Circadian Clock Regulates Metabolism and Cardiac Myocyte Survival During Cardiovascular Stress

By

Matthew J Love

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Abstract

Disruption of the normal circadian clock has been associated with greater incidence of cardiovascular disease in shift workers. While the underlying mechanisms for this phenomenon remains poorly understood, recent evidence from our laboratory has identified a novel signaling axis that functionally connects the mechanistic target of rapamycin (mTOR), a critical regulator of cardiac hypertrophy and autophagy to the circadian gene Circadian locomotor output cycles kaput (*Clock*). Herein, I show that in contrast to normal cardiac myocytes, post-natal cardiac myocytes genetically deficient for Clock gene (*Clock* -/-) exhibited impaired mTOR signaling and autophagy gene expression in response to different cellular stresses, including hypoxia, hypertrophy, and amino acid starvation. This is highlighted by significant reduction in mTOR activity and its upstream regulators, Ras homolog enriched in brain (Rheb) and AMP activated protein kinase (AMPK), as well as its downstream targets, Unc-51-like kinase (ULK1), 4E-BP1 (eIF4E binding protein 1), and S6 kinase 1 (S6). Additionally, impaired mTOR signalling in *Clock* -/- cardiac myocytes was accompanied by deregulated autophagy gene activation and decreased cell viability. Interestingly, cardiac myocytes subjected to nutrient stress through absence of amino acids also displayed similar autophagy regulation deficiency. Gain of function of *Clock* normalized autophagy gene expression through AMPK decrease and stabilization of the mTOR pathway which increased cell survival. Western blot analysis verified that circadian clock overexpressed resulted in activation of mTOR activity that coincided with a reduction in AMPK while ULK1 levels were normalized in a nutrient stressed model. Further mutations of *Clock* failed to activate mTOR and stabilize autophagy and cell survival in cardiac myocytes. To our knowledge my data provides the first evidence that mechanistically links *Clock* to amino acid

starvation response in cardiac myocytes via the mTOR pathway. This data suggests interventions that mitigate circadian disruption may prove beneficial in improving amino acid metabolism and cardiovascular disease burden in shift workers or individuals with sleep disorders.

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List of Figures

Abbreviation	Definition
ΔΨM	Mitochondrial membrane potential
4E-BP1	eIF4E binding protein 1
Akt	Protein kinase B
AMPK	AMP protein activated kinase
ATG	Autophagy relating gene
bHLH	Basic -helix-loop-helix domain
BMAL1	Brain and muscle Arnt-like protein 1
BP	Bottom panel
CVDs	Cardiovascular diseases
CCN	Core-clock network
Clock	Circadian locomotor output cycles kaput
Cry	Cryptochrome
DFSF	DF serum free media
DMEM	Dulbecco's Modified Eagle Medium
DMEM0	Dulbecco's Modified Eagle Medium without glucose
eIF4E	Initiation factor 4E
FOXO	Factor forkhead box O
GCN2	General control nonderepressible 2
HBSS	Hanks balanced salt solution
HIF1α	Hypoxia inducible factor 1 alpha
HPX	Hypoxia
HR	Heart rate
IGF	Insulin-like growth factors
IR	Ischemia reperfusion
ISO	Isoproterenol
LP	Left panel
MI	Myocardial infarction
MP	Middle panel
mTOR	Mammalian/Mechanistic target of rapamycin
NAD+/NADH	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltranferase
NIH	National Institutes of Health
NMX	Normoxia
PE	Phenylephrine
Per	Period

PCDC4	Programmed cell death 4
PI3K	Phosphoinositide kinase 3
RAG	Recombination-activating gene
Raptor	Regulator associated protein of mammalian target of rapamycin
Rheb	Ras homolog enriched in brain
RP	Right panel
S6	S6 kinase 1
SCN	Suprachiasmatic nucleus
SIRT	Sirtuin
TFEB	Transport of transcript factor EB
TAC	Transverse aortic constriction
TP	Top panel
TSC1/2	Tuberous sclerosis protein 1/2
ULK	Unc-51-like-autophagy activating kinase
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive intestinal polypeptide
WT	Wild type

Introduction

1.1 Circadian and the cardiovascular system

The circadian clock is responsible for regulating the various cellular, metabolic, and physiological processes associated with transitioning between sleep/wake periods. Stimuli such as light, nutrient availability, and other environmental cues can lead to changes in circadian driven proteins that are found in virtually every cell type in the body (Lowery & Takahashi 2011), (Bell-Pederson et al, 2005), (Mistry et al, 2017), (Takahashi et al, 2008), (Richards & Gomz, 2013). In mammals, the circadian clock is regulated centrally by the hypothalamic pacemaker found in the suprachiasmatic nucleus (SCN) and coordinates circadian oscillations peripherally to other systems (Moore, 2013), (Lowery & Takahashi, 2011). A key component of the molecular circadian machinery is the heterodimerization of two basic -helix-loop-helix domain proteins (bHLH) compromised of the Circadian locomotor output cycles kaput (*Clock*) and brain and muscle Arnt-like protein 1 (BMAL1). The CLOCK:BMAL1 dimer allows the complex to bind to cis' acting E box elements on responsive promoters and transcriptionally activate circadian output genes (Huang et al, 2012), (Lowery & Takahashi, 2011). This activates a transcriptional core-clock network (CCN) comprised of other circadian regulators that include Period (Per) and Cryptochrome (Cry) proteins which serve as a negative feedback loop on the CLOCK:BMAL1 dimer, supressing their activity. The CLOCK:BMAL1 complex also activates other circadian genes such as ROR and REV-ERB and similarly feedback to regulate the expression of the BMAL1 activator RORE. The binding of REV-ERB to RORE represses expression of BMAL1 activation while RORs positively regulate it (Huang et al, 2012), (Lowery & Takahashi, 2011). Disruption of this intricate feedback cycle such as jet lag (Arendt et al, 1997), shift work (Knutsson et al, 1986), or sleep disorders (Bradley & Floras 2009), (Kasai &

Bradley, 2011) can be detrimental to the pathophysiology of cancer, metabolism, and notably for the cardiovascular system.

It is well known that components of the cardiovascular system including vascular smooth muscle, aorta and endothelial cells have their own molecular clocks in addition to input from the SCN (Storch et al, 2002). Previous research has pointed towards this strong association between circadian clock and the cardiovascular system. Most notably, their relationship is highlighted by the circadian influence on blood pressure and heart rate (HR); where both parameters peak in morning and progressively fall throughout the day (Durgan & Young, 2010), (Takeda & Maemura, 2011). This association is important physiologically because those who deviate from this diurnal oscillation have reported increased risk of cardiovascular diseases (CVDs). It has been shown that individuals without the characteristic nighttime fall in blood pressure are at a higher risk for CVDs (Verdecchia et al, 1990) (Kairo et al, 2001) (Takeda & Maemura, 2011). Circadian genes such as BMAL1 and CRY were shown to cause blood pressure levels to oscillate and the deletion of PPARγ, a known activator of BMAL1 resulted in reduced diurnal variation in heart rate (Masuki et al, 2005), (Curtis et al, 2007), (Wang et al, 2008). Additionally, myocardial infarction (MI) and other CVDs such as stroke, arrhythmias, and heart failure are more likely to occur in the morning partly due to the circadian controlled genetic and immune responses in morning compared to any other time of day (Bennardo et al, 2016). MI in murine models strengthen the understanding of the circadian:cardiac relationship by showing that short term disruption of diurnal rhythms worsens cardiac conditions resulting in increased cardiac scarring and left ventricular dysfunction, ultimately leading to less efficient ejection fraction compared to mice without circadian disruption (Alibhai et al, 2014). Appropriate inflammatory responses allow for removal of dead tissue and remodelling of myocardium; however, the diurnal

disruption leads to an altered inflammatory responses and wound healing which triggers maladaptive cardiac remodelling (Frangogiannis, 2012), (Mistry et al, 2017). Therefore, the importance of a normal intact circadian rhythm is necessary for prevention of CVDs.

Another CVD of interest to circadian rhythm is ischemia reperfusion (IR) injury, a condition where coronary artery flow is compromised resulting in decreased oxygen delivery to the heart muscle. This causes tissue hypoxia that ultimately leads to cardiac dysfunction from increased cardiac cell death (Carden & Granger, 2000) (Rabinovich-Nikitin et al, 2019). Despite promising therapies for treating IR injury such as revascularization and cardiac catherization, the risk for adverse cardiac remodelling is extremely high and may lead to heart failure (Schirone et al, 2017). One of the major underlying causes of cardiac dysfunction is cardiomyocyte death; while there are many types of cell death, several involve biological cues from the mitochondria – a highly dynamic organelle which constantly undergoes fusion and fission (Mughal & Kirshenbaum, 2011) (Sciarretta et al 2018). Mitochondria change their morphology depending on nutrient and ATP availability, with long elongated fused mitochondria associated with an abundance of usable ATP for oxygen production while fragmented mitochondria are associated with lower ATP levels and reduced oxygen reserve capacity (Gomes et al, 2011). Under normal physiological conditions, mitochondria oxidize glucose and fatty acids to produce ATP essential for continuous contraction of cardiac myocytes, however CVDs such as IR disrupt calcium homeostasis resulting in the production of reactive oxygen species (ROS) and mitochondria mediated cell death (Hoppel et al, 2008) (Szydlowska & Tymoanski, 2010), (Biala & Kirshenbaum, 2014).

1.2 The mTOR Pathway and the Cardiovascular System

A key player that links mitochondria function and circadian rhythm is the mammalian/mechanistic target of rapamycin (mTOR) protein which is an evolutionary conserved serine/threonine kinase involved in cellular metabolism and growth (Sciarretta, Volpe, Sadoshima, 2014), (Saxton & Sabatini, 2017). mTOR forms two multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Sciarretta et al, 2019). These two complexes both contain mTOR, mammalian lethal with se13 protein 8 (Kim et al, 2003), the inhibitory DEP domain containing mTOR interacting protein (Peterson et al, 2009) and Tel two interacting protein 1 (Kaizuka et al, 2010). mTORC1 contains the regulator associated protein of mammalian target of rapamycin (Raptor) (Kim et al, 2002) (Hara et al, 2002) and Proline-rich AKT substrate of 40kDa (Sancak et al, 2007) while mTORC2 has the rapamycin insensitive companion of mTOR (Sarbassov et al, 2004), mammalian stress activated MAP kinase-interacting protein 1, and Proteins observed with rictor 1 and 2 (Sciarretta et al, 2019). These complexes both regulate anabolic processes such as protein, nucleotide, and lipid synthesis while inhibition of the mTOR complexes leads to catabolic pathways such as autophagy (Wullschleger, Loewith & Hall, 2006), (Saxon & Sabatini, 2017).

Several proteins interact with the mTOR complex including protein kinase B (Akt), AMP protein activated kinase (AMPK), tuberous sclerosis protein 1/2 (TSC-1/2), and Ras homolog enriched in brain (Rheb). Both AMPK and TSC-1/2 regulate mTOR activity in response to changes in nutrient availability, hypoxia, DNA damage or cytokines. Specifically, TSC2 was shown to inhibit the small GTPase Rheb- found on lysosomal membranes, from activating mTORC1 in cardiomyocytes (Inoki et al, 2003), (Menson et al, 2014), (Lim et al, 2017). When AMPK is active, it not only inactivates mTOR by phosphorylating TSC-2, but it also inhibits

RAPTOR—a critical regular of mTOR (Inoki, Zhu, Guan, 2003), (Gwinn, 2008). Conversely, both phosphorylation of Akt and presence of growth factors dissociate TSC1/2 from the lysosomal membrane therefor inhibiting TSC1/2 thus allowing Rheb to activate mTOR promoting cell growth (Lee et al, 2007) (Menson et al, 2014).

Important downstream targets of mTOR are S6-kinase1 (S6) and elF4E binding protein 1 (4E-BP1) which promote cell growth when activated (Wullschleger, Loewith, & Hall, 2006), (Laplante & Sabatini, 2012). Particularly important to cardiomyocytes, mTORC1 phosphorylates 4E-BP1 thus inhibiting interaction with initiation factor 4E (elF4E) ultimately allowing for protein translation as inhibition of mTOR resulted in reduced protein synthesis and 4E-BP1 accumulation (Zhang et al, 2010), (Laplante & Sabatini, 2012). In contrast, when mTOR phosphorylates S6, cell growth is promoted by activating eIF4E leading to protein translation while programmed cell death 4 (PDCD4) is inhibited (Dorello et al, 2006). It is also noteworthy that cardiomyocytes undergoing cellular stress from hypoxia or nutrient deprivation experience reduced mTOR expression. It was shown that nutrient stress increased AMPK levels which was reported to interact with mTORC1 directly through RAPTOR or indirectly via TSC-1/2 thereby inhibiting mTOR (Inoki et al, 2006), (Gwinn et al, 2008). During oxidative stress in cardiomyocytes it was found that Thioredoxin-1 binds to mTOR reducing its activity while simultaneously maintaining the complex for cell survival under beneficial conditions (Oka et al, 2017). Due to the important role mTOR and the supporting upstream and downstream proteins involved in regulating cell growth and autophagy, it is not surprising that cardiomyocytes heavily rely on this complex for normal development.

Both mTORC1 and mTORC2 are identified as vital contributors of cardiac and vascular growth starting from embryonic development. Notably, complete genetic ablation of mTOR in

mouse models resulted in cardiovascular developmental abnormalities and metabolic defects in utero (Zhu et al, 2010). Cardio-specific deletion of mTOR and Raptor resulted in similar cardiac defects in adult mouse hearts that included sarcomere disorganization, apoptosis, and autophagy (Zhang et al, 2010). Cardiomyopathies were also found when Rheb was deleted from these mice leading to mortality after 10 days because of the loss of fundamental protein production in cardiomyocytes (Zhang et al, 2010). Simultaneous deletion of 4E-BP1 rescued the catastrophic phenotype seen with deletion of mTOR, RAPTOR, and Rheb demonstrating that the mTOR pathway was heavily involved (Zhang et al, 2010), (Shende et al, 2011), (Tamai et al, 2013). Postnatal mice with cardiac mTOR deletion also displayed cardiac dilation, fibrosis, and apoptosis leading to heart failure with the mice dying within three weeks (Mazelin et al, 2016). These data show that genetic disruption of the mTOR pathway during development or in post-natal hearts triggers metabolic and aberrant defects in cardiac growth.

Although complete genetic ablation of the mTOR complex is incompatible with both normal cardiac development and cardiac function in adulthood, partial ablation of mTOR has been suggested to be beneficial to the cardiovascular system (Wu et al, 2013). This is because the partial inhibition of mTOR allows for less pro-growth proteins to be activated, leading to a decrease in energy expenditure, misfolded proteins and activation of autophagy (Sciarretta et al, 2019). These factors have been linked to a decrease in cardiac aging by lowering heart inflammation and fibroblast accumulation (Flynn et al, 2013). The beneficial effects of partial ablation could be reversed when both mTOR and Rheb were overexpressed because they triggered cardiac hypertrophy and sarcomere disorder in cardiomyocytes (Zhou et al, 2013). This implies that partial inhibition of the mTOR pathway is an adaptive response in

cardiomyocytes that upholds key mechanisms such as energy preservation, and proper protein folding leading to cardiac cell survival, and autophagy.

