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Nutrient Sensing Pathways Mediating IGFBP1 Phosphorylation in FGR

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Abstract

Impairment of fetal oxygen levels and nutrient delivery contributes to fetal growth restriction (FGR), which affects 20% of pregnancies. Such cellular stress induces hepatic Insulin-like Growth Factor Binding Protein 1 (IGFBP1) phosphorylation, which sequesters Insulin-like Growth Factor 1 (IGF-I) and markedly reduces fetal growth signaling. IGFBP1 hyperphosphoryaltion in hypoxia is mediated through the mTOR signaling pathway and through the Amino Acid Response (AAR) pathway during amino acid deprivation. Hypoxia stimulates upstream mTORC1 regulators, AMPK and REDD1 which are well-established upstream regulators of one of the two mTOR complexes, mTORC1. The molecular mechanisms by which upstream mTORC1-driven processes regulate IGFBP1 phosphorylation in hypoxia are unknown. We hypothesized that AMPK impacts IGFBP1 phosphorylation by modulating mTORC1 signaling due to hypoxia – a key factor in the development of reduced fetal growth *in utero*. Our results indicated that upregulation of AMPK phosphorylation at Thr172 via chemical activators leads to greater IGFBP1 phosphorylation. Additionally, we investigated the effects of combined hypoxia and amino acid deprivation (specifically leucine) on pIGFBP1 levels. We hypothesized that combined hypoxia and leucine deprivation results in greater IGFBP1 phosphorylation than either treatment alone, however we found that the combined conditions lead to IGFBP1 phosphorylation similar to leucine deprivation alone. The investigations in this study of nutrient sensing proteins (AMPK-MTORC1) and multiple cellular stressors (nutrient deprivation and hypoxia) mediating IGFBP1 hyperphosphorylation help provide greater insight of the underlying mechanisms regulating FGR.

Keywords

IGFBP1, hypoxia, mTOR, AAR, AMPK, REDD1, IGF1, HepG2 cells, Western Blot.

Summary for lay-person

An estimated 20% of all pregnancies are affected by fetal growth restriction (FGR), a condition characterized by improper nutrient flow to the developing fetus, usually resulting in infants with low birthweight. The onset of FGR during pregnancy can also negatively impact physical and neurological health throughout childhood and even into adulthood. These children are disproportionately affected by learning disorders such as ADHD and are at a higher risk for the development of diabetes, obesity and cardiac diseases in later life. Thus, the effects of FGR have life-long health implications making better diagnosis and subsequent therapeutic intervention during pregnancy imperative.

Current diagnostic methods aim to identify physical manifestations of disease through evaluation of physical markers of proper fetal growth such as fetal height and maternal weight, as well as using radiological techniques to visualize the developing baby. However, these methods often identify FGR symptoms late into pregnancy, when symptoms have fully manifested and are irreversible. Our study aims to help identify the onset of FGR earlier on, through the detection of a network of proteins and their signaling activity at the cellular level. The hepatic protein Insulin-like Growth Factor Binding Protein 1 has been highly associated with FGR and is known to mitigate specific protein pathways crucial to fetal growth, especially when phosphorylated.

In this study we hypothesized that IGFBP1 protein and its' phosphorylation is regulated through a network of proteins reliant on AMPK protein in low oxygen conditions, we also hypothesized that conditions of nutrient deprivation combined with low oxygen would amplify IGFBP phosphorylation and increase the levels of IGFBP1. We found that AMPK is among a network of several oxygen sensing proteins involved with increasing IGFBP1 protein and phosphorylation levels. We also found that conditions of low oxygen and nutrient restriction did not amplify the levels of IGFBP1 protein and phosphorylation levels greater than that observed when only one of the conditions is present. These findings help to further shed light about the network of proteins and conditions involved in manifesting FGR at cellular level and helps us to better understand the nature of the disease.

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Lastly, I would like to thank my grandma Rahela Begum who passed away in September 2017 and my grandpa Abdul Wahab Bhuiyan who passed away recently in July 2018. It has been a very difficult year losing you both in such a short span of time. I will always remember your love, kindness and affection.

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List of Abbreviations

°C	Degrees centigrade
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
A	Amps
AAR	Amino acid response
Akt	Protein kinase B
AMPK	AMP-Activated Kinase
ANOVA	Analysis of variance
ATF4	Activating Transcription Factor 4
CAMMKK2	Calcium/Calmodulin-dependent protein kinase 2
DEPTOR	DEP domain-containing mTOR interacting protein
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced chemiluminescent reagent
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4E
EIF2A	Eukaryotic translation initiation factor 2A
FBS	Fetal bovine serum
FGR	Fetal growth restriction
FKBP12	FK506 binding protein
GH	Human growth hormone
HepG2	Human hepatocellular carcinoma
HIF	Hypoxia-inducible factor x
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 receptor

IGF-IIR	Insulin-like growth factor 2 receptor
IGFBP	Insulin-like growth factor binding protein
L	Litre
LKB1	Liver Kinase B1
MAPK	Mitogen-activated protein kinase
mg	milligram
ml	Milliliter
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
nm	Nanometer
nM	Nanomolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Dulbecco's Phosphate Buffered Saline
PERK	Protein Kinase R-like ER Kinase
PP2A	Protein Phosphatase 2A
PP2C	Protein Phosphatase 2C
PPM1E	Mg^{2+}/Mn^{2+} dependent protein phosphatase 1E
raptor	Regulatory-associated protein of mTOR
REDD1	DNA damage inducible transcription factor 1
Rheb	Ras homologue enriched in the brain
rictor	Rapamycin-insensitive companion of mTOR
RPM	Revolutions per minute
S6K	P70S6 kinase

SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TAK1	TGFβ-activated kinase 1
TBS	Tris-buffered saline
TSC1/2	Tuberous sclerosis complex 1⁄2
µg	Microgram
µl	Microliter
UPR	Unfolded protein response
V	Volts

1 Introduction

1.1 Fetal Growth Restriction

Fetal Growth Restriction (FGR), also known as Intrauterine Growth Restriction (IUGR) is a growth disorder characterized by attenuation of fetal growth during pregnancy. The fetal growth potential is determined by a combination of fetal and maternal genetic factors as well as external and environmental factors.[1] During pregnancy factors such as maternal diet, exercise, diseases, infections, air quality and even socioeconomic status have an important role in determining fetal growth.[2] A crucial component in fetal development is the transfer of nutrients and gases from the mother to the fetus via the placenta.[3,4] Abnormalities or complications in any of these areas can potentially lead to FGR. Deterioration in placental function during pregnancy limits the transfer of oxygen and nutrients to the fetus contributing to FGR.[5] Conditions such as preeclampsia, poor nutrition, smoking, hypertension or blood disorders in the mother often result in placental insufficiency–often found concurrently with FGR.[6] The consequences of FGR not only impair early development of the fetus but go on to produce life-long complications in the overall health of the individual such as learning disorders, higher prevalence of cardiac diseases, and obesity.[7,8]

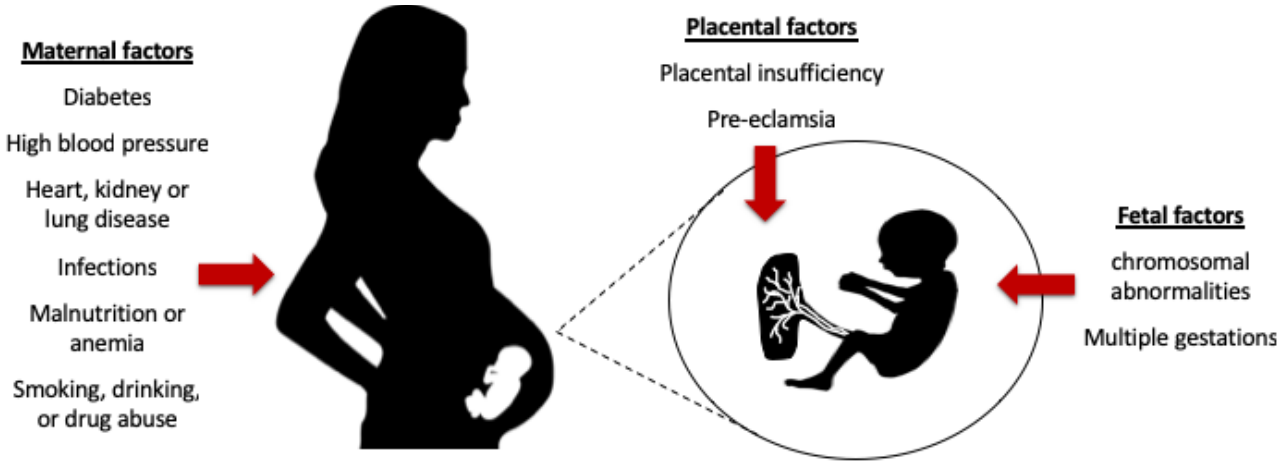

Figure 1: Factors contributing to FGR/IUGR. Factors such as diabetes, high blood pressure, infections, preeclampsia, chromosomal abnormalities, smoking and nutrition are a few of several contributing factors in the development of FGR/IUGR.[31]

1.2 Fetal Growth Restriction Diagnosis

FGR affects 8-10% of all pregnancies and are often associated with higher rates of neonatal mortality and morbidities. Infants affected by FGR are identified based on birth weight below the 10th percentile for gestational age.[1] However, it is often difficult to differentiate newborn infants afflicted with FGR with from Small for Gestational Age (SGA) newborn infants who often lack the physiological comorbidities associated with FGR infants. Therefore, other physical features in addition to weight must be considered.[4] The FGR infants have been noted to have more pathological features such as asymmetry in parts of the body, especially in cranial skeletal structure. The limited transfer of nutrients to the fetus also leads to permanent alteration of gene expression in cells such as adipocytes, myocytes, pancreatic beta cells and hepatocytes. Such alterations in gene expression can vastly change metabolic and endocrine functions leading to greater risk of cardiac diseases, diabetes and obesity in adulthood.[9] Infants born with FGR often grow up to be more susceptible to developmental and learning disorders during early years.[6, 10]

Onset of the disease may be caused by a number of factors such maternal, fetal or placental factors. Reduced maternal-fetal nutrient and gas exchange as a consequence of placental defects is often one of the most critical and common factors contributing to FGR. Placental insufficiency is often diagnosed concurrently with FGR in affected fetuses. The attenuation of oxygen and nutrient supply lead to changes in metabolic proteins and gene expression prior to the physical manifestation of FGR. The changes in protein expression may occur as a result of the oxygen and nutrient stress or genetically predetermined factors, both leading to reduced growth signaling activity. This has been determined in the past through identification of nutrient sensing pathways which are downregulated as a result of the stressors such as Insulin-like Growth Factor 1 Receptor (IGF1R) and Mechanistic Target of Rapamycin (MTOR) signaling cascade, crucial regulators of fetal growth.[11] Conversely, cellular stress responsive signaling pathways are upregulated as a result of nutrient deficiency such as the Amino Acid Response (AAR) and Unfolded Protein Response (UPR) pathways.[12] Investigation of these pathways and their role in the pathophysiology of FGR may provide greater insight in the detection and manifestation of the disease, allowing for the development for better medical interventions and therapeutics for FGR.[9,13]

Current methods of diagnosis primarily focus on identifying maternal risk factors such as blood sugar levels, potential infections, blood pressure, nutrient intake, maternal abdomen circumference, as well as determining fetal physical markers of development such as size and weight through sonographic evaluation.[9] The flow of blood between the fetus and placenta is also monitored using uterine arterial Doppler evaluation of the umbilical artery. However, these methods can often lead to unclear understanding of the fetal growth level.[14,15] In addition, physiological and metabolic changes in the fetus occur much earlier than the manifestation of the physical symptoms of FGR, making it crucial to find methods that can evaluate the onset of FGR symptoms prior to the manifestation of growth attenuation. Effects of early onset FGR are detectable at 34-week gestational age however changes in fetal protein and gene expression levels occur prior to that and are often irreversible even with interventions.[16] In addition to the difficulty in establishing early intervention of FGR pregnancies, differentiation of FGR infants from small for gestational age infants (SGA) is often very difficult to decipher. Approximately 70% of all SGA infants lack comorbidities found in FGR cases. These infants are often healthy but simply have lower body/mass index due to factors such as ethnicity, sex and genetics. Thus, these current markers of identification are largely qualitative and manifest beyond stages of effective intervention, making the need for effective pathophysiological analysis earlier in the onset of FGR.[16]

1.3 IGF1R, IGF1 and IGFBP1 signaling cascade

The Insulin-like Growth Factor (IGF) system of proteins, receptors and ligands are crucial to mediating growth signaling activity and facilitating proper fetal development. The IGF system consists of three different receptors: Insulin-like Growth Factor I Receptor (IGF-IR), Insulin Receptor (IR) and Mannose-6-phosphate (M6P) / Insulin-like Growth Factor II Receptor (IGF-IIR).[17] Although all three receptors are important facilitators of metabolic and mitogenic activity, the IGF-IR complex is of particular interest to us in this study as it facilitates mitogenic activity through IGF-I and IGF-II ligand binding, which is significantly crucial to fetal development.[17] The IR and M6P/IGF-IIR receptor complexes also bind to IGF-I and IGF-II ligands but to a much lower affinity than IGF-IR. As their name suggests, IR has a strong affinity for insulin-binding, while M6P/IGF-IIR receptor has a strong affinity for mannose-6-phosphates as ligands. The Insulin Receptor helps mediate mitogenic activities via binding to IGF-II, progressing the cell from G0/G1 to S and G2/M phases of the cell cycle. The M6P/IGF-

IIR receptor mainly targets M6P residues on newly synthesized lysosomal enzymes from the Golgi apparatus to the lysosomes, ensuring delivery of extracellular lysosomal enzymes to the appropriate cell compartment. Additionally, M6P/IGF-IIR receptor also binds to M6P residues on IGFs but has a much greater affinity for IGF-II than IGF-I. The M6P/IGF-IIR plays a crucial role in the internalization and degradation of IGF-II, making it an important process in limiting growth, and especially of tumorigenic growth. [17]

Both IGF-I and IGF-II ligands share strong similarities in sequence with insulin, as well as structural similarities with proinsulin.[18,19] Although both IGF-I and II are important regulators of fetal growth, IGF-II plays crucial role during early gestational age in neural and postnatal development. IGF-I however plays an important from mid to late gestational age. [17] Both IGF-I and II bind to strongly to IGF-IR initiating mitogenic signaling and proliferation. IGF-I is also found to be correlated in low concentrations in fetal umbilical cord in growth restricted infants and conversely in higher concentration in healthy infants. Recent studies have also implicated human Growth Hormone (GH) in mediating growth globally similar to IGF-I. Both proteins are important in endocrine signaling, promoting the growth globally, as well as in an autocrine and paracrine manner, promoting growth of the liver. [17] While both IGF1 and GH mediate postnatal development, IGF1 has been shown to demonstrate similar effects as GH and are secreted at higher concentrations than GH, which are present in low levels. Defects in IGF-I structure lead to more severe growth restriction while GH defects lead to mild growth restriction. This further indicates the importance of the role of IGF-I ligand in IGF-IR activation as well as promotion of overall growth in fetuses. [18,19, 20]

The bioavailability of IGF-I ligand is impacted by the presence of insulin-like growth factor binding proteins (IGFBP) which bind to IGF-I, extending IGF-I half-life and sequestering it from circulation. The IGF system consists of six different IGFBPs (numbered 1 to 6) which are expressed in various tissues and organs throughout the body.[21,22] All IGFBPs contain great structural homology specifically around the N and C terminus regions, and contain a flexible linker domain common to all IGFBPs.[23] Mutagenic studies of *IGFBP1* gene corelating to the N-terminuses lead to the lack of IGFBP1-IGF-I binding, suggesting that the N-terminus structure of IGFBP1 is crucial for binding. [21,22,23] The importance of the IGFBP1 N-terminus in IGF-I was initially predicted based on nuclear magnetic resonance studies of the region, however site-directed mutagenesis of the region further clarified the important role the N-

terminus region has in IGFBP1-IGF-I binding. Though IGFBPs can bind to IGF-Is, they have varying effects depending on the type of IGFBP binding to IGF-I and the location of where the binding is occurring. [21,22,20]

The IGFBPs can be found circulating globally and locally in specific tissues. In circulation, IGFBPs act as transport proteins carrying IGF-Is, this is often the case with IGFBP3.[17] However, in local tissues, IGFBPs can act as IGF1 regulators, binding to them and sequestering them from circulation. [17,21,22,20] Other IGFBPs can even act independently of the IGF-IR-IGF-I system. The circulating levels of some IGFBPs increase in response to cellular or nutrient stress. Such is the case for IGFBP1 which increases in response to low oxygen, amino acids and increased endoplasmic reticulum stress; this effect on IGFBP1 can be most prominently observed in hepatocytes. The increased levels of IGFBPs in the cell bind to the IGF-Is, mitigating IGF-IR signaling activity, impeding the cell growth and proliferation processes. [16,24]

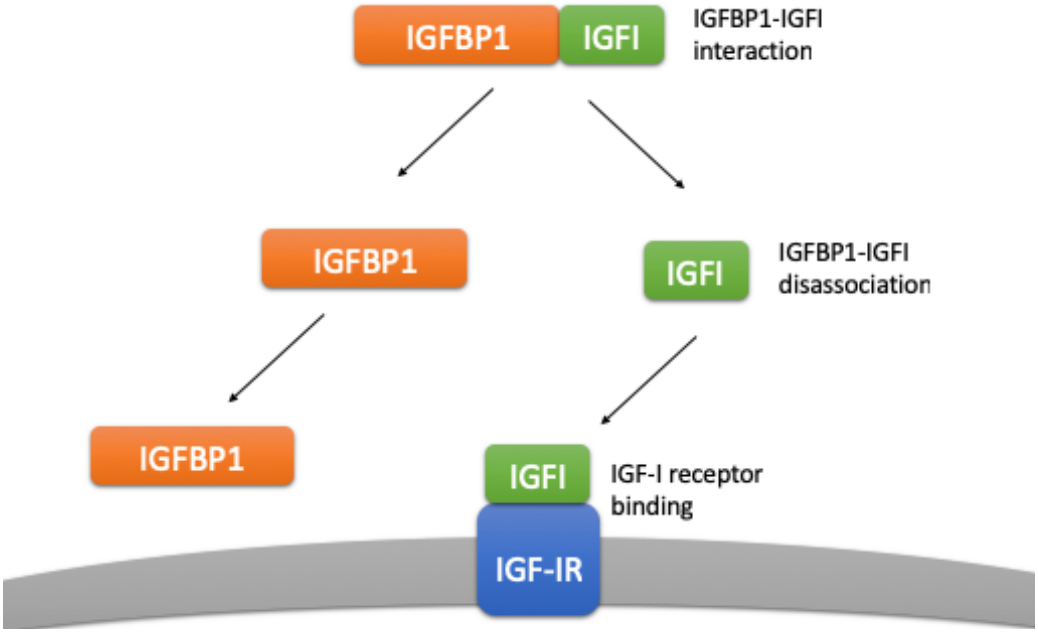

Figure 2: IGF-I-IGF-IR and IGF-I-IGFBP1 binding. IGF-I binds to IGF-IR activating growth signaling cascade, however in the presence of IGFBP1, IGF-I-IGFBP1 binding sequesters IGF-I bioavailability, mitigating the IGF-IR signaling cascade.[25]

Often the obstruction of vascular nutrient and gas exchange from maternal blood to the fetus results in conditions of lowered fetal oxygen levels (hypoxia) and amino acid levels in FGR. These nutrient-deprived conditions upregulate secretion and phosphorylation of IGFBP1 in the

fetal liver- the main site of IGFBP1 expression during fetal development. Although other IGFBP's, such as IGFBP3, are also expressed in the liver, IGFBP1 has the most pronounced expression and upregulation in response to hypoxia, starvation and cellular stress.[26] The fetal liver mediates crucial metabolic and physiological functions in early fetal development.[16, 25] The conditions of hypoxia and nutrient deprivation result in increased phosphorylation at several Serine residues (Ser 169, 119, 101, 98) in IGFBP1. [16] Currently, we are able to detect phosphorylation changes on Ser 169, 119 and 101 through immunoblot analysis through the use and development of customized antibodies targeted for these sites.[27] Various types of cellular stress such as hypoxia, nutrient deprivation and even drugs such as rapamycin has varying degrees of change in the phosphorylation levels at each site.[18] However, the overall increase in IGFBP1phosphorylation results in greater IGFBP1-IGF-I binding affinity. At healthy, physiological oxygen levels (normoxia), IGFBP1 phosphorylation can lead to a 6-8-fold increase in IGFBP1-IGF-I binding affinity. [13,16] Alternatively, hypoxia leads to greater phosphorylation of IGFBP1 protein, which can lead to a 200-300-fold increase in IGFBP1-IGF-I binding affinity. [13,16] The increased IGFBP1-IGF-I binding affinity during cellular stress and nutrient deprivations leads to reduced IGF-I bioavailability, attenuating IGF-IR signaling and subsequently fetal growth. [13,16, 25]

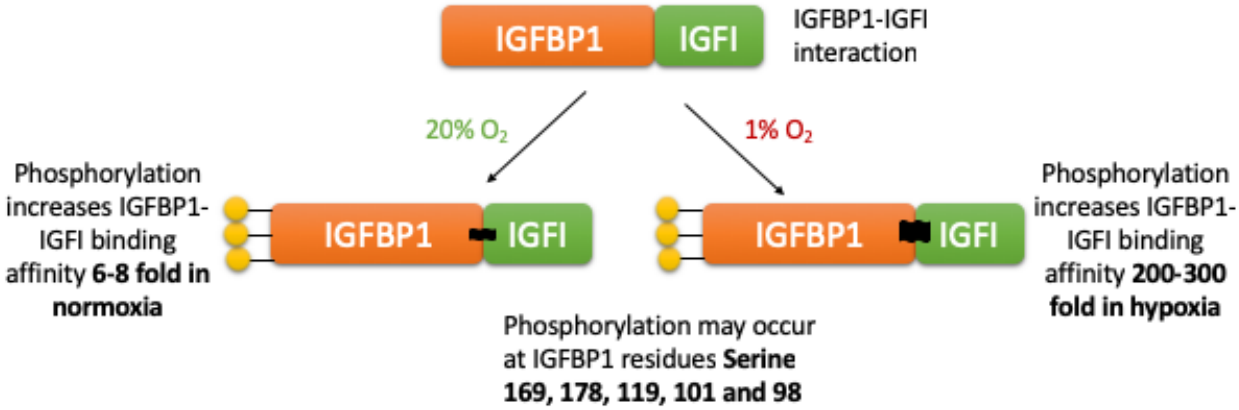

Figure 3: Phosphorylation of IGFBP1. Phosphorylation of IGFBP1 leads to increase in IGFBP1-IGF-I binding affinity in normoxia (6-8-fold) and hypoxia (200-300-fold). [13,16, 25]

1.4 Overview of nutrient sensing pathways regulating IGFBP1 secretion and phosphorylation

The biomolecular mechanisms mediating IGFBP1 phosphorylation during FGR is complex involving numerus pathways. Some of these pathways such as the AAR and mTOR signaling pathway have been shown to effectively regulate IGFBP phosphorylation during nutritional deprivation. In addition, the AAR and MTOR pathways overlap with the UPR signaling, indicating that growth attenuation via IGFBP1 phosphorylation in cellular stress or nutrient deprivation mediates a global response in cells. Previous studies in our lab indicated that hypoxia mediates IGFBP1 secretion and phosphorylation via down regulation of MTOR complexes in human hepatoma cells (HepG2).[18] We also determined that amino acid deprivation, specifically leucine deprivation, leads to increased IGFBP1 secretion and phosphorylation via upregulation of AAR pathway.[19] Current studies in literature indicate that the UPR pathway proteins may be linked to MTOR downregulation and AAR upregulation during cellular stress.[28] However, the role of UPR pathway in regulation of IGFBP1 phosphorylation is unclear. [28,29,30] Additionally, the exact mechanism of how upstream MTOR regulators mediate hypoxic signaling to increase in IGFBP1 secretion or phosphorylation is also unclear.[31] The downregulation of MTOR is indicated to be mediated through complex sequence of events through hypoxia sensing inducible factors (HIFs) and other proteins such as adenosine monophosphate kinase (AMPK) and DNA damage inducible transcription factor 4 (DDIT4/REDD1).[31,32] The role of HIF, AMPK and REDD1 in regulation of IGFBP1secretion and phosphorylation requires greater investigation.[32,33,34]

Overall, the metabolic factors contributing to fetal growth restriction in multifactorial. Several pathways such as MTOR, AAR and UPR collectively contribute to IGFBP1 hyperphosphorylation and subsequently reducing IGF1 bioavailability and sequestering IGF1R activity – a crucial signaling pathway in promoting fetal growth. Our hope is that by closely examining the protein activity contributing to IGFBP1 hyperphosphorylation, we may potentially pave the way to better able to identify the metabolic profile associated with fetal growth restriction and subsequently better prognosis of the disease.

