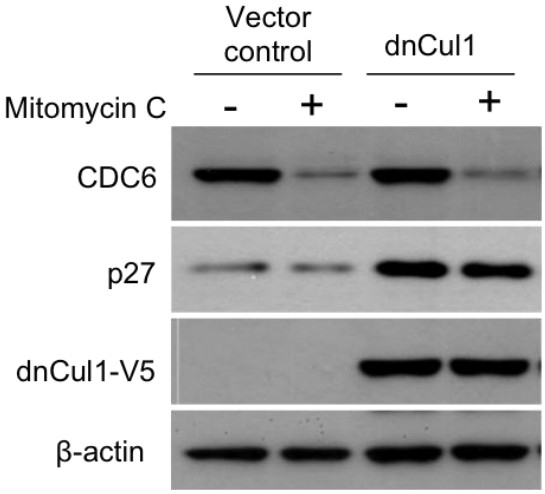


Figure 116. *CDC6 degradation upon mitomycin C treatment is independent of Cul1 E3 Ligase.* HEK293 cells were transfected with dnCul1-V5 pcDNA3 (1 μg) or pcDNA3 vector control (1 μg) for 3 days followed by mitomycin C (10 μg/μl) treatment for 8 hours and cell lysis.

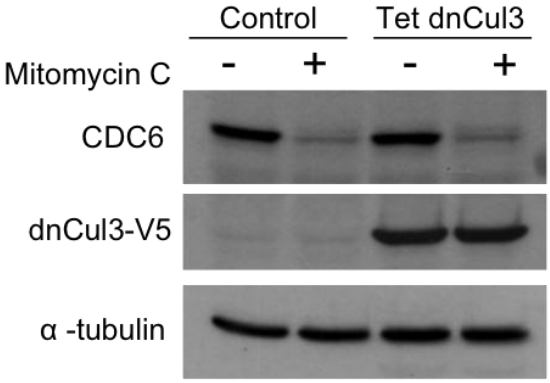


Figure 117. *CDC6 degradation upon mitomycin C treatment is independent of Cul3 E3 Ligase.* HEK293 cells stably expressing dnCu3-V5 were induced with 1 μg/μl tetracycline for 24 hours followed by mitomycin C (10 μg/μl) treatment for 8 hours and cell lysis.

There are two mammalian Cul4 isoforms, Cul4a and Cul4b, which are known to share extensive sequence homology and possibly functional redundancy. Hence, to test if Cul4 is involved, overexpression of dnCul4a-V5 (amino acids 1 to 439) and dnCul4b-FLAG (amino acids 1-594) individually (Figure 118) or co-expression of the two Cul4 isoforms (Figure 119) were performed in HEK293 cells. Overexpression of dnCul4a and/or dnCul4b had no significant effect on mitomycin C induced CDC6 protein degradation, thus indicating that CDC6 is not a substrate of Cul4 during mitomycin C dependent DNA damage.

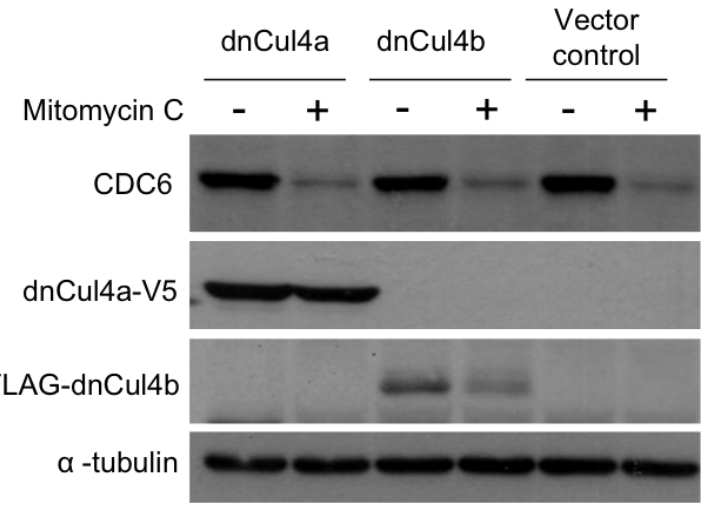


Figure 118. *CDC6 degradation upon mitomycin C treatment independent of Cul4a and Cul4b E3 Ligases.* HEK293 cells transfected with 0.4 μg pcDNA3 vector control, dnCul4a-V5 or dnCul4b-FLAG for 3 days were treated with 10 μg/μl mitomycin C for 8 hours before cell lysis.