1.3 Mitochondrial Induced Autophagy and mTOR in the Cardiovascular System

In the heart, autophagy is necessary for maintaining cardiac homeostasis, function, and cell survival. (Sciarretta et al, 2018). Autophagy allows cells to re-cycle and/or discard damaged organelles, and macromolecular structures during metabolic stress imposed by CVDs such as IR injury, MI, and heart failure (Matsui et al, 2007), (Kubali et al, 2013), (Ikeda et al, 2015), (Eisenberg et al, 2016). Autophagy is activated in response to nutrient stress or when cellular ATP falls below a threshold resulting in AMP/ADP accumulation. The catabolism of damaged macromolecules through this highly specialized lysosomal process allows for amino acids, fatty acids, and carbohydrates to be re-cycled as an energy source to maintain cellular ATP levels (Nakai et al, 2007). This is especially important to adult cardiomyocytes when taking into account their lost replicative ability and their role in cardiac contraction. Cardiomyocytes use ATP for contraction and therefor, during starvation autophagy becomes a vital process to ensure cells have enough energy to survive nutrient deprivation (Ravikumar, 2010), (Haseli et al, 2019). Although autophagy is viewed as a protective mechanism, excessive or de-regulated autophagy can be detrimental and exacerbate injury resulting in cell death (Yu et al, 2006)

Autophagy is a highly regulated process that involves the lysosomal digestion of autophagosomes and their cargo within to ensure cells are performing optimally. Autophagosomes are formed with the activation and recruitment of unc-51-like-autophagy activating kinase (ULK) to cellular cargo create the phagophore— the outer membrane of the eventual autophagosome. ULK1 in particular phosphorylates Beclin 1 activating the Vps34

kinase complex leading to formation of the developing autophagosome. Autophagy-related genes (ATG) allow the autophagosome to mature and subsequently fuse with the lysosome associated N-ethylmaleimide-sensitive fusion proteins attachment protein receptor (SNARE) to form the autolysosome. Interestingly, circadian plays a major role in regulating autophagy within the cardiovascular system. This was first discovered through the observed diurnal change in autophagic vacuoles (Pfiefer & Struass, 1981) and was further supported by the display of autophagy genes following a similar diurnal pattern. In recent years, the mTOR complex is seen as a critical regulator of the circadian:autophagy interaction highlighted by mTOR (Ramanathan et al, 2018), AMPK (Lamia et al, 2009), and ULK1 (Ma, Panda, Lin, 2011) being manipulated by circadian machinery.

Notably, mTOR inhibits autophagy by phosphorylating and negatively regulating ULK1 thereby inhibiting the downstream activation of vsp34 and autophagosome formation. Conversely, when mTOR is inactive such as during nutrient stress conditions, autophagosomes are able to form and fuse with lysosomes with help from autophagic machinery including the Transport of transcript factor EB (TFEB) which drives lysosome function and cell homeostasis (Napilotano & Ballabio, 2016). TFEB's role in cell clearance has been highlighted by being an important therapeutic point in mice with lysosomal storage disorders, accumulation of cell toxins, and undigested autophagosomes (Spampanato et al, 2013). In the presence of nutrient abundance mTOR phosphorylates TFEB and retains it in the cytoplasm thereby inhibiting lysosomal synthesis (Settembre et al, 2012) and thereby inhibting lysosomal functioning. In contrast, starvation inactivates mTOR allowing nuclear translocation of TFEB and lysosomal synthesis to occur which promote autophagic processes (Napilotano & Ballabio, 2016). In this

way, mTOR plays a central role in autophagy by regulating not only regulating the formation of the autophagosomes but also lysosomal biogenesis.

1.4 Cardiac Hypertrophy and mTOR

Cardiac hypertrophy is an important physiological process that allows the heart to adapt to increased mechanical load during exercise or after ischemic injury. However, cardiac hypertrophy and ventricular remodelling can also be pathological and impair diastolic function resulting in heart failure. mTOR has been known to play a key role in regulating pathological hypertrophy because it's role in generating proteins involved in sarcomere formation. This is best illustrated by the fact that mTORC1 was activated by both physiological and pathological hypertrophy induced by transverse aortic constriction (TAC) (Sciaretta, Volpe, Sadoshima, 2014). Disruption of the mTOR pathway in mice impaired the heart's ability to adapt to cardiac stress demonstrated by absence of compensatory cardiac hypertrophy that was accompanied by apoptotic and autoptic cell death (Zhang et al, 2010). Conversely, pathological cardiac hypertrophy can be inhibited through partial ablation of mTOR without compromising the hearts ability to adapt to increased mechanical loading (Sciaretta, Volpe, Sadoshima, 2014). Several studies manipulated cardiac hypertrophy by introducing mTOR regulators such as rapamycin (McMullen et al, 2004), Rheb (Wu et al, 2013), and PRAS40 (Volkers et al, 2013) demonstrating that mTOR is vitally linked to cardiac hypertrophy. Notably, mTOR activation alone is not sufficient for induction of pathological cardiac hypertrophy, as Sciaretta et al highlights that hypertrophy can be induced by a variety of convergent signaling pathways which contribute to increased cardiomyocyte size and volume. Additionally, cardiac myocytes over-expressing mTOR did not exhibit increased pathological remodelling when exposed to pressure overload

and TAC, supporting the notion that mTOR favours cardioprotective pathways that lead to cardiac hypertrophy (Sciaretta, Volpe, Sadoshima, 2014).

In this regard, investigations directed toward understanding cardiac hypertrophy at the cellular levels have utilized β-adrenergic agonists such as Isoproterenol (ISO) to stimulate cardiac growth. This β 1 specific adrenergic receptor agonist can increase cardiac output by stimulating cardiac hypertrophy in different experimental models. Many studies have used ISO to understand the role of autophagy in hypertrophy and continues to be debated today (Beesley, Noguchi, Welsh, 2016), (Zhang et al, 2017). A 2016 study determined that ISO induced hypertrophy in neonatal rat cardiomyocytes which resulted in reduction of autophagic activity (Lu et al, 2016). The authors showed in that study that sirtuin (SIRT) 6 was able to restore autophagy and protect against the maladaptive effects of hypertrophy through the transcriptional activation of the factor forkhead box O (FOXO) which presumably reduces Akt and thus curtailing mTOR activity in favour of autophagy (Menson et al, 2014) (Lu et al, 2016). ISO has also been used to uncover information on how circadian genes interact with cardiac myocytes. It was found that hearts exposed to β- adrenergic stimulation had elevated amplitude of *Per2* providing a link between cardiac hypertrophy and circadian (Beesley, Noguchi, Welsh, 2016). Phenylephrine (PE), meanwhile specifically binds to α-adrenergic receptors and stimulates vasoconstriction and cardiac preload and thereby offering some benefit to hypotensive patients (Richards, Lopez, Maani, 2020). However, due to the increase in cardiac workload and increased ventricular stretch imposed by systemic vasoconstriction, cardiac cell hypertrophy can ultimately transition to heart failure. PE was also found to cause significant activation of mTOR by increasing both phosphorylation of S6 and 4E-BP1 suggesting that inhibiting mTOR via TSC-2 increase leads to beneficial cardiac remodelling from pathological hypertrophy (Morales et al,

2016). The same group has reported that time of day affects the presence of absence of autophagic flux with early timepoints promoting autophagy and later ones inhibiting autophagy suggesting once again that circadian may be involved (Xie et al, 2014). Notably, risk of cardiac hypertrophy seems to increase when misfolded proteins and damaged organelles accumulate inside the cell. This suggests that the regulation of autophagy and mTOR are vital to diminishing pathological hypertrophy and that circadian likely contributes to this growth: recycle dichotomy.

1.5 Circadian Interaction with Metabolism

As previous alluded to, circadian rhythm is important for ensuring proper metabolic status. During sleep, the body is in a state of nutrient deprivation and does not require metabolic complexes such as the mTOR pathway to be activated. This means that it is evolutionarily advantageous for metabolism to be in communication with circadian. For example, the insulin signalling pathway has become one of the most investigated topics for circadian and metabolic interaction. These studies highlight the importance of circadian controlled oscillations of insulin-like growth factors (IGFs) activating PIK3-Akt-mTOR pathway stimulating growth (Zhang et al, 2014). While these studies provide exciting advancements in CCN proteins effect on metabolism, there is literature that highlights the bidirectional relationship between circadian clock and metabolism from mTOR signalling, resynchronizing the SCN.

Cao et al found that light pulses at night activate the mTORC1/S6 pathway within the SCN causing photic entrainment and later found that mTORC1 activated 4E-BP1 which transcribed mRNA of vasoactive intestinal polypeptide (VIP) which also caused circadian realignment (Cao et al, 2010) (Cao et al, 2013). Additionally, it was found that *Drosophila* with elevated AKT and mTOR expression induces longer circadian periods; conversely, reducing these genes shortens circadian periods (Zheng & Sehgal, 2010) (Kijak & Pyza, 2017). In mice,

those with knocked down mTOR showed impaired circadian behaviour and decreased synchronization amount SCN cells (Cao et al, 2013).

In hepatocytes, lentiviral shRNA mTOR knockdown increased period length in mice and was reversed when Rheb mutations overexpressed mTOR. This in turn could be reversed by inhibition of mTOR via rapamycin, torin1 and PP242 which all act as negative regulators of mTORC1&2 (Ramanathan et al, 2018). Additionally, CCN proteins CLOCK, BMAL1, and Cry1 were all elevated when TSC-2 was knocked down compared to when it was fully functional, however the effects of TSC-2 knockdown overexpression could be inhibited by rapamycin (Ramanathan et al, 2018). Studies have looked into the affect circadian has on other autophagic regulators such as TFEB and show how it localizes in the nucleus through a cyclical oscillation. Additionally, overexpression of TFEB leads to increased REV-ERB which inhibits autophagic flux suggesting that TFEB works together with circadian genes to create its own autophagic rhythm (Pastore et al, 2019). The following results point to a complex interaction between autophagy related proteins such as mTOR and TFEB with circadian genes, however, more can be uncovered by exploring if these proteins are still interacting with circadian proteins in cardiomyocytes.

1.6 SIRT1 as a Potential Mediator Between Circadian Rhythm, mTOR, and the Cardiovascular System

Potential mediators for the circadian/mTOR interaction in the cardiovascular system come from the SIRT protein family, which are a family of seven proteins encoded by the Silent Information Regulatory gene (Haigis & Sinclair, 2010), (Covington & Bajpeyi, 2016). SIRT proteins can be found in the cytoplasm or nucleus and deacetylate a wide range of proteins.

(Yamamoto et al, 2007), (Haigis & Sinclair, 2010), (Covington & Bajpeyi, 2016), (Mayo et al, 2017). Certain SIRT proteins such as SIRT3 can play an important role in mitochondria biogenesis, metabolism of both lipids and glucose, and insulin sensitivity (Yang et al, 2006), (Yamamoto, Schoonjans, & Auwerx 2007), (Poulose & Raju, 2015), (Bindu, Pillai, & Gupta, 2016). SIRT3 along with SIRT5 and SIRT6, have protective roles in IR injury (Koentges et al, 2016), (Boylston et al, 2015), (Wang et al, 2016) while SIRT2 and SIRT3 have similar positive effects on atherosclerosis and heart failure respectively (Grillon et al, 2012) (Zhang, Ma, & Xiang, 2018). SIRT proteins 2 through 7 may contribute to casual disease mechanisms in the heart, none touch on circadian, metabolic, and cardiovascular health quite like SIRT1 which has been studied extensively for its vital role as a multifunctional protein in all three systems.

In both the brain and peripheral clocks, SIRT1 alters the expression of many CCN proteins. In particular, SIRT1 directly deacetylates BMAL1 on E-Box containing promotors which antagonize the heterodimerization of CLOCK and BMAL1 (Nakahata et al, 2012). This deacetylation also seems to be circadian controlled, implying that SIRT1 also regulates circadian rhythm (Asher et al, 2008), (Nakahata et al, 2012). Additionally, mice deficient in SIRT1 had improper regulation of CCN proteins such as *per1, per2, cry1*, and *cry2* with *per2* specifically repressing SIRT1 activity (Russell, Finick, & Kelly, 2005), (Soni et al). Much like these CCN genes, SIRT1 is another regulator of metabolic health status. SIRT1 can also function as an energy sensor, consuming (NAD+) which is a metabolite of nicotinamide phosphoribosyltranferase (NAMPT) (Mayo et al, 2017). SIRT1 was shown to be activated by NAD+ in caloric restricted yeast leading to extended lifespan (Anderson et al, 2003) (Anderson et al, 2017). Another study found that NAD+ depletion is a major cause of cell death highlighting the importance of SIRT1 to cell survival (Yang et al, 2007). Caloric restriction also increases

SIRT1 expression in a plethora of tissues (Chang & Guarente, 2014) while high fat diet and obesity decrease SIRT1 (Chalkkiadaki & Guarente, 2012). Within prostate cancer cells, SIRT1 was upregulated leading to resistance towards oxidative stress increasing cell proliferation (Wang et al, 2011) (Jung-Hynes et al, 2011).

With this body of information regarding the underlying relationship between SIRT1 and metabolism, we can begin to appreciate an association between mTOR and SIRT1. The previously mentioned energy currency produced by the mitochondria such as ATP and NADH need to be tightly regulated to ensure depletion does not occur, causing aforementioned mitochondrial induced cell death. The mTOR pathway detects the metabolic end products through upstream targets such as AMPK, which becomes activated by low cellular levels of ATP. (Nogueiras et al, 2012). SIRT1 utilizes NAD+ for enzymatic activity and sensing NAD+/NADH levels, therefore supporting a role for SIRT1 in mitochondrial quality control and thus mTOR complex activity (Andersson et al, 2003) (Nogueiras et al, 2012). AMPK increases SIRT1 by increasing levels of NAD+, while at the same time activating fatty acid oxidation thereby indirectly activating AMPK (Feige et al, 2008) (Canto et al, 2009) (Um et al, 2010) The polyphenol resveratrol-most notably known for providing cardiovascular benefits, was found to be dependent on SIRT1 for mediating resverastrol's activation of AMPK and NAD+ for improving mitochondrial function (Price et al, 2012).

As previously mentioned, SIRT6 is found to associate with FOXO, however SIRT1 also has a relevant relationship with FOXO. SIRT1 has been shown to catalyze the deacetylation of FOXO during oxidative stress preventing FOXO induced cell death (Brunet et al, 2004) (Motta et al, 2004) (Greer & Brunet, 2005). While intriguing on its own, this circles back to mTOR because the upstream mTOR regulator Akt along with its upstream partner phosphoinositide

kinase 3 (PI3K), phosphorylate FOXO transcription factors in the presence of growth factors such as IGF leading to cell survival through the Akt-mTOR pathway (Brunet et al, 1999) (Rena et al, 1999) (Greer & Brunet, 2005) (Dharaneeswaran et al, 2014). Additionally, PI3K and Akt contribute to the activation of mTORC1 to promote physiological cardiac hypertrophy (Mcmullen et al, 2003). Notably, FOXO1 overexpression resulted in Akt-mTOR mediated cell growth while deficiency of FOXO1 led to the loss of Akt-mTOR feedback loop (Dharaneeswaran et al, 2014). We see here that SIRT1 has the potential to be a major site for interaction between key upstream mTOR targets such as AMPK and Akt and circadian rhythm.

SIRT1 in the heart functions similarly as its role in circadian and metabolic physiology by deacetylating histone and non-histone proteins (D'Onofrio, Servillo L, & Balestrieri, 2018). SIRT1 can be induced by phytoalexin which has been shown to improve cardiac function after CVDs such as IR-injury, atherosclerosis, and cardiac aging (Lancake & Price, 1997), (Stein & Matter, 2011), (Cote et al, 2015). Similarly to the mTOR pathway, SIRT1 expression is also increased during embryonic cardiac development in mice suggesting a role in both maintenance and development of the heart (Sakamoto et al, 2004). It was found that low to moderate overexpression of SIRT1 leads to antioxidant enzyme activation which inhibits apoptotic pathways mentioned previously. Higher levels of SIRT1was found to have negative effects by activating Akt thus leading to Akt mediated cardiac hypertrophy likely through the mTOR pathway (Alcendor et al, 2007), (Soni et al, 2021). Despite this it has been shown that cardiac aging reduces levels of SIRT1 showing that SIRT1 may be linked to autophagy because of the important role autophagy plays in cardiac aging.