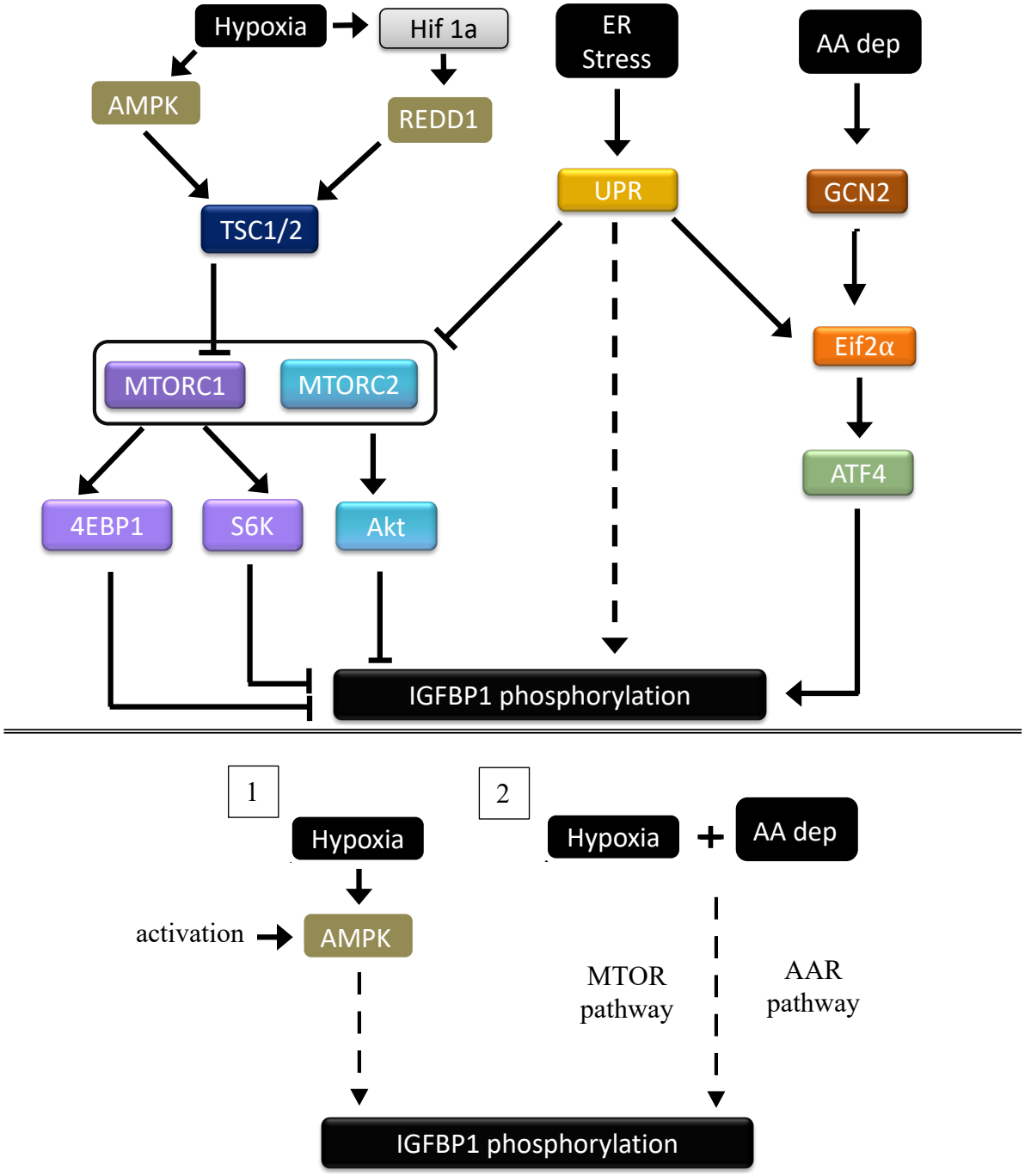

Figure 4: Nutrient sensing pathways mediating IGFBP1 phosphorylation. The MTOR, AAR and UPR pathways respond to nutritional deprivation and cellular stress, leading to an upregulation of IGFBP1 phosphorylation. In this research study we examine (1) the relationship between AMPK activation and IGFBP1 hyperphosphorylation in hypoxia, as well as (2) the impact of combining conditions of hypoxia and amino acid deprivation on IGFBP1 hyperphosphorylation.

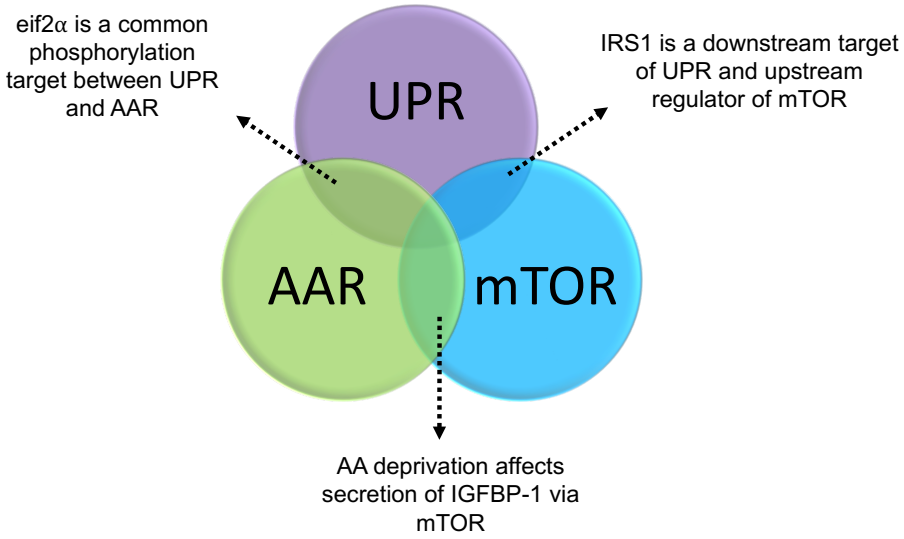

Figure 5: Overlap in nutrient sensing pathways mediating IGFBP1 phosphorylation. The MTOR, AAR and UPR pathways have significant overlap when mediating IGFBP1 phosphorylation in response to nutritional deprivation and cellular stress.

1.5 MTOR pathway

The mammalian target of rapamycin (MTOR), also known as the mechanistic target of rapamycin is a serine/threonine kinase that is a major growth and proliferation mediating signaling pathway in human cells. MTOR is a highly sensitive nutrient sensing kinase that is affected by cellular energy levels, nutrient deprivation, oxygen and cellular stress. The MTOR complex is comprised of 2 subunits MTOR complex 1(C1) and 2 (C2).[18, 35] MTORC1 is comprised of 5 different complexes, with MTOR as the main complex, a regulatory associated protein of MTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8) and a Proline-rich AKT1 substrate 40 and DEP domain-containing mTOR-interacting protein (DEPTOR) component. The last two are non-core components. MTORC2 is comprised of more than 5 different complexes as well, and like MTORC1, C2 also has MTOR is as the main complex.[11, 18, 36] MTORC2 also contains a rapamycin-insensitive companion of MTOR (RICTOR), an MLST8 (GβL) component, a DEPTOR component. MTORC2 has unique components such as mammalian stress-activated protein kinase interacting protein 1 (mSin1), Protor ½, Telomere length regulation protein 2 and Tti1.[35]

Since MTOR is essentially a kinase that is affected by cellular conditions in the promotion of growth, several different downstream proteins are activated by the MTOR kinase in response to those conditions. Past studies have shown that inhibition of MTOR complexes leads to reduced cell growth and size. The MTOR complexes play an important role in cell autophagy, nutrient metabolism, ribosomal activity, and subsequently mRNA translation. The relative activity of the MTORC1 kinase has been well established through the evaluation of downstream effectors the Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and p70S6 kinase (p70-S6k or S6k). [35,36] The phosphorylation of 4E-BP1 and S6k are upregulated in response to increased MTORC1 activity due to factors such as nutrition and oxygen levels. Subsequently, they are downregulated in the absence of nutrition and oxygen. The inhibition of upstream MTORC1 negative regulator Tuberous Sclerosis Complex 2 (TSC2) has been shown to upregulate MTORC1 downstream activity but not MTORC2 activity. [35,36,37] The MTORC2 activity is observed through the phosphorylation of downstream proteins Protein Kinase C alpha (PKC alpha), Serum and Glucocorticoid-regulated Kinase 1 (SGK1) Protein Kinase B (also known as Akt). Akt activity is prominently downregulated in response to MTORC2 inhibition during cellular stress.[38]

The activation of downstream functional readouts of MTORC1/2 lead to the promotion several growth promoting pathways. S6k activation leads to increased protein translation of ribosomal proteins and also increase self-phosphorylation of S6k. Phosphorylation of 4E-BP1 via MTORC1 functions to promote global protein synthesis.[11] When not activated 4E-BP1 binds to the eukaryotic translation initiation factor 4E (eIF4E) however, phosphorylation of 4E-BP1 at Thr70 by mTORC1 prevents 4E-BP1 from binding eIF4E, allowing it to promote cap-dependent translation of proteins. In this way, active mTORC1 signaling is able to promote cell growth through specific pathways via S6K phosphorylation, as well as broader scale changes to protein synthesis via 4E-BP1 phosphorylation.[11,39] MTORC2 functions are more elusive than MTORC1. MTORC2 functions to upregulate cellular growth, survival and proliferation, as well as metabolism. [36] Additionally, MTORC2 plays an important role in regulation of cellular actin cytoskeleton. The activation of Akt is mediated through MTORC1 through phosphorylation at Ser473. [36] The activation of Akt allows for activation of several downstream proteins in facilitating gene expression of metabolic genes, cell-cycle arrest, and death.[38]

Past studies in our lab indicated that hypoxia mediated downregulation of MTORC1 and 2 leads to greater IGFBP1 phosphorylation.[18] However, the regulation of upstream MTORC1 negative regulator TSC1/2 via AMP-Activated Kinase (AMPK) and DNA Damage Response 1/DNA Damage Inducible Transcription Factor 4 (REDD1/DDIT4) in hypoxic conditions need to be further investigated.[40]

Figure 6: Components of MTORC1 and MTORC2. The MTOR complexes are comprised of several units that together regulate and activate different growth and proliferative activities in the cell.[36]

1.6 AMPK mediated downregulation of MTOR

Cellular stress such as low oxygen levels (hypoxia) activates a diverse profile of proteins and enzymes that relay growth attenuation signaling in the cell. Our previous studies indicated the MTOR complexes to be a crucial mediator of growth attenuation. Hypoxic conditions activate a series of upstream MTOR modulators that lead to MTORC1 attenuation, these upstream MTORC1 regulators are known as AMP-Activated Kinase (AMPK) and DNA Damage Response 1/DNA Damage Inducible Transcription Factor 4 (REDD1/DDIT4). Both AMPK and REDD1 are activated in response to cellular stress.[41,42,32] The secretion of REDD1 is increased in hypoxia in a Hypoxia Inducible Factor 1 alpha (HIF1a) dependent manner.[40] The phosphorylation of AMPK is increased in response to hypoxia, due to changes in the AMP to RTP ratio. Both AMPK and REDD1 converge on the activation of MTORC1 negative regulator TSC1/2.[43,44] REDD1 activates TSC1/2 in a parallel and AMPK-independent manner. However, unlike AMPK that responds to cellular energy levels, REDD1 is largely activated in hypoxic conditions through hypoxia sensing molecules such as Hif1a. [31,45] Conversely AMPK is activated by several different nutrient activated proteins. In this study we will examine the role AMPK in MTORC1 attenuation in hypoxia.

There are twelve different types of AMPK in mammals that are found in a tissue specific manner.[46] These proteins are regulated allosterically through competitive binding and possibly post-translational modifications, however, the exact activity of the various types of AMPK or factors that affect the localization of the protein are not fully understood. Activation of AMPK is thought to be mediated when allosteric binding to the AMPK gamma subunit allows for phosphorylation of the Thr172 residue on the subunit. Phosphorylation of AMPK at Thr172 is upregulated in conditions of low energy in tissues, which leads to an upregulation of Adenosine Monophosphate (AMP) to Adenosine diphosphate (ADP) ratio. [47] AMPK phosphorylation in these conditions is mediated through upstream AMPK kinase (AMPKK).[39] Various proteins, enzymes and even chemicals have been implicated to activate, as well as deactivate AMPK in response to cellular stress and nutrition levels.

Known activators of AMPK are Liver Kinase B1 (LKB1), Calcium/Calmodulin-dependent protein kinase 2 (CAMKK2), TGFβ-activated kinase 1 (TAK1), as well as AMPKK. Activation of AMPK can be mediated through chemicals such as Aminoimidazole-4-carboxamide ribonucleotide (AICAR) which is acts as an AMP analog. Sensing an upregulation of the AMP analog AICAR, AMPK is phosphorylated by AMPKK. In addition, AICAR is a chemical intermediate in the generation of Inosine Monophosphate (IMP), an important step in purine metabolism, thus, AICAR is a more natural stimulator of AMPK.[48] Aside from AICAR, Acetyl Salicylic Acid (ASA) or commercially known as Aspirin, has been shown to activate AMPK signaling pathway in various models.[32,47] ASA is converted into its active form salicylate partially in low pH found in the stomach but more so by non-specific esterase found in hepatic tissue. ASA is able to activate AMPK by directly binding to the protein to cause inhibit dephosphorylation of AMPK activation site at Thr172.[49] Activation of AMPK is one of the pathways alongside REDD1 that can lead to activation of TSC1/2. The activation of TSC1/2 leads to inhibition of MTORC1 and subsequently attenuation of global cellular growth and proliferation.[50] AMPK is also known to directly inhibit MTORC2, leading to an overall attenuation of growth signaling activity. [40,43]

Proteins known to promote AMPK dephosphorylation are Protein Phosphatase 2A (PP2A), Protein Phosphatase 2C (PP2C) and Mg^{2+}/Mn^{2+} dependent protein phosphatase 1E (PPM1E).[47] As well, the compound Dorsomorphin/Compound C has also been shown to potentially attenuate AMPK phosphorylation in hepatocytes.[51] In human hepatocytes, downregulation of

AMPK may lead to attenuation of TSC1/2 complex, an MTORC1 inhibitors. Reduced TSC1/2 activity may increase MTORC1 signaling activity and subsequently increased growth signaling activity.[43] Although the role of AMPK as an upstream MTORC1 regulator has been established, the greater role of AMPK in affecting IGFBP1 phosphorylation during cellular stress has yet to be investigated. [50, 52]

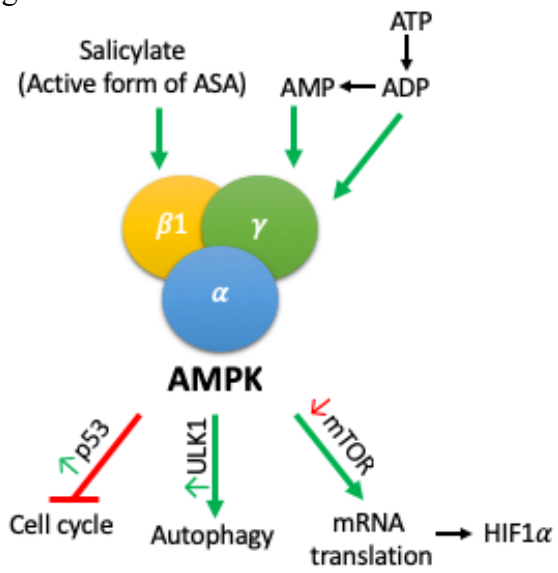

Figure 7: Activation of AMPK via salicylate and AICAR. Salicylate activates AMPK via binding at the β1 subunit of the protein, while AICAR acts as an AMP analog activates AMPK via upregulation of the AMP to ATP ratio. [47]

1.7 Amino acid deprivation

There are 20 amino acids, of which nine are essential amino acids (EAAs). These are leucine, isoleucine, valine, lysine, threonine, tryptophan, phenylalanine, methionine, histidine.[19] These amino acids are crucial to growth, development and maintenance of bodily functions. However, EAAs are not synthesized inside the human and must be acquired externally.[19,53] Excess EAAs however are not stored and are expelled from the body. Therefore, an adequate and continuous supply of EAAs are essential to the development of a healthy fetus (fetogenesis).[54,53] During fetal development, the supply of EAAs are transported from the maternal blood to the fetus via the placenta. Often FGR pregnancies have reduction trans-placental EAA transport. Down-regulation of the expression of placental amino acid transporters such as System L (leucine) transporter, Taurine transporter (TAUT), and System A transporter lead reduced fetal EAA levels in FGR.[55] Damage or abnormalities to the placenta

lead to reduced fetal access to EAAs, in addition to maternal malnutrition which can also reduce maternal and fetal supply of EAAs.[55] In cases where some form of placental dysfunction reduces limits the EAA supply, the levels of EAA in maternal blood increases whilst fetal EAA levels remain low. [55]

The reduced fetal amino acid availability leads to a range of problems in fetal development.[12] Amino acid deprivation studied on animal models indicated that maladaptation to the limited nutrition manifests as quickly as 48 hours, resulting in attenuation of growth. [12] In addition, reduced maternal nutritional intake or fasting, especially in the third trimester can lead to impaired gluconeogenesis. The severe need to provide energy in fetus impairs process mediating fetal growth. Reduction in placental amino acid transfer increases fetal protein catabolism, concentrations of certain fetal tissue amino acids increase in human FGR. Several studies have indicated that the fetal response to amino acid deprivation leads to long term repercussions on offspring health in addition to attenuation of fetal growth. [12]

In an experimental model using rats for investigating the long-term effects of FGR, nutrient restricted pregnant rats showed significantly lower levels of circulating essential branched chain amino acids. This led to the onset of FGR in the off-springs, which had severe glucose intolerance and atherosclerosis in their adult life.[28] In human FGR cases, in approximately 70-80% of FGR infants, metabolic stress due to amino acid deprivation and subsequently low energy supply causes the fetus to prioritize preservation of the functions of vital organs such as the heart, brain and placenta whilst compromising of the full maturation of other organs.[19,56] This frequently leads to FGR infants that are typically born with asymmetrical growth and larger head to body mass ratios. [56] Additionally, maturation of several fetal organs, even the brain is also compromised leading compromised function in later life. [15,57,58]

1.8 Amino Acid Response pathway

The amino acid response (AAR) pathway is activated in response to decreased essential amino acid concentrations and is a cellular stress response mechanism in mammalian cells.[12] The AAR pathway is a part of the integrated stress response pathways (ISR) that are comprised of several proteins that relay and activate stress signaling.[19] When the transfer of nutrients from the maternal blood to fetus placenta is obstructed, a series of stress responsive pathways are

triggered resulting in attenuation of fetal growth. These conditions elicit an activation of amino acid response pathway.[54,59] The amino acid response is an evolutionarily conserved mechanism that can be observed in various species in the manifestation of FGR.

Activation of the amino acid response starts with the phosphorylation of general control nonderepressible 2 (GCN2) protein, which then leads to the phosphorylation of eukaryotic translation initiation factor 2 A (EIF2A).[60,61] The upregulation of EIF2A leads to the increased expression of activating transcription factor 4 (ATF4).[62] Conditions of amino acid starvation promote the accumulation of uncharged tRNA molecules. These uncharged tRNA molecules are detected and sensed by GCN2. The tRNA binds to GCN2 initiating the AAR sequence signaling activity.[63,64] The binding of uncharged tRNA molecules to GCN2 leads to dimerization of the latter, and subsequent autophosphorylation at Thr898.[65] GCN2 is serine/threonine kinase and is one of four different kinases that lead to the activation of the second step in the AAR, activation of EIF2A.[53]

Although EIF2A is the only identified downstream target for GCN2, EIF2A is phosphorylated by the Unfolded Protein Response (UPR) pathway protein PKR-like endoplasmic reticulum (ER) kinase (PERK).[30, 66] The UPR pathway is activated in response to unfolded proteins, endoplasmic reticulum stress and even hypoxia. Additionally, EIF2A is activated by heme-regulated inhibitor kinase (HRI) in response to fluctuation in hemoglobin levels, and double-stranded RNA-activated protein kinase (PKR) in response to viral infections. Although all four kinases activate EIF2A, the downstream effects on proteins and gene expression is varied depending the kinase that phosphorylates EIF2A.[67,68] Activation of EIF2A via AAR and UPR both lead to the increased expression of Activating Transcription Factor 4 (ATF4), that mediates changes and global translation and gene expression. However, ATF4 has varied downstream affects when activated by AAR and UPR.[62, 69] The upregulation of ATF4 protein expression allows ATF4 to allow for the transcription of genes that promote growth attenuation.

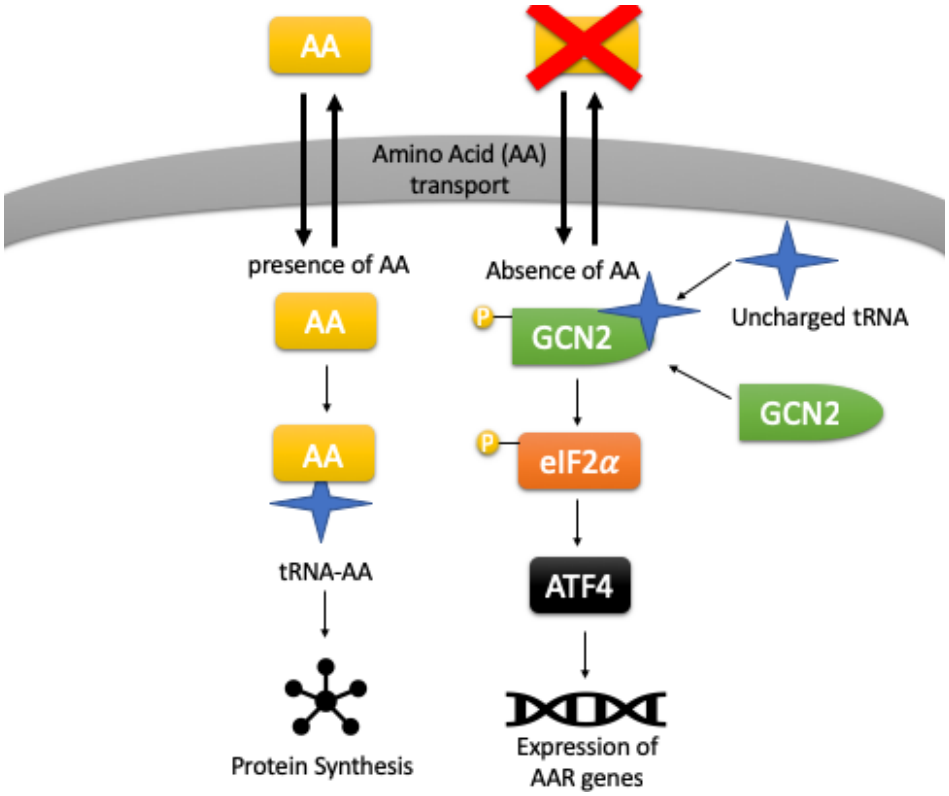

Figure 8: Amino acid deprivation activates the AAR pathway signaling. The AAR pathway signal is relayed through phosphorylation of GCN2, EIF2a and increased expression of ATF4 which promotes expression of AAR genes.[54]

Previous studies in our lab indicated that the AAR pathway can be triggered by depriving HepG2 cells of branched amino acid leucine.[13,19] Conditions of leucine deprivation resulted in hyperphosphorylation of IGFBP1 via the upregulation AAR signaling proteins. However, disruption in the transfer of nutrients through the placenta results in fetal deprivation of both amino acids and oxygen. [13,19] The condition of hypoxia and amino acid deprivation are often concurrently present and have yet to be investigated.

1.9 Rationale, Hypotheses and Objectives for overall study

The condition of Fetal Growth Restriction induces changes the drastically alter gene and protein expression. Our goal is to investigate this phenomenon using HepG2 cells as a model for fetal hepatocyte – a major site of hormone and metabolite secretion essential to fetal development. We sectioned this study into two parts – firstly, (1) investigating the role of upstream MTOR regulator AMPK in mediating IGFBP1 hyperphosphorylation in hypoxia; secondly, (2) we investigate the novel effect multiple stressors (hypoxia and amino acid deprivation) have on protein pathways that we have identified in previous studies as contributors to IGFBP1 hyperphosphorylation. The reason we investigate both protein regulators and cellular stress stimuli in mediating IGFBP1 phosphorylation, is because both of these factors contribute to the inhibition of cellular growth. Although we look deeper into the rationale for these aspects separately in the sections below (**Section 1.9.1** for AMPK and **Section 1.9.2** for combined stressors) our overall goal is to understand the varying metabolic changes occurring during fetal growth restriction, by studying the IGBP1 mediated attenuation of IGF1-IGF1R activity. We hypothesize that growth attenuation is a result of multifactorial cellular stress, activating several proteins upstream of major growth signaling pathways such as MTOR and AAR, resulting in a diverse metabolic profile contributing towards IGFBP1 hyperphosphorylation and growth attenuation in large.