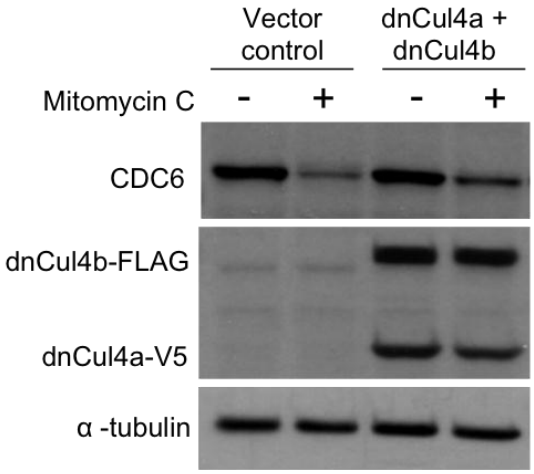


Figure 119. *CDC6 degradation upon mitomycin C treatment is independent of Cul4a and Cul4b E3 Ligases.* HEK293 cells were transfected with pcDNA3 vector control (0.6 μg) as control or co-transfected with dnCul4a-V5 (0.3 μg) and dnCul4b-FLAG (0.3 μg) for 3 days followed by mitomycin C (10 μg/μl) treatment for 8 hours and cell lysis.

Because dnCul2 and dnCul5 are expressed at much lower levels compared to dominant-negative forms of other cullins (Chew and Hagen, 2007), I used an alternative approach to inhibit Cul2 and Cul5 based E3 ligases. Both Cul2 and Cul5 utilize Elongin B/C as an adaptor to recruit substrate receptors. Hence, to test for involvement of Cul2 and Cul5, siRNA mediated silencing of Elongin C was performed. I first validated the efficiency of the Elongin C siRNAs by testing the expression of the known

Cul2 substrate HIF-1α. As expected, with the knock down of Elongin C, HIF-1α protein was stabilized (Figure 120). To further confirm this, HEK293 cells were incubated at 1 % O_2 to stabilize HIF-1α protein followed by reoxygenation to induce the degradation of the HIF-1α protein. As can be observed in Figure 121, in cells transfected with control siRNAs, HIF-1α protein is stable in hypoxia and rapidly (within 7 min) degraded upon reoxygenation. However, with Elongin C knockdown, HIF-1α degradation was markedly delayed upon reoxygenation (Figure 121). To determine the involvement of Cul2 and Cul5 in CDC6 regulation, I next tested if Elongin C knockdown affects CDC6 stability. No effect on CDC6 stability was observed with Elongin C knock down in cells treated with mitomycin C (Figure 122). This indicates that Cul2 and Cul5 do not regulate CDC6 protein stability upon mitomycin C treatment.

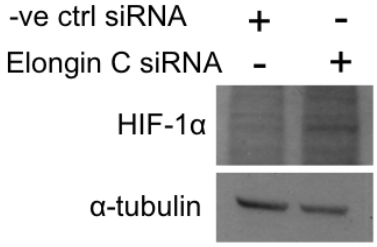


Figure 120. *siRNA mediated silencing of Elongin C stabilizes HIF1α.* HEK293 cells were transfected with 20 nM control or Elongin C siRNAs for 3 days before cell lysis.

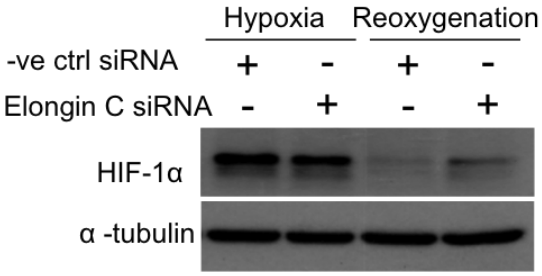


Figure 121. *siRNA mediated silencing of Elongin C stabilizes HIF1α.* HEK293 cells transfected with 20 nM control or Elongin C siRNAs for 3 days were incubated at 1 % O_2 for 4 hours followed by cell lysis immediately or 7 mins after reoxygenation in normoxia.