1.7 mTOR and Starvation

Human metabolism was evolutionary influenced through periods of famine, and thus our bodies evolved ways to adapt to periods of starvation. Glucose is often considered the primary fuel for organismal life, however during times when glucose is sparse, specific mechanisms such as gluconeogenesis and glycogenolysis become activated to ensure sufficient cellular glucose is available for cell survival (Rothman et al, 1991), (Lepriver & Roblat, 2020). As nutrient availability depletes, triglycerides release from lipid stores and allow fats to become the primary energy source and thus allows for the preservation of proteins. This highlights the importance of glucose sensing for starvation, and it should come as no surprise that mTOR acts as a glucose sensor. When glucose is present, mTOR is activated and can be used towards anabolic processes while glucose depletion leads to catabolic processes including autophagy as mentioned above (Liu & Sabatini, 2020). The synthesis of proteins, lipids, and nucleotides are extremely ATP demanding, thus the ability to control mTOR when glucose levels are low is critical to avoid ATP depletion (Buttgereit & Brand, 1995), (Lepriver & Roblat, 2020). Notably, dysregulated mTOR has been observed in a number of metabolic related diseases such as diabetes, obesity, and metabolic syndrome (Saxon & Sabatini, 2017).

Depleted glucose levels causes the upstream negative mTOR regulators AMPK and TSC-1/2 to activate by detecting low levels of ATP rather than AMP and ADP (Hardie, Ross, Hawley, 2012) (Lepriver & Roblat, 2020). It was found that the observed pathway of TSC-1/2 phosphorylating AMPK to inhibit mTOR allowed energy depleted cells to control cell size, reduce protein synthesis, and prevent apoptosis (Inoki, Zhu, Guan, 2003). TSC knockout cells were unable to adapt to glucose deprivation resulting in the increase of a known apoptosis inducer: p53. This led to cell death, however when mTOR was inhibited ATP levels were

restored and protected against apoptosis via TSC1/2 and AMPK (Lee et al, 2007) (Choo et al, 2010). When nutrients are present, the tight association between the pro-autophagic protein ULK1 and AMPK is disrupted by mTOR phosphorylating ULK1, keeping autophagy inactivated suggesting the importance of autophagy in cell longevity (Kim et al, 2011), (Egan et al, 2011). Many studies have used amino acid deficient Hanks balanced buffer solution (HBSS) as a way to investigate the effect starvation have on this particular pathway (Shang & Wang, 2011). While it is understood that starvation causes the dissociation between mTOR and ULK1 leading to autophagic activity, this mechanism is thought to be independent of AMPK (Alers et al, 2012). Since circadian is heavily tied to nutrient availability, which is linked to the mTOR complex, understanding the role of circadian rhythm in this nutrient sensitive protein network could provide new insight in treating starved cardiomyocytes.

Previously, many studies have defined a bi-directional relationship between mTOR and CNN genes while there are several pieces of literature exploring mTOR's role in the cardiovascular system. With mTOR's role in hypertrophy and starvation, I hope to learn more about how CCN genes interact with mTOR in cardiomyocytes under different stress conditions. Based on the previous literature mentioned above, I hypothesize that Rheb, SIRT1,Akt1, Ulk1, AMPK, and TSC-2 will be important players in regulation the interaction between circadian and the mTOR pathway during both hypertrophy and starvation.

2.0 Materials and Methods

2.1 Neonatal Cardiac Myocyte Isolation and Cell Culture

Neonatal ventricular myocytes were isolated from 1-2-day old Sprague-Dawley rats pups. Pups were sacrificed by cervical dislocation and the isolated myocytes were plated at a density of $1x10^6$ per 35 mm dish or $3.2x10^5$ per coverslip. Cells were incubated in at 37 ° Celsius (C) overnight in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DF) supplemented with 17mM HEPES, 3mM NaHCO3, 2mM L-glutamine, 50μg/mL gentamicin and 10% fetal bovine serum (FBS), then the myocytes were transferred to DF serum-free (DFSF) media.

Male homozygote *ClockΔ19/Δ19* and wild type (WT) littermates on a C57Bl/6 J background used for this study were obtained from Dr. Tami Martino University of Guelph. The $Clock^{\Delta 19/\Delta 19}$ mice contain a mutation on the CLOCK protein that disrupts dimerization with its cognate partner BMAL1, and thus disturbing the transcription of circadian controlled genes. Transverse aortic constriction (TAC) were inflicted onto rats to induce pressure overload in the left ventricle creating cardiac hypertrophy, under light anesthesia. Sham-operated animals underwent the same surgical procedure, except the ligature was not tightened (Podobed et al, 2014). All animal experiments conducted in this study were approved by animal care committee of University of Manitoba as well as by University of Guelph and are in accordance with the guidelines laid for protection of animals used for scientific purposes by Canadian Council for Animal Care, directive 2010/63/EU, and National Institutes of Health (NIH). Animals were housed at an animal facility with standard laboratory conditions, 12/12 hours light/dark cycle. Rodent chow and water were accessible at *ad libitum*.

2.2 Plasmids, Transfection, and Adenovirus

Cultured cells were transfected with expression plasmids using Effectene reagent (Qiagen, Inc.) or infected with adenovirus carrying the desired gene 24h after plating under either DFSF or HBSS serum, as earlier described. The plasmids used include: *Clock* Δ19, a gift from Carrie L Partch and pcDNA HA flag plasmid that was used as vehicle control. The adenoviruses used are: AdCMV), Ad*Clock*, Ad*Clock* V315R, Ad*Clock* L57E, Ad*Clock* L57E/L113E/W284 (*Clock* 3xMut). For *Clock* adenovirus studies, cells were infected for 24 hours at a multiplicity of infection of 10–20 and placed in DFSF or HBSS.

2.3 Western Blot Analysis

Mice were anaesthetized with isofluroane at 24 hours post-reperfusion, sacrificed by 5% isoflurane and cervical dislocation, hearts and cells were lysed in RIPA buffer (1.0% deoxycholate, 140 mM NaCl, 10 mM Tris-HCl, 1% Triton X-100 and 0.1% SDS) with the addition of protease inhibitors (10µl of NaF, 10µl of PI, 10µl of Na3VO4, and 3.4µl PMSF per 1mL of RIPA). Lysate was then measured for protein concentration through bicinchoninic acid (BCA). Protein cell lysate (12-20 µg) were then denatured for 5 minutes at 100°C and resolved on a denaturation sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel at 80 volts for 20 minutes followed by 100 volts for ~1 hour. The protein lysate was transferred to a nitrocellulose membrane at 100 volts for one hour at 4°C. Membranes were blocked for 1 hr in 5% skim milk in TBS-T (50mM Tris-HCl, 150mM NaCl, 0.3% Tween- 20, pH 7.4) at room temperature. Membranes were incubated with primary IgG antibodies directed at proteins relating to the mTOR: Circadian interaction including phosphorylation of mTOR(p-mTOR) (NEB#5536) , mTOR (NEB#2972S), CLOCK(NEB#5157S), Rheb (NEB#13879S), phosphorylation of AKT

(p-akt) (NEB#9271S) ,AKT, (NEB#2314S) , SIRT1 (NEB#8469S) , ULK1(NEB#8054S) , P62 (NEB#5114S), LC3(NEB#2775S), TSC-2 (NEB#3990S), phosphorylation of AMPK (p-AMPK) (NEB#2535S), TFEB (INV#75572), S6 (NEB#2217S) BNIP3(Kirshebaum Lab), Actin (Sigma #A2172-.2ML) at 1:1000 dilution overnight at 4°C. Following incubation, membranes were washed three times with 1x TBS-T for 10 mins each and incubated with specific secondary antibodies for 1 hr at room temperature. Chemiluminescence reaction using horseradishes peroxidase (HRP) conjugated antibody with enhanced chemiluminescence (ECL) reagents (GE Healthcare) was used to detect bound proteins. Membranes were visualized using Amersham Imager 680 (GE Healthcare).

2.4 Cell Viability Assay:

Cell viability was examined through epifluorescent microscopy where myocytes were stained with 2μM of Calcein acetoxymethylester (Calcein-AM, Invitrogen) to stain live cells coloured green, and 2 μM of ethidium homodimer-1 (VWR) to inspect dead cells coloured red. After treatment, cells on coverslips were washed with PBS and then incubated for 30 minutes at 37°C using DFSF media or Hanks Balanced Salt Solution (HBSS) if cells were starved. Chemical compounds such as PE and ISO were added to their respective media at 50μM. At the end of the 30-minute incubation, coverslips were inverted onto glass slides and visualized using Olympus AX-70 research fluorescent microscope at x200 magnification. Image J software was utilized to quantify the pictures taken from the microscope. An n=5 myocyte isolation were tested and at least 200 cells for each condition were analyzed.

2.5 Mitochondrial membrane potential (ΔΨM)

Cells were incubated with tetra-methylrhodamine methyl ester perchlorate (TMRM, Molecular Probes, 50 nM) and visualized by epifluorescence microscopy. The program ImageJ was used to measure cell florescence intensity as mitochondria under normal conditions fluoresce bright red, while a fainter colour indicates dissipation of mitochondrial membrane potential.

2.6 Statistical Analysis

Multiple comparisons within groups were calculated via one-way ANOVA and Tukey post hoc test using the graphpad computer program. Differences were considered to be statistically significant if *P<0.05, ΔΨM and cell viability experiments had their means taken from n=2 myocyte isolations. Western data had their mean fluorescence taken from ImageJ and incorporated fluorescence of background and actin to make a final score. This score was then ratioed to the score the differences between our control and treatment groups.

3.0 Results

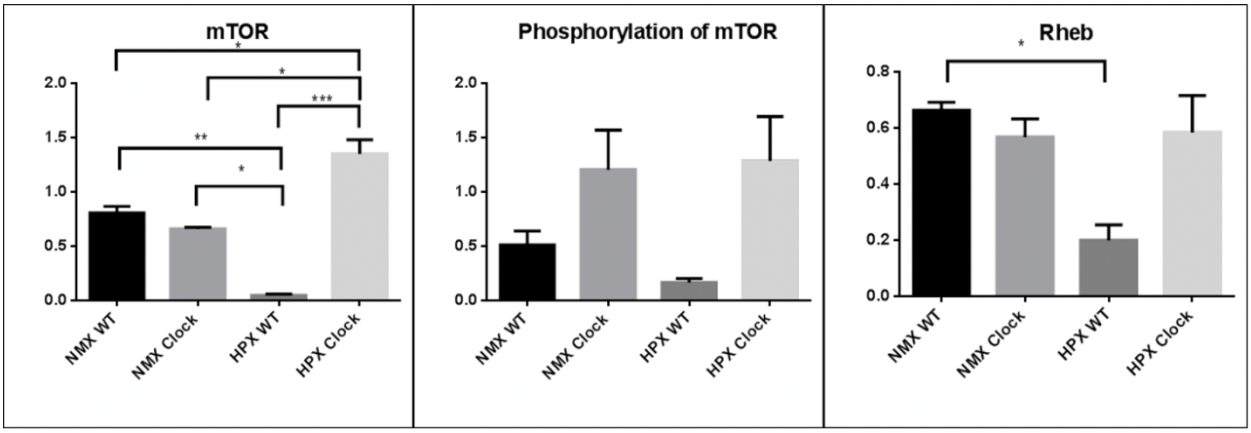
3.1 *Clock* effects mTOR expression in Cardiac Myocytes during Normoxia and Hypoxia.

To begin to assess how cardiomyocytes regulate the mTOR pathway during *Clock* overexpression, we underwent western blot analysis to investigate how *Clock* drives p-mTOR, mTOR, and Rheb expression in normoxic (NMX) and hypoxic (HPX) environments. We then infected cells with Cytomegalovirus (AdCMV) as a control or *Clock*. As Figure 1 suggests we see a slight but non-significant (P=0.8312) decrease in Rheb expression in NMX *Clock* compared to the control group, NMX. However, during HPX we see a significant decrease in Rheb expression compared to the control group (P=0.0482). Despite poor Rheb expression in the

HPX WT, the HPX *Clock* condition increased Rheb levels to a similar expression as NMX conditions. This was seen by no detection differences in Rheb expression for HPX *Clock* compared to NMX WT (P=0.8902) and NMX *Clock* (P=0.9988). However, when comparing Rheb expression between HPX WT and HPX *Clock*, there was no significant difference in means (P=0.0862) (Figure 1 A).

When looking at kinase activity of mTOR, NMX *Clock* showed more expression quantitively compared to NMX WT (Figure 1B), however it was considered non-significant (P=0.4143). HPX WT had similar scores for their expression of p-mTOR, as reflected by the relatively high p value (P=0.8209) between WT conditions. Additionally, both the NMX *Clock* and HPX *Clock* had very similar expression for p-mTOR (P=0.9967) while having relatively strong yet non-significant increases in p-mTOR compared to HPX WT (P= 0.1794 & 0.1486 respectively). However, once we took the expression calculations and divided them by the control, the model became significant with the mean expression of NMX *Clock* and HPX *Clock* having a substantial difference compared to HPX *Clock* (P=0.0038 & 0.0031 respectively)(Supplemental Figure 1). mTOR expression meanwhile, had a significant overall ANOVA model (P=0.0011) indicating significant differences among mTOR activity means. This was reflected with the HPX *Clock* group, which had significant increases in mTOR expression compared to NMX WT and HPX WT (P=0.0208 & 0.0008 respectively). While we do see a similar significant increase with mTOR in NMX *Clock* compared to HPX WT (P=0.0137), there is no longer an observed difference when comparing NMX *Clock* to NMX WT (P=0.5342). HPX *Clock* also showed a greater mTOR increase compared to NMX *Clock* (P=0.0087) while the HPX WT had significantly lowered with all treatments suggesting that *Clock* overexpression increases mTOR in hypoxia.

A



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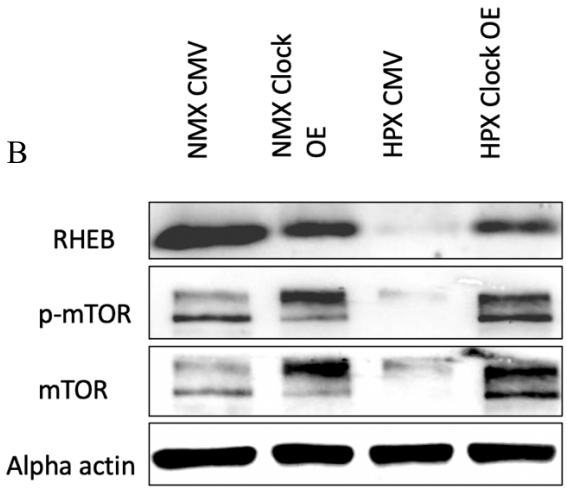


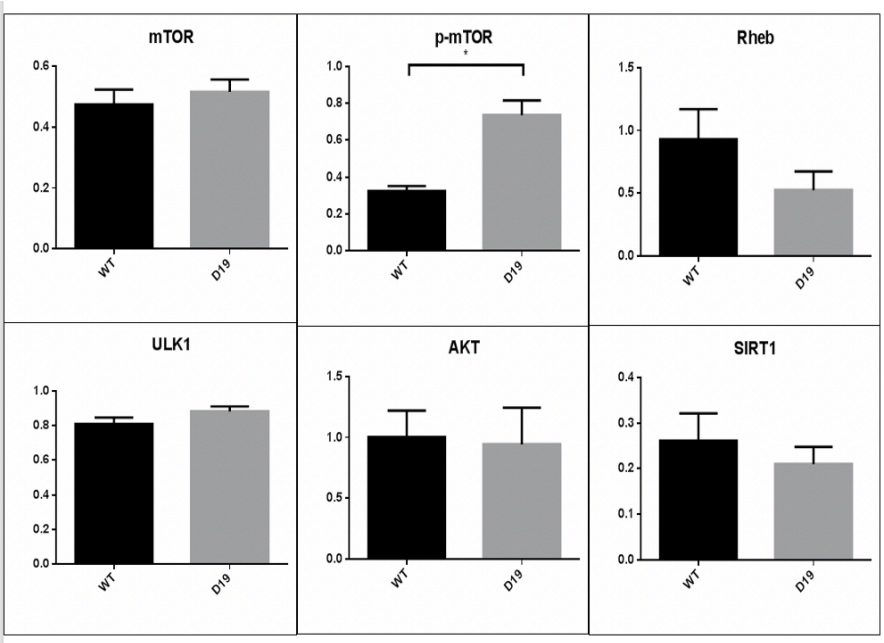
Figure 1 **Hypoxia reduces mTOR function via Rheb in cardiac myocytes** A) Western blot analysis of mammalian target of rapamycin (mTOR), phospho-mTOR (p-mTOR), and Ras homolog enriched in brain (Rheb) in normoxic (NMX) and hypoxic (HPX) conditions infected with either Cytomegalovirus (AdCMV) or Circadian locomotor output cycles kaput (Clock) (X-axis) and ratio of probed protein to α sarcomeric actin (Actin) (Y-axis). B) Qualitative representation of mTOR, p-mTOR, and Rheb expression along with the loading control actin.

