6-22-2015 12:00 AM

Molecular Mechanisms Linking Amino Acid (Leucine) Deprivation to IGFBP-1 Hyperphosphorylation in Fetal Growth Restriction

Niyati M. Malkani
The University of Western Ontario

Supervisor
Dr. Madhulika Gupta
The University of Western Ontario

Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

MOLECULAR MECHANISMS LINKING AMINO ACID (LEUCINE) DEPRIVATION TO IGFBP-1 HYPERPHOSPHORYLATION IN FETAL GROWTH RESTRICTION

An Integrated-Article Thesis

by

Niyati Malkani

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

In this study, we explore the molecular mechanisms linking amino acid (leucine) deprivation to IGFBP-1 hyperphosphorylation *in vitro*. During pregnancy, a maladaptive fetal response to *in utero* amino acid deprivation leads toFetal Growth Restriction (FGR). FGR infants display elevated phosphorylatedIGFBP-1, which is associated with decreased IGF-I bioavailability. Leucine deprivation inhibits mechanistic target of rapamycin (mTOR) signaling and stimulates the amino acid response (AAR). Using HepG2 cells, a model for fetal hepatocytes, we demonstrate that in leucine deprivation, the AAR modulates total and phosphorylated IGFBP-1 while mTOR mediates total IGFBP-1 secretion only. We also reveal that protein kinases CK2 and PKC mediate IGFBP-1 phosphorylation and subsequent IGF-I bioactivity in leucine deprivation. Together, our findings implicate fetal hepatic AAR and CK2 activation as key mechanistic links between amino acid deprivation and decreased IGF-I bioavailability in FGR and suggest a novel role for PKC in modulating IGFBP-1 phosphorylation *in vitro*.

Keywords

Amino acid deprivation

Amino acid response

Activating transcription factor 4 (ATF4)

Eukaryotic initiation factor 2 (eIF2)

Fetal growth

Fetal growth restriction (FGR)

General control non-derepressible 2 (GCN2)

Insulin-like growth factor (IGF)

Insulin-like growth factor binding protein-1 (IGFBP-1)

Insulin-like growth factor binding protein-1 phosphorylation

Integrated stress response

Intrauterine growth restriction (IUGR)

Leucine deprivation

Mechanistic target of rapamycin (mTOR)

Nutrient deprivation

Protein kinase A (PKA)

Protein kinase C (PKC)

Protein kinase CK2 (CK2)

Co-Authorship Statement

All chapters were written and figures were prepared primarily by me, and reviewed under the direction of Dr. Madhulika Gupta. Dr. Madhulika Gupta prepared the subsection detailing phosphosite-specific IGFBP-1 antibody validation (Chapter 2, Section 2.2.7). The CK2 activity assay (Chapter 3, Section 3.2.8) and corresponding data analyses (Chapter 3, Section 3.3.6) was conducted by Majida Abu Shehab in collaboration with the Litchfield lab.

Acknowledgments

First and foremost, I would like to thank my supervisor Dr. Madhulika Gupta for her support, direction, and guidance over the course of my project. Dr. Gupta's patience, diligence, and dedication to my progress hasled to the successful completion of this thesis, which is a shared accomplishment with her.

I would also like to extend my appreciation to the members of Dr. Gupta's lab, with a special thanks to Majida Abu Shehab for training and assisting me in all aspects of the benchwork required for the experiments presented in this thesis as well as her companionshipover the years. A special thank you to my former lab mate, Ian Damerill, for his friendship and company in the lab. Thank you to Dr. David Litchfield for access to the tools and facilities used for our CK2 activity assay, and to Majida for performing the assay.

I would like to acknowledge the support of my advisory committee, Drs. David O'Gorman and Susan Meakin for their ideas and input in my project, and to extend my gratitude to Barb Green and the rest of the administrive team at the Department of Biochemistry for their support over the years. Thank you to the Department of Pediatrics for their financial assistance in the form of the Graduate Studentship and travel grant which were awarded to me. I would like to extend my appreciation to Gabriella Parker, Megan MacLeod and Paula Nopper at Children's Health Research Institute (CHRI) for their administrative support, and to CHRI for their financial support (travel grant awarded to me). Thank you to the Lawson Healh Research Institute (LHRI), NSERC, and NIH for their funding to MBG which made this work possible.