1.9.1 Rationale, Hypothesis and Objectives for AMPK modulation

Rationale: Previous studies have indicated that hypoxic conditions increase AMPK phosphorylation at Thr172 and upregulate REDD1 total protein, which subsequently lead to MTORC1 downregulation through the activation of MTORC1-inhibitor TSC1/2.[35] Previous studies in our lab indicated that MTOR inhibition is a key step in promoting IGFBP1 hyperphosphorylation.[18] However, it is unknown if AMPK modulation via hypoxia or chemical activators can directly impact IGFBP1 hyperphosphorylation via inhibition of the MTOR pathway.

A detailed schematic of the proposed pathway is shown in **Figure 9** in conditions of normoxia (left) and hypoxia (right).

Hypothesis: Hypoxic induction of AMPK increases IGFBP1 phosphorylation via negative regulation of MTORC1 *in vitro* using HepG2 cells as a model for fetal hepatocytes.

Aim: In this study, I will determine the upstream regulation of MTORC1 via AMPK in mediating IGFBP1 phosphorylation during hypoxia.

Objectives:

(1) Determine if AMPK or REDD1 is stimulated during hypoxia.

(2) Determine if AMPK modulation of MTORC1 in hypoxia can induce IGFBP1 phosphorylation.

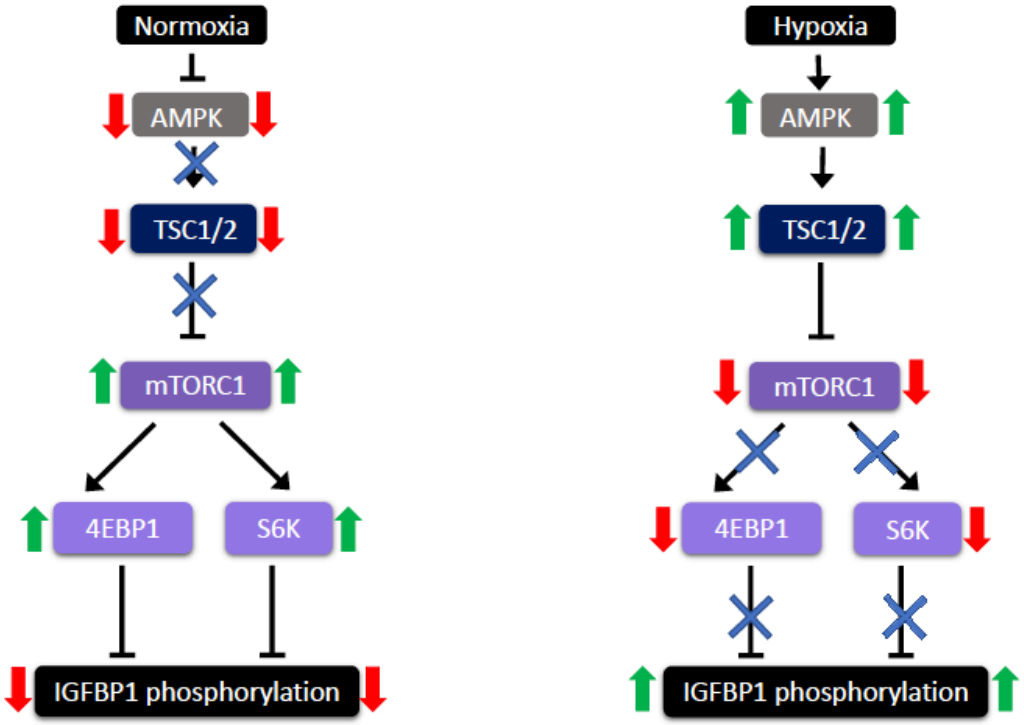

Figure 9: Schematic for hypothesized AMPK mediated upregulation of IGFBP1 hyperphosphorylation via downregulation of MTORC1. We hypothesize that hypoxic signaling relays AMPK mediated downregulation of MTORC1 and subsequently leading to downregulation of 4E-BP1 phosphorylation and p70-S6K (S6K) phosphorylation. All of these steps may collectively be leading to an increase of IGFBP1 phosphorylation, a major protein in facilitating fetal growth restriction.

1.9.2 Rationale, Hypothesis and Objectives for combined hypoxia and leucine deprivation treatment

Rationale: Condition of hypoxia elicit IGFBP1 hyperphosphorylation via downregulation of MTOR,[18] while conditions of nutrient deprivation (specifically leucine) elicit IGFBP1 hyperphosphorylation through the upregulation of AAR pathway.[19] However, these conditions are often found simultaneously in FGR and the proteomic activity in combined conditions of hypoxia with leucine deprivation has yet to be investigated. [13,19]

Hypothesis: Hypoxia mediated downregulation of MTOR and nutrient deprivation mediated upregulation of AAR pathways occur simultaneously during combined conditions of hypoxia with nutrient deprivation *in vitro* using HepG2 cells. The combined conditions hypoxia with leucine deprivation lead to greater levels of IGFBP1 phosphorylation than either treatment alone due to the activation of cellular stress response on IGFBP1 hyperphosphorylation through both MTOR and AAR pathways.

Aim: Determine regulation of IGFBP1 phosphorylation via MTOR and AAR in combined conditions of hypoxia and amino acid deprivation.

Objectives:

(1) Determine the levels of IGFBP1 phosphorylation in combined hypoxia with leucine deprivation in comparison to either hypoxia or leucine deprivation.

(2) Determine the activity levels of MTOR and AAR pathways in combined hypoxia with leucine deprivation in comparison to either hypoxia or leucine deprivation.

1.9.3 Rationale for using HepG2 cell model

Although IGFBP1 can be found circulating in the blood and in various parts of the body, it is highly expressed in the liver during growth restriction.[17,21,] During fetal development the liver acts major metabolic driver of growth through the secretion of various growth hormones and proteins such as IGF-I. For that reason, our goal was to select a model based on the human fetal liver. The HepG2 cells exhibit protein characteristics and gene expression patterns similar to primary human fetal hepatocytes are widely used as a model for fetal hepatocytes.[70,71] Like fetal hepatocytes, HepG2 cells also mainly express fetal isoenzymes of aldolase, pyruvate kinase, with limited cell surface receptor for asialoglycoproteins.[72] Previous studies in our lab indicated HepG2 cells to exhibit similar patterns of IGFBP1 phosphorylation and MTOR downregulation during nutrient deprivation as fetal baboon primary hepatocytes.[16] Therefore HepG2 cells were highly preferred for this study.

2 Methods

2.1 Cell Culture

In this study, human hepatocellular carcinoma cells (HepG2) were used as a model for fetal hepatocytes. The HepG2 (American Type Cell Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with nutrient mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp, Carlsbad, CA) and placed in an incubator at 37°C in 20% O_2 and 5% CO_2. The HepG2 cells were grown to 80% confluence in T-75 flasks (Invitrogen) prior to being plated in 6-well polystyrene culture dishes (Invitrogen) using 3 mL of 0.5% trypsin-EDTA of 1:10 dilution (Life Technologies, Carlsbad, CA) for 5-8 mins at 37°C. Cells were then re-suspended in 10% FBS, DMEM/F12 media to achieve a concentration of $3x10^5$ cells/mL . A total of 2 mL of the cell suspension was aliquoted to each well in the 6-well polystyrene culture dishes and placed in an incubator at 37°C in 20% O_2 and 5% CO_2 for 24 hours, after which the cells were starved in either serum-free DMEM/F12 or 2% FBS DMEM/F12 overnight. After starvation, cells were treated with various treatments and subsequently placed back in the incubator at 37°C in 20% O_2 and 5% CO_2 for 24 hours.

2.2 Hypoxia Treatment

HepG2 cells starved with 2% FBS with DMEM/F12 overnight were placed in a modular semi-sealed chamber (Billups-Rothenberg Inc., Del Mar, CA) that was flushed with 1% O_2 and 5% CO_2 for 15 mins to stimulate hypoxia. The hypoxia flushed chamber with HepG2 cells was subsequently sealed to prevent any gases from leaving or entering. The chamber was then placed on a gentle shaker in 37°C for 24 hrs. At the same time, another set of HepG2 cells starved with 2% FBS with DMEM/F12 overnight, were placed in 20% O_2 and 5% CO_2 for 24 hours to stimulate conditions of normoxia.

In past studies in our lab, the oxygen content in the hypoxic chamber was verified to be 1% O_2 at 12 hour intervals using a Hudson 5590 Oxygen Monitor (Hudson, Ventronics Division, Temecula, CA).[13] Additionally, the pH and partial pressure of O_2 and CO_2 were monitored

using an ABL700 series blood gas analyzer (Radiometer, Copenhagen, Denmark). The pO_2 was determined to be 43.3 torr or 1.53 mm Hg for 1% O_2 and 133.3 torr or 7.36 mm Hg for 20% O_2 at the end of the hypoxia treatment period.[13,73] The pH level was determined to be between 4.5 and 5.5, while decreases between 0.7 to 0.9 were observed in hypoxic treatments.[13] The pCO_2 remained stable between control and hypoxic treatments.[13]

2.3 Optimization of AMPK activation and inhibition

Modulation of AMPK was mediated through select activators (acetylsalicylic acid/ASA and 5-aminoimidazole-4-carboxamide ribonucleotide, (Caymen Chemicals, Ann Arbor, MI)) and inhibitor (Dorsomorphin (Caymen Chemicals, Ann Arbor, MI)). In order to determine the optimal concentration of AICAR, ASA and Dorsomorphin for the treatment of HepG2 cells, a dose response experiment was conducted for each chemical. Concentrations of 3 and 5 uM acetylsalicylic acid were tested for optimal levels of IGFBP1 secretion and phosphorylation. Concentrations of 0.10, 0.25, 0.50, 0.75 and 1.00 mM was tested for AICAR. And lastly concentrations of 1.0, 2.5, 4.0, 5.0 and 10 uM were tested for Dorsomorphin.

HepG2 cells were treated using optimal concentrations of 15.5 mM for ASA, 0.25 uM for AICAR and 2.5 uM for Dorsomorphin in 2% FBS with DMEM/F12 for 24 hours, following overnight starvation in 2% FBS with DMEM/F12. The cells were either treated in either normoxia or hypoxia as described in **Section 2.2**. After 24 hours of treatment, cell media and cell lysate were collected as described in **Section 2.5.**

Table 1. Activators and inhibitors of AMPK

Activators	Inhibitors
Acetyl salicylic acid (ASA)	Dorsomorphin (Compound C)
AICAR	

2.4 Leucine deprivation with hypoxia

Special DMEM/F-12 media selectively deprived of essential amino acids leucine, methionine, lysine, glutamine, as well as various salts (Sigma-Aldrich Corp., St. Louis, MO) was used for the preparation of Leucine 450 mM media. All essential amino acids, except leucine, were

added to the special DMEM/F-12 media to levels normally found in regular DMEM/F-12 media. Biological grade leucine (Sigma-Aldrich Corp., St. Louis, MO) was added to the special media at a concentration of 450 mM (leucine enriched media, used as control) or 0 mM (Leucine deprived media). Once HepG2 cells were plated and starved overnight, cells were washed with 1X PBS and subsequently treated with either Leucine deprived media or Leucine enriched media (2 mL/well). The cells were then stored in the incubator at 37°C in 20% O_2 and 5% CO hours at 37°C, in either normoxia or treated with hypoxia as listed in Section 2.5.

2.5 Cell media and lysate preparation

Following a 24-hour treatment period in the treatment-specific media, cells were removed from 37°C incubator and placed on ice. The cell media was collected from each well into labelled tubes and placed in -20°C. The cells adhering to 6-well polystyrene culture plate were washed using 200 uL of ice cold 1X Phosphate Buffered Saline (Invitrogen, Carlsbad, CA) and subsequently treated with 150 uL of cell lysis buffer solution (1:1000 dilution of protease inhibitor cocktail, 1:1000 dilution of phosphatase inhibitor cocktail 2, 1:1000 dilution of phosphatase inhibitor cocktail 3 and 1:100 dilution of cell lysis buffer stock, (Sigma Aldrich, St.Louis, MI)). The cells were subsequently scraped from the 6-well polystyrene culture plates using the cell lysis buffer solution and a plastic scraper (Diamed, Mississauga, ON) and stored into labelled tubes at -80°C overnight. The cell lysate samples were removed from -80°C the next day and sonicated for 15 secs, prior to being centrifuged at 13000 rpm, at 4 °C for 30 mins.

2.6 Bradford protein assay

After sonication and centrifugation of the cell lysate sample, 3 uL of each sample was added to 42 uL double deionized water (ddH_2O) to make a 1:15 dilution. Samples were plated in 10 uL aliquots, in triplicates on 96-well Microplates (VWR). Additionally, protein standard samples with concentrations of 0, 25, 125, 250 and 500 ug/mL (ThermoFisher Scientific, Waltham, MA) were also plated on 10 uL aliquots, in duplicates. The protein standard samples and their relative absorbance values are used to create a graph of various concentrations (0, 25, 125, 250 and 500 ug/mL), the concentrations of the unknown samples are correlated to be a

value within in that range based on the relative absorbance.

A 200 uL aliquot of Bradford Reagent (BioRad) was added to each sample and standard using a multi-channel pipette prior to irradiation. The 96-well Microplates with protein samples and Bradford reagent was then placed on the Thermo Labsystems Multiskan Ascent Photometer (ThermoFisher Scientific) and irradiated with 595 nm light after an initial shake at 960 rpm. The relative absorbance resulting from the exposure was used to correlate the protein concentration in ug/uL

$$\boldsymbol{Protein\ concentration}\left(\frac{\boldsymbol{ug}}{\boldsymbol{uL}}\right) = (\boldsymbol{20\ ug})/(\frac{\boldsymbol{absorbance}}{\boldsymbol{1000\ uL}})$$

2.7 Cell viability assay

Cells were grown and plated as described in section 2.1 in 96-well polystyrene plates. A total of 10 uL of the cell stock was added to each well and topped off with 10% FBS with DMEM/F12 to have a total of 100 ul of media per well. Cells were grown in 37 degrees Celsius incubator overnight and starved with 2% FBS or 0% FBS (depending on treatment specific starvation) for a minimum of 12 hours. The cells in the 96-well plate were rinsed with 100 uL 1X PBS and treated as described in either sections 2.2, 2.3 or 2.4 for 24 hours. Following treatment, 10 uL of Dojindo Molecular Technologies Cell Counting Kit-8 (Washington, DC) was added to each well and left in incubator for one hour. After incubated plates were taken to Thermo Labsystems Multiskan Ascent Photometer (ThermoFisher Scientific) and irradiated with 495 nm of light. The relative absorbance values were recorded and normalized to wells containing only 2% FBS containing media for deduction of background light. The absorbance values for treatments were than normalized with control (cell treated with only 2% FBS) and graphically represented using GraphPad Prism via One-way analysis of variance with Dunnetts Multiple Comparison Post-Test. The values were graphically presented with a Standard Error of Mean (±SEM) for each sample. Significance values were depicted via (*) $p< 0.05$, (**)$p< 0.003$ and (***)$p< 0.001$.

2.8 Western blotting

Using SDS-PAGE, equal amounts of cell lysate (20 ug) from treatments were loaded to determine the level of protein phosphorylation, total protein and action expression of mTOR,

AAR and AMPK functional readouts. All antibodies for mTOR and AAR functional readouts were purchased from Cell Signaling Technologies (Danvers, MA). All antibodies for AMPK functional readouts were purchased from Santacruz Biotechnology (Dallas, TX). Additional details regarding antibody dilution and preparation are included in the **Supplemental Table 1.1.** The secretion of IGFBP1 in the HepG2 cell media as well as the phosphorylation sites Ser101, 119 and 169 were determined using 5 ul and 25 uL of cell media respectively. After samples were loaded and run in SDS-PAGE, the gel was transferred to a nitrocellulose membrane, which was washed twice with 1X Tris Buffered Saline (TBS) and blocked using 5% skim milk or BSA in TBS with 0.1% Tween (TBST, BioShop) for 1 hr. After washing off the blocking buffer twice with TBS, antibodies prepared in 1:1000 dilution in TBST, 5% skim milk or BSA were applied to the nitrocellulose membrane and placed on a shaker at 4°C overnight. Following the antibody incubation, the membrane was washed twice with TBS and incubated with goat-anti-rabbit or goat-anti-mouse secondary antibody (BioShop, Burlington, ON) in 1:10000 ratio for 1 hour. The membrane was washed twice with TBST before being visualized using ECL Clarity solution (BioRad, Hercules, CA), via chemiluminescence on the Molecular Imager VersaDoc (BioRad). All antibodies were purchased from cell signalling except for IGFBP1 (6303, Medix Biochemica, Espoo, Finland) and IGFBP1 phosphosites (YenZym Antibodies, San Francisco, CA). Westernblot analysis of proteins that are phosphorylated are stripped and re-incubated in an antibody corresponding to the total protein of the phosphorylated protein. After imaging both the phosphorylated and total protein levels on the same blot, the blot is once again stripped and re-incubated in beta-actin to examine loading controls.

2.9 Data analysis

The intensity of the bands on the visualized blots where analyzed using Image Lab software (BioRad, Hercules, CA). The following band intensity values normalized to the control identified in the set. Additionally, for blots that were probed for phosphorylated, total and actin protein, band intensity values were normalized by taking the phosphorylated protein bands, dividing that by the total protein bands and further dividing that value by the actin protein bands.

$$relative\ phospho-protein\ expression = \frac{\left(\frac{phosphorylated\ protein}{total\ protein}\right)}{actin\ protein}$$

Following normalization, statistical analysis and graphing were done using GraphPad Prism via One-way analysis of variance with Dunnetts Multiple Comparison Post-Test. The values were graphically presented with a Standard Error of Mean (±SEM) for each sample. Significance values were depicted via (*) $p< 0.05$, (***)$p< 0.003$ and (****)$p< 0.0001$.

3 Results

3.1 Activation of AMPK leads to upregulation of IGFBP1 secretion and phosphorylation in hypoxia

The role of AMPK activation in hypoxia resulting in MTOR downregulation has been extensively studied in various human and mouse models.[74] However in this study we attempt to understand the relationship that AMPK activation in hypoxia has on IGFBP1 hyperphosphorylation at Ser101, Ser119 and 169. The increase of IGFBP1 phosphorylation at these sites has previously been correlated to sequestering IGF1 and subsequently IGF1R downregulation.[18,19,27] In this section we investigate the larger impact AMPK phosphorylation has on IGFBP1 and on growth signaling pathways such as MTOR in hepatocytes which is a major important component of fetal growth.

3.1.1 Hypoxia induces AMPK phosphorylation

Currently, several AMPK studies strongly suggest that cellular stress such as hypoxia leads to greater AMPK phosphorylation at the Thr172 residue on the gamma subunit **(Section 1.6)**. We confirmed these findings to be significant through immunoblot analysis **(Section 2.8-2.9)** using phospho-AMPK^{Thr172} targeting antibodies, of cell lysate from hypoxia treated (**Section 2.2)** HepG2 cells. twenty-four-hour hypoxic treatment of the cells induced a 3 to 4-fold increase of AMPK phosphorylation at Thr172 **(Figure 10A)**. We also determined that, though levels of REDD1 protein increase 2 to 3-fold in hypoxic conditions in HepG2 cells, the increase is not detected to be significant **(Figure 10B)**. Overall, the upregulation of AMPK phosphorylation in hypoxia was detected to highly significant and was chosen for further investigation in this study.

Figure 10: Hypoxic conditions lead to upregulation of AMPK phosphorylation and levels of REDD1 protein in HepG2 cells.

A-B. Representative western blots of AMPK protein at phosphorylation site Thr172 **(Figure 10A)** and REDD1 protein levels **(Figure 10B)** from the cell lysate of hypoxia **(H)** treated and control **(C)** HepG2 cells (n=3 each) using equal aliquots of the cell lysate (20 ug). Hypoxic treatment of HepG2 cells increase AMPK phosphorylation and total REDD1 protein levels. Values are displayed as mean + SEM, NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p <$

0.0001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

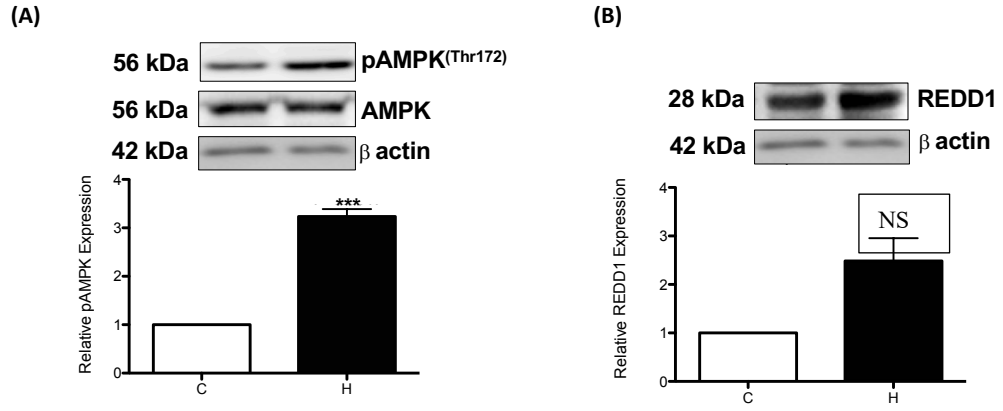

Figure 10. Hypoxia induces AMPK phosphorylation and REDD1 secretion in HepG2 cells.

3.1.2 Chemical induction of AMPK activation increases IGFBP1 phosphorylation and secretion

Next, we determined the effect of AMPK activation via AMPK chemical inducers AICAR and ASA **(Section 1.6)** on IGFBP1 secretion and phosphorylation at sites Ser101, 119 and 169. These sites are selected due to the availability of customized, targeted antibodies **(Section 1.3, Figure 2)** for IGFBP1 phosphorylation sites**.** We tested the effects of five different AICAR concentrations on HepG2 cells – 0.10, 0.25, 0.50, 0.75 and 1 mM **(Figure 11)** in normoxic conditions (**Section 2.1)**. Total IGFBP-1 protein and phosphorylation levels were significantly increased at 0.25 mM AICAR **(Figure 11)**. For ASA, we tested two different concentrations of 15.5 mM (3 ug/mL) and 25.8 mM (5 ug/mL) and found significantly optimal IGFBP1 total protein and phosphorylation levels to be at 15.5 mM **(Figure 12)** in normoxic conditions (**Section 2.1)**. The level of total protein and phosphorylation of IGFBP1 at Ser101,119 and 169 were detected using immunoblot analysis for both AMPK inducer treatments.

Figure 11. Optimal levels of total and phosphorylated IGFBP1 protein found at 0.25 mM AICAR in dose response.

A-D. Representative western blot of IGFBP-1 from the cell media of AICAR treated and non-treated (control) HepG2 cells (n=3 each) using equal aliquots of the treated cell media. AICAR treatment of HepG2 cells increased IGFBP-1 secretion (**Figure 11A)** and phosphorylation at Ser101 (**Figure 11B)**, Ser119 (**Figure 11C)**, and Ser169 (**Figure 11D)**. The most significant induction of IGFBP-1 secretion and phosphorylation are seen at 0.25 mM AICAR treatment. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p <$ 0 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

Figure 12. Optimal levels of total and phosphorylated IGFBP1 protein found at 15.5 mM ASA in dose response.