3.2 **Cardiac Myocytes Exposed to the Δ19 *Clock* Mutation**

The *Clock* Δ19 mutation is a deletion in the 19th exon of the mouse *Clock* gene and shows how cardiomyocytes perform when *Clock* is non-functional. This is reflected in Figure 2A as the

neonatal cells with the *Clock* mutation show less activation of Rheb and mTOR. However, only p-mTOR had a significant increase in the *Clock*Δ19 treatment (P=0.0124) On the other hand, WT cardiomyocytes no change in the expression of the autophagosome inducer ULK1 compared to the *Clock* Δ19 mutants. Other upstream targets of mTOR such as AKT and SIRT1 also had no significant changes between WT and *Clock* Δ19 conditions.

A



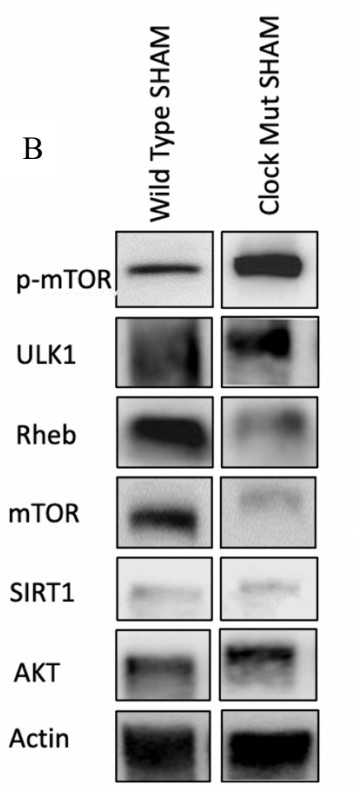


Figure 2 **Clock Δ19 disrupts mTOR signalling** A) Western blot analysis quantifying the expression of mTOR, p-mTOR, Rheb, Unc-51 like autophagy activating kinase (ULK1), Protein kinase b (AKT), and sirtuin 1(SIRT1) in cardiac myocytes with the Clock Δ 19 mutation (Clock Mut) (X-axis) and ratio of probed protein to actin (Y-axis) . B) Visual representation of the western blot analysis of mTOR, Rheb, ULK1, AKT, SIRT1 and the loading control actin.

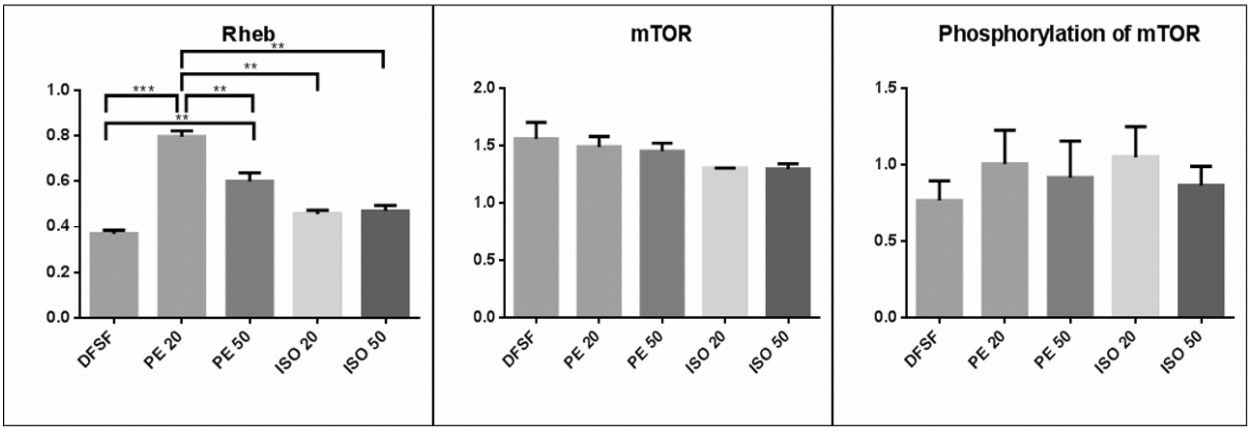
3.3 **Cardiomyocytes Exposed to Hypertrophy and Starvation Media**

The pro-growth inducers PE and ISO were placed in media at both 20 and 50 μg/mL. p-mTOR expression had no significant change between any of the treatment groups compared to control (Figure 3A). There was additionally no change in the expression between treatment groups, however, quantitatively there appears to be slightly greater expression in the 20μg groups. We also did not detect any differences between PE and ISO in this experiment at any dosage. This pattern held true for total mTOR with no significant changes between the control

and treatments groups, and among the treatments themselves. PE did show greater Rheb expression compared to the control at both 20μg (P=0.0004) and 50μg (P=0.0072). Meanwhile, ISO did not show any difference in Rheb expression from control but were lower than their dosage matched counterparts (Figure 3B).

Cardiomyocytes were exposed to different degrees of media which induced starvation for 24 hours including DFSF as a control, DMEM (Dulbecco's Modified Eagle Medium), DMEM without glucose (DMEM0), and HBSS. When analyzing the keystone proteins of the mTOR pathway, DFSF showed the highest expression of p-mTOR compared to all other observed treatments (Figure 4A) and was statistically different from HBSS and DMEM0. The DMEM treatment showed significantly higher expression of p-mTOR compared to DMEM0 (P=0.018); DMEM0 displayed no expression of p-mTOR (Figure 4B). After observing total mTOR there was no significant difference between DFSF, DMEM, and HBSS—however, DMEM0 was significantly different from DFSF and DMEM as this treatment had little observed mTOR expression present (Figure 4B). However, HBSS was the only treatment group not to have a statistically higher expression than DMEM0 (Figure 4A). TSC-2 and *Clock* did not show any significance between groups, however once the controls were accounted for, DMEM0 had greater expression of TSC-2 compared to DMEM and HBSS treatments (Supplemental Figure 3). Notably, *Clock* was downregulated upon HBSS induced starvation compared to DMEM (P=0.0152), but DMEM0 remained similar to DMEM. Additionally, once the expression scores were compared to control, p-mTOR expression in DMEM had a statistically significant lower expression compared to HBSS (P=0.0113).

A



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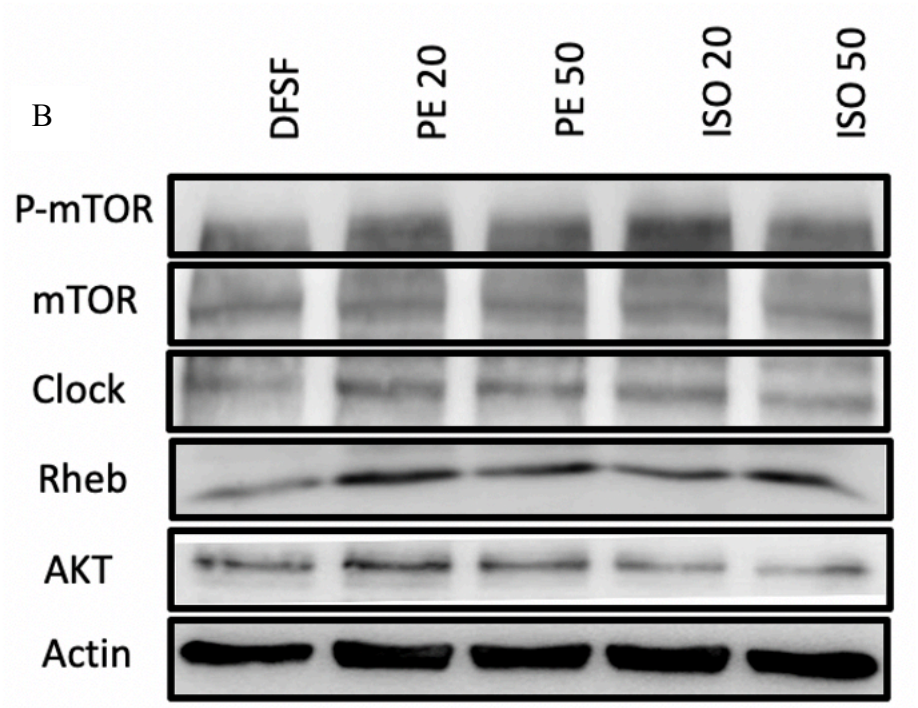
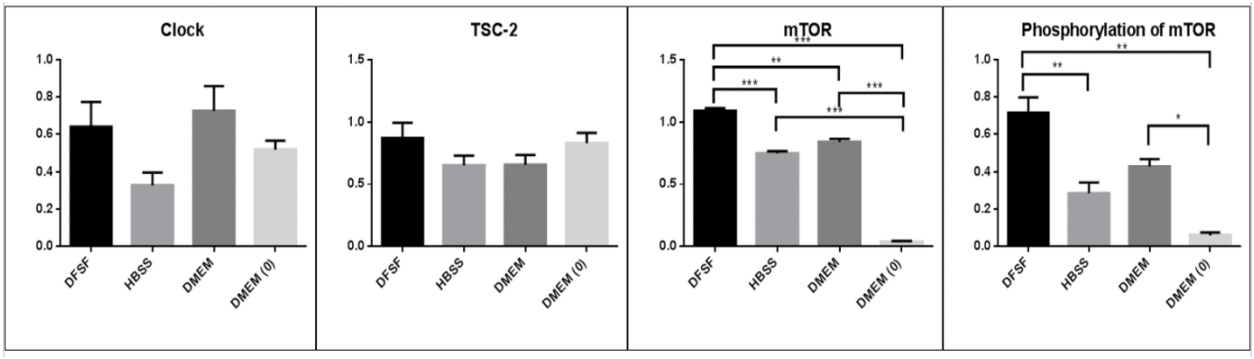


Figure 3 **Hypertrophy inducers PE and ISO increase Rheb in cardiac myocytes** A) Western blot analysis quantifying the expression of p-mTOR, mTOR, Clock, Rheb, and AKT in DF serum-free (DFSF), 20 μg/μM of phenylephrine (PE 20), 50 μg/μM of phenylephrine (PE 50), 20 μg/μM of isoproterenol (ISO 20), and 50 μg/μM of isoproterenol (ISO 50) (X-axis) and ratio of probed protein to actin (Y-axis). B) Visual representation for the western blot analysis of mTOR, Rheb, Clock, and TSC-2 and the loading control actin.

A



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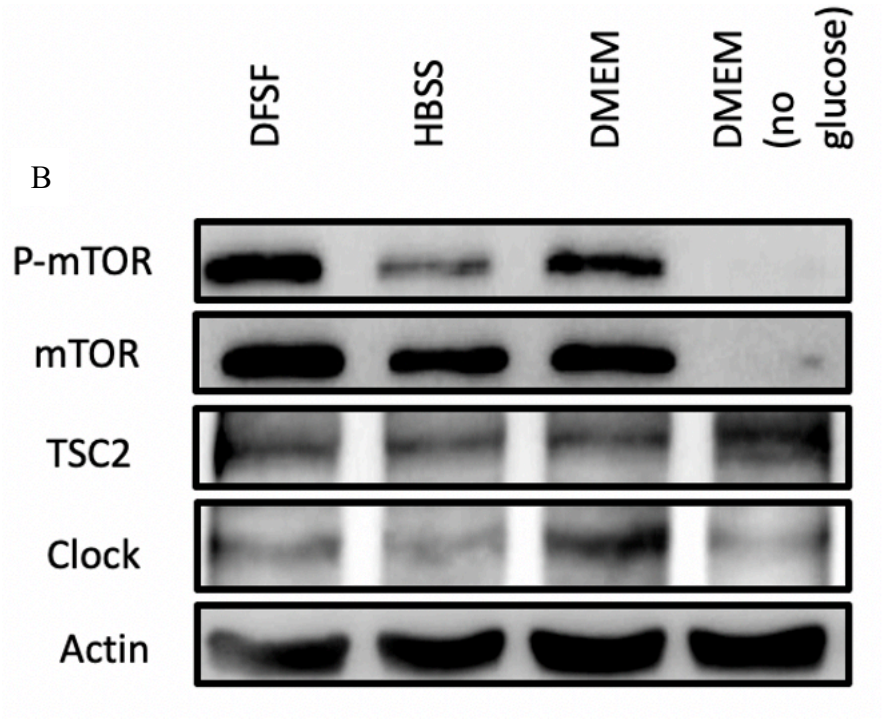


Figure 4 **Nutrient stress disrupts mTOR phosphorylation** A) Western blot analysis quantifying the expression of p-mTOR, mTOR, Clock, and Tuberous Sclerosis Complex 2 (TSC-2) in DF serum-free (DFSF), Hanks Balanced Salt Solution (HBSS), Gibco Dulbecco's Modified Eagle Medium (DMEM), and DMEM without glucose (DMEM0) and ratio of probed protein to actin (Y-axis). B) Visual representation for the western blot analysis of mTOR, Rheb, Clock, and TSC-2 and the loading control α sarcomeric actin.

3.4 *Clock* Overexpression Effect on ΔΨM and Cell Viability in Starvation and Hypertrophic Media

Based on the observance of reduced mTOR activity in starved cardiomyocytes (Figure 4) and the increased Rheb activity seen in cardiomyocytes exposed to hypertrophic compounds (Figure 3), we tested the impact of these cellular stresses on mitochondrial function and cell viability via TMRM and live/dead staining (Figure 5). Loss of ΔΨM was observed with starvation induced by HBSS with respect to all other treatments, most notably DFSF and HBSS+ *Clock* (P=0.0001 for both). This was consistent with the cell viability analysis as there was a significant rise in cell death for cardiac myocytes within the HBSS group compared to DFSF (P=.0001). Infection via *Clock* adenovirus within the HBSS starved cardiomyocyte treatment significantly raised ΔΨM which translated into rescuing mitochondrial activity (P=0.1205) and cell death percentage (P=0.9951) back down to levels similar to the DFSF treatment.

The hypertrophic agent's PE and ISO did not show any major differences between control for either ΔΨM or cell viability. The addition of *Clock* to both of the compounds did not provide any significant difference between the control group. Qualitatively, *Clock* did have a slight protective but insignificant effect on ΔΨM and cell viability most noticeable with respect to PE (Figure 6). As expected, all hypertrophy treatments had significantly high ΔΨM or cellular survival compared to cells starved in HBSS (Figure 6).