Finally, I would like to thank my parents, Mohit and Ritika Malkani, my brother Kabir, and my grand-parents Moti and Sarla Shahani for their emotional support and for never loosing faith in me. Thank you.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

AGA	Appropriate for Gestational Age
ALS	Acid Labile Subunit Glycoprotein
ATF4	Activating Transcription Factor 4
BCAA	Branched-chain Amino Acid
BIS	Bisindolylemaleimide II
DEPTOR	DEP domain-contain mTOR-interacting protein (DEPTOR)
DMEM/F12	Dubelco's Modified Eagle Serum with Ham's nutrient mixture F12
EAA	Essential Amino Acid
eIF2	Eukaryotic Initiation Factor 2
FGR	Fetal Growth Restriction
GCN2	General Control Non-Derepressible 2
GDP	Guanine di-phosphate
GTP	Guanine tri-phosphate
HR	IGF-I/insulin Hybrid Receptor
IGF	Insulin-like Growth Factor
IGF-1R	Insulin-like Growth Factor Receptor Type 1
IGF-2R	Insulin-like Growth Factor Receptor Type 2
IGFBP	Insulin-like Growth Factor Binding Protein
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate 1
ISR	Integrated Stress Response

LC-MS/MS	Liquid Chromatography/Tandem Mass Spectrometry
MAPK	Mitogen-Activated Protein Kinase
MEK	MAPK Kinase
MKK	MEK Kinase
MNR	Maternal Nutrient Restricted
mTOR	Mechanistic Target of Rapamycin
μM	micro molar
μL	micro liter
nM	nano molar
PERK	PKR-like endoplasmic reticulum (ER) kinase
PKA	cAMP-dependant Protein Kinase A
PKC	Protein Kinase C
PKI	Protein Kinase Inhibitor
RAPTOR	Regulatory-associated protein of mammalian target of rapamycin
RICTOR	Rapamycin-insensitive companion of mammalian target of rapamycin
p70-S6K	p70-ribosomal S6 Kinase
Ser	Serine
SGA	Small for Gestational Age
TBB	4,5,6,7-tetrabromobenzotriazole
Thr	Threonine
Tyr	Tyrosine
tRNAs	Transfer ribonucleic acids

Chapter 1

Introduction

1.1 Fetal Growth Restriction (FGR)

1.1.1 Definition, diagnosis and outcomes

Fetal Growth Restriction (FGR) is a perigestational growth disorder whereby the fetus fails to achieve its full, genetically-determined growth potential, and is linked to an increased risk of neonatal death as well as several perinatal and adult morbidities[1,2]. Affecting 5-7% of pregnancies[3], FGR infants are born under the 10th percentile birth-weight expected for gestational age[4], though some more stringent definitions classify FGR infants as those born under the 5th or 3rd percentiles[5] of expected birth-weight. FGR infants are notoriously difficult to identify among small for gestational age (SGA) babies – a classification accorded to all neonates born under the 10th percentile expected birth-weight, including those who are healthy and constitutively small due to factors such as ethnicity, sex, and body/mass index and those infants affected by other growth-restricting pathologies. In fact, 70% of SGA infants are at no increased risk for perinatal or adult morbidity[6]. There are presently no reliable tools for the diagnosis and management of FGR[7] among SGA infants. Currently, likelihood of FGR onset is determined prenatally based on suspicion of maternal risk factors, followed by clinical assessments of various maternal aspects (e.g. maternal abdominal circumference, amniotic fluid volume, and estimated fetal weight) as well as sonographic screening of the fetus[7,8]. Uterine arterial Doppler velocimetry is a useful measure of placental function, as it indicates blood flow between the fetus and placenta[9], and Doppler values of the umbilical artery, in addition to fetal heart rate, are useful in detecting the fetal cardiac manifestations of FGR[7,10]. However, clinically, these tools have been proven to be highly unreliable in consistently predicting FGR[7, 9, 11] and therapeutic strategies against FGR have also been ineffective[12].

The importance of perigestational influences on the development of adult diseases is becoming increasingly recognized in a paradigm known as the “developmental origins of health and disease” or “fetal programming”[13,14]. Indeed, FGR is associated with perinatal mortality and morbidity[1,15] as well as severe adult neurological, metabolic, and cardiovascular complications[1,2,15-17], even for infants who have achieved post-natal “catch-up growth”[18]. The lifetime implications of FGR for the individual patient, and the

subsequent implications of the disorder on public health system, make it an important area for investigation.

1.1.2 Insufficient placental nutrient (amino acid) transfer in FGR

The underlying etiologies of FGR are diverse: fetal genetics, perigestational viral infections, and disturbances in maternal health account for 30-50% of FGR cases[19-21]. However, the majority of FGR incidences are attributed to placental factors which compromise the ability of the placenta to adequately transfer nutrients from the mother to the fetus during pregnancy[4,22,23]. Perigestational nutritional supply is a dominant influence on the fetal programming of adult diseases[387,388]. The adequate availability of nutrients to the fetus is directly linked to fetal growth and is dependant on adequate placental function[389].