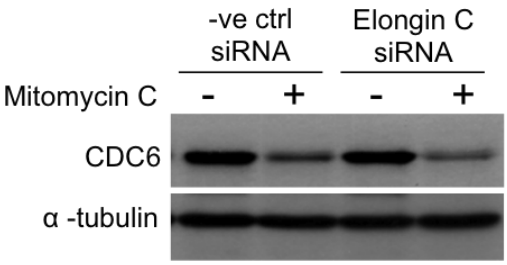


Figure 122. *CDC6 degradation upon mitomycin C treatment is not mediated by Cul2 and Cul5 E3 Ligases.* HEK293 transfected with 20 nM control or Elongin C siRNAs for 3 days were treated with mitomycin C (10 μg/μl) for 8 hours before cell lysis.

My results suggest that none of the well characterized Cullin E3 ligases regulates CDC6 stability upon mitomycin C treatment. To confirm these results, I utilized an alternative approach. Hence, to test the involvement of Cullin E3 ligases directly, siRNA mediated silencing of Rbx1, a subunit of all Cullin E3 ligases, was carried out. The reported Cullin E3 ligase substrates HIF-1α and SLBP were stabilized with Rbx1 silencing (Figure 123). It has been reported that HIF-1α protein synthesis is inhibited by mitomycin C treatment (Lou et al., 2010), hence explaining the reduced HIF-1α protein levels in the presence of the DNA damaging agent in both control and Rbx1 knockdown cells (Figure 123). Knockdown of Rbx1 also increased the basal CDC6 protein concentration. This is likely secondary to effects of Cullin E3 ligase inhibition on cell cycle progression, as described above. Knockdown of Rbx1 did not affect CDC6 stability when cells were treated with mitomycin C (Figure 123). This result indicates that mitomycin C induced CDC6 degradation is not regulated by Cullin E3 ligases.

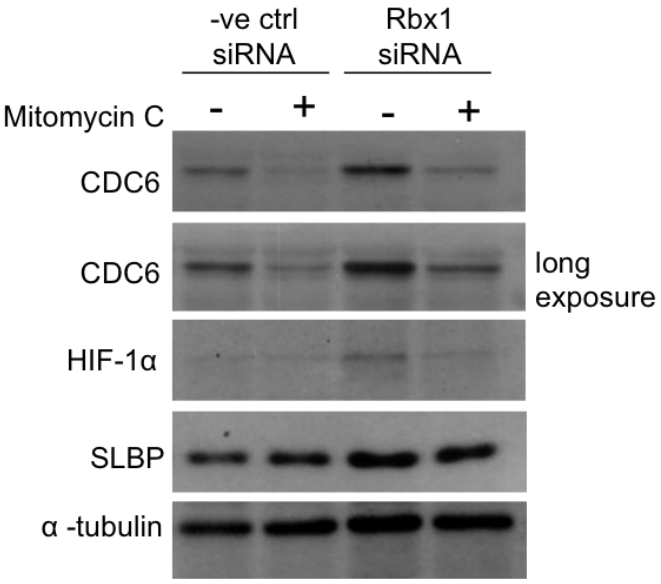


Figure 123. *CDC6 degradation upon mitomycin C treatment is not mediated by Cullin E3 Ligases.* HEK293 cells were transfected with 20 nM control or Rbx1 siRNAs for 3 days followed by mitomycin C (10 μg/μl) treatment for 8 hours and cell lysis.

Inhibition of the neddylation pathway with MLN4924 treatment or induction of dnUbc12 expression blocks CDC6 degradation. This indicates that the Nedd8 pathway is involved in the mitomycin C induced CDC6 protein degradation. However, the effect of inhibiting the Nedd8 pathway is independent of Cullin E3 ligases. These results suggest that the Nedd8 pathway mediates mitomycin C induced CDC6 degradation via an alternative target. It has been reported that the MDM2 RING E3 ubiquitin ligase promotes Nedd8 modification of the p53 tumor suppressor protein as well as its own neddylation. This function is independent of Cullin E3 ligase activity (Xirodimas et al., 2004). Hence, I next tested if MDM2 regulates CDC6 stability in the presence of mitomycin C. As can be observed, overexpression of MDM2 did not have any significant effect on CDC6 levels upon mitomycin C treatment (Figure 124). This indicates that MDM2 does not mediate the mitomycin C induced CDC6 degradation. My results suggest that neddylation affects CDC6 stability via a novel mechanism and further studies are needed

to characterize the mechanisms through which neddylation affects CDC6 stability upon DNA damage.