A-D. A representative western blot of IGFBP-1 from the cell media of ASA treated and non-treated (control) HepG2 cells (n=3 each) using equal aliquots of the treated cell media. ASA treatment of HepG2 cells resulted in increase of the levels of total IGFBP-1 (**Figure 12A)** and phosphorylated IGFBP-1 at Ser101 (**Figure 12B)**, Ser119 (**Figure 12C)**, and Ser169 (**Figure 12D)** relative to control. The most significant induction of total and phosphorylated IGFBP-1 levels are seen at 15.5 mM treatment of ASA. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

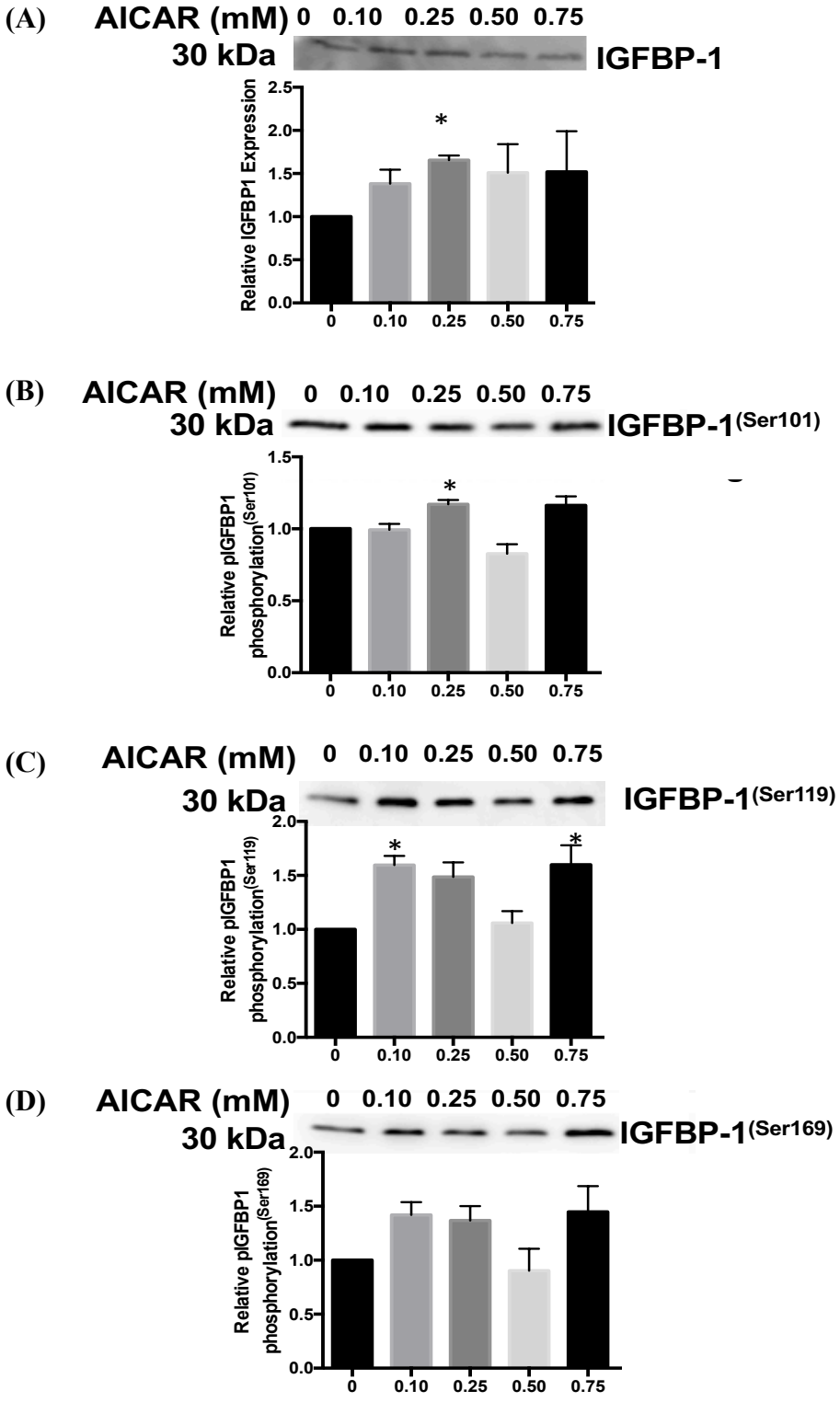

Figure 11. Optimal levels of total and phosphorylated IGFBP1 protein found at 0.25 mM AICAR in dose response.

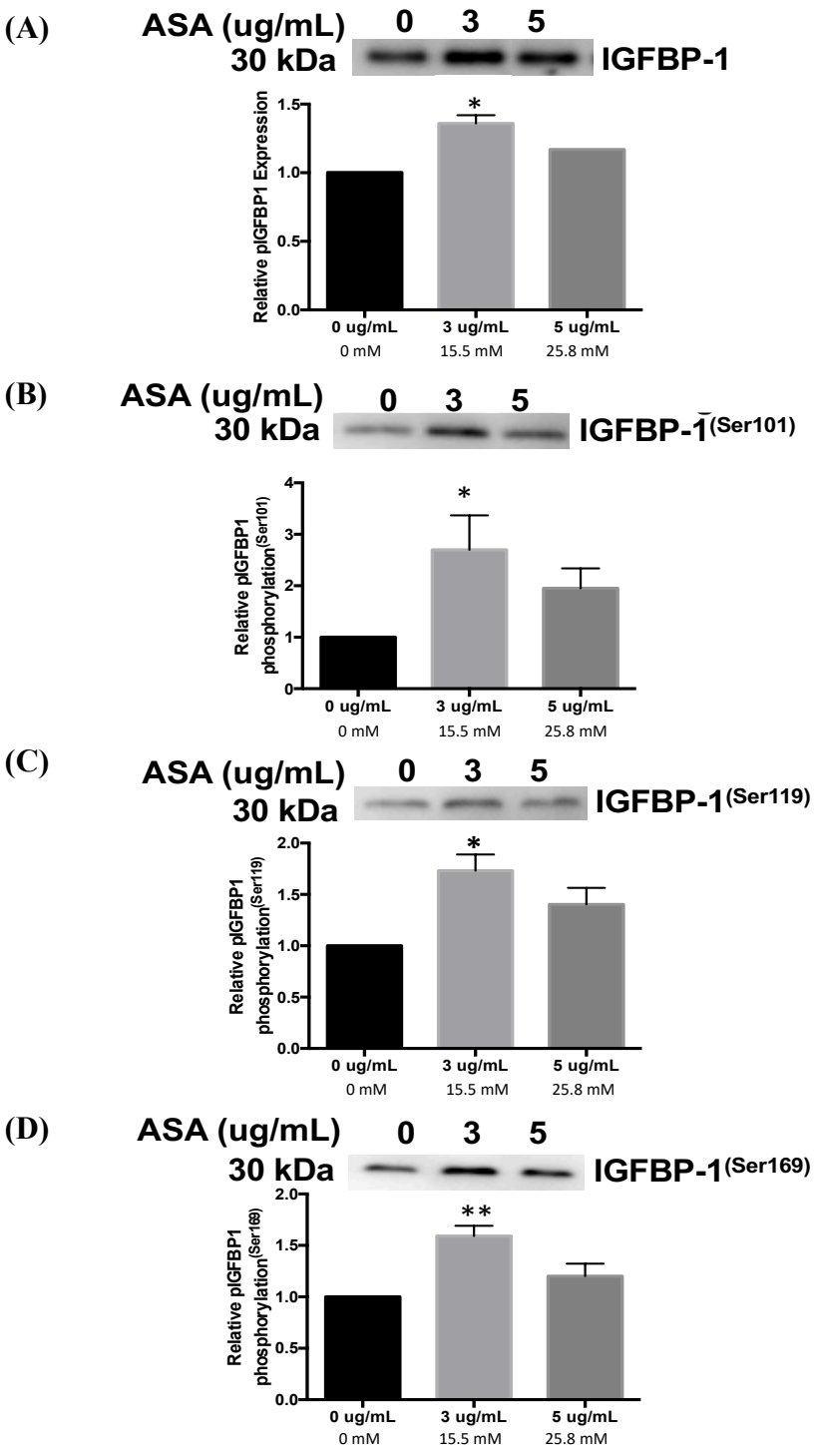

Figure 12. Optimal levels of total and phosphorylated IGFBP1 protein found at 3 ug/mL or 15.5 mM ASA in dose response.

3.1.3 Dorsomorphin treatment of HepG2 cells leads to decrease in IGFBP1 phosphorylation and secretion in normoxia

After achieving effective activation of AMPK using AICAR and ASA, we wanted to investigate possible ways to inhibit AMPK and reverse hypoxic upregulation of IGFBP1. Inhibition of AMPK was attempted using a chemical inhibitor dorsomorphin **(Section 1.6)**. Current literature has indicated Dorsomorphin as an effective inhibitor of cellular AMPK for short durations, however AMPK inhibition for longer incubation periods (24 hours) has yet to be tested. Additionally, Dorsomorphin in high concentrations can have and inhibitory effect on MTOR activity (discussed in further detail in **Section 4.1.5**), thus a dose response of Dorsomorphin is conducted in this section is to find a concentration that is low enough to not inhibit MTOR activity but high enough to actually suppress AMPK activity. The effect of dorsomorphin mediated inhibition of AMPK on total and phosphorylated IGFBP1 protein levels were determined using immunoblot analysis **(Section 2.8-2.9)** of Dorsomorphin treated HepG2 cells. Five different concentrations of dorsomorphin were tested on HepG2 cells – 1.0, 2.5, 4.0, 5.0, and 10 uM **(Figure 13)** in normoxic conditions **(Section 2.1)**. The lowest levels of total and phosphorylated IGFBP-1 compared to control were found to be at 2.5 uM dorsomorphin **(Figure 13).** Although the effects of Dorsomorphin on AMPK inhibition are not clear, a decrease in IGFBP1 hyperphosphorylation can be seen as result of Dorsomorphin treatment of HepG2 cells. IGFBP1 phosphorylation at Ser101,119 and 169 were detected using immunoblot analysis for dorsomorphin and determined to be reduced compared to control by 30-50%.

Figure 13. Total and phosphorylated levels of IGFBP1 are significantly reduced in the dorsomorphin dose response.

A-D. Representative western blot of total and phosphorylated IGFBP-1 from the cell media of dorsomorphin treated and non-treated (control) HepG2 cells (n=3 each) using equal aliquots of the treated cell media. Dorsomorphin treatment of HepG2 cells decreased levels of total IGFBP1(**Figure 13A)** and phosphorylated IGFBP-1 at Ser101 (**Figure 13B)**, Ser119 (**Figure 13C)**, and Ser169 (**Figure 13D)**. The most significant reduction of total and phosphorylated IGFBP-1 was seen at 2.5 uM dorsomorphin treatment. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

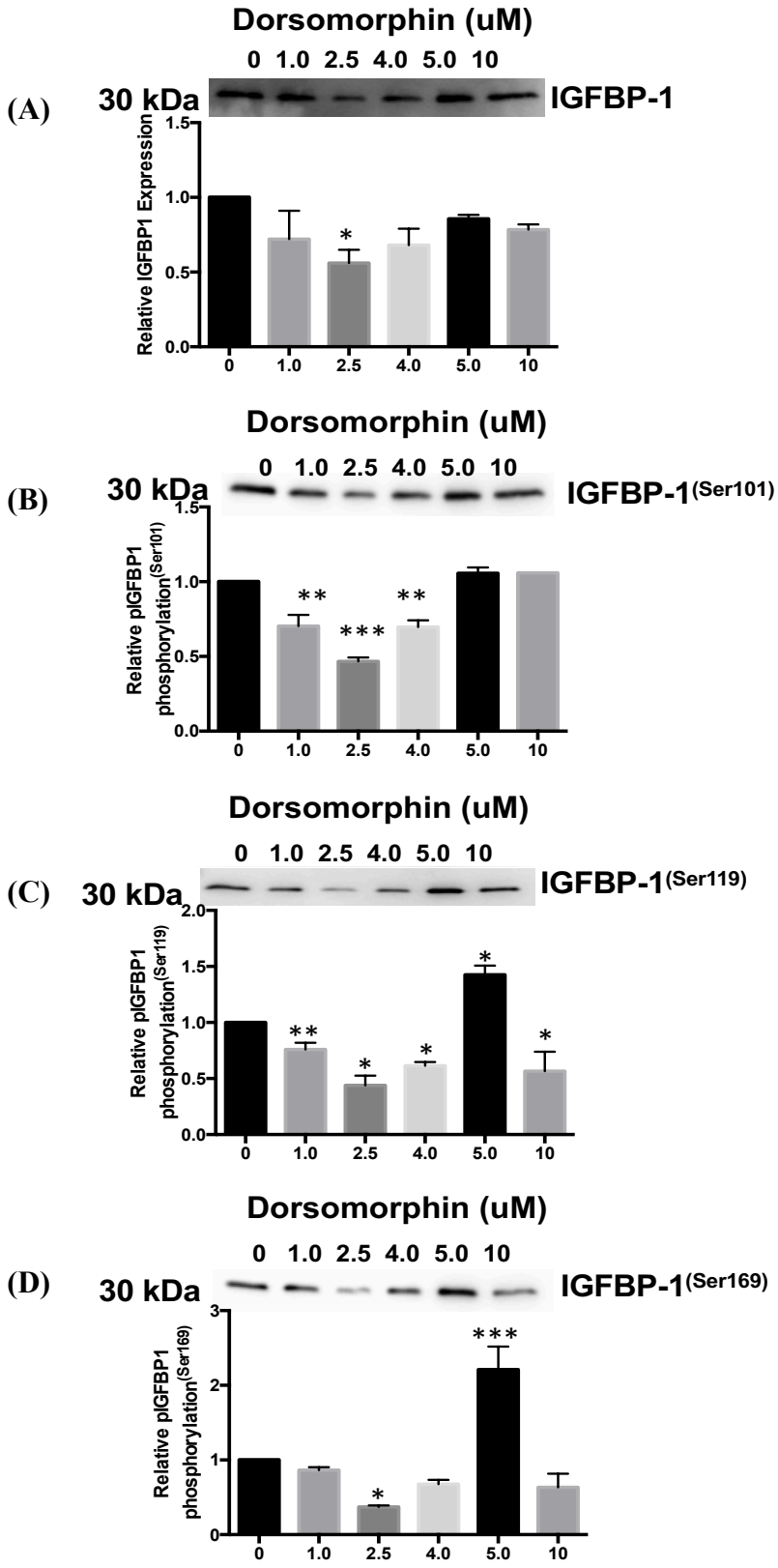

Figure 13. Total and phosphorylated levels of IGFBP1 are significantly reduced using dorsomorphin mediated inhibition of AMPK in dorsomorphin dose response.

3.1.4 AMPK phosphorylation is upregulated via AICAR or ASA.

Next, we determined the effect AICAR, ASA and Dorsomorphin has on AMPK activation via examination of the phosphorylation site at Thr172. AMPK is activated in AICAR (0.25 mM) or ASA (15.5 mM) treated HepG2 cells, leading to a 2-fold and 1.2-fold increase in AMPK phosphorylation at Thr172, respectively **(Figure 14)**. The phosphorylation of AMPK at Thr172 is not significantly decreased in dorsomorphin treated HepG2 cells **(Figure 14).** Additionally, treatment of HepG2 cells through AMPK activation via ASA (As) or AICAR (Ac) and inhibition via Dorsomorphin (Dm) has no significant effect on cell viability based on Cell Counting Kit -8 assay **(Figure 15)**.

Figure 14. Activation of AMPK via ASA and AICAR increases AMPK phosphorylation at Thr172.

A. Representative western immunoblots of AMPK phosphorylation at Thr172 **(Figure 14)** from the cell lysate of Dorsomorphin (Dm, 2.5 uM), AICAR (Ac, 0.25 mM), ASA (As, 15.5 mM) treated and non-treated (control) HepG2 cells (n=3 each) using equal aliquots of the cell lysate (20 ug). Inhibition of AMPK in HepG2 cells via Dorsomorphin decreased phospho-AMPK 48-52% **(Figure 14)**. While AMPK activation via ASA and AICAR increase phospho-AMPK signaling 1.2-fold and 2-fold respectively. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

Figure 15. Modulation of AMPK via dorsomorphin, ASA and AICAR with and without hypoxia (1% O_2), has no significant effect on cell viability.

A. Representative graphs of relative absorbance values of HepG2 cells treated with AICAR (Ac), ASA (As), Dorosomorphin (Dm) with and without hypoxia using CCK-8 assay at 450 nm. Equal number HepG2 cells ($3.5x10^5$ cells/mL) were plated in each well. No significant changes in cell viability observed. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

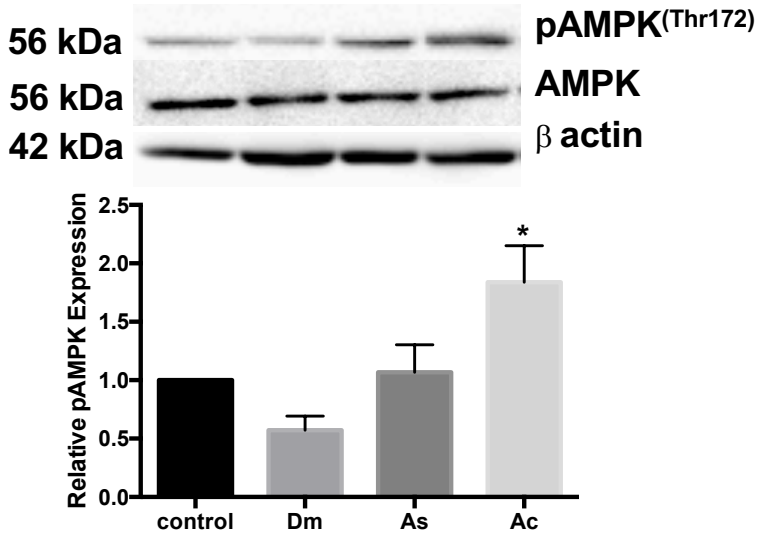

Figure 14. Activation of AMPK via ASA and AICAR increases AMPK phosphorylation at Thr172.

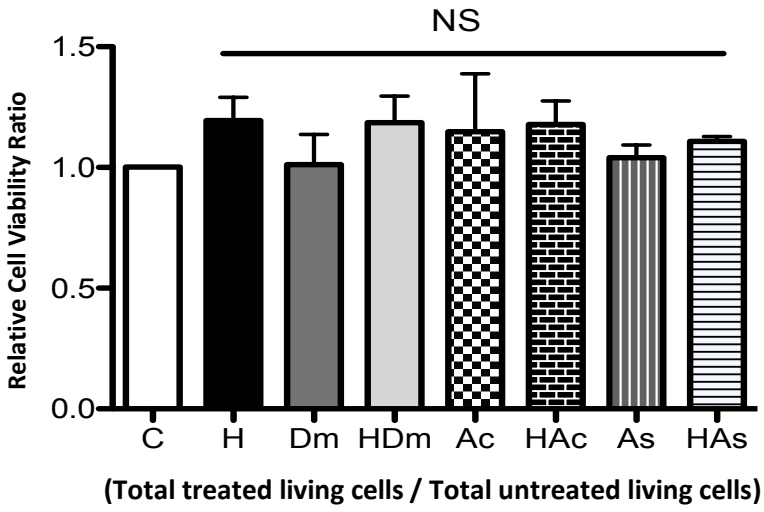

Figure 15. AMPK inhibition and activation via dorsomorphin, ASA and AICAR with and without hypoxia has negligible effects on cell viability (CCK-8 assay).

3.1.5 AMPK activation via AICAR or ASA in hypoxia leads to levels of IGFBP1 phosphorylation similar to hypoxia alone

After establishing a mode of chemical activation of AMPK, we examined the results of combining AMPK activation and hypoxia, to determine if the levels of IGFBP1 hyperphosphorylation observed in hypoxia alone is similar to that of hypoxia with AMPK activation to suggest overlap of activity. We compared the levels of IGFBP1 phosphorylation resulting from AMPK activation via AICAR (0.25 mM) or ASA (15.5 mM) in normoxia (N) and hypoxia (H) with that resulting from hypoxia alone. We found that the greatest level of total **(Figure 16A)** and phosphorylated IGFBP1 at all sites (Ser101, 119, 160 – **Figure 16B, C and D**) resulted from AICAR treatment alone (Ac) compared to normoxia (control), hypoxia (H) or AICAR with hypoxia (HAc). A similar level of total and phosphorylated IGFBP1 resulted from ASA treatment with (HAs) and without hypoxia (As) compared to hypoxia alone (H) at all IGFBP1 phosphorylation sites (Ser101, 119, 160). Both AICAR with hypoxia (HAc) and ASA with hypoxia (HAs) treatment resulted in similar levels of total and phosphorylated IGFBP1 at all sites (Ser101, 119, 160) in comparison to hypoxia alone.

It is important to note however that the Ser101 **(Figure 16C)** site differed in that there was a significant difference was observed between AICAR and AICAR with hypoxia, defying the trend observed with the other sites. Although this is surprising, we have found in past studies as well that variations in the level of phosphorylation differ in response to different stimuli such as Rapamycin , hypoxia or leucine deprivation.[18,19] In this specific case Ser101 site is highly sensitive to both AICAR and hypoxia, and combining the two stimuli does not have an additive effect but instead reduces the the Ser101 phosphorylation level to a lower amount. This is an interesting phenomenon we observe throughout this study for other combined conditions as well, where a combination treatment leads to reduced IGFBP1 signaling as opposed having an additive effect. This may be due to a threshold or optimal limit to the level of phosphorylation each IGFBP1 phosphorylation site can generate in response to stimuli. This is discussed in greater detail in **Section 4.1.3**

Figure 16. HepG2 cells treated with AICAR alone resulted in the greatest amount of total and phosphorylated IGFBP1.

A-D. Representative western blots of total and phosphorylated (Ser101, 119, 160) IGFBP-1 from the cell media of hypoxia (H) , AICAR (Ac, 0.25 mM), AICAR with hypoxia (HAc), ASA (As, 15.5 mM) and ASA with hypoxia (HAs) treated HepG2 cells alongside normoxia treated (N, used as control) HepG2 cells (n=3 each) using equal aliquots of the treated cell media. AICAR treatment of HepG2 cells increased total IGFBP-1 secretion (**Figure 16A)** and phosphorylation at Ser101 (**Figure 16B)**, Ser119 (**Figure 16C)**, and Ser169 (**Figure 16D)** drastically. However, AICAR with hypoxia treated cells resulted in IGFBP1 phosphorylation similar to hypoxia alone. Values are displayed as mean + Standard error of mean. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

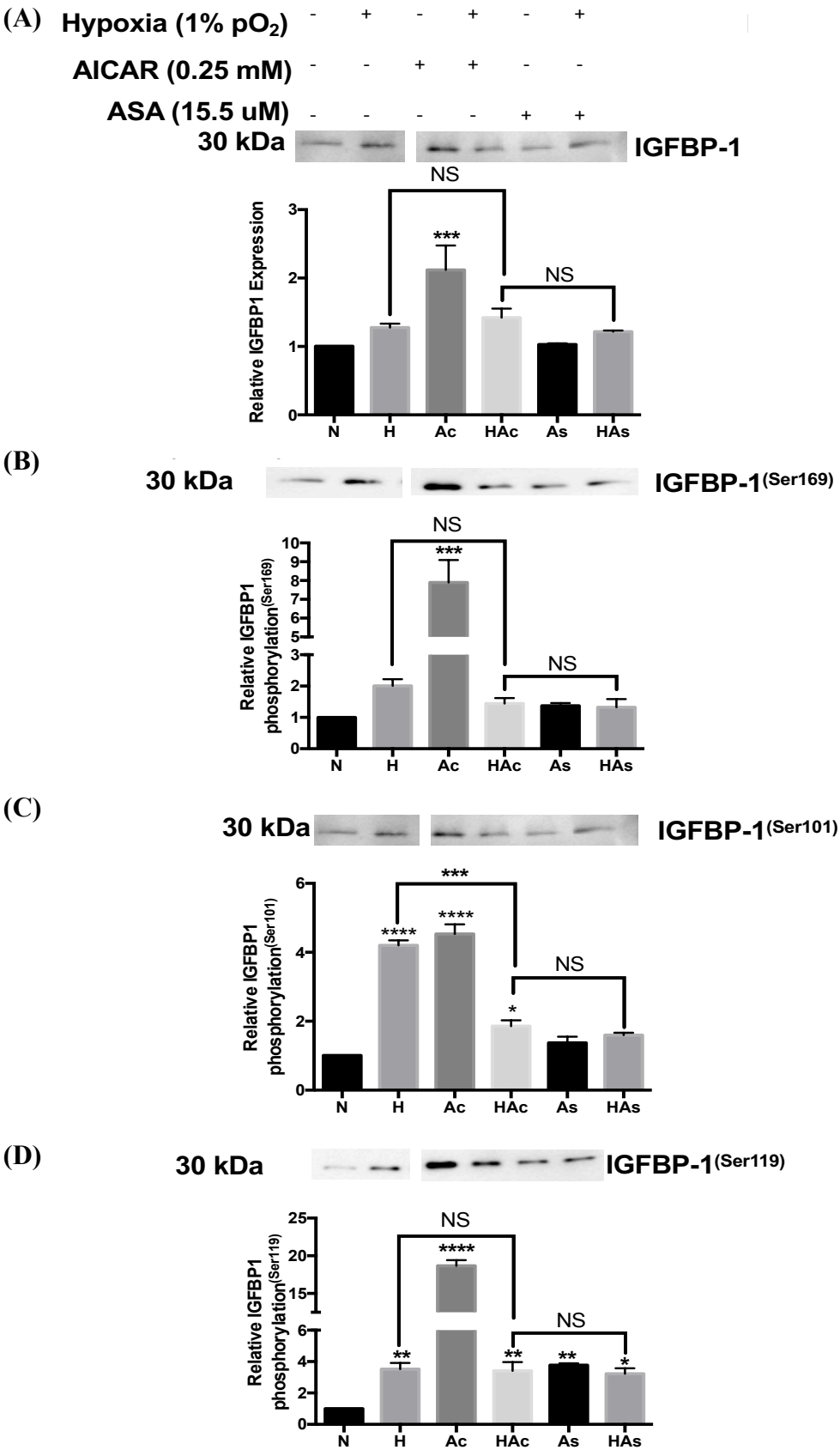

Figure 16. AICAR treatment with hypoxia results in IGFBP1 phosphorylation analogous to hypoxia alone at Ser 119 and 101 of IGFBP1.