LP RP

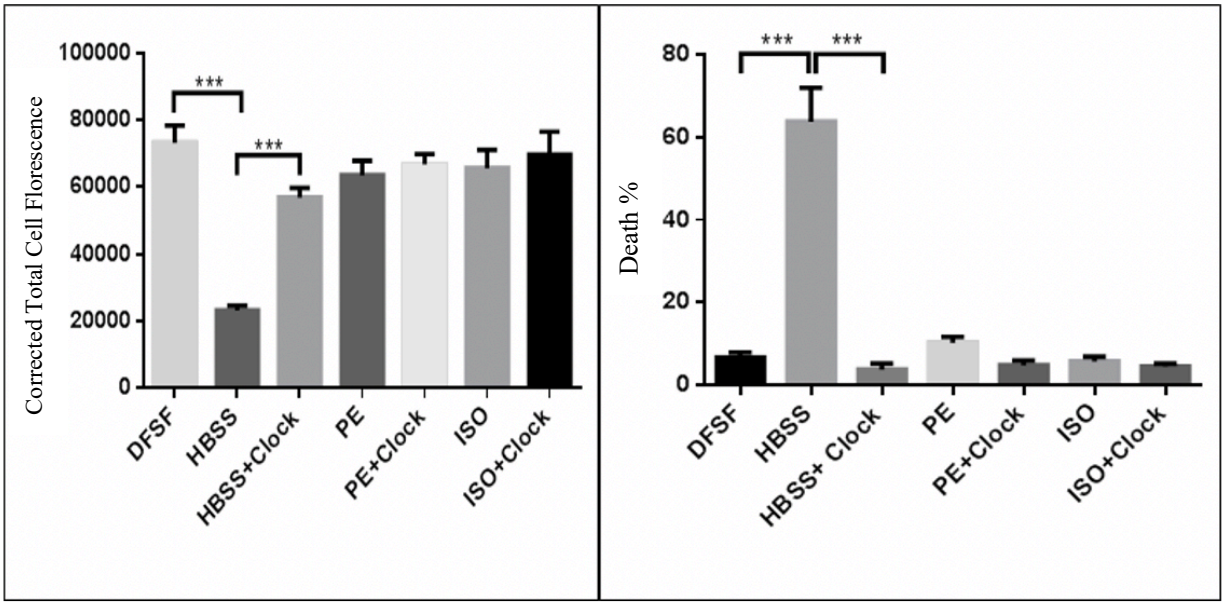


Figure 5 **Clock restores mitochondrial function and cell survival in amino acid starved cardiac myocytes**. Mitochondrial membrane potential (ΔΨM) (LP) and Cell viability analysis (RP) in cardiac myocytes placed in DFSF, HBSS, HBSS+Clock, 20 µg/µM of phenylephrine (PE), PE+Clock, 20 µg/µM of isoproterenol (ISO), ISO+Clock.

LP RP

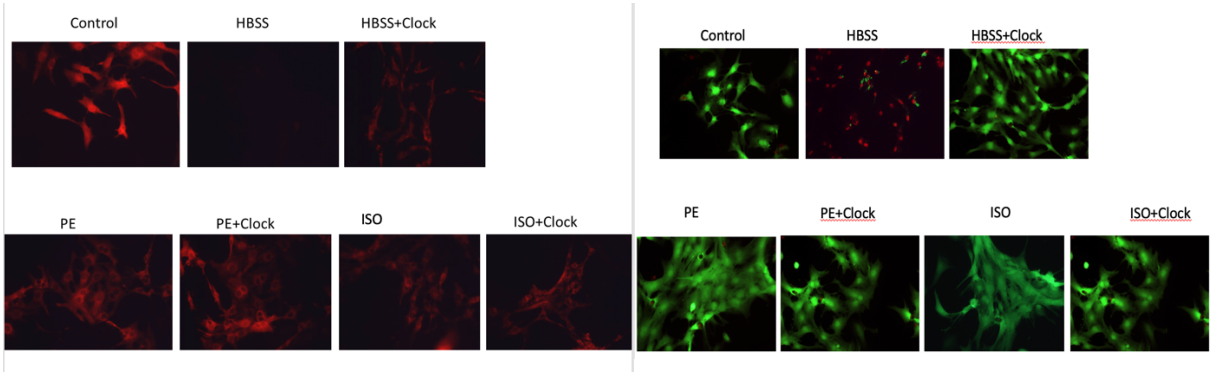


Figure 6 **Staining pictures representing data of Figure 5**. Microscope pictures of ΔΨM (LP) Cell viability (RP) staining for cardiac myocytes in DFSF, HBSS, HBSS+Clock, PE, PE+Clock, ISO, and ISO+Clock

3.5 Western blot analysis of *Clock* Overexpression Under Amino Acid Starvation

After observing how *Clock* rescued starvation induced death from the HBSS, we then looked into if any proteins involved in the mTOR complex had changed when *Clock* was introduced (Figure 7A). We saw a significant increase in kinase activity of mTOR as the HBSS+*Clock* treatment significantly raised expression of p-mTOR compared to just the HBSS treatment (P=0.023). While there was a similar noticeable increase in p-4e-BP1 in the HBSS+*Clock* treatment compared to HBSS, this was not met with significance (P=0.1254). However, HBSS+*Clock* had similar expression means as the control: DFSF (P=0.334) and this was not replicated in the HBSS treatment as there was a significant decrease in p-4e-BP1 compared to DFSF (P=0.0089). Notably, the HBSS treatment had significantly less expression of both p-mTOR and p-4E-BP1 compared to the control (Figure 7A). This pattern was not seen in total mTOR expression, as there was no significant change in mTOR between DFSF, HBSS, and HBSS+*Clock* despite the observed increase seen with regard to the HBSS+*Clock* treatment (Figure 7B). However, when dividing the mean mTOR expression by DFSF, we see that there is a significant difference between HBSS and HBSS+*Clock* (P= 0.0355) (Supplemental Figure 4). Total 4e-BP1 similarly showed no significant changes among the three treatments, however this time dividing by the control gave no improved model.

Infecting *Clock* in the HBSS media appeared to cause a significant change in the mTOR activator Rheb as well (Figure 7B). However, after analyzing Rheb expression means there was no significant changes despite the visualization of HBSS+*Clock* increasing Rheb (P=0.6218). Sirt1 was another protein that was not significantly upregulated within the HBSS and HBSS+*Clock* treatments (P=0.8931). No change was also detected in the kinase activity of the upstream mTOR target: p-AKT, however total Akt was overexpressed in both HBSS and

HBSS+*Clock* compared to control (P=0.0107 & 0.0051 respectively). S6 was another downstream target we investigated, however we did not find any significant differences between the conditons. Downstream of mTOR, ULK1 did show a variety of expression between the three treatments. Cardiac myocytes exposed to HBSS had significant increase of ULK1 compared to DFSF (P=0.001) and to HBSS+*Clock* (P=0.0019). Kinase activity of another autophagy inducer:p62, did show a significant increase in the HBSS treatment compared to HBSS+*Clock* once expression scores were relative to control (P=0.0061) Meanwhile, there was no difference in means detected between DFSF and HBSS+*Clock* (P=0.9791). This trend was also upheld after probing for the kinase activity of the mTOR inhibitor AMPK which was also significantly lower in the HBSS+*Clock* compared to HBSS (P=0.0005). Although here we observed that HBSS+*Clock* was significantly lower than DFSF (P=0.0005) while HBSS and DFSF were identical (P=0.999).

3.6 Western Blot Analysis of *Clock* Overexpression on Hypertrophy Media

The hypertrophic compound PE did not show significantly higher expression of anabolic proteins such as 4E-BP1 (P=0.9446), AKT (P=0.4227), and Rheb (P=0.9863) compared to control (Figure 7A). Kinase activity of either 4e-BP1 and AKT did not have any significant difference compared to control (P=0.9985 & 0.9738 respectively). Additionally, we did not detect any significant difference in mTOR for either total or phospho (P=0.4734). Catabolic proteins such as ULK1 and AMPK, however, did show a significant decrease in the PE treatment when compared to control (P=0.0262 & 0.0014 respectively). Adding *Clock* to PE led to increased significant expression in both total 4E-BP1 (P=0.0158) and AKT (P=0.0026) when compared to control. Total and phospho mTOR were found not to be significantly different from

control with the addition of *Clock* (P=0.5469 & 0.464) and this was consistent for SIRT1(P=0.2225), p-4e-BP1 (P=0.3399), p-AKT (P=0.9738), Rheb (P=0.6735), ULK1 (P=0.9934) and p-AMPK(P=0.0513).

The addition of *Clock* to PE led to certain significant changes of protein expression compared to PE alone, including total 4e-BP1 (P=0.0471), AKT (P=0.0215), and ULK1 (P=0.0133) with the *Clock* treatment upregulating the selected proteins. When incorporating both treatments expression relative to the control in their comparison to each other, we see significant differences in both SIRT1 (P=0.0012) and mTOR (P=0.0015). PE did not show any significant difference in the expression of any desired proteins with respect to ISO except for p-AMPK which was downregulated in PE (P=0.0034). This was mostly kept uniform for PE+*Clock* and ISO+*Clock* as well, however there were key differences found in SIRT1 (P=0.003), AKT (P=0.001), and p-mTOR (P=0.0307) expression (Supplemental Figure 5).

As seen in Figure 7, ISO on its own did not show any significant changes from DFSF with respect to any of the investigated proteins involved in the mTOR pathway. The addition of *Clock* did not provide any significant changes compared to control with respect to any of the observed proteins except for p-AMPK. However, we did see a substantial fall of the upstream mTOR inhibitor compared to the control (P=0.0248). This non-significant protein expression held true for the comparison between ISO and ISO+*Clock* treatments with exception of AKT which had lowered in response to ISO+*Clock* (P=0.0185). Adjusting for the control showed a slight but significant increase to mTOR expression and decrease to p-p62 expression with the addition of *Clock* in ISO compared to ISO alone (P=0.0233 & .0084 respectively). PE and ISO varied in their response to p-AMPK (P=0.0034), with PE having lower expression among the hypertrophic compounds. Notably, the addition of *Clock* did mediate different protein expression

responses from each of the compounds. For example, SIRT1 (P=0.0409), AKT (P=0.001), p-mTOR (P=0.0307), and p-p62 (P=0.0004) were all upregulated with PE + *Clock* compared to ISO + *Clock*. After taking the control into account for the one-way ANOVA table, mTOR also was significantly overexpressed in the PE + *Clock* compared to ISO + *Clock* (P=0.0216).

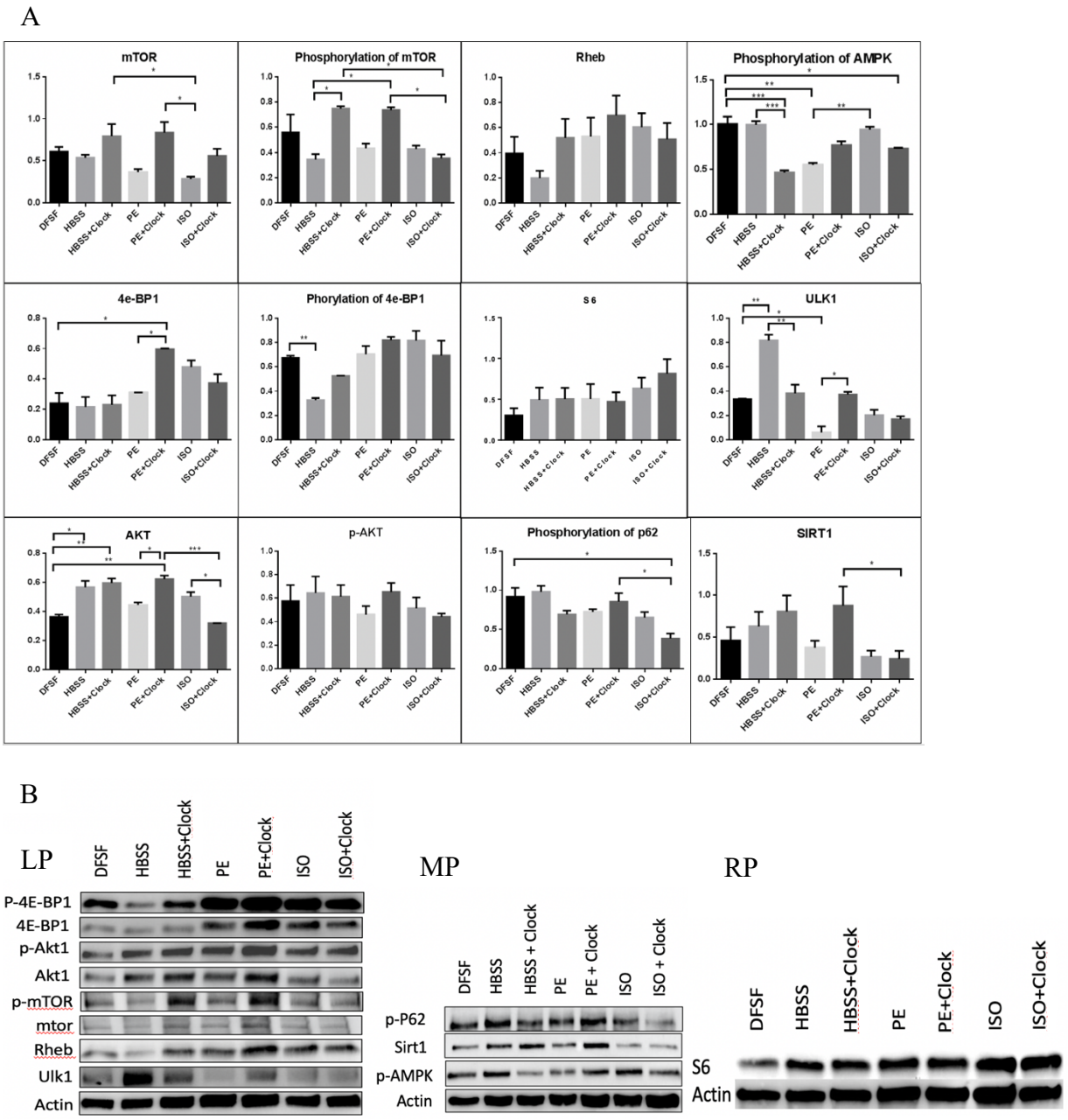


Figure 7 **Clock restores mTOR complex activity via downregulation of p-AMP**K A) Western blot analysis of cardiac myocytes treated with DFSF, HBSS, HBSS+Clock, PE, PE+Clock, ISO, ISO+Clock. Quantified proteins include: mTOR, p-mTOR, Rheb, p-AMPK, 4E-BP1, p-4E-BP1, S6, ULK1, AKT, p-AKT, p-p62, and SIRT1 (X-axis) and ratio of probed protein to actin (Y-axis). B) Visual representation of western blot analysis for mTOR, p-mTOR, Rheb, 4E-BP1, p-4E-BP1, ULK1, AKT, p-AKT (LP), p-AMPK, p-p62, SIRT1 (MP) and S6 (RP). Actin was used as loading control.