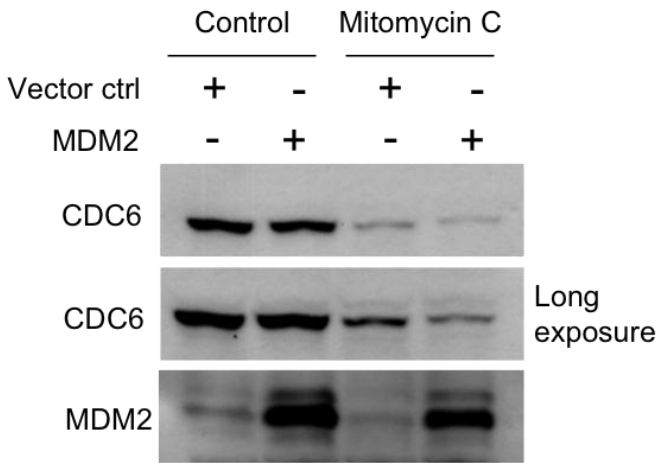


Figure 124. *CDC6 degradation upon mitomycin C treatment is not mediated by MDM2.* HEK293 cells were transfected with 0.4 μg pcDNA3 vector control or MDM2 for 3 days before treatment with mitomycin C (10 μg/μl) for 8 hours and cell lysis.

5.3 Discussion

CDC6 is an essential regulator of DNA replication in eukaryotic cells and its timely degradation is important for normal cell cycle progression. In addition, DNA damage induced CDC6 degradation is likely to play an important role to prevent rereplication and block cell cycle progression by promoting DNA damage checkpoint functions. A major pathway through which CDC6 protein stability is regulated is via cell cycle dependent ubiquitination by the APC^{Cdh1} E3 ubiquitin ligase. In this study, I characterized potential alternative mechanisms that regulate CDC6 protein stability. Cullin E3 ligases are an important class of cellular E3 ubiquitin ligases that have been implicated in CDC6 ubiquitination in budding and fission yeast. Cullin E3 ligases have also been suggested to play a role in mediating CDC6 degradation in mammalian cells. Here I demonstrate that although MLN4924, which inhibits all Cullin E3 ligases, led to marked CDC6 accumulation, CDC6 stability is not regulated by Cullin E3 ligases. The effect of the Cullin E3 ligase inhibitor is a consequence of a delay in cell cycle progression whereby the majority of the MLN4924 treated cells were arrested in G1 phase.

To study DNA damage induced CDC6 degradation, I used the DNA crosslinking agent mitomycin C. This drug exerted a pronounced inhibitory effect on cellular CDC6 protein levels. It has been reported that DNA damage induced by UV irradiation and MMS leads to CDC6 ubiquitination and degradation mediated by the HUWE1 E3 ubiquitin ligase (Hall et al., 2007).

Notably, I found that the effect of mitomycin C on CDC6 is not mediated by the HUWE1 E3 ligase, indicating that there are other ubiquitin ligases that regulate CDC6 stability. Furthermore, I also observed that the known CDC6 regulator APC^{Cdh1} does not mediate CDC6 ubiquitination and degradation upon mitomycin C treatment. Interestingly, treatment of cells with MLN4924 or induction of dnUbc12 expression prevents the CDC6 downregulation by mitomycin C. This indicates that a functional Nedd8 pathway is required for mitomycin C induced CDC6 degradation. The best characterized targets of the Nedd8 pathway are Cullin E3 ligases. However, my studies indicate that Cullin E3 ligases are not involved in mitomycin C induced CDC6 degradation. Thus, inhibition of Cullin E3 ligases using different approaches was without effect on CDC6 protein stability in mitomycin C treated cells. Hence, my results suggest that CDC6 stability in response to mitomycin C is regulated by a neddylation dependent mechanism that does not involve Cullin E3 ligases. This suggests that Nedd8 can also exert important cellular effects in a Cullin independent manner. In line with this, Cullin independent, RNF111 E3 ubiquitin ligase dependent neddylation has recently been reported to play an important role in the DNA damage response (Ma et al., 2013).