3.1.6 AMPK activation in hypoxia leads to MTORC1/2 inhibition similar to hypoxic inhibition of MTORC1/2

Following the examination of IGFBP1 levels as a result of AMPK activation in hypoxia, we investigated the affect AMPK activation has on MTORC1/2 activity, through the western blot analysis of MTOR functional readouts. We found that the greatest level of MTOR inhibition based on the levels of downstream functional readouts was seen in AICAR with hypoxia treatment. In the previous section we noted that AICAR with hypoxia lead to similar levels of IGFBP1 hyperphosphorylation as hypoxia alone but, here we are seeing a strong level of MTORC1 inhibition in the combined conditions. This may indicate the combined conditions have an additive inhibition on the MTOR pathway which does not necessarily correspond to high levels of IGFBP1 hyperphosphorylation. However, MTOR activity is a strong indicator for growth signaling activity, however in past studies we have seen that inhibition of this pathway does lead to significant increase in IGFBP1 hyperphosphorylation, the level of phosphorylation produced is not pronounced a those seen other stimuli such as amino acid deprivation.[18,19] This indicates that observing IGFBP1 phosphorylation alone may not be sufficient to understand the underlying growth attenuation activity during cellular stress, instead, IGFBP1 hyperphosphorylation data must be taken in consideration with a profile of growth signaling proteins such as those found in the MTOR pathway.

Activation of AMPK via hypoxia (1% O_2) or AICAR (0.25 mM) or ASA (15.5 mM) attenuation of MTORC1 and MTORC2 downstream signaling. Both AICAR and ASA treatment of HepG2 cells reduces phosphorylation of Akt at Ser473 **(Figure 17A)**, p70 S6k at Thr389 **(Figure 17B)** and 4E-BP1 at Thr70 **(Figure 17C)** in normoxic conditions as well as hypoxic. However, the greatest reduction of phosphorylation is seen in the levels of Akt phosphorylation **(Figure 17C)** in AICAR or ASA treated HepG2 cells with hypoxia compared to control (normoxia). No significant difference is seen between the levels of p70 S6k phosphorylation produced in response to hypoxia and hypoxia with AICAR or ASA. However, significantly lower levels of Akt and 4E-BP1 phosphorylation are seen in treatments of AICAR with hypoxia compared to hypoxia alone. The levels of Akt and 4E-BP1 phosphorylation seen in treatments of AICAR with hypoxia and ASA with hypoxia are similar when compared to each other.

Figure 17. MTORC1 and 2 inhibition via AICAR or ASA with hypoxia leads to greater attenuation of MTORC1/2 signaling than either treatments alone.

A-C. Representative western blots of Akt at Ser473 **(Figure 17A)**, p70 S6K at Thr389 **(Figure 17B)**, 4E-BP1 at Thr70 **(Figure 17C)** from the cell lysate of hypoxia (H) , AICAR (Ac, 0.25 mM), AICAR with hypoxia(HAc), ASA (As, 15.5 mM) and ASA with hypoxia (HAs) treated HepG2 cells alongside normoxia treated (N, used as control) HepG2 cells (n=3 each) using equal aliquots of the cell lysate (20 ug). Treatment of HepG2 cells with hypoxia lead to 30-40% decrease of phosphorylation of Akt and S6k, as well as 50% decrease of 4E-BP1 phosphorylation. Treatment of HepG2 cells via AICAR and ASA reduce phosphorylation of Akt, p70 S6k and 4E-BP1 by at least 50% in normoxic and hypoxic conditions. However, Akt phosphorylation is inhibited far greater in treatments with both AICAR and hypoxia or ASA and hypoxia compared to hypoxia, AICAR or ASA alone. Phosphorylation of Akt in treatments of AICAR and ASA in normoxia resulted in 30-40% reduction compared to control. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

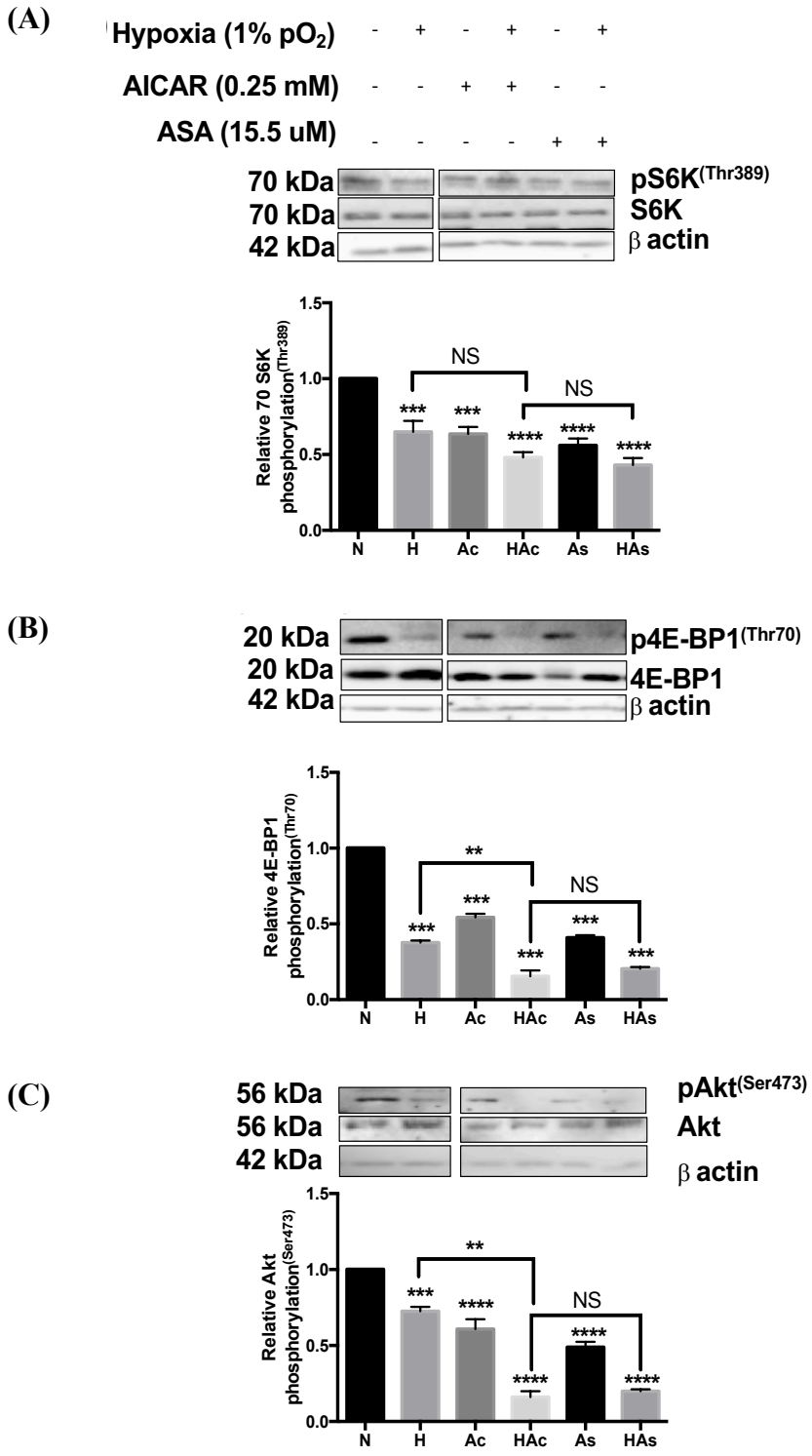

Figure 17. MTORC1 and 2 inhibition via AICAR or ASA with hypoxia leads to greater attenuation of MTORC1/2 signaling than either treatments alone .

3.1.7 IGFBP1 hyperphosphorylation in hypoxia is unaffected by dorsomorphin treatment

Next we determined if Dorsomorphin treatment is able to reduce IGFBP1 hyperphosphorylation in hypoxia. The initial purpose of utilizing Dorsomorphin was to inhibit AMPK, however our previous results (**Section 3.1.3**) indicate that although Dorsomorphin treatment reduces IGFBP1 hyperphosphorylation, the effect of this compound on AMPK activity is unclear. In this section, we found that treatment of HepG2 cells with Dorsomorphin in hypoxic conditions does not effectively reduce IGFBP1 hyperphosphorylation. Attempted inhibition of AMPK via Dorsomorphin (2.5 uM) leads to similar levels of total **(Figure 18A)** and phosphorylated IGFBP1 **(Figure 18B-D)** at all three sites (Ser101, 119, 169) in normoxia compared to untreated (control) HepG2 cells. As well, Dorsomorphin treatment of the cells lead to lower levels of total and phosphorylated IGFBP1 compared to hypoxia treatments. However, treatment of HepG2 cells with Dorsomorphin in hypoxia restored levels of total and phosphorylated IGFBP1 analogous to levels found in treatments of hypoxia alone. Thus, Dorsomorphin attempted inhibition of AMPK is ineffective in repressing hypoxia induced IGFBP1 secretion and hyperphosphorylation in HepG2 cells.

Figure 18. Dorsomorphin treatment of HepG2 cells in hypoxia results in IGFBP1 hyper phosphorylation analogous to hypoxia alone.

A-D. Representative western immunoblots of total and phosphorylated IGFBP-1 from the cell media of hypoxia (H) , Dorsomorphin (Dm, 2.5 uM), Dorsomorphin with hypoxia (HDm) treated HepG2 cells alongside normoxia treated (N, used as control) HepG2 cells (n=3 each) using equal aliquots of the treated cell media. Dorsomorphin treatment of HepG2 cells stabilized IGFBP-1 secretion (**Figure 18A)** and phosphorylation at Ser101 (**Figure 18B)**, Ser119 (**Figure 18C)**, and Ser169 (**Figure 18D)** similar to levels found in untreated (control) cells. However, Dorsomorphin treatment with hypoxia resulted in IGFBP1 phosphorylation greater than hypoxia alone. Values are displayed as mean + Standard error of mean. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

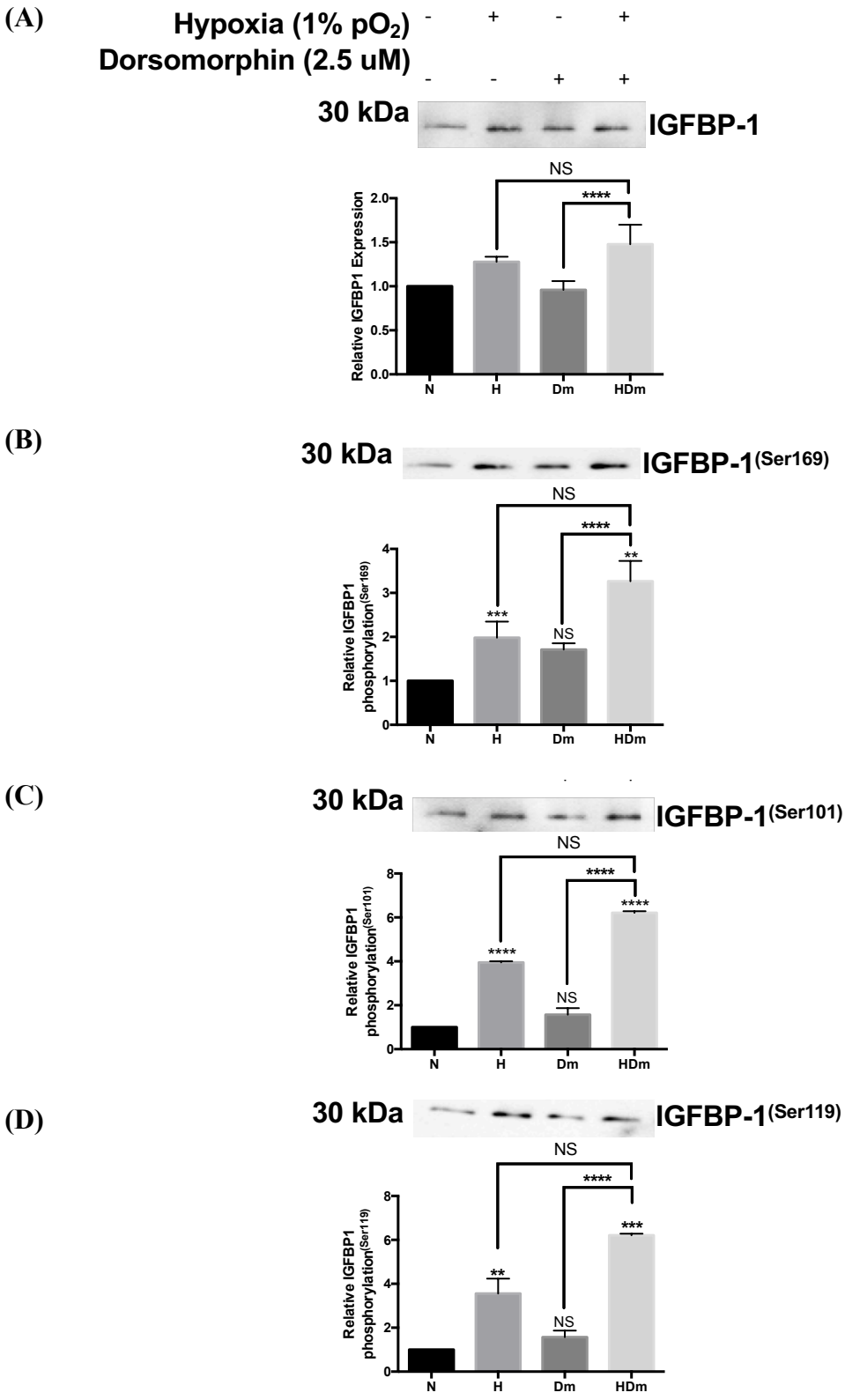

Figure 18. Dorsomorphin treatment of HepG2 cells in hypoxia results in IGFBP1 hyper phosphorylation analogous to hypoxia alone.

3.1.8 Dorsomorphin treatment of HepG2 cells in hypoxia does not rescue MTORC1/2 attenuation in hypoxia

After observing the effect of dorsomorphin on IGFBP1 hyperphosphorylation, we investigated the effect dorsomorphin has on MTORC1/2 activity, through the evaluation of MTORC1/2 downstream readouts. Inhibition of AMPK via Dorsomorphin (2.5 uM) in normoxia maintains MTORC1 **(Figure 19A-B)** and 2 **(Figure 19C)** downstream signaling levels similar to that of untreated HepG2 cells (control). However, Dorsomorphin with hypoxia (1% O_2) leads to greater attenuation of both MTORC1 and 2 downstream signaling (phosphorylation of p70 S6k at Thr389, 4E-BP1 at Thr70, and Akt at Ser473) than hypoxia alone. This may suggest that Dorsomorphin behavior is affected by hypoxia or hypoxia mediates MTOR downregulation in ways independent of AMPK.

Figure 19. Attempted **AMPK inhibition via dorsomorphin with hypoxia leads to attenuation of MTORC1 and 2 signaling.**

A-C. Representative western blots of p70 S6K at Thr389 **(Figure 19A)**, 4E-BP1 at Thr70 **(Figure 19B)**, Akt at Ser473 **(Figure 19C)** from the cell lysate of hypoxia (H) , Dorsomorphin (Dm, 2.5 uM), Dorsomorphin with hypoxia(HDm) treated HepG2 cells alongside normoxia treated (N, used as control) HepG2 cells (n=3 each), using equal aliquots of the cell lysate (20 ug). The treatment of HepG2 cells with hypoxia lead to 30-40% decrease of Akt and S6k phosphorylation and 50% decrease of 4E-BP1phosphorylation. The treatment of HepG2 cells with dorsomorphin lead to similar levels of Akt, p70 S6k and 4E-BP1 phosphorylation as control. However, the treatment of Dorsomorphin with hypoxia lead to 30% reduction of p70-S6k and 4E-BP1 phosphorylation, as well as 60% decrease in Akt phosphorylation compared to control. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

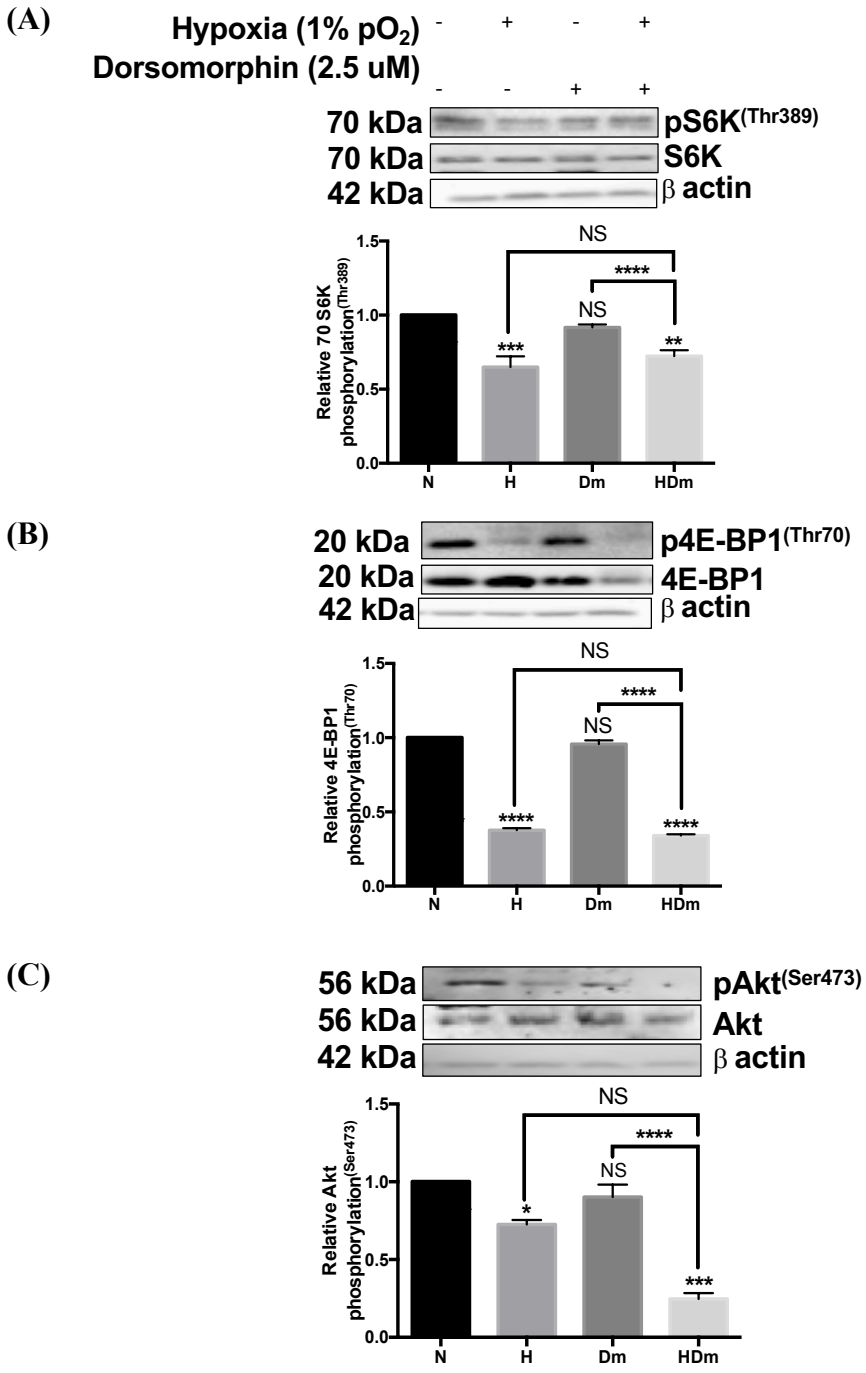

Figure 19. Attempted AMPK inhibition via dorsomorphin with hypoxia leads to attenuation of MTORC1 and 2 signaling.

3.2 IGFBP1 secretion and phosphorylation induction via combined hypoxia and leucine deprivation

In the second part of this study, we investigated the effect of combining both hypoxia and leucine deprivation on IGFBP1 hyperphosphorylation. Previous studies have examined the impact of these stressors separately on HepG2 cells. However, in an effort to better understand the mechanisms of FGR, we will be combining these two stressors in order to better simulate conditions that fetuses afflicted with FGR would experience. In this portion of the study we look closely at both the levels of IGFBP1 hyperphosphorylation at all three sites, as well we investigate the MTOR and AAR pathway activity via western blot analysis of their downstream functional readouts.

3.2.1 Combined treatment of hypoxia and leucine deprivation induces lower levels of IGFBP1 secretion and phosphorylation than leucine deprivation alone

First, we examine the effect combined conditions of hypoxia and leucine deprivation have on total and phosphorylated IGFBP1 levels. HepG2 cells are treated with leucine enriched media (450 uM), leucine deprived media (0 uM), leucine enriched media (450 uM) with hypoxia (1% O_2) and leucine deprived media (0 uM) with hypoxia (1% O_2). The treatment of cells with leucine deprived media results in the greatest increase in total and phosphorylated (Ser101, 119, 169) IGFBP1 levels **(Figure20A-D)**. The treatment of leucine deprivation with hypoxia leads to increased levels of total **(Figure 20A)** and phosphorylated IGFBP1 **(Figure 20B-D)** significantly greater than untreated (control) HepG2 cells, but significantly less than the levels observed in leucine deprivation treatment alone. The combined treatment of leucine deprivation with hypoxia was significantly greater than the leucine supplemented with hypoxia treated HepG2 cells at IGFBP1 phosphorylation sites Ser101 **(Figure 20B)** and 119 **(Figure 20C)**. However, the increase seen at Ser 169 **(Figure 20D)** and total IGFBP1 levels **(Figure 20A)** between leucine deprivation with hypoxia and leucine supplemented with hypoxia are insignificant.

Figure 20. Treatment of HepG2 cells with leucine deprivation with hypoxia (LH0) result in lower levels of IGFBP1 phosphorylation and secretion as leucine deprivation alone (L0).

A-D. Representative western blots of total and phosphorylated IGFBP-1 from the cell media of leucine 450 uM (control), leucine 0 uM, leucine 450 uM with hypoxia (1% O_2) and leucine 0 uM with hypoxia (1% O_2) treated HepG2 cells (n=3 each) using equal aliquots of the treated cell media. Leucine 0 uM treatment leads 2-fold increase in total IGFBP1 **(Figure 20A),** 5-fold increase in phosphorylation at Ser101 **(Figure 20B)**, 23-fold increase of phosphorylation at Ser119 **(Figure 20C)** and 11-fold increase in phosphorylation at Ser169 (**Figure 20D)**. Leucine 0 uM with hypoxia treatment results in 5-fold increase in IGFBP1 phosphorylation at Ser101, 17-fold at Ser119, 8-fold at Ser169 compared to untreated HepG2 cells (control). Leucine 0 uM with hypoxia treatment lead to 1.5-fold increase in total IGFBP1 secretion compared to control. Values are displayed as mean + SEM NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$ versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3

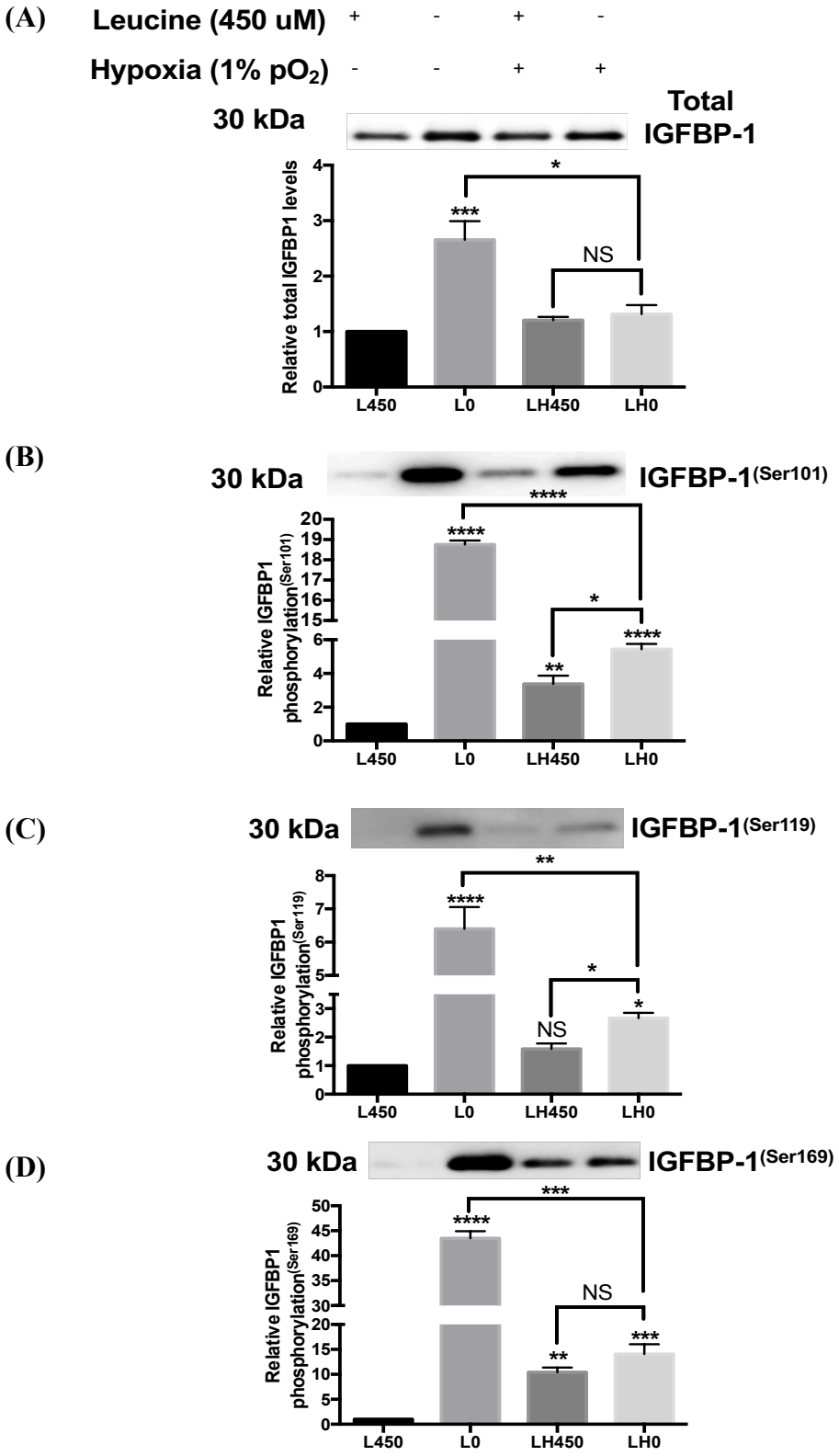

Figure 20. Treatment of HepG2 cells with leucine deprivation with hypoxia (LH0) results in lower levels of IGFBP1 phosphorylation and secretion than leucine deprivation alone (L0).