3.7 Cell Viability and Mitochondrial Membrane Potential of Circadian Mutations

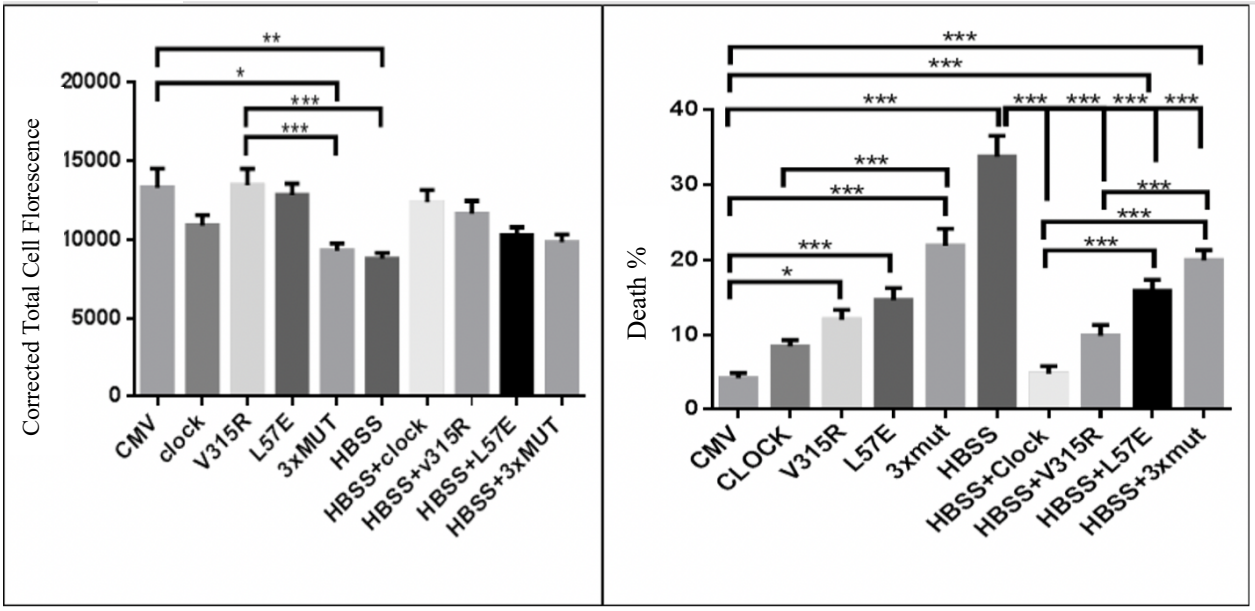
In order to ensure that *Clock* was the true culprit behind the rescue of starvation induced death of cardiac myocytes, we repeated the ΔΨM and cell viability experiment except with different mutations on the *Clock* gene meant to stimulate circadian misalignment (Figure 8). Mitochondrial membrane potential followed a similar trend as control showed the highest florescence among treatments. HBSS continued to have reduced ΔΨM compared to the control (P=.003) which was once again brought back up with the addition of *Clock* (Figure 8A), however this was not significant (P=0.0649). The triple mutant also displayed a decreased ΔΨM compared to CMV (P=0.05). We also observed that once again the triple mutant had the lowest mitochondrial activity among the *Clock* mutations, with significantly lower ΔΨM compared to both V315R and L57E (P=0.0008 & 0.012 respectively). With respect to HBSS, none of the *Clock* mutations provided any significant decrease in ΔΨM compared to HBSS alone (Figure 8A).

In the cell viability analysis, the adenovirus control: CMV, showed no significant change from the cell media control of DFSF (P=0.999). All 3 mutations had a significantly different death rate compared to the control and *Clock* by itself, with 3XMUT showing the greatest significance (P=0.0001). Once again, HBSS had a significantly higher death rate compared to the control (P=0.0001) and was rescued by the addition of *Clock* (P=0.9999) similar to the previous experiment. *Clock* in control media did not show any difference from the true control (P=0.675), however, the addition of *Clock* mutations V315R and L57E did not provide any significant difference from *Clock* for cell viability. The triple mutation was the only circadian misalignment that displayed a significant increase in death rate compared to *Clock* (P=0.0001). Notably, the mutations hindered *Clock*'s ability to rescue cardiac myocytes with all mutations in HBSS

having a greater death rate compared to HBSS +*Clock* (Figure 8A). Despite this, only L57E and 3XMUT in HBSS had significantly greater death rate compared to *Clock* in HBSS. This is most prominent in the triple mutation treatment (P=.0001) which had the death rate closest to HBSS.

Meanwhile, cardiac myocytes with *Clock* Δ19 were transfected to understand how a completely defective *Clock* gene inhibits a cardiac myocytes mitochondrial activity. ΔΨM for the *Clock* Δ19 and the *Clock* Δ19+HBSS were significantly less than PCDNA (P=0.0001 for all). However, we did not see any significant differences among treatment groups (Figure 9A). The death rate observed in cardiac myocytes transfected with *Clock* Δ19 was not significantly greater than PCDNA (P=0.5716). Once the cells were starved via HBSS, the combination of HBSS+ the *Clock* Δ19 had massive increases in cell death that was significantly greater than the other two groups (P=0.0001 for both). Additionally, having a completely defective *Clock* gene in HBSS had a significantly greater death rate compared to just HBSS alone (P=0.001) (Figure 9B).

A LP RP



B

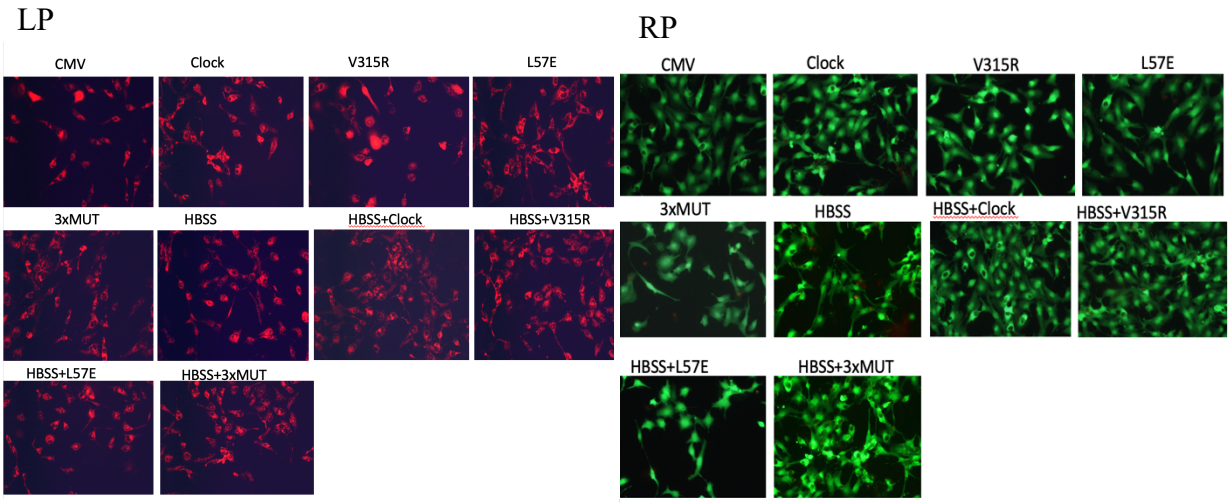
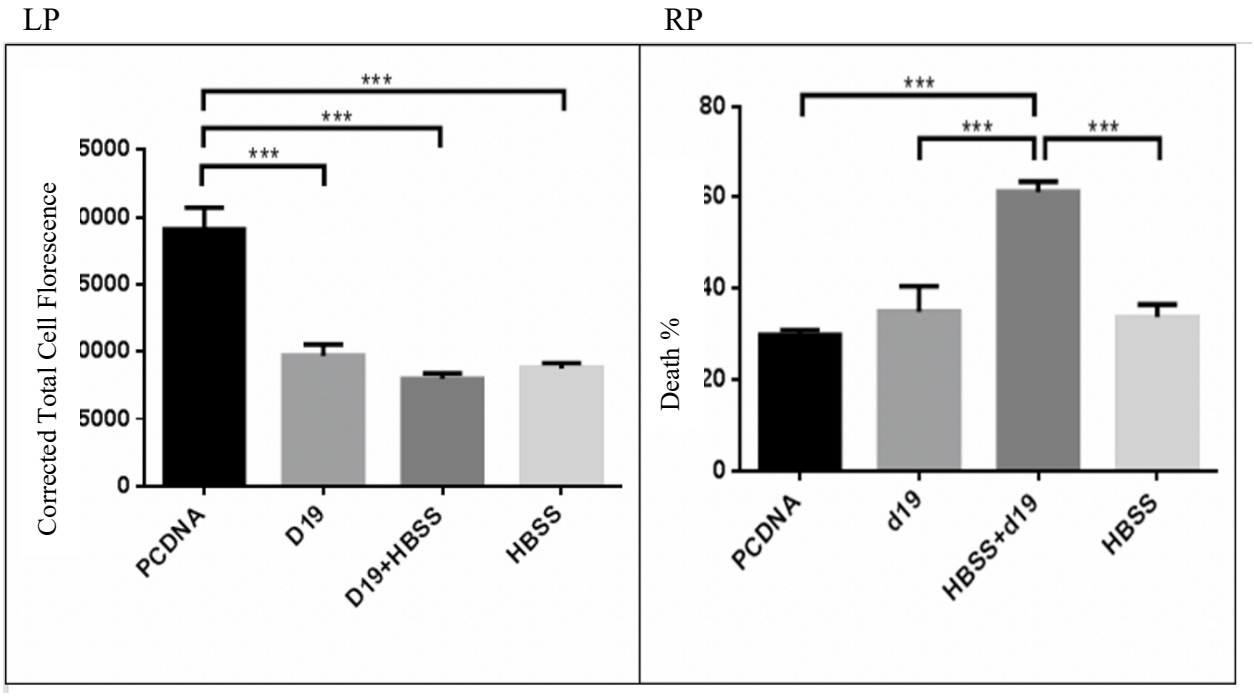


Figure 8) **Clock mutations inhibit rescue observed in HBSS+Clock** A) Quantifying ΔΨM (LP) and cell viability (RP) analysis for cardiac myocytes exposed to CMV, Clock, V315R, L57E, 3xMUT, HBSS, HBSS+Clock, HBSS+V315R, HBSS+L57E, HBSS+3xMUT conditions. B) Visual representation of the ΔΨM (LP) and Cell viability (RP) staining.

A



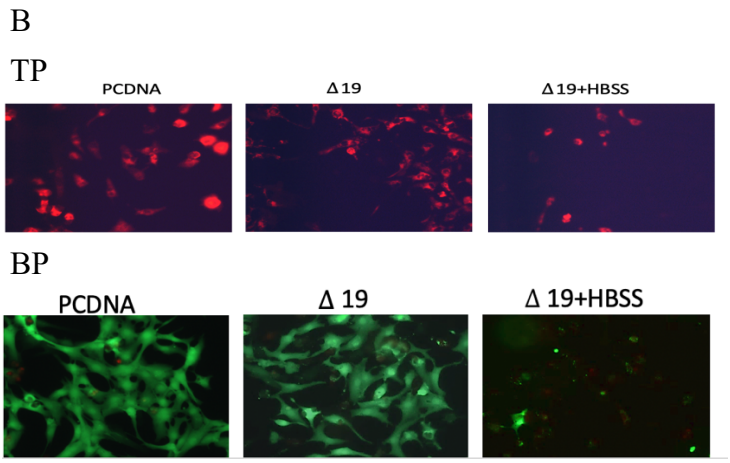


Figure 9 **Clock Δ19 exaggerates mitochondrial dysfunction and cell death in amino acid starved cardiac myocytes**. A) Quantifying ΔΨM (LP) and Cell viability (RP) analysis for cardiac myocytes exposed to PCDNA, Clock Δ19, and Clock Δ19+HBSS. B) Visual representation of the ΔΨM (TP) and the Cell viability (BP) staining's.

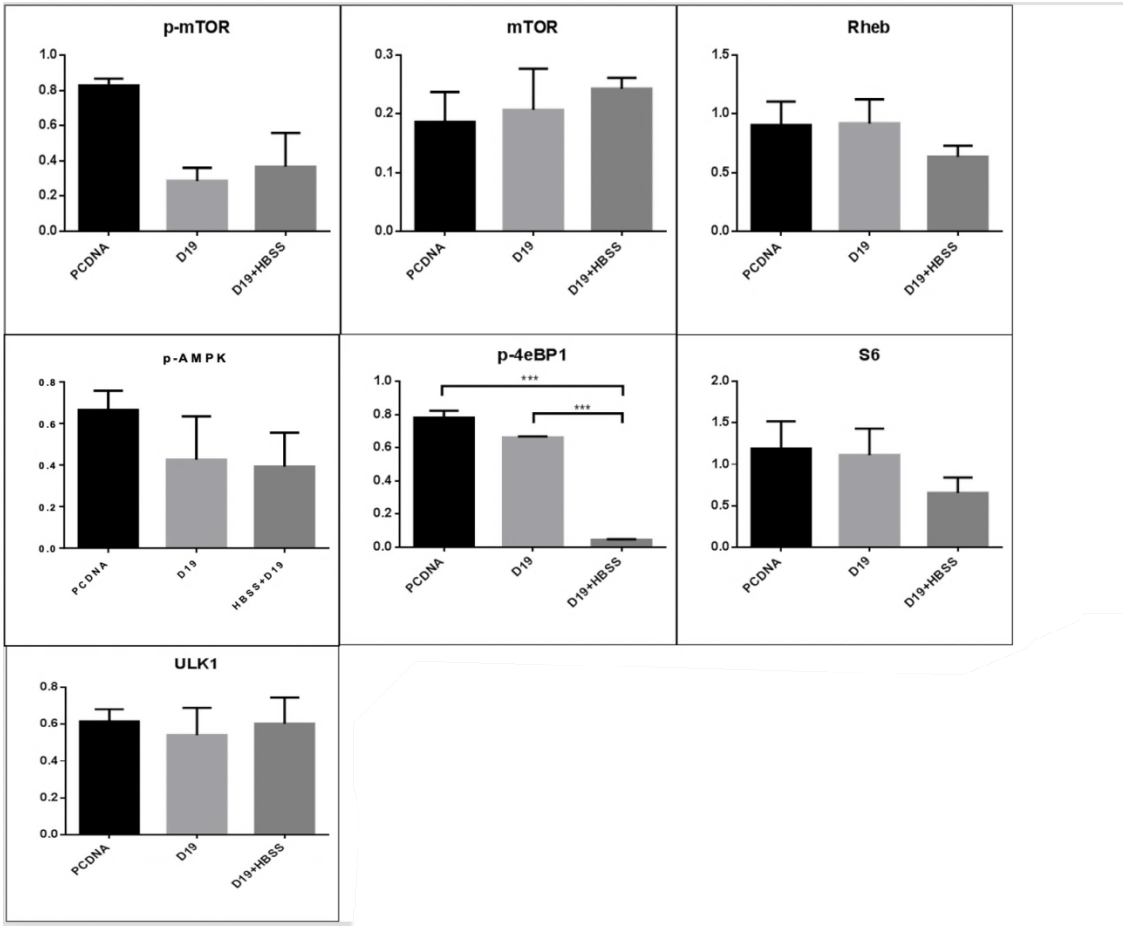
3.8 Western Blot Analysis of *Clock* Δ19 and *Clock* Δ19+HBSS

Due to the mass decrease in cell viability and ΔΨM observed in the Δ19+HBSS treatment, we took to western blot to investigate how the mTOR pathway responded to the circadian deletion coupled with amino acid starvation (Figure 10). Starting with total mTOR, we did not see any significant differences in mTOR expression between PCDNA, *Clock* Δ19, and Δ19+HBSS. Although this held true for p-mTOR, we did observe a decrease in p-mTOR expression in both Δ19 and Δ19+HBSS (Figure 10B), however this was non-significant (Figure 10A). Meanwhile, the upstream mTOR activator Rheb also did not show any significant differences between any of the observed groups (Figure 10A) despite the qualitative appearance of under expression in the Δ19+HBSS group (Figure 10B). Once we compared both treatment groups by the control, we did observe a slight significant decrease of Rheb expression in the Δ19+HBSS compared to Δ19 alone (P=0.029) (Supplemental Figure 7). We then looked at the kinase activity of the known mTOR upstream inhibitor AMPK. Expression levels of p-AMPK were greater in the Δ19+HBSS (Figure 10B), however this was not considered significant until

we accounted for PCDNA. After looking at the treatment group:control ratio, there was a significant increase in Δ19+HBSS compared to Δ19 (Supplemental Figure 7).