In conclusion, my studies provide novel insight into the mechanisms underlying ubiquitin dependent regulation of CDC6 protein stability. My results indicate that contrary to budding and fission yeast, Cullin E3 ligases are not involved in CDC6 degradation during the normal cell cycle in mammalian cells. It is likely that in mammalian cells APC^{Cdh1} is the exclusive ligase responsible for cell cycle dependent regulation of CDC6. DNA damage

induces an alternative mechanism of CDC6 degradation including a novel pathway that involves a Nedd8 dependent but Cullin E3 ligase independent degradation pathway. My results provide evidence that the neddylation cascade can exert cullin independent cellular functions.

6.0 Conclusions and future studies

My project aimed to characterize the different mechanisms through which cells respond to hypoxic stress. The cellular response to hypoxic stress is complicated and various different mechanisms are activated in response to low oxygen concentrations. My thesis focused on how the mTORC1 pathway is regulated through changes in oxygen concentrations. I also studied the post-translational regulation of the important hypoxia-induced regulator of the mTORC1 pathway REDD1 as well as of the key player of replication in the cell cycle CDC6.

Changes in oxygen concentrations regulate mTORC1 activity in a highly dynamic manner whereby the inhibition of mTORC1 in hypoxia is rapidly reversed upon reoxygenation. My results show that the rapid response of mTORC1 to changes in oxygen concentrations is not mediated by the HIF transcription factor or its transcriptional targets REDD1 and BNIP3. Furthermore, I also show that mTORC1 inhibition in hypoxia is independent of transcription and new protein synthesis, suggesting a post-translational regulation of mTORC1 activity in response to changes in oxygen concentrations. Lastly, my results indicate that hypoxia regulates mTORC1 directly at the level of mTORC1 and my preliminary results showed that this may involve a heme containing protein. In future studies, it would be interesting to identify the heme containing protein that regulates mTORC1 activity in hypoxia and reoxygenation.

REDD1 is a negative regulator of mTORC1 in hypoxia that is highly unstable with a very short half-life. In my study to characterize the post-translational regulation of REDD1, I have identified that mTORC1 regulates REDD1 protein stability in a mTORC1-REDD1 feedback loop manner. Contrary to a previous study, my results indicated that REDD1 is not ubiquitinated by Cul4a or other Cullin RING E3 ubiquitin ligases. Furthermore, the ubiquitination and degradation of REDD1 is not dependent on phosphorylation by GSK3β. Although the silencing of HUWE1 E3 ubiquitin ligase led to increased REDD1 protein levels, HUWE1 does not regulate REDD1 protein stability. Hence, the E3 ligase that mediates REDD1 ubiquitination is currently still unknown and its identification would be an important task.

CDC6 is a key regulator of DNA replication in the cell cycle as it is an essential component of the preRC. We initially hypothesized that the CDC6 protein is regulated in an oxygen dependent manner. However, my studies indicate that the CDC6 protein level is not affected by hypoxia. Hence, I characterized the post-translational regulation of CDC6 in normoxia. In my study, I found that in contrast to reports of CDC6 regulation by Cullin E3 ligases in budding and fission yeast, regulation of CDC6 stability in mammalian cells is independent of Cullin E3 ligases. Stabilization of CDC6 protein upon inhibition of Cullin E3 ligases is a secondary consequence of a delay in cell cycle progression. Furthermore, the treatment of cells with the DNA damage inducing agent mitomycin C induced CDC6 degradation independent of the known regulators of CDC6 protein stability HUWE1 or

APC. Instead, an alternative mechanism involving a Nedd8 dependent, Cullin E3 ligases independent degradation pathway is involved. It will be very interesting to characterize this novel mechanism in future work.

In all three parts of my project, I did not find out the exact mechanisms involved. With regards to the identification of the molecular mechanism of mTORC1 regulation in hypoxia, I used multiple candidate approaches as well as a candidate siRNA screen approach. In future studies, it would likely be necessary to devise non-biased, genetic cellular screening methods to obtain more detailed insights. With regards to the mechanism of REDD1 and CDC6 ubiquitinations, the identification of E3 ubiquitin ligases for ubiquitin-proteasome substrates is notoriously difficult and non-biased biochemical or siRNA based approaches would be necessary in future work. In my work, I have, however, eliminated a number of possible mechanisms or pathways that were reported to or could potentially regulate the mTORC1 pathway or REDD1 and CDC6 protein stability. These results are likely to greatly promote and help in the design of future mechanistic studies to characterize the involved mechanisms further.

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