3.2.2 Combined hypoxia and leucine deprivation treatment leads to MTORC1 and 2 inhibition similar to that of leucine deprivation alone

Following the examination of IGFBP1 hyperphosphorylation levels as a result of combined treatments, we examine the effect the treatments have on MTORC1/2 activity through evaluation of their downstream functional readouts. HepG2 cells are treated with leucine enriched media (450 uM), leucine deprived media (0 uM), leucine enriched media (450 uM) with hypoxia (1% O_2) and leucine deprived media (0 uM) with hypoxia (1% O_2). Both the leucine deprivation (0 uM) treatment and leucine deprivation (0 uM) with hypoxia (1% O_2) treatment lead to the significant decrease in phosphorylation of the MTORC1 downstream functional readouts p70-S6K at Thr389 **(Figure 21A)** and 4E-BP1 at Thr70 **(Figure 21B)**. Only slight decreases in phosphorylation of MTORC2 functional readout Akt at Ser473 **(Figure 21C)** is seen for leucine deprivation and leucine deprivation with hypoxia treatments. Leucine supplemented with hypoxia treatment lead to significant decrease in phosphorylation of p70-S6K at Thr389 (**Figure 21A)**, 4E-BP1 at Thr70 (**Figure 21B)**, and Akt at Ser473 (**Figure 21C**) as well, however the variation between leucine supplemented with hypoxia and leucine deprived with hypoxia is insignificant **(Figure 21A-C)**.

Figure 21. Leucine deprivation with hypoxia (LH0) treatment of HepG2 cells result in similar levels of MTORC1 downregulation at p70-S6K and p4EBP1 as leucine deprivation alone (L0) but not in MTORC2 downregulation (pAkt).

A-C. Representative western blots of p70-S6K at Thr389 (**Figure 21A**), 4E-BP1 at Thr70 **(Figure 21B)** and Akt at Ser473 **(Figure 21C)** from leucine 450 uM (control), leucine 0 uM, leucine 450 uM with hypoxia and leucine 0 uM with hypoxia treated HepG2 cells (n=3 each) using equal aliquots of the treated cell lysate (20 ug). Leucine 0 uM treatment leads to a 50% of decrease p70-S6K at Thr389, 25% decrease in phosphorylation of 4E-BP1 at Thr70 and 20% of Akt at Ser473. Leucine 450 uM with hypoxia lead to 28% decrease in p70-S6K phosphorylation at Thr389 (**Figure 21A)**, 15% decrease in 4E-BP1 phosphorylation at Thr70 (**Figure 21B)**, and 26% decrease in Akt phosphorylation at Ser473 (**Figure 21C**). Leucine 0 uM with hypoxia results in a 60% decrease in phosphorylation of p70-S6K at Thr389, 35% decrease in phosphorylation of 4E-BP1 at Thr70 and 30% of Akt at Ser473. Values are

displayed as mean + SEM. NS p≥0.05, *p< 0.05, **p≤ 0.01, ***p≤ 0.001, ****p < 0.0001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

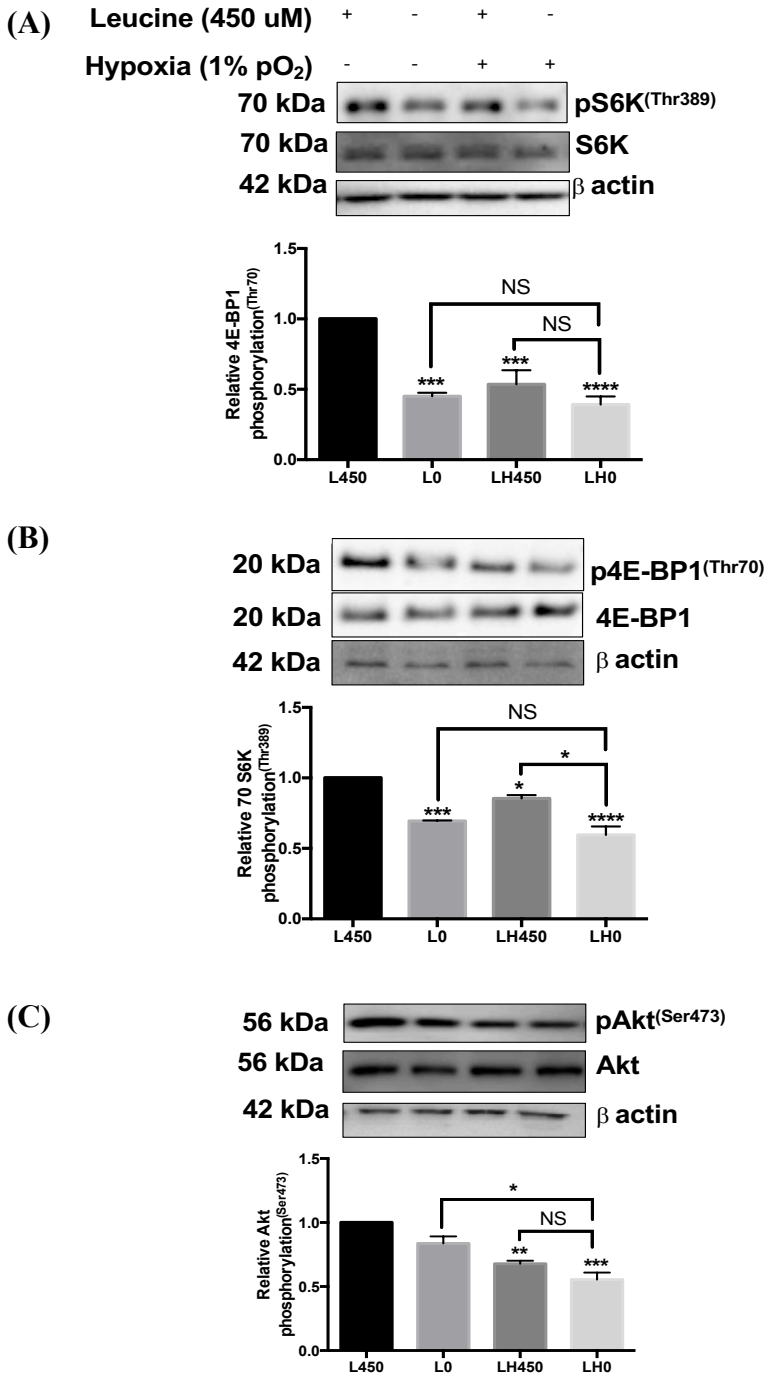

Figure 21. Leucine deprivation with hypoxia (LH0) treatment of HepG2 cells result in similar levels of MTORC1 downregulation at p70-S6K and p4EBP1 as leucine deprivation alone (L0) but not in MTORC2 downregulation (pAkt).

3.2.3 Combined treatment of hypoxia and leucine deprivation leads to upregulation of AAR pathway

We also examined the effect of hypoxia with leucine deprivation treatment of HepG2 cells has on AAR pathway by examining downstream functional readouts of AAR pathways using Western blot analysis. HepG2 cells are treated with leucine enriched media (450 uM), leucine deprived media (0 uM), leucine enriched media (450 uM) with hypoxia (1% O_2) and leucine deprived media (0 uM) with hypoxia (1% O_2). The treatment of cells with leucine deprived media and leucine deprived with hypoxia resulted in the greatest increase in phosphorylation of AAR pathway functional readouts GCN2 at Thr898 and EIF2a at Ser51. HepG2 cells treated with leucine deprivation with hypoxia lead to 5.5-fold increase in phosphorylation of GCN2 at Thr898 and 11-fold increase in phosphorylation of EIF2a at Ser51. The increase in GCN2 phosphorylation seen in leucine deprivation with hypoxia treatment of HepG2 cells is much greater than the 4-fold increase in GCN2 phosphorylation seen in the leucine deprivation treatment alone or the 2-fold increase seen in leucine enriched media with hypoxia. The combined treatment also leads to EIF2A phosphorylation at Ser51 greater than that the 2-fold increase seen in the leucine deprivation treatment alone, as well as greater than the 10-fold increase seen in the leucine enriched media with hypoxia treatment.

Figure 22. Treatment of HepG2 cells in leucine deprivation with hypoxia results in greater levels of GCN2 and EIF2A phosphorylation upregulation than either leucine deprivation or hypoxic treatments of HepG2 cells.

A-B. Representative western blots of GCN2 **(Figure 22A)** at Thr898 and EIF2a at Ser51 **(Figure 22B)** from the cell lysate of leucine 450 uM (control), leucine 0 uM, leucine 450 uM with hypoxia (1% O_2) and leucine 0 uM with hypoxia (1% O_2) treated HepG2 cells (n=3 each) using equal aliquots. Leucine 0 uM treatment leads to 4-fold increase of phosphorylation of GCN2 at Thr898 and 2-fold increase in phosphorylation of EIF2a at Ser51. Leucine 0 uM with hypoxia leads to 5.5-fold increase of phosphorylation of GCN2 at Thr898 and 11-fold increase in phosphorylation of EIF2a at Ser51. Leucine enriched media with hypoxia leads to 2.5-fold increase in of phosphorylation of GCN2 at Thr898 and 2-fold increase in phosphorylation of EIF2a at Ser51. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

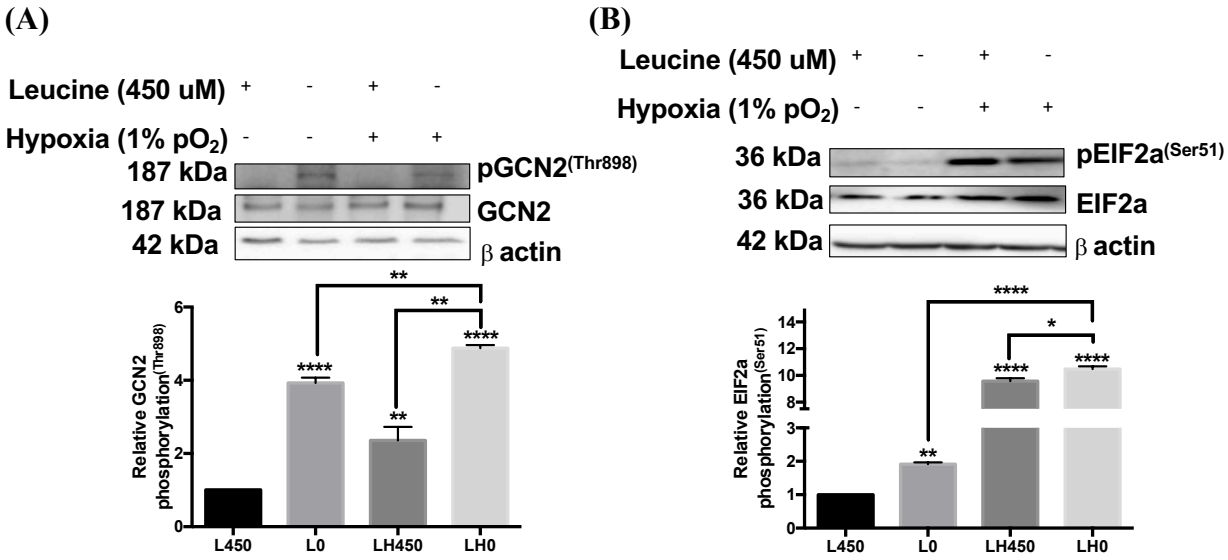

Figure 22. Treatment of HepG2 cells in leucine deprivation with hypoxia (LH0) results in greater levels of GCN2 and EIF2A phosphorylation upregulation than either leucine deprivation (L0) or hypoxic (LH450) treatments of HepG2 cells.

3.2.4 Combined treatment of hypoxia and leucine deprivation leads to minimal upregulation of ATF4

Next we examined the effect of combined hypoxia and leucine deprivation on ATF4 – a protein indicated to be active in both the AAR and UPR pathway. Current literature suggests that conditions of hypoxia can elicit activation of ATF4 via the UPR pathway.[62] However as previously discussed **(Section 1.8)**, ATF4 activation can vary based on the type of stimulus that contributing toward ATF4 activation - namely hypoxia, leucine deprivation or amino acid deprivation. The treatment of cells with leucine deprived media (0 uM) resulted in the greatest increase of AAR pathway functional readout ATF4 both at 12 and 24 hours after application of treatment media. Interestingly, ATF4 levels decreased when treated with combined hypoxia and leucine deprivation. This may be due to the various upstream protein activators of ATF4 that react to multiple stimuli, leading to a more subtle expression of total ATF4 levels but increased metabolic regulation through AAR, and UPR. This is discussed in further detail in **Section 4.2.4.**

The ATF4 protein was detected via immunoblotting technique using equal amounts of cell lysate from treated HepG2 cells. The combination of leucine deprivation (0 uM) with hypoxia (1% O_2) lead to 1.5-fold increase in ATF4 expression 12 hours after the beginning of the treatment. However, changes in ATF4 expression for leucine enriched (450 uM) with hypoxia and leucine deprivation (0 uM) with hypoxia was difficult to identify at 24 hours. However, a 12-hour incubation period revealed leucine rich media (450 uM) with hypoxia to have a 2-fold increase in ATF4 expression and leucine deprived (0 uM) with hypoxia to have 1.5-fold increase in ATF4 expression. Leucine deprivation (0 uM) alone produced 24-fold increase in ATF4 expression at 12 hours and 25-fold at 24 hours.

Figure 23. Leucine deprivation with hypoxia (LH0) treatment of HepG2 cells results in 1.5-fold increase in ATF4 expression, whereas leucine deprivation alone (L0) results in 24-25-fold increase in ATF4 expression.

A-B. Representative western blots of ATF4 at 12 **(Figure 23A)** and 24 hours **(Figure 23B)** after application of media from the cell lysate of leucine 450 uM (control), leucine 0 uM, leucine 450 uM with hypoxia and leucine 0 uM with hypoxia treated HepG2 cells (n=3 each) using equal aliquots. Leucine 0 uM treatment leads to 24-fold increase of ATF4 expression at 12 hours and 25-fold increase at 24 hours. Leucine enriched with hypoxia leads to a 2-fold increase in ATF4 expression at 12 hours and 1.2-fold increase in ATF4 expression at 24 hours after application of treatment media Leucine 0 uM with hypoxia lead to 1.5-fold increase of ATF4 expression at 12 hours and minimal change at 24 hours. Values are displayed as mean + SEM. NS $p \geq 0.05$, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

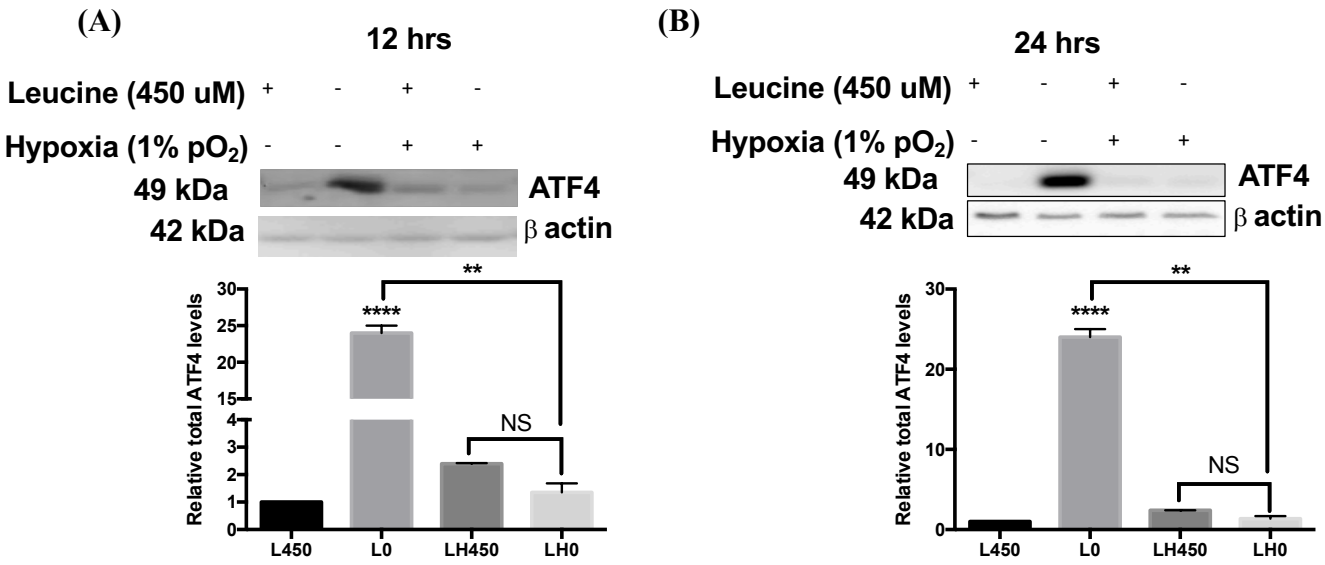

Figure 23. Leucine deprivation with hypoxia (LH0) treatment of HepG2 cells results in 1.5-fold increase in ATF4 expression, whereas leucine deprivation (L0) alone results in 24-25-fold increase in ATF4 expression.

4 Discussion

4.1 IGFBP1 hyperphosphorylation via AMPK-mediated suppression of MTORC1 in hypoxia

4.1.1 Summary of key findings in upstream regulation of MTORC1 via AMPK

In the first part of this study we investigated the possibility of AMPK mediated regulation of IGFBP1 secretion and phosphorylation. We hypothesized that activation of AMPK via AICAR or ASA would lead to increased total and phosphorylated IGFBP1 levels. We determined, through chemical activation and inhibition of AMPK in hypoxia, that though AMPK activation leads to increased levels of total and phosphorylated IGFBP1, inhibition of AMPK does not restore IGBP1 secretion and phosphorylation to normal levels in hypoxic conditions. Our investigations also showed that AMPK is significantly activated and subsequently MTORC1 downregulated **(Figure 17A-C)** in conditions of hypoxia alone. Additionally, hypoxia induces a wide range of nutrient sensing proteins, such as REDD1 **(Figure 10B)** to impede MTORC1 activity and facilitate the hypoxia mediated stress response, however, the level of overall of REDD1 protein upregulation is not significant.

Activation of AMPK using AICAR or Aspirin leads to increased IGFBP1 secretion and phosphorylation **(Figure 16A-D)**, as well as downregulation in the phosphorylation of MTORC1 downstream functional readouts 4E-BP1 and p70-S6K **(Figure 17A-B)**. This indicated that AMPK modulation alone can trigger IGFBP1 response **(Figure 16A-D)**. When AMPK activation using AICAR or Aspirin was combined with hypoxia, minimal differences were seen in IGFBP1 secretion and phosphorylation between AMPK activation with hypoxia and hypoxia alone **(Figure 16A-D)**. AMPK activation via AICAR showed the highest level of IGFBP1 secretion and phosphorylation, however AICAR treatment was combined with hypoxia, levels of IGFBP1 secretion and phosphorylation decreased. These findings indicate that there be a limited threshold to the level IGFBP1 upregulation that can be achieved through hypoxia, compared to the level of IGFBP1 upregulation achieved through the use of AMPK chemical inducers such as AICAR. However, interestingly, the downregulation of MTOR complexes were observed to be greater in AMPK activation with hypoxia than AMPK activation alone or hypoxia alone **(Figure 17A-B)**. It is important to note that though MTOR

down-regulation is a key factor in IGFBP1 hyperphosphorylation, there are several nutrient sensing pathways such as AAR and UPR that may collectively contribute towards greater IGFBP1 hyperphosphorylation than MTOR downregulation alone. Our studies have even previously indicated that upregulation of AAR (e.g. via leucine deprivation treatment)[19] results in far greater IGFBP1 hyperphosphorylation than treatments that target MTOR downregulation (e.g. hypoxia or rapamycin)[18].

Lastly, we observed that inhibition of AMPK via Dorsomorphin in hypoxia did not impede the increase in IGFBP1 phosphorylation and secretion observed during hypoxia **(Figure 18A-D)**. Similarly, Dorsomorphin mediated inhibition of AMPK in hypoxia was not able reverse the downregulation of MTORC1 activity as observed though phosphorylation levels of 4E-BP1 and p70-S6K **(Figure 19A-D)**. These findings indicated that although AMPK relays hypoxia signaling to induce MTORC1 down regulation and IGFBP1 phosphorylation, inactivation of AMPK alone does not impede hypoxia mediated signaling activity. Further investigation of the AMPK and REDD1 modulation of upstream MTOR activity is needed, specifically the upstream regulators of REDD1 such as Hif1α and LKB1 in future studies.

4.1.2 In-vitro model of hypoxia

In this study, conditions of fetal oxygen restriction were modeled in-vitro by placing HepG2 cells with 1% O_2 in a sealed chamber.[13,75] Alternatively, healthy oxygen levels were modeled in-vitro by placing HepG2 cells with 20% O_2 (normoxia) in a sealed chamber **(Section 2.2)**. In addition to the several studies in current and past literature that use similar models of hypoxia, low oxygen tension in tissues has been shown to be a low as 3%.[76,73] Fetal lambs with low oxygen tension have been shown to have O_2 levels as low as 1-2% in the descending aorta, as opposed to normal oxygen levels which range from 4-12.1% (depending on the tissue type).[77] However, the model of healthy oxygen levels using 21% O_2 may in fact be better described as hyperoxia, it is commonly used for most cell lines to produce optimal cell viability.[31] In addition, previous studies in our lab have consistently modelled normoxic and hypoxic conditions using such O_2 levels.[18,19]

4.1.3 Investigating methods of achieving optimal levels of total and phosphorylated IGFBP1 via chemical induction of AMPK

In **Section 3.1.2**, we determined the optimal levels of AMPK activators AICAR and ASA needed in order to promote maximum increase in total and phosphorylated IGFBP1 without jeopardizing cell viability (**Figure 15)**. Although, AMPK is activated in hypoxic conditions, our goal in activating AMPK was not to mimic hypoxia but to investigate the extent to which AMPK modulation can promote IGFBP1 increase and hyperphosphorylation. Identifying effective AMPK modulation can allow us to understand if AMPK modulation is a significant regulatory step in IGFBP1 regulation and subsequently attenuation of cell growth. Based on the data collected from treatment of HepG2 cells with AMPK activators in **Figure 11-12**, we determined that maximum levels of total and phosphorylated IGFBP1 can be achieved using 0.25 mM of AICAR and 15.5 mM of ASA. Interestingly, we observed that each IGFBP1 phosphorylation site differed in their level of phosphorylation in reaction to the various AMPK activators. Each IGFBP1 phosphorylation site is affected in a unique manner in response to various stimuli such as hypoxia, leucine deprivation or AMPK activation. This finding is something we have consistently seen in past studies in our lab as well, when investigating the effects of hypoxia[18], leucine deprivation[19] or rapamycin[27] on IGFBP1 hyperphosphorylation.[13] Following the determination of optimal concentrations of AICAR and ASA, we investigated if those concentrations increase AMPK phosphorylation as well in **Figure 14**. We ensured that those concentrations were not lethal to cell viability in **Figure 15**. Cell viability remained relatively stable across all treatments using a CCK kit. Additionally, we examined the cell culture for signs of cell death based on qualitative observations of the cell media and cell growth throughout the treatment and study.

Alongside AMPK activation, we also investigated AMPK inhibition in HepG2 cells using Dorsomorphin or Compound C in **Section 3.1.3**. We found that the use of dorsomorphin effectively reduced total and phosphorylated IGFBP1 levels at 2.5 μM in normal conditions (21% O_2). We determined that Dorsomorphin effectively reduced AMPK phosphorylation without mitigating cell viability in **Figure 14** and **15** respectively. However, Dorsomorphin was not effective in reducing levels of total and phosphorylated IGFBP1 in hypoxia **(Figure 13)**. We utilised the Dorsomorphin treatment of HepG2 cells as a quick and effective way to determine if AMPK inhibition alone can attenuate IGFBP1 signaling, however effective

inhibition can also be utilised using targeted small-interfering RNA (siRNA). We initiated the process of exploring effective AMPK silencing but were unable to observe successful silencing through commercially available siRNA in HepG2 cells. Effective silencing may however be achieved through highly targeted smart pool of siRNA in future studies. Regardless of the method of AMPK inhibition utilised, it is clear that AMPK inhibition alone cannot prevent IGFBP1 upregulation and hyperphosphorylation, as several proteins are involved in the upstream MTOR regulation of IGFBP1.