Downstream mTOR targets such as ULK1 surprisingly did not provide any significant changes both quantitatively (Figure 10A) and qualitatively (Figure 10B). Despite this, other mTOR downstream targets such as kinase activity of 4eBP1 did have strong expression in both PCDNA and Δ19 which were significantly higher compared to Δ19+HBSS (P=0.005 & 0.008 respectively). In a similar manner, expression of S6 also had a decrease in the Δ19+HBSS treatment (Figure 10B), however this was not significant until both treatment groups were compared to PCDNA (P=0.009) (Supplemental Figure 7).

A



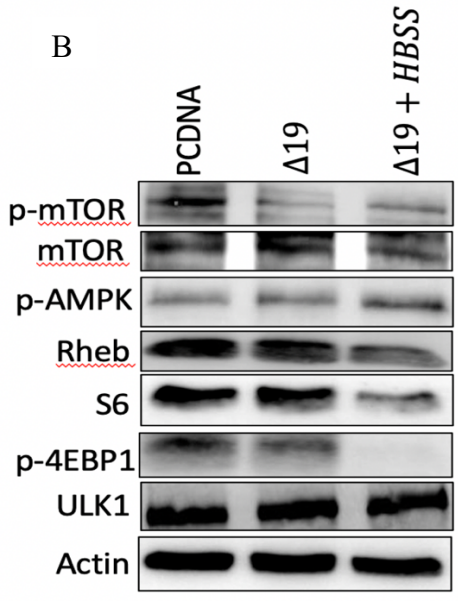


Figure 10 Clock Δ19 prevents proper regulation of AMPK during amino acid starvation in cardiac myocytes. A) Western blot analysis of cardiac myocytes transfected with PCDNA, Clock Δ19, and Clock Δ19+HBSS. Quantified proteins include: mTOR, p-mTOR, Rheb, p-AMPK, p-4E-BP1, S6, ULK1 (X-axis) and ratio of probed protein to actin (Y-axis). B) Visual representation of western blot analysis for mTOR, p-mTOR, Rheb, p-AMPK, p-4E-BP1, S6, ULK1. Actin was used as loading control.

4.0 Discussion

4.1 Hypoxia Regulates mTOR in Cardiac Myocytes

This study provides a comprehensive assessment of *Clock* and its manipulation of mTOR during cardiac myocyte stress. We first investigated hypoxia as cellular stress because of mTOR's sensitivity to O_2 deprivation. Other studies have shown that hypoxia can block mTOR activation through a variety of mechanisms including activation of upstream inhibitors, autophagy promotion, and Rheb inactivation via BNIP3 (Li et al, 2007), (Wouters & Koritzinksy, 2008), (Blagoskonny, 2013). As shown in Figure 1, I illustrate that hypoxia inhibits

mTOR activity response both through an observed decrease in expression in total mTOR, kinase activity of mTORS2448, and impaired Rheb activation. This shows that disrupting mTOR expression occurs in cardiac myocytes. Unexpectedly, I detected a profound increase in the total and phosphorylated mTOR levels when *Clock* was overexpressed in cardiac myocytes subjected to hypoxia. Cardiac myocytes respond to hypoxia by activating the hypoxia inducible transcription factor 1 α (HIF1α), which is responsible for switching metabolism from oxidative metabolism of fatty acids to glycolysis as well as promoting angiogenesis through the activation of vascular endothelial growth factor (VEGF) as pro-survival mechanisms (Airley and Mobasheri, 2007), (Burroughs et al, 2013). *Clock* is known to communicate with hypoxic responses via HIF1α by negatively regulating the transcription factor through activation of *Cry*1 (Dimova et al, 2019). Since the data and previous studies have shown that proteins involved in the activation of the mTOR complex are disrupted by hypoxia, the dramatic rise in the HPX+*Clock* treatment suggests that *Clock* directly or indirectly influences mTOR activity. Future research is needed to conclude whether this is through HIF1α, upstream mTOR targets, or another method.

4.2 *Clock* Δ19 Influences the mTOR pathway in Cardiac Myocytes

Cardiovascular aging is considered a major variable for predicting CVDs and can result in heart failure (Thomas & Rich, 2007). Not only is heart failure a common reason for hospitalization among individuals over the age of 65, but half of the admitted patients die after 5 years of the diagnosis (Mistry et al, 2017). Disrupting the *Clock* gene via the *Clock* Δ19 mutation leads to a variety of issues pertaining to cardiac myocytes including hypertrophy, fibrosis, and reduced cardiovascular contractility (Durgan, 2011), while other studies have found that the

Clock Δ19 mutation promote variables leading to heart failure in an age-dependent manner (Alibhai et al, 2017). Since mTOR is another pathway heavily involved in reversing age-dependent cardiovascular pathology (Dia et al, 2014), I wanted to investigate if mTOR was still functional throughout the *Clock* Δ19 mutation treated cells to potential find evidence towards their relationship. As indicated by Figure 2 B, I found more p-mTOR and less Rheb in the *Clock* Δ19 mutation. Although only the increase in p-mTOR was considered significant, this suggests that a disturbed *Clock* gene is activating p-mTOR. However, due to the relatively low expression of Rheb, this is likely through a pathway independent of Rheb. Interestingly, total mTOR is not significantly greater than the WT condition which highlights the dysregulation of mTOR in the *Clock* Δ19 infected cardiac myocytes.

The study by Dai et al displayed that aged hearts are rescued through mTOR regulation; however, my data suggests that by mitigating *Clock* through the *Clock* Δ19 mutation, the mTOR pathway is both disorganized and inefficient. Taken together, the data highlights that the *Clock* Δ19 mutation may be deregulating proper mTOR activity and leading to persistent and unregulated mTOR activation in cardiac myocytes (Sciaretta et al, 2021). This may result in the observed age dependent increase of heart failure in aging shift workers. ULK1 promotes autophagy and several studies have shown that circadian contributes to proper autophagy regulation. Autophagy is vital to preventing CVDs because of its role in mitochondrial quality control within cardiomyocytes and was found to be impaired by the *Clock* Δ19 mutation (Rhyzikov et al, 2019) via mitochondrial DNA damage and ROS production (Chen, Kroemer, Kepp, 2020). Thus, with a non-functional *Clock* gene, I expected the mTOR:ULK1 interaction to be impaired. However, I did not find any significant change in ULK1 expression between WT and *Clock* Δ19 treatments. While the observed decrease in *Clock* Δ19 infected cardiac myocytes

were marginal, more work is needed to understand how autophagy responds to a defective *Clock* gene. However, based on my results ULK1 is not a major contributing factor in *Clock* Δ19 induced pathology contradicting my original hypothesis.

4.3 The Effects of Phenylephrine and Isoproterenol on the mTOR pathway in Cardiac Myocytes

Following this, cardiac myocytes were subjected to hypertrophy through the use of PE and ISO at two different protein concentrations. Comparing PE to ISO, Figure 3 indicates that PE has a greater effect on mTOR. This is especially highlighted in Rheb where PE at 20ug provides the greatest expression among treatments. Recent literature involving PE and ISO on the mTOR complex is sparely populated; however this western blot supports the hypothesis that α adrenergic activity manipulates the mTOR complex to a greater degree compared to β adrenergic activity, specifically Rheb. Since mTOR is consistently synonymous with growth, I weas surprised to see that the Rheb had the greatest increase compared to the control. Rheb was first found as an mTOR activator within the brain, however overexpression in cardiac myocytes increase infarct size and disturbs autophagy (Sciaretta et al, 2012) (Wu et al, 2013). Additionally, mTOR and Rheb are hypothesized to have different roles in hypertrophy response (Blackwood et al, 2018). mTOR overexpression was found to not result in cardiac mass increase in mouse hearts, and there is growing belief that this is due to multiple signalling pathways which contribute to the enlargement of cardiac myocyte volume during pathological hypertrophy (Sciaretta, Volpe, Sadoshima, 2014). While there were expectations that mTOR would be raised with the hypertrophic compounds PE and ISO, my findings contradict this and support the hypothesis for the involvement of other signalling pathways in non-physiological hypertrophy.

However, future studies are required to investigate Rheb's role in initiating pathological cardiac hypertrophy.

4.4 The Effects of Nutrient Stress on the mTOR pathway and *Clock* in Cardiac Myocytes

The next experiment begun by subjecting cardiac myocytes to different media types with or without certain vital ingredients that prevent nutrient stress. DFSF is typically used for culturing cardiac myocytes from neonatal rat pups as the absence of serum allows the media to act as a true baseline and prevent any serum induced growth which may increase mTOR complex proteins. DMEM meanwhile has an abundance of growth factors, vitamins, amino acids, and 4500mg/L of glucose making it widely applicable for culturing a variety of cell types. HBSS lacks both growth factors and amino acids allowing cells subjected to the media to experience nutrient stress. The last type of media used was DMEM but without the 4500g/L of glucose (DMEM0) to provide a different kind of nutrient stress than HBSS. The first observation noticed was that both phospho and total mTOR had the highest expression under baseline DFSF conditions. This was expected in the nutrient stressed conditions of HBSS and DMEM0 as they were without key ingredients needed to trigger mTOR activation. Notably, DMEM0 did not show any total or phospho mTOR expression and was even significantly lower compared to HBSS. During glucose starvation, expression of mTOR is completely inhibited, potentially as a survival mechanism to prevent any waste of energy. This adaptation was proposed for a cancer cell model in recent work by Leprivier & Rotblat who propose the idea of inhibiting mTOR blockers to prevent tumor survival (Leprivier & Rotblat, 2020). The upregulation of the mTOR inhibitor TSC-2 seen in Figure 4 in the DMEM0 group further supports their hypothesis. However, in the cardiovascular model, the shutdown of mTOR raises concerns for patients with fluctuating glucose levels from metabolic disorders like diabetes. Herein, I highlight the danger

of glucose starvation while prior studies have shown that overabundance of glucose can be countered via partial mTOR blockage (Das et al, 2014).

Amino acid starvation induced by HBSS was notable because while total mTOR activity was relatively similar to DMEM, p-mTOR was significantly inhibited compared to DMEM once dividing by the control. Therefore, the relative downregulation of p-mTOR suggests that amino acid starvation may be responsible for the inactivity of upstream pro-mTOR kinases resulting in low mTOR kinase activity. This is supported by previous studies indicating that amino acids have the vital role of promoting the formation of Recombination-activating gene (RAG) GTP complex which allow mTOR to localize at the lysosome. Amino acid starvation prevents the active configuration of the RAG GTP complex resulting in mTOR to diffuse through the cytosol preventing the Rheb:mTOR interaction (Sancak et al, 2008) (Jewell, Russell, Guan, 2013). The downregulation of p-mTOR in the HBSS treatment further supports this mechanism within the cardiovascular system.

Amino acid starvation also decreased *Clock* compared to all other treatments. Circadian *Clock* and nutrients have thought to be interconnected due to metabolites such as amino acids undergoing circadian controlled oscillations throughout the day. Additionally, misaligned circadian clocks are risk factors for developing metabolic related disorders such as diabetes (Turek et al, 2005) (Dallmann et al, 2012). This was further supported by a recent study showing that *per* knockout mice have issues metabolizing amino acids such as tryptophan and valine leading to starvation susceptibility (Schablet et al, 2020). Herein, my data provides evidence that this relationship is likely bidirectional, as amino acid starvation does reduce *Clock* expression. Following this experiment, $\Delta\Psi M$ and cell viability staining were used to understand how decreases in p-mTOR and *Clock* translate to mitochondrial dysfunction and cell survival.

4.5 *Clock* Overexpression Rescues Cardiac Myocytes From Amino Acid Starvation Through Downregulation of AMPK

The ΔΨM and cell viability analysis showed significant decreases in both HBSS staining's compared to control. This suggests that the amino acid induced nutrient stress is resulting in mitochondrial dysfunction followed up by an increase in cell death. Nutrient stress has long been a topic of discussion as a hypothesis for the increase risk of diabetic cardiomyopathies, with fatty acids producing pathological inflammation that reduce adaptation to cardiac stress (Ko et al, 2009). Herein, my data shows that absence of amino acids provides a similarly stressful environment as nutrient overload that does not support physiological ΔΨM and cell viability. Notably, the addition of *Clock* rescues both parameters to levels that were similar to control. This is interesting because the understanding of amino acid deficiency adaptation is well defined in the heart. Deficiency of amino acids leads to the activation of non-charged transfer RNA (tRNA) and activates the general control nonderepressible 2 (GCN2) kinase which then activates proper response (Dong et al, 2000) (Qin et al, 2017). Seeing how the addition of *Clock* restored ΔΨM and cell viability, these findings support the hypothesis that circadian helps elicits the amino acid deficiency response.

After finding that *Clock* induced restoration of ΔΨM and cell viability, I took to western blot to analyze how the mTOR complex was manipulated by the overexpression of *Clock* within an amino acid deficient environment. As Figure 5A and B show, a significant increase in p-mTOR activity was detected once *Clock* was overexpressed in amino acid starvation compared to HBSS alone. mTOR is a critical regulator of autophagy which is a vital part of amino acid

deficiency adaptation (Qin et al, 2017). Dysfunctional phospho mTOR may initiate the subpar cell quality control mechanisms leading to mitochondrial impairment and cell death. This is further supported by the rescue of both Rheb (although not significant) and p-4E-BP1 expression in the HBSS+*Clock* treatment compared to HBSS. Additionally, AMPK and ULK1 were brought down in the HBSS+*Clock* group. However, AMPK is brought down to levels that were significantly less than HBSS and the control group while ULK1 levels were similar to basal expression with *Clock* overexpression. This suggests that autophagy levels are rising in the amino acid starved myocytes and might be contributing to the loss of ΔΨM and cell viability. This is supported by prior studies which rescue PC-12 cells from amino acid starvation induced cell death through autophagy inhibitor 3-methyladenine (Sadasivan et al, 2006).

Furthermore, this shows *Clock* is communicating with mTOR upstream through AMPK and downregulating it to bring cell quality control mechanisms back to physiological measures. Lamia et al findings show that AMPK manipulates circadian through Cry and Per genes, while later work suggests that AMPK localization is circadian controlled (Lamia et al, 2009), (Jordan & Lamia, 2012). Another piece of evidence was that only kinase activity of 4E-BP1 changed with the addition of *Clock*. mTOR, when active, phosphorylates 4E-BP1 inhibiting it from binding with eIF4E allowing protein synthesis to occur (Laplante & Sabatini, 2012). Meanwhile there was no significant change found in SIRT1 suggesting that despite the original hypothesis, SIRT1 may not be involved in the response to amino acid starvation. Taken together, these results further support that *Clock* interacts with AMPK leading to functional and physiological mTOR complex and proper regulation of p-4E-BP1 and ULK1 levels resulting in restoration of ΔΨM and cell viability in amino acid induced nutrient stress.

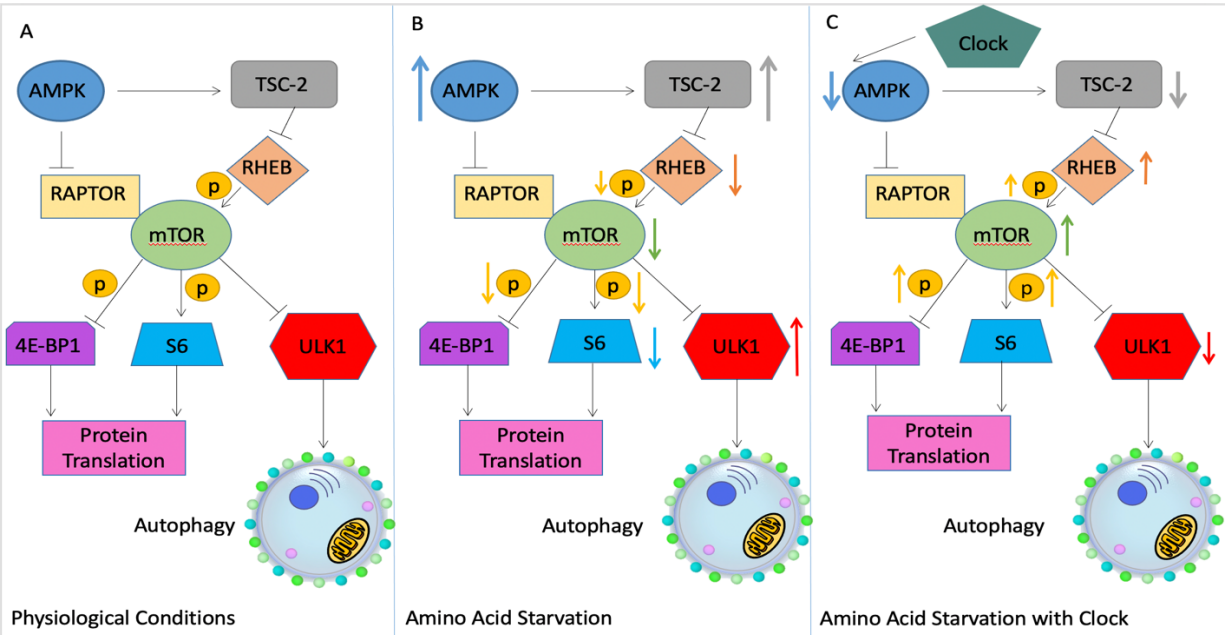


Figure 11 **Clock regulates the mTOR complex via AMPK during amino acid starvation** A) mTOR regulation under physiological conditions showing AMPK initiating TSC-2 induced Rheb inhibition and inhibition of the mTOR complex which phosphorylates S6 and 4E-BP1 leading to protein translation and inhibits ULK1. B) Amino acid starvation creates a nutrient stress that causes increases in AMPK leading to decrease of Rheb, mTOR, p-4E-BP1, S6 and increases TSC-2 and ULK1. C) Overexpressed Clock leads to downregulation of AMPK resulting in mTOR increase and stabilization of ULK1.

4.6 Differences Between the Effects of *Clock* on Phenylephrine and Isoproterenol in Cardiac Myocytes

Conversely, the ΔΨM and cell viability analysis did not show any significant differences between the control and any of the hypertrophy inducing agents. Often cardiac hypertrophy is seen as a major risk factor for heart failure because of its role in contractile dysfunction and ventricular dilation (Papademetriu, 2004) (Abel & Doenset, 2011). If this were the only case, it would be expected that there would be a reduced ΔΨM and cell viability among cardiac myocytes. However, when hypertrophy elicits a non-pathological response, it is considered cardioprotective and explains why endurance athletes often display cardiac hypertrophy and why

the cardiac myocytes were able to survive under PE and ISO conditions (McMullen & Jennings, 2007). Therefore, the presence of ΔΨM and lack of cell death from 20μg of PE and ISO did not elicit pathological cardiovascular hypertrophy.

Overexpression of *Clock* in PE and ISO did not initiate any significant changes to ΔΨM and cell viability. However, investigation of protein expression by western blot analysis showed that *Clock* was manipulating the mTOR complex in a hypertrophy model. Notably, this manipulation only took place in the PE treatment once again supporting that α adrenergic activity is linked to mTOR. α adrenergic receptors help regulate hypertrophy in the heart through mTOR, with prior studies showing that PE induces mTOR kinase activity despite activation of AMPK (Mihaylova & Shaw, 2011) (Sato et al, 2018). The fact that mTOR activity (both total and kinase), Rheb, p-4E-BP1, S6, and Akt were all considered similar to control while AMPK was still present. The addition of *Clock* overexpression only highlights this relationship as mTOR, Rheb, and AMPK all increase slightly. Additionally, ULK1 which was heavily under expressed in the PE was significantly raised with the addition of *Clock*, indicating that circadian may promote positive hypertrophic responses via rescuing autophagy. Chronic or excessive autophagy can cause cellular death as seen previously with amino acid starvation, but autophagy inhibition has been identified as a product of Calcineurin induced cardiac hypertrophy (Maiuri et al, 2011) (He et al, 2014).

This same pattern was not found with ISO, as the western blot indicated that *Clock* is not strongly affecting mTOR, Rheb, 4E-BP1, or SIRT1 in the presence of the β adrenergic agent. Plenty of studies have explored how mTOR is affected by ISO due to the hypertrophic compound's ability to initiate a variety of CVDs. A 2018 study found that a pharmaceutical protected against ISO induced CVDs through autophagy inhibition by activating AKT and

mTOR (Fan et al, 2019). Therefore, I expected to see a downregulation of AKT and mTOR in the ISO treatment, but this was not the case. However, this is likely explained by the concentration used not being enough to elicit pathological responses. It is very likely the *Clock* does not interact with β adrenergic activity at the dosage used in this experiment. This is because the only changes *Clock* overexpression induced were slight significant decreases in AKT and p-p62 and an increase to total mTOR. Despite this, the only evidence of *Clock* providing any influence on ISO response in cardiac myocytes is the significant decrease seen in the autophagy receptor: p-p62.

4.7 *Clock* Mutants Reduce Cell Viability Among Cardiac Myocytes Exposed to Amino Acid Starvation

To ensure that *Clock* was the true culprit behind the changes observed between HBSS and HBSS+*Clock*, another staining took place to investigate ΔΨM and cell viability. This time, a series of circadian mutations were added to simulate circadian misalignments and to investigate if the rescue from amino acid starvation still occurred. The mutations utilized resulted in a specific impairment to the Clock:BMAL dimer and the eventual binding to E-box. The V315R mutation interferes with dimerization activity of Clock and BMAL, while L57E hinders the dimers ability to be E-Box bound. 3xMUT takes advantage of inhibiting both dimerization and binding to E-Box (Huang et al, 2012). In similar fashion to the last ΔΨM analysis, HBSS continued to have poor mitochondrial activity and cell viability rate, but the addition of *Clock* brought both parameters back up to similar levels as control. Additionally, the circadian mutation V315R and L57E showed similar ΔΨM to fully functional *Clock* and the control. It was the triple mutant where a significant dip in ΔΨM was noticed that was similar to HBSS. However, there

was not the expected significant drop in any of the HBSS treatments paired with a *Clock* mutation. Cell viability was a different story, as the addition of *Clock* mutations hindered *Clock*'s ability to save cardiac myocytes. This was demonstrated with the L57E and 3XMUT mutations which had significantly higher death rates compared to HBSS+*Clock* and HBSS+V315R (3XMUT only). Despite this, HBSS alone still carried the highest death rate which indicates that these *Clock* mutations are either not fully blocking *Clock*'s rescuing ability in amino acid nutrient stress or *Clock* is only contributing to the rescue. The former is supported by the fact that HBSS+V315R acted in a similar fashion to HBSS+*Clock* with respect to ΔΨM and cell viability while the triple mutant resulted in greater mitochondrial dysfunction and cell death.

4.8 *Clock* Δ19 Fails to Rescue Cardiac Myocytes From Amino Acid Starvation via Dysregulation of p-AMPK

This experiment was repeated with the utilization of the *Clock* Δ19 mutation within HBSS. The pairing of *Clock* Δ19 with HBSS resulted in the hypothesized drop in ΔΨM and cell viability. The observed mitochondrial dysfunction was significantly different than the control of PCDNA but were similar to both HBSS and *Clock* Δ19 alone. Circadian and amino acid metabolism have become tightly linked in recent years, with many key metabolic actions such as amino acid digestion (Barattini et al, 1993), absorption (Pan & Hussain, 2009), and tissue utilization (Durgan et al, 2011) depending on time of day. Therefore, it was not surprising to see *Clock* Δ19 in nutrient sufficient media display poor mitochondrial activity. Since amino acid metabolism was already impaired from a dysfunctional *Clock* gene, this helps explain why *Clock* Δ19+HBSS did not drastically worsen ΔΨM levels. Conversely, the HBSS+ *Clock* Δ19 treatment displayed a copious amount of cell death which was significantly greater than all other

treatments. The drastic rise in cell death along with the lack of cell survivorship indicates that the pairing of amino acid starvation *Clock* Δ19 is non-habitable for cardiac myocytes.

Herein, a fully dysfunctional *Clock* gene mitigates HBSS+*Clock* rescue of ΔΨM and cell viability, which supports my earlier findings suggesting that *Clock* is contributing to amino acid starvation rescue. Of note, AMPK expression in the HBSS+*Clock* Δ19 was not significantly different than PCDNA or *Clock* Δ19 supporting the hypothesis that *Clock* rescues through AMPK during amino acid starvation. Even though AMPK was under expressed, there was no expected rise in the p-mTOR and Rheb. In fact, both p-mTOR and Rheb levels were less than control and resulted in reduction in p-4E-BP1 and S6 while ULK1 expression went unchanged from control. This deviates from the previously defined relationship between AMPK and the mTOR complex but provides additional evidence that *Clock* dysfunction leads to improper mTOR regulation in response to amino acid starvation. To our knowledge, this is the first experiment that functionally links the *Clock* gene to nutrient stress adaption from amino acid starvation likely through the means of reducing AMPK and stabilizing autophagic activity.

5.0 Conclusion

In this study, cardiac myocytes underwent multiple forms of stress which helped explore how the role of the mTOR complex is regulated by *Clock*. Through nutrient stress initiated by amino acid starvation a novel relationship between *Clock* and the mTOR pathway was identified through downregulation of AMPK. Future studies are needed to reveal whether this is mediated through downstream circadian regulators such as *per* or *cry* which have been identified for their role in regulating AMPK in non-amino acid deficient environments (Lamalia et al, 2009). Additionally, *Clock*'s role in mTOR regulation for both hypoxia and glucose induced starvation remain to be

explored. Deficiency of amino acid metabolism is a major characteristic in those with metabolic disorders (Mangge et al, 2014) (Tobias et al, 2018) (Grajeda-Iglesias & Aviram, 2018). Herein, this data provides exciting evidence that interventions which restabilize *Clock* may restore cardiac myocyte adaptation to amino acid starvation.

6.0 References

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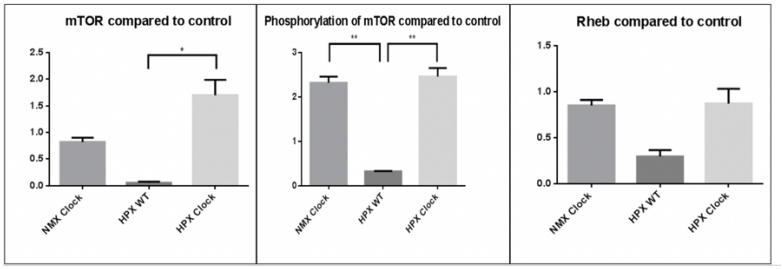
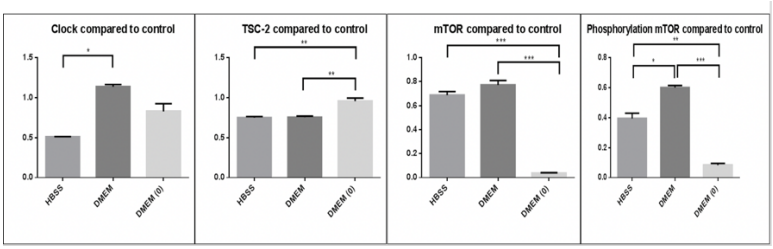
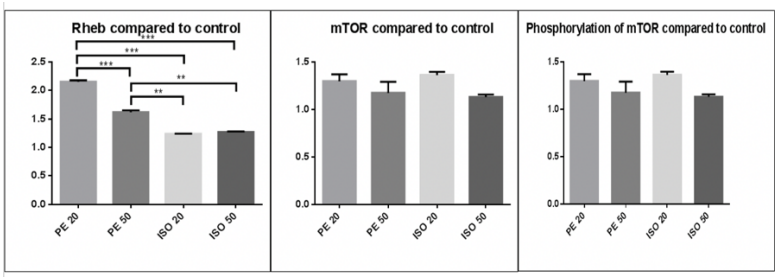
7.0 Supplemental Material



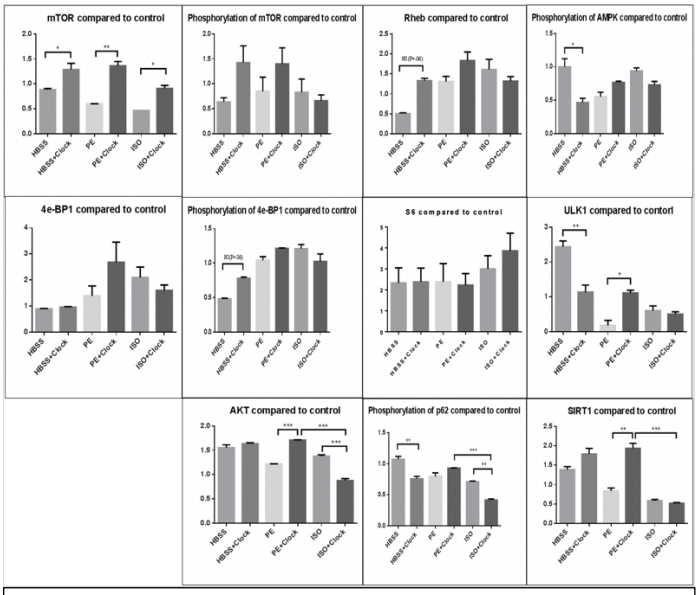
Supplemental Figure 1. Figure 1 A) compared to control



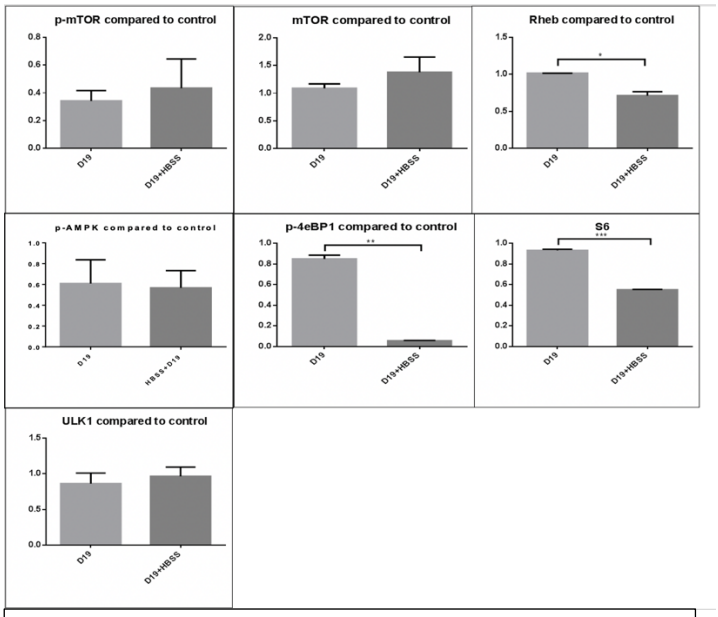
Supplemental Figure 2. Figure 3 A) compared to control



Supplemental Figure 3. Figure 4 A) compared to control



Supplemental Figure 4. Figure 7 A) compared to control



Supplemental Figure 5. Figure 10 A) compared to control