4.1.4 Regulation of IGFBP1 hyperphosphorylation via AMPK mediated suppression of MTORC1 and MTORC2 in hypoxia

We compared the levels of IGFBP1 upregulation and hyperphosphorylation that resulted from hypoxia to that from AMPK activation via AICAR or Aspirin, as well as both hypoxia and AMPK activation. We found that AMPK activation via AICAR or Aspirin (**Section 3.1.2**) mediates upregulation of IGFBP1 phosphorylation in hypoxia similar to hypoxia alone. However, AICAR treatment alone produced the greatest increase in IGFBP1 upregulation as well as hyperphosphorylation at all three sites (Ser101, 119 and 169). This is not surprising considering that we selected the dose of AICAR that produced the greatest levels of total and phosphorylated IGFBP1. As well AICAR is an endogenous cellular molecule that is produced as an intermediate during purine synthesis [47] as opposed to ASA or hypoxia (1% O_2) which are chemical components not normally found in the cell. We believe this endogenous characteristic of AICAR allows the compound to be better accepted by the cells as opposed to ASA or hypoxia which in excess can be lethal, activating pathways that promote cell death.[47] Conditions of hypoxia and hypoxia with AICAR and hypoxia with ASA lead to similar levels of IGFBP1 secretion and phosphorylation, with the exception of phosphorylation at Ser101. In past studies of IGFBP1 phosphorylation, it was noted that different treatments would lead to differentially activated phosphorylation sites and that not all sites were equally activated in response to same treatment, thus it is not unexpected for Ser101 to vary in phosphorylation compare to Ser119 and 169.[18,19]

The fact that simultaneous exposure of hypoxia and AMPK activation leads to a lower levels of IGFBP1 phosphorylation and higher level of MTORC1 downregulation than hypoxia alone may suggest that AMPK activation has an important and highly regulated role in hypoxic signaling and that perhaps there is a threshold to AMPK activation during multiple cellular

stimuli. Another factor may be due to the fact that hypoxia is known to activate a number of stress responsive pathways such as the Unfolded Protein Response (UPR) pathway thus with the addition of AICAR may elicit an alternative multi-pathway reaction which may not necessarily have an impact on IGFBP1 phosphorylation but instead may restrict growth through other mechanisms. It is also interesting to note, that all three stimulators of AMPK (hypoxia, AICAR and Aspirin) lead to similar levels of MTOR downregulation despite AICAR resulting in much more IGFBP1 secretion and phosphorylation. It is possible that there is also a threshold to how much MTOR can be downregulated using multiple cellular stimuli before growth restriction leads to cell death. In our studies, we ensured that none of the levels of cellular stimuli, whether AMPK activators or hypoxia lead to the severity of cell death via cell viability assays. Thus, upstream MTOR mediated upregulation of IGFBP1 may be more complex and may require greater investigation.

4.1.5 Investigating methods of decreasing total and phosphorylated IGFBP1 via AMPK inhibition in hypoxia

In order to further understand the role of AMPK in IGFBP1 regulation, we utilized AMPK inhibitor Dorsomorphin or Compound C **(Figure 14)**. Inhibition of AMPK was used to determine if hypoxic downregulation of MTOR activity and upregulation IGFBP1 phosphorylation and secretion could be prevented. Although there was no significant difference between untreated cells and Dorsomorphin treated cells in terms of MTOR activity **(Figure 19A-C)** and IGFBP1 secretion and phosphorylation **(Figure 18A-D)**, Dorsomorphin was not able to rescue cells from the impact of hypoxic treatment. This is not surprising due to the fact that even with inhibition of AMPK, REDD1 expression along with various Hypoxia inducible factors (HIF) in response to hypoxia **(Figure 10B)** leads to increased TSC2 activation and subsequently inhibition of MTORC1.

Inhibition of AMPK in hypoxia results in decreased IGFBP1 secretion and phosphorylation at Ser169 and Ser119. Modulation of AMPK Dorsomorphin did not interfere with cell proliferation and viability. Although Dorsomorphin is a potent inhibitor of AMPK **(Figure 14)**, dorsomorphin treatment does not reverse hypoxia induced MTORC1 downregulation and upregulation of IGFBP1 secretion and phosphorylation. MTORC1/2 activity is still sufficiently inhibited even with AMPK inhibition in hypoxia. This suggests that other proteins mediating hypoxic inhibition of MTOR such as REDD1 and HIF which converge on TSC2 activation,

may also be involved in IGFBP1 increase and hyperphosphorylation. Further investigations on the role of REDD1 and HIF in hypoxic inhibition of MTOR may provide a better understanding of upstream MTOR mediated regulation of IGFBP1.

Additionally, we observed that high doses of Dorsomorphin treatment leads to greater IGFBP1 phosphorylation **(Figure 13A-D),** the opposite of our intended use of the drug. We found that although dorsomorphin naturally promotes growth through AMPK inhibition, studies indicate that higher doses of the compound leads to downregulation of Akt, an important growth regulator.[78] The downregulation of Akt by Dorsomorphin leads to downregulation of MTOR and its functional activities and subsequently IGFBP1 upregulation **(Figure 13A-D)**. Therefore, exceeding appropriate Dorsomorphin doses can prove to be detrimental to cell growth and proliferation.[48]

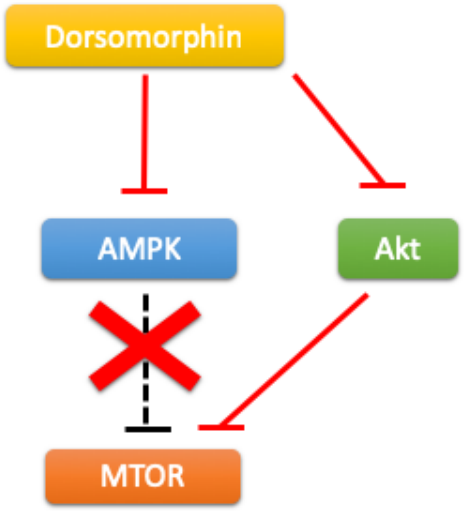

Figure 24. Dorsomorphin mediated MTOR downregulation. Though inhibition of AMPK by Dorsomorphin/Compound C prohibits AMPK mediated MTOR downregulation, Dorsomorphin/Compound C can also directly attenuate MTOR signaling via Akt downregulation.

4.1.6 Error analysis for AMPK mediated increase of total and phosphorylated IGFBP1

In this study, we indicated that hypoxic activation of AMPK and upregulation of REDD1 led to TSC2 mediated downregulation of MTORC1. However, there were two problems with this, first one being that REDD1 upregulation was not significant with our hypoxic incubation. Our methods of introducing hypoxia involve a 24-hour period in which HepG2 cells are exposed to 1% O_2, it is possible the significant levels of increase are seen at an earlier time point, as various other studies have demonstrated that hypoxia significantly elucidates REDD1 increase at shorter time intervals.[52] The second problem was that once AMPK is activated, AMPK phosphorylates TSC2 at Ser1387 and Thr1271, however when we attempted to show this through Western blot analysis of the Ser1387 and Thr1271 site of TSC2 , we found that not only were the antibodies difficult to obtain commercially, but the commercially available antibodies were not able to properly detect the phosphorylation at those sites.[79] This is was due to the fact that TSC2 has multiple phosphorylation sites that are activated by a wide range of proteins, AMPK targets 2 of those sites. TSC2 activation sites are sensitive to kinases that react to various cellular conditions and global health of the cell. As such the specific activation of Ser1387 and Thr1271 may occur transiently and thus difficult to characterize through immunoblotting methods. [44] Using MS-MS to target detection of phosphorylation is a possibly more effective yet expensive alternative to detection of TSC2 activation.

Another area of concern was the degradation of Aspirin during cellular treatment. Aspirin or ASA is chemical that is foreign to human cells and as overtime is metabolized and excreted from the body.[47] Interestingly, this occurs in the stomach and liver of humans. In this study we used human hepatoma cells, making Aspirin metabolism over time a concern. Regardless, Aspirin was able to significantly successfully induce IGFBP1 hyperphosphorylation and secretion, as well as MTOR downregulation **(Figure 11A-D, 13A-D** and **Figure 16A-C).** However, we observed that Aspirin was often not as effective as AICAR or hypoxia in IGFBP1 induction or MTOR downregulation. In future studies, a time dependent examination of MTOR downregulation via Aspirin may help to better understand the full effects of the chemical prior to degradation. Additionally, in order to insure that all treatments resulted in viable cells, a cell viability test using a CCK-8 kit was done **(Figure 15).** The CCK-8 test indicated all treatments resulted in cell viability that was similar to the untreated HepG2 cells.

4.1.7 Conclusions and future directions for AMPK mediated upregulation of IGFBP1 hyperphosphorylation

Previous studies have indicated the role of both AMPK and REDD1 in mediating TSC2 upregulation in hypoxia during short intervals (1-2 hours).[40,80] Current literature indicates that both AMPK and REDD1 relay hypoxic signaling via TSC2, while previous studies conducted in our lab indicate that TSC2 activation leads to MTOR downregulation in hypoxia - a crucial step in mediating IGFBP1 hyperphosphorylation.[81] In this study our novel findings showed that, AMPK is an important instigator of IGBP1 hyperphosphorylation observed during hypoxia over a 24 hour period. The hypoxia mediated AMPK activation is shown to correlate to greater MTORC1 downregulation. However, further investigation needs to be done regarding the role of REDD1 and HIF proteins in modulating upstream MTOR regulation.

While we explored the AMPK mode of TSC2 activation in this study, investigating the role of HIFs and REDD1 can provide a broader idea of the various pathways involved in the cellular stress response involved in growth attenuation. In future studies, TSC2 modulation via REDD1 and HIF proteins maybe determined through echinomycin mediated activation of REDD1 and inhibition via PD184352 REDD1 inhibitor.[81,82] As well, the effects of dual inhibition or activation of AMPK and REDD1 on TSC2 mediated MTORC1 downregulation and subsequently IGFBP1 hyperphosphorylation may be investigated. The dual inhibition of AMPK and REDD1 should be tested both in hypoxic and normoxic conditions, in order to determine their role in mediating hypoxia induced IGFBP1 hyperphosphorylation. Concurrent inhibition of AMPK and REDD1 in hypoxia, will help to determine if hypoxia induced IGFBP1 hyperphosphorylation can be prevented through these branches of TSC2 regulation. As well, concurrent activation of AMPK and REDD1 in normoxic conditions will allow us to determine if hypoxia-like IGFBP1 hyperphosphorylation can be stimulated through modulation of these proteins. In addition to chemical activation and inhibition, small interfering RNA (siRNA) mediated silencing of AMPK and REDD1 may also help to further examine and understand their role in IGFBP1 hyperphosphorylation and subsequently growth attenuation.

4.2 Determination of IGFBP1 hyperphosphorylation in combined conditions of hypoxia and leucine deprivation

In the second part of this study we hypothesized that combined treatment of hypoxia and leucine deprivation in HepG2 cells would lead to synergistic downregulation of MTOR and upregulation of AAR pathway signaling, leading to greater levels of IGFBP1 hyperphosphorylation than either treatment alone. However, through further investigation I observed that combined treatment of hypoxia and leucine deprivation lead to equal less IGFBP1 secretion and phosphorylation than either treatment at Ser101, Ser119 and Ser169 **(Figure 20A-D)**. Downregulation of MTOR activity in combined conditions was similar to hypoxia alone **(Figure 21A-C)**. This was determined through examination of phosphorylation of MTOR downstream functional readouts p70-S6K, 4E-BP1 and Akt. Whereas upregulation of AAR in combined conditions was similar to Leucine deprivation alone **(Figure 22A-B and Figure 23A-B)**. This was determined through evaluation of increased phosphorylation of AAR signaling proteins GCN2 and EIF2a. As well the total protein levels of AAR signaling protein ATF4 increased similarly in Leucine deprivation and Leucine deprivation with hypoxia. Overall the resulting IGFBP1 secretion and hyperphosphorylation was not synergistic as I had anticipated but rather maintained at an optimal threshold to promote growth attenuation.

4.2.1 In-vitro model of nutrient deprivation

In this study amino acid deprivation was modeled using leucine deprived cell media. Leucine is a branched chain amino acid (BCAA) and has been shown to be critical for fetal development.[83] In cases of FGR/IUGR, lower levels of leucine seemed to be prevalent in addition to other branched amino acids. Analysis of placental nutrient transfer after 20 weeks of gestation indicate Leucine transfer to be particularly increased in human pregnancies. In addition, studies have also shown that Leucine and other BCAAs such as Phenylalanine are able to rapidly cross the placenta from mother to fetus. [4, 19,84] Past studies have indicated Leucine to play an important role in intercellular signaling. Leucine plays an important role in the synthesis of growth factors in the hepatocyte as well as hepatocellular regeneration. [4] Additionally, the branched structure of leucine allows it to play an important role in maintain protein structure.[85] Oral supplementation of leucine has been shown to be able to induce increased hepatic protein signaling, specifically in MTOR. Past studies in our lab have also indicated leucine depletion to have the most severe growth attenuation in HepG2 cells

compared to other BCAAs.[13,19] For these reasons, we have also opted to use leucine deprivation for the stimulation of amino acid deprivation in this study.

4.2.2 Reduced levels of total and phosphorylated IGFBP1 in combined conditions of hypoxia and leucine deprivation compared to leucine deprivation alone

In our previous studies, we established that individual treatments of hypoxia, as well as leucine deprivation lead to upregulated levels IGFBP1 and hyperphosphorylation at Ser101, 119 and 169.[18,19] In this study, we found that, in side by side comparisons, leucine deprivation elicits a drastically stronger IGFBP1 response than hypoxia **(Figure 20A-D)**. However, when we combined hypoxia and leucine deprivation, we found the resulting levels of IGFBP1 upregulation and hyperphosphorylation to be higher than the levels observed in hypoxia but less than that observed in leucine deprivation. This contradicted our initial hypothesis in which we expected the combination of both treatments would to lead to greater IGFBP1 upregulation than either individual treatments. In fact, when we compare the IGFBP1 response from leucine deprivation treatment alone to the combined leucine deprivation with hypoxia, the latter seems to have a reduced IGFBP1 response. This may be due to the fact that hypoxia activates a number of pathways such as UPR that contribute to growth attenuation but do not necessarily lead to IGFBP1 hyperphosphorylation.[66] The complex overlap and regulation of pathways that contribute to IGFBP1 phosphorylation are not fully understood. It is possible that there is a hypoxia induced hyperphosphorylation has a limit or threshold of IGFBP1 phosphorylation levels compared to the levels of IGFBP1 generated from leucine deprivation. Both current and previous studies have confirmed that IGFBP1 hyperphosphorylation is upregulated at different levels dependent on the cellular stress conditions (e.g. hypoxia, rapamycin, or leucine deprivation)[18,19], however it is not clear, how cells differentiate between the IGFBP1 generated from hypoxia (via MTOR suppression) and that from leucine dep (AAR activation). Moreover, it is unclear how cells enforce restrictions or maintain IGFBP1 levels based on the type of stimuli (hypoxia, leucine deprivation or both). In the next few sections, we investigate the some of the biomolecular pathways mediating IGFBP1 via immunoblot analysis of MTOR and AAR signaling pathways. Further investigation of the overlap of additional network of pathways that relay hypoxic signaling, such as UPR may help us to understand exactly how hypoxic signaling in IGFBP1 regulation and growth restriction related mechanisms work (**Section 4.2.4**).

4.2.3 Investigation of MTORC1 and MTORC2 downregulation in hypoxia and leucine deprivation dual treatment

When investigating MTOR downregulation, all three treatments (leucine deprivation, hypoxia, hypoxia with leucine deprivation) result in similar levels of 4E-BP1, p70-S6k and Akt phosphorylation. However, MTOR downregulation is small snapshot of one of many growth modulating pathways in play during cellular stress, it is possible stress signals are mediated globally throughout the cell. The fact that the addition of hypoxia to leucine deprivation treatment of HepG2 cells lowers the total and phosphorylated IGFBP1 levels compared to leucine deprivation alone indicates that the metabolic pathways responsible for sensing and relaying hypoxic conditions are far more complex than our current understanding of these pathways. Earlier in this study, we discussed Hypoxia Inducible Factors (HIFs) and their role in MTOR downregulation and IGFBP1 upregulation in response to hypoxia. However, it is important to note that though much of upstream MTOR regulation has yet to be fully understood, upstream MTOR regulators have been shown to interact with the Unfolded Protein Response (UPR) pathway. The UPR pathway, in addition to affecting upstream MTOR regulation, also reacts to hypoxia- activating a cascade of proteins (e.g. PERK, IRE1, ATF4), which may or may not lead to IGFBP1 upregulation. Thus, there are multiple metabolic responses to hypoxia in cells, of which only some have been identified in their role in promoting IGFBP1 hyperphosphorylation. Further investigation of the UPR pathway may help to improve our understanding of mechanisms that modulate IGFBP1 signaling.

In addition to the complex array of metabolic pathways that respond to hypoxia, it is possible that there is a threshold or limit to the extent hypoxic conditions elicit IGFBP1 signaling. Perhaps there are specific regulatory factors in place within the cellular metabolic systems that help regulate hypoxic survival given that low oxygen conditions are something humans naturally experience, such as those living in high altitudes or experiencing in multiple gestations. For example, at a fundamental level we know that oxygen is required as an electron acceptor in cellular respiration in the mitochondria to generate the energy (ATP) required to run cellular processes but we also know that in the absence of oxygen, anaerobic respiration can take place in the cytoplasm to generate ATP as well, although not as efficient. Thus, even in low oxygen conditions, cellular processes can cope somewhat due to alternative mechanisms in place in order to generate the necessary energy. We are not introducing conditions

completely lacking oxygen (anoxia), just reduced oxygen (hypoxia). Conditions of anoxia can obviously be dangerous and lethal to the cell and thus avoided in our studies.

4.2.4 Investigation of AAR pathway upregulation in hypoxia and leucine deprivation dual treatment

Detection of AAR pathway signaling proteins phospho-EIF2a and phospho-GCN2 via immunoblot analysis in **Figures 22-23** depicts a greater increase in phosphorylation of these proteins in conditions of leucine deprivation with hypoxia than individual treatments of either leucine deprivation or hypoxia. These findings in conjunction with immunoblot analysis of MTOR signaling proteins in **Figure 21** indicates that combined conditions of leucine deprivation and hypoxia mediate a collective response through both MTOR and AAR pathway, despite the IGFBP1phorsphorylation levels being relatively lower in combined treatments compared with individual treatments. On possible explanation could be that though multiple cellular stress conditions may elicit moderate increases of IGFBP1 hyperphosphorylation, the array of biomolecular activity may still be incredibly diverse and active. The increase of IGFBP1 hyperphosphorylation is a strong indicator of growth attenuation but does not substitute as detailed purview of the complex metabolic activities taking place during nutrient stress; for understanding detailed molecular underpinnings of nutrient stress, an in-depth proteomic analysis of signaling proteins associated with various nutrient sensing pathways provides better insight.

The detection of another AAR pathway signaling protein ATF4 through immunoblot analysis showed greater upregulation in the leucine deprivation only treatment but comparatively lower in the leucine deprivation and hypoxia treatment. This finding contrasts the trend observed with the other AAR pathway functional readouts, EIF2a and GCN2 which are most elevated in combined hypoxia and leucine deprivation treatment. Studies have indicated that the levels of ATF4 increase in response to EIF2a phosphorylation,[86] however as previously discussed, EIF2a has several different kinases which react to different stimuli (shown in **Figure 25** below).[61] The following downstream activity as a result of the EIF2a activation is uniquely dependent on the various protein kinases activating EIF2a. During Leucine deprivation and hypoxia, more than one of these kinases could have been activated, some of which resulted in ATF4 increase, others which may have resulted in increased translations of growth attenuating genes.[87]

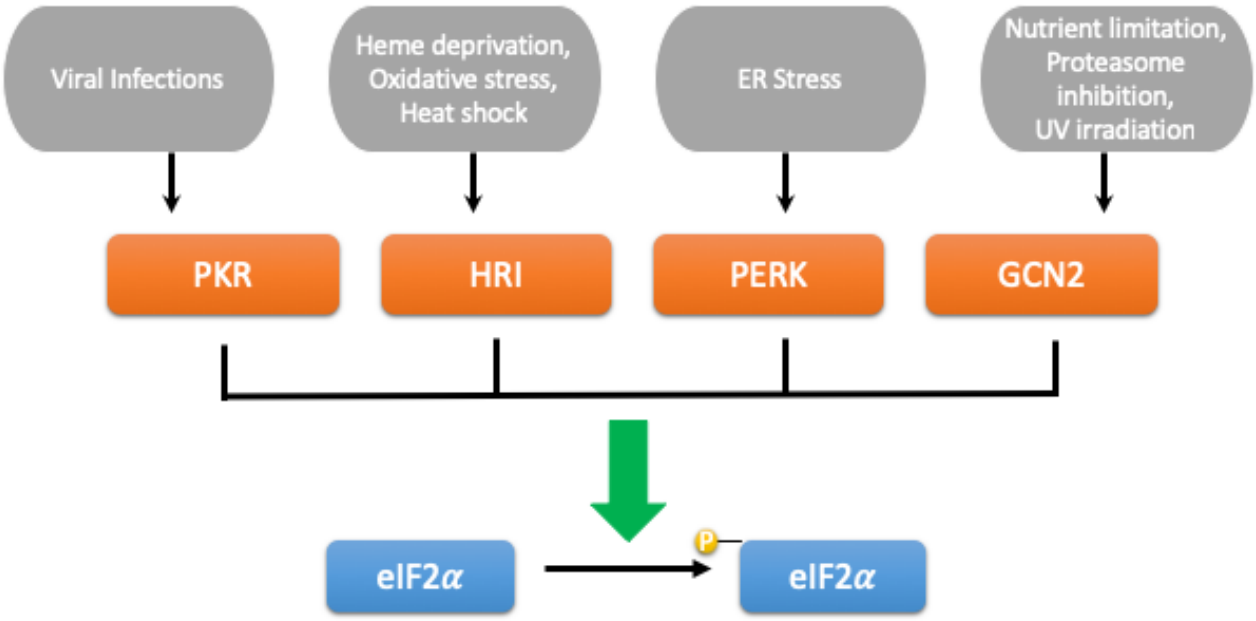

Figure 25. Different kinases lead to EIF2a activation. Activation of EIF2a is mediated through four different kinases, each eliciting activation of unique set of genes. [87]

The findings in this study indicate that inhibition of MTOR and activation of AAR signaling in conditions of hypoxia and leucine deprivation occur independently of one another. However certain proteins such as ATF4 are affected by multiple nutrient sensing pathways (ie. AAR and UPR). Proteins that are cross implicated in multiple pathways require further investigation regarding their transcriptional activity and co-localization through experiments such as immunohistochemistry (IHC). The effect multiple pathways have on the activation of the nutrient responsive proteins in the growth signaling cascade is crucial to understanding the metabolic network mediating IGFBP1 hyperphosphorylation. **Figure 26** depicts how proteins activated from both amino acid deprivation via AAR pathway and ER stress via UPR pathway converge on the activation of ATF4.[30] Further investigations in the role of the UPR signaling pathway in modulating AAR signaling proteins as well as any other nutrient sensing pathways affecting IGFBP1 secretion and phosphorylation is needed to better understand the role of these metabolic markers.[29] Systematic activation and inhibition of the UPR pathway proteins such as Protein Kinase R like ER kinase (PERK) could help evaluate the role of the UPR pathway in IGFBP1 hyperphosphorylation, as well as the larger interconnected network regulating growth attenuation in FGR.[68]

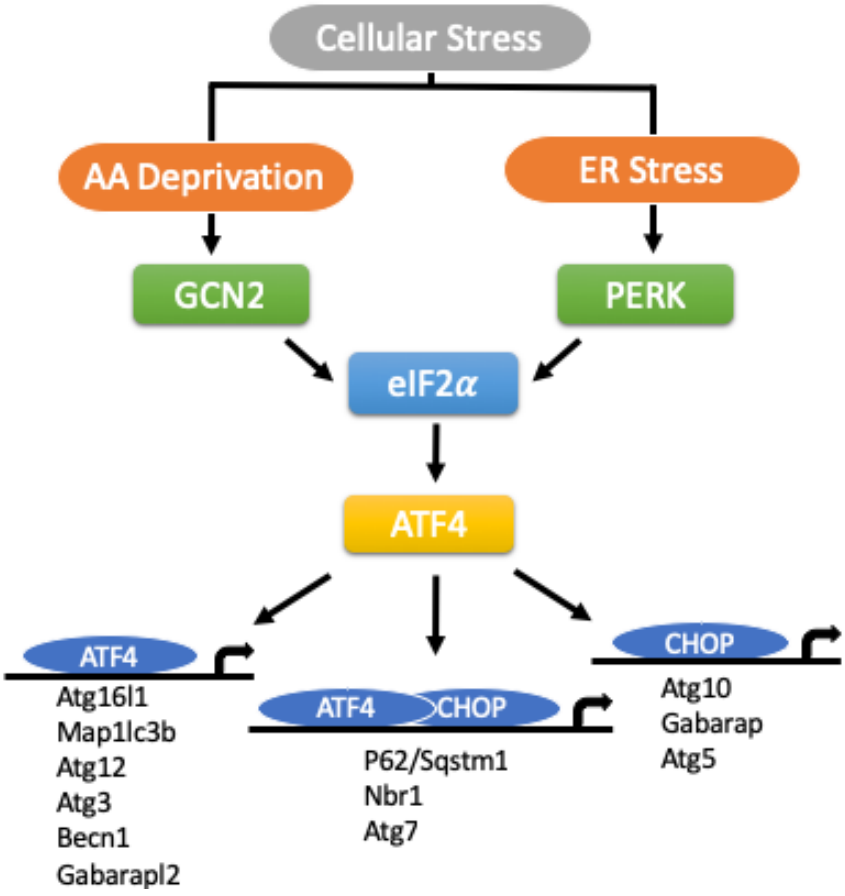

Figure 26. AAR and UPR pathway converge on EIF2a activation. Activation of EIF2a is mediated through AAR and UPR, each eliciting activation of unique set of genes.[30]

4.2.5 Error analysis for hypoxia and leucine deprivation mediated increase of total and phosphorylated IGFBP1

One problem we encountered in conducting immunoblot assays of the cell media proteins such as the total and phosphorylated IGFBP1, was the lack of a proper loading control. Several attempts were made to use fibrinogen as a loading control; however, fibrinogen levels are adversely affected in hypoxic conditions, making it an unreliable loading control for our experiments.[88] Furthermore, we attempted ponceau staining, however since levels of proteins such as IGFBP1 are increased in response to hypoxia and growth indicating proteins such as fibrinogen or collagen are increased, ponceau staining does not offer insight into total quantity or protein being loaded independent of oxygen levels. Some studies have indicated the detection of cytochrome-c from the cell media as a viable loading control, which may be worth investigating in future studies.[89] Future immunoblot assays could be followed up with cell

viability assays or cell counting assays to indicate the presence of a consistent number of cells in each replicate well. However, reagents applied to cell culture wells such as trypan blue can often interfere with detection of the IGFBP1 protein from the immunoblot membranes. A possible alternative may be to try the CCK-8 viability assay, which has been indicated by the manufacturers to have low interference in subsequent use in immunoassays.

4.2.6 Conclusions and future directions for hypoxia and leucine deprivation mediated upregulation of IGFBP1 hyperphosphorylation

Treatment of HepG2 cells with hypoxia and leucine deprivation leads to induction of IGFBP1 phosphorylation analogous to individual conditions of either hypoxia or leucine deprivation at Ser101, however some reduced levels were seen at Ser169, Ser119 and total IGFBP1. The combined treatment leads to inhibition of MTOR signaling pathway at levels similar to hypoxia alone. However, the conditions of hypoxia and leucine deprivation lead to an upregulation of phosphorylation of the AAR signaling proteins EIF2a and GCN2 in combined conditions compared leucine deprivation alone. Activation of EIF2a is mediated through several different kinases such as PERK through the UPR pathway. Therefore, further investigation of the role of UPR pathway in mediating IGFBP1 hyperphosphorylation and growth attenuation is required.

One interesting aspect to note is that through-out this study, the addition of multiple cellular stressors such hypoxia with AMPK activation or hypoxia with nutrient deprivation, did not lead to an increased or additive effect on IGFBP1 levels compared to individual treatments. Increased levels of IGFBP1 protein and hyperphosphorylation may be a crucial aspect in signaling growth attenuation, but we showed that cells are able to effectively slow growth and proliferation (via detection of MTOR downregulation) with even 1-2-fold increases in IGFBP1. The metabolic sensitivity to IGFBP1 may be greater than that we anticipated during cellular stress. Further investigation of the overlap in cellular nutrient sensing pathways that lead to increased IGFBP1 hyperphosphorylation may help us to better understand the physiological changes in growth experienced in conditions of FGR.

4.3 Overall conclusions

The goal of this study was to better understand the pathophysiology of FGR using HepG2 cells as model for fetal hepatocytes. We did this by examining the impact multiple cellular stressors can have on IGFBP1 hyperphosphorylation in addition to examining the proteins involved in relaying the cellular stress to IGFBP1 hyperphosphorylation. Previous studies using HepG2 model have shown the effect hypoxia or nutrient deprivation has on IGFBP1 hyperphosphorylation individually, however, in this study we combined both treatments to better simulate the multitude of conditions associated with FGR. We found that combined conditions led to the simultaneous modulation of nutrient sensing pathways associated with both hypoxia and nutrient deprivation. Combined cellular stress conditions led to lower levels of IGFBP1 hyperphosphorylation than nutrient deprivation alone but when compared to hypoxia, the level of hyperphosphorylation was the same or greater. This indicates that nutrient deprivation is a major modulator of total and phosphorylated IGFBP1 levels in HepG2 cells and that additionally, there is a threshold to the level of IGFBP1 hyperphosphorylation that occurs based on the type of cellular stress. Additionally, we examined the impact of direct modulation of hypoxia responsive protein AMPK has on the total and phosphorylated levels of IGFBP1 in HepG2 cells. Previous studies have indicated that AMPK activation in hypoxia is an important step in the attenuation of growth signaling activity, however a direct correlation between AMPK and IGFBP1 hyperphosphorylation had not been investigated. We found that chemical activation of AMPK leads to increased IGFBP1 phosphorylation, however inhibition of AMPK alone may not be enough to reverse the effects of hypoxia. This is due to the fact AMPK relays hypoxic signaling to growth signaling pathways alongside a network of other proteins that need to be equally investigated. Overall, this study attempts to shed light on both the cellular conditions and protein pathways that result in the metabolic changes seen in FGR. Understanding the mechanism of FGR disease may help to develop better prognosis and therapeutic treatments.

4.4 Supplemental Figures

Supplemental Table 1.1. Antibody concentrations

Antibody	DTT	Gel %	Blocking (1xTBS)	Primary antibody (1°) dilution	1° dilution buffer (1xTBS)	Secondary antibody (2°)	2° dilution buffer (1xTBS)
α IGFBP-1 (6303)	**no**	**12%**	5% BSA	1: 10,000	1 X TBS	α mouse HRP 1: 10,000	1X TBS
Phospho 101-IGFBP-1	**yes**	**12%**	5% milk, 0.1% Tween	1:1000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
Phospho 169-IGFBP-1	**yes**	**12%**	5% milk, 0.1% Tween	1:1000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
Phospho 119-IGFBP-2	**yes**	**12%**	5% milk, 0.1% Tween	1:1000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
α actin (Pan Actin)	**yes**	**10%**	5% milk, 0.1% Tween	1: 3,000	5% BSA , 0.1 % tween	α mouse HRP 1: 10,000	5% BSA, 0.1 % tween
α p70-S6K	**yes**	**8%**	5% milk, 0.1% Tween	1: 1,000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
α phospho p70-S6K	**yes**	**8%**	5% milk, 0.1% Tween	1: 1,000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
α Phospho 4E-Bp-1	**yes**	**15%**	5% milk, 0.1% Tween	1: 1,000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween

phospho AKT	**yes**	**10%**	5% milk, 0.1% Tween	1:1,000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
AKT	**yes**	**10%**	5% milk, 0.1% Tween	1:1,000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
pGCN2/total GCN2	**yes**	**8%**	5% milk, 0.1% Tween	1:1000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
eIF2α/peIF2α	**yes**	**10%**	5% milk, 0.1% Tween	1:1000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
ATF4	**yes**	**10%**	5% milk, 0.1% Tween	1:1000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
pAMPK	**yes**	**10%**	5% milk, 0.1% Tween	1:1000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween
REDD1	**yes**	**10%**	5% milk, 0.1% Tween	1:1000	5% BSA , 0.1 % tween	α Rabbit HRP 1: 10,000	5% BSA, 0.1 % tween

References

1. Sankaran, S. & Kyle, P. M. Aetiology and Pathogenesis of IUGR. *Best Pract. Res. Clin. Obstet. Gynaecol.* **23**, 765–777 (2009).

2. Randhawa, R. & Cohen, P. The role of the insulin-like growth factor system in prenatal growth. *Mol. Genet. Metab.* **86**, 84–90 (2005).

3. Laskowska, M., Laskowska, K. & Oleszczuk, J. Interleukin-18 concentrations in pregnancies complicated by preeclampsia with and without IUGR: A comparison with normotensive pregnant women with isolated IUGR and healthy pregnant women. *Pregnancy Hypertens.* **1**, 206–212 (2011).

4. Abd El-Wahed, M. A., El-Farghali, O. G., ElAbd, H. S. A., El-Desouky, E. D. & Hassan, S. M. Metabolic derangements in IUGR neonates detected at birth using UPLC-MS. *Egypt. J. Med. Hum. Genet.* **18**, 281–287 (2017).

5. Marzioni, D. *et al.* Alterations of maternal plasma HTRA1 level in preeclampsia complicated by IUGR. *Placenta* **33**, 1036–1038 (2012).

6. Guo, L. *et al.* Differentially expressed microRNAs and affected biological pathways revealed by modulated modularity clustering (MMC) analysis of human preeclamptic and IUGR placentas. *Placenta* **34**, 599–605 (2013).

7. Ruiz-González, M. D. *et al.* Morbility, clinical data and proteomic analysis of IUGR and AGA newborns at different gestational ages. *Data Br.* **9**, 438–447 (2016).

8. Gaccioli, F. & Lager, S. Placental nutrient transport and intrauterine growth restriction. *Front. Physiol.* **7**, 1–8 (2016).

9. Unterscheider, J. *et al.* Predictable progressive Doppler deterioration in IUGR: Does it really exist? *Am. J. Obstet. Gynecol.* **209**, 1–7 (2013).

10. Hutter, S. *et al.* Fetal gender specific expression of tandem-repeat galectins in placental tissue from normally progressed human pregnancies and intrauterine growth restriction (IUGR). *Placenta* **36**, 1352–1361 (2015).

11. Sengupta, S., Peterson, T. R. & Sabatini, D. M. Regulation of the mTOR Complex 1 Pathway by Nutrients, Growth Factors, and Stress. *Mol. Cell* **40**, 310–322 (2010).

12. Di Giulio, A. M. *et al.* Plasma amino acid concentrations throughout normal pregnancy and early stages of intrauterine growth restricted pregnancy. *J. Matern. Neonatal Med.* **15**, 356–362 (2004).

13. Seferovic, M. D. *et al.* Hypoxia and leucine deprivation induce human insulin-like growth factor binding protein-1 hyperphosphorylation and increase its biological activity. *Endocrinology* **150**, 220–231 (2009).

14. Ergaz, Z., Avgil, M. & Ornoy, A. Intrauterine growth restriction - Etiology and consequences: What do we know about the human situation and experimental animal models? *Reprod. Toxicol.* **20**, 301–322 (2005).

15. Gatford, K. L., Simmons, R. A., De Blasio, M. J., Robinson, J. S. & Owens, J. A. Review: Placental Programming of Postnatal Diabetes and Impaired Insulin Action after IUGR. *Placenta* **31**, S60–S65 (2010).

16. Gupta, M. B. The role and regulation of IGFBP-1 phosphorylation in fetal growth restriction. **136**, 111–123 (2015).

17. Houston, M. Sue, Holly, Jeffrey M. P., Feldman, E. L. *IGF and Nutrition in Health and Disease*. (Humana Press, 2004).

18. Damerill, I. REGULATION OF IGFBP-1 PHOSPHORYLATION IN HYPOXIA VIA MTOR SIGNALING. (University of Western Ontario, 2014).

19. Malkani, N. Molecular Mechanisms Linking Amino Acid (Leucine) Deprivation to IGFBP-1 Hyperphosphorylation in Fetal Growth Restriction. (University of Western Ontario, 2015).

20. Klammt, J., Kiess, W. & Pfäffle, R. IGF1R mutations as cause of SGA. *Best Pract. Res. Clin. Endocrinol. Metab.* **25**, 191–206 (2011).

21. K., O. *et al.* Intra-placental insulin like growth factor (IGF-1) therapy reprograms

altered gene expression in insulin signalling pathway of IUGR liver. *Am. J. Obstet. Gynecol.* **208**, S185 (2013).

22. Klammt, J., Pfäffle, R., Werner, H. & Kiess, W. IGF signaling defects as causes of growth failure and IUGR. *Trends Endocrinol. Metab.* **19**, 197–205 (2008).

23. Alsat, E., Guibourdenche, J., Couturier, A. & Evain-Brion, D. Physiological role of human placental growth hormone. *Mol. Cell. Endocrinol.* **140**, 121–127 (1998).

24. Lang, C. H., Nystrom, G. J. & Frost, R. A. Regulation of IGF binding protein-1 in HepG2 cells by cytokines and reactive oxygen species. *Am J Physiol Gastrointest Liver Physiol* **276**, G719–G727 (1999).

25. Wheatcroft, Stephen B. (University of Leeds) , Kearney, M. T. IGF-dependent and IGF-independent actions of IGF-binding protein-1 and -2: implications for metabolic homeostasis. *Trends Endocrinol. Metab.* **20**, 153–162 (2009).

26. Allard, J. B. & Duan, C. IGF-binding proteins: Why do they exist and why are there so many? *Front. Endocrinol. (Lausanne).* **9**, 1–12 (2018).

27. Abu Shehab, M., Khosravi, J., Han, V. K. M., Shilton, B. H. & Gupta, M. B. Site-specific IGFBP-1 hyper-phosphorylation in fetal growth restriction: Clinical and functional relevance. *J. Proteome Res.* **9**, 1873–1881 (2010).

28. Vandewynckel, Y.-P. *et al.* Placental growth factor inhibition modulates the interplay between hypoxia and unfolded protein response in hepatocellular carcinoma. *Int. J. Cell Biol.* **16**, 519–529 (2016).

29. Ron, D. & Walter, P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **8**, 519–529 (2007).

30. Oslowski, C. M. & Urano, F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods Enzymol.* **490**, 71–92 (2013).

31. Giaccia, A. J., Simon, M. C. & Johnson, R. The biology of hypoxia: The role of oxygen sensing in development, normal function, and disease. *Genes Dev.* **18**, 2183–

2194 (2004).

32. He, Z. *et al.* Aspirin regulates hepatocellular lipid metabolism by activating AMPK signaling pathway. *J. Toxicol. Sci.* **40**, 127–136 (2015).

33. Sofer, A., Lei, K., Johannessen, C. M. & Ellisen, L. W. Regulation of mTOR and Cell Growth in Response to Energy Stress by REDD1 Regulation of mTOR and Cell Growth in Response to Energy Stress by REDD1. **25**, 5834–5845 (2005).

34. Campos, T. *et al.* Rapamycin requires AMPK activity and p27 expression for promoting autophagy-dependent Tsc2-null cell survival. *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 1200–1207 (2016).

35. Foster, K. G. & Fingar, D. C. Mammalian target of rapamycin (mTOR): Conducting the cellular signaling symphony. *J. Biol. Chem.* **285**, 14071–14077 (2010).

36. Mao, Z. & Zhang, W. Role of mTOR in Glucose and Lipid Metabolism. *Int. J. Mol. Sci.* **19**, 2043 (2018).

37. Hara, K. *et al.* Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* (2002). doi:10.1016/S0092-8674(02)00833-4

38. Holczer, M. *et al.* Akt determines cell fate through the negative regulation of the PERK-eIF2α phosphorylation pathway Zineb. *Cell Death Differ.* **19**, 310–320 (2012).

39. Dunlop, E. A. & Tee, A. R. The kinase triad, AMPK, mTORC1 and ULK1, maintains energy and nutrient homoeostasis. *Biochem. Soc. Trans.* **41**, 939–943 (2013).

40. Lee, M. *et al.* AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *J. Biol. Chem.* **278**, 39653–39661 (2003).

41. Laderoute, K. R. *et al.* 5'-AMP-Activated Protein Kinase (AMPK) Is Induced by Low-Oxygen and Glucose Deprivation Conditions Found in Solid-Tumor Microenvironments. *Mol. Cell. Biol.* **26**, 5336–5347 (2006).

42. Appenzeller-Herzog, C. *et al.* NIH Public Access. *J. Biol. Chem.* **40**, 10188–10193

(2012).

43. Wolff, N. C. *et al.* Cell-Type-Dependent Regulation of mTORC1 by REDD1 and the Tumor Suppressors TSC1/TSC2 and LKB1 in Response to Hypoxia. *Mol. Cell. Biol.* **31**, 1870–1884 (2011).

44. Inoki, K., Zhu, T. & Guan, K.-L. TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell* **115**, 577–590 (2003).

45. Rantanen, K. *et al.* p62/SQSTM1 regulates cellular oxygen sensing by attenuating PHD3 activity through aggregate sequestration and enhanced degradation. *J. Cell Sci.* **126**, 1144–1154 (2013).

46. Xiao, B. *et al.* Structure of mammalian AMPK and its regulation by ADP. *Nature* (2011). doi:10.1038/nature09932

47. Steinberg, G. R., Dandapani, M. & Hardie, D. G. AMPK: mediating the metabolic effects of salicylate-based drugs? *Trends Endocrinol. Metab.* **24**, 481–487 (2013).

48. Wade, M., Li, Y.-C. & M. Wahl, G. The AMPK inhibitor Compound C is a potent AMPK-independent anti-glioma agent. *Mol. Cancer Ther.* **13**, 596–605 (2013).

49. Kim, J., Yang, G., Kim, Y., Kim, J. & Ha, J. AMPK activators: Mechanisms of action and physiological activities. *Exp. Mol. Med.* **48**, e224-12 (2016).

50. Hay, N. & Sonenberg, N. Upstream and downstream of mTOR. *Genes and Development* (2004). doi:10.1101/gad.1212704

51. Sinnett, S. E. & Brenman, J. E. Past Strategies and Future Directions for Identifying AMP- Activated Protein Kinase (AMPK) Modulators. **143**, 111–118 (2015).

52. Hay, W. W. *et al.* Regulation of mTOR and Cell Growth in Response to Energy Stress by REDD1 Regulation of mTOR and Cell Growth in Response to Energy Stress by REDD1. *J. Physiol.* **150**, 220–231 (2009).

53. Harding, H. P. *et al.* An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* **11**, 619–633 (2003).

54. Kilberg, M. S., Balasubramanian, M., Fu, L. & Shan, J. The Transcription Factor Network Associated With the Amino Acid Response in Mammalian Cells 1 , 2. *Amer* **3**, 295–306 (2012).

55. Ijichi, C., Matsumura, T., Tsuji, T. & Eto, Y. Branched-chain amino acids promote albumin synthesis in rat primary hepatocytes through the mTOR signal transduction system. *Biochem. Biophys. Res. Commun.* **303**, 59–64 (2003).

56. Hunter, D. S. *et al.* Programming the brain: Common outcomes and gaps in knowledge from animal studies of IUGR. *Physiol. Behav.* **164**, 233–248 (2016).

57. Xie, L. *et al.* The frontal cortex IGF system is down regulated in the term, intrauterine growth restricted fetal baboon. *Growth Horm. IGF Res.* **23**, 187–192 (2013).

58. Biron-Shental, T. *et al.* Telomeres are shorter in placental trophoblasts of pregnancies complicated with intrauterine growth restriction (IUGR). *Early Hum. Dev.* **86**, 451–456 (2010).

59. Dann, S. G. & Thomas, G. The amino acid sensitive TOR pathway from yeast to mammals. *FEBS Letters* (2006). doi:10.1016/j.febslet.2006.04.068

60. Ye, J. *et al.* GCN2 sustains mTORC1 suppression upon amino acid deprivation by inducing Sestrin2. *Genes Dev.* (2015). doi:10.1101/gad.269324.115

61. Castilho, B. A. *et al.* Keeping the eIF2 alpha kinase Gcn2 in check. *Biochimica et Biophysica Acta - Molecular Cell Research* (2014). doi:10.1016/j.bbamcr.2014.04.006

62. Kilberg, M. S., Shan, J. & Su, N. ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends in Endocrinology and Metabolism* (2009). doi:10.1016/j.tem.2009.05.008

63. Taylor, P. M. Role of amino acid transporters in amino acid sensing. *Am. J. Clin. Nutr.* (2014). doi:10.3945/ajcn.113.070086

64. Hundal, H. S. & Taylor, P. M. Amino acid transceptors: gate keepers of nutrient exchange and regulators of nutrient signaling. *AJP Endocrinol. Metab.* (2009).

doi:10.1152/ajpendo.91002.2008

65. Sood, R., Porter, A. C., Olsen, D. A., Cavener, D. R. & Wek, R. C. A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2α. *Genetics* (2000).

66. Vandewynckel, Y.-P. *et al.* Placental growth factor inhibition modulates the interplay between hypoxia and unfolded protein response in hepatocellular carcinoma. *BMC Cancer* **16**, 9 (2016).

67. Uppala, J. K., Gani, A. R. & Ramaiah, K. V. A. Chemical chaperone, TUDCA unlike PBA, mitigates protein aggregation efficiently and resists ER and non-ER stress induced HepG2 cell death. *Sci. Rep.* **7**, 1–13 (2017).

68. Harding, H. P. *et al.* Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* (2000). doi:10.1016/S1097-2765(00)00108-8

69. Ye, J. *et al.* The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. *EMBO J.* (2010). doi:10.1038/emboj.2010.81

70. Costantini, S., Di Bernardo, G., Cammarota, M., Castello, G. & Colonna, G. Gene expression signature of human HepG2 cell line. *Gene* (2013). doi:10.1016/j.gene.2012.12.106

71. Rowe, C. *et al.* Proteome-wide analyses of human hepatocytes during differentiation and dedifferentiation. *Hepatology* (2013). doi:10.1002/hep.26414

72. Guillouzo, A. *et al.* The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem. Biol. Interact.* (2007). doi:10.1016/j.cbi.2006.12.003

73. Seferovic, M. D., Chen, S., Pinto, D. M. & Gupta, M. B. Altered Liver Secretion of Vascular Regulatory Proteins in Hypoxic Pregnancies Stimulate Angiogenesis in vitro. *J. Proteome Res.* **10**, 1495–1504 (2011).

74. Zhou, G. *et al.* Role of AMP-activated protein kinase in mechanism of metformin action. *J. Clin. Invest.* (2001). doi:10.1172/JCI13505

75. Wu, D. & Yotnda, P. Induction and Testing of Hypoxia in Cell Culture. *J. Vis. Exp.* 4–7 (2011). doi:10.3791/2899

76. Firth, S. M. & Baxter, R. C. Cellular actions of the insulin-like growth factor binding proteins. *Endocrine Reviews* (2002). doi:10.1210/er.2001-0033

77. Simon, M. C. & Keith, B. The role of oxygen availability in embryonic development and stem cell function. *Nat. Rev. Mol. Cell Biol.* (2008). doi:10.1038/nrm2354

78. Vucicevic, L. *et al.* Compound C induces protective autophagy in cancer cells through AMPK inhibition-independent blockade of Akt/mTOR pathway. *Autophagy* **7**, 40–50 (2011).

79. Huang, Jingxiang; Manning, B. D. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. *Biochem. J.* **290**, 1717–1721 (2009).

80. Sofer, A., Lei, K., Johannessen, C. M. & Ellisen, L. W. Regulation of mTOR and Cell Growth in Response to Energy Stress by REDD1. *Mol. Cell. Biol.* (2005). doi:10.1128/MCB.25.14.5834-5845.2005

81. Vega-Rubin-de-Celis, S. *et al.* Structural analysis and functional implications of the negative mTORC1 regulator REDD1. *Biochemistry* (2010). doi:10.1021/bi902135e

82. Britto, F. A. *et al.* REDD1 deletion prevents dexamethasone-induced skeletal muscle atrophy. *AJP Endocrinol. Metab.* (2014). doi:10.1152/ajpendo.00234.2014

83. Teodoro, G. F. R. *et al.* Leucine Is Essential for Attenuating Fetal Growth Restriction Caused by a Protein-Restricted Diet in Rats. *J. Nutr.* **142**, 924–930 (2012).

84. Han, J. M. *et al.* Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. *Cell* (2012). doi:10.1016/j.cell.2012.02.044

85. Anthony, T. G. *et al.* Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eIF2

kinase GCN2. *J. Biol. Chem.* (2004). doi:10.1074/jbc.M404559200

86. Bröer, S. & Bröer, A. Amino acid homeostasis and signalling in mammalian cells and organisms. *Biochem. J.* (2017). doi:10.1042/BCJ20160822

87. Staschke, K. a *et al.* Integration of general amino acid control and TOR regulatory pathways in nitrogen assimilation in yeast. *J. Biol. Chem.* **285**, 16893–16911 (2010).

88. Yan, S. F., Mackman, N., Kisiel, W., Stern, D. M. & Pinsky, D. J. Hypoxia/hypoxemia-induced activation of the procoagulant pathways and the pathogenesis of ischemia-associated thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **19**, 2029–2035 (1999).

89. Jinesh, G. *et al.* Mitochondrial oligomers boost glycolysis in cancer stem cells to facilitate blebbishield-mediated transformation after apoptosis. *Cell Death Discov.* **2**, (2016).

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