As the feto-maternal interface, adequate placental function is essential in controlling appropriate resource allocation between mother and fetus, which is integral to reproductive success. The placenta matures during pregnancy to meet increasing fetal nutritional demands[10], which intensify as fetal growth rate increases over the course of gestation with the fetus experiencing maximum overall growth during the third trimester[24]. Pathological placental function, such as due to abnormal placental angiogenesis leading to decreased utero-placental blood flow, decreases placental capacity for nutrient transfer[25,26]. Both mother and fetus physiologically adapt to fluctuations in nutrient availability based on nutritional cues derived from the placenta[13], and placental function itself is metabolically consuming and dependent on hormonal cues from both the mother and fetus[27,28]. Evidence is emerging for placental nutrient sensing as a key factor in healthy fetal growth[13], suggesting that the placenta serves as much more than a passive conduit for nutrients from the mother to fetus. Placental nutrient sensing involves a complex of signaling mechanisms which serve to integrate nutritional cues from both mother[29-34] and fetus[27,35-38] to regulate nutrient transportin an effort to optimize both maternal and fetal outcomes[13,39]. Insufficiency in any aspect of this process which results in the fetus not receiving the appropriate quantity of nutrients, such as essential

amino acids, necessary for its healthy development can trigger a maladaptive fetal response leading to FGR[40,41].

Excess amino acids are not stored in humans[54], so the developing fetus requires a constant, reliable supply of essential amino acids (EAAs) from the placenta in order to sustain fetogenesis. Of the 20 amino acids, nine (leucine, isoleucine, valine, lysine, threonine, tryptophan, phenylalanine, methionine, histidine) are considered "essential" as they are not internally synthesized[54]. Decreased fetal/maternal "enrichment" ratios of various EAAs – which areindicative of reduced trans-placental EAA transport – have been detected in FGR pregnancies[46-48]. This is largely due to the down-regulated expression of placental amino acid transporters (System L (leucine) transporter, Taurine transporter (TAUT), and System A transporter) in FGR[22,49-52]. Inadequate placental function leads to increased amino acid concentrations in the maternal circulation and decreased circulating amino acids in the fetuses of FGR pregnancies[42]. Induced FGR via placental embolization in an ovine model reduced fetal plasma and amniotic fluid concentrations of all amino acids by 15%[43], and altered amino acid profiles are observed in the plasma of women who experience FGR versus healthy pregnancies[44].Maladaptative responses by the fetus to perigestational amino acid deficiency likely induce a host of developmental complications, including FGR[53].

1.1.3 Fetal response to deficient nutrient (amino acid) supply

Decreased fetal amino acid uptake is characteristic of FGR[55] and fetal circulating EAAs are decreased in growth-restricted fetuses[4]. The ability of a single EAA, leucine, to attenuate induced FGR[45] suggests that amino acid availability is implicated in FGR pathogenesis. Bajoria et. al. assessed amino acid plasma concentrations at birth in human twins who experienced discordant growth, where one infant was FGR and one was appropriate for gestational age (AGA)[56]. The FGR twin demonstrated decreased plasma venous EAAs, especially the branched-chain amino acids (BCAAs – leucine, isoleucine, valinel) compared to the AGA twin[56]. Teodoro et. al. demonstrated that amino acid supplementation of maternal-protein-restricted rats restored growth and organ mass in offspring[45], which were otherwise reduced by restricted maternal diets.

In the fetus, an intrinsic maladaptive response after suffering amino acid deprivation likely manifests in FGR[53]. Fetuses are highly sensitive to fluctuations in circulating maternal amino acid availability – in fact, 48 hours of restricted maternal protein intake was sufficient to produce growth-restricted offspring in a mouse model[57]. Maternal fasting led to decreased insulin, increased glucagon and potentiated activity of key gluconeogenic factors in the livers of growth-restricted rats[57]. Girard et. al. demonstrated that decreased nutrient supply from the mother during the third trimester induces premature fetal gluconeogenesis, likely leading to impaired fetal growth at the cost of endogenous fuel provision[57]. The need for the fetus to endogenously provide energy in amino acid-restricted conditions is further demonstrated by the observation that diminished placental amino acid transfer elicits increased fetal protein catabolism, as observed via increased concentrations of certain fetal tissue amino acids, and concomitant placental dysfunction, in human FGR[28,58,59].

There is strong evidence that the fetal response to amino acid unavailability *in utero*inflicts long term repercussions on offspring health in addition to confining fetal growth[7,45,56,60]. In an experimental model for FGR, pregnant rats fed nutrient restricted diets exhibited significantly decreased circulating levels of the three essential BCAAs, which led to FGR in off-springs and correlated to severe glucose intolerance and atherosclerosis in adult life[60]. In approximately 70-80% of human FGR infants[61,62], the metabolic stress due to amino acid insufficiency causes the fetus to prioritize its limited energy supply towards preserving the functions of vital organs such as the heart, brain and placenta at the compromise of the full maturation of remaining fetal organs[63-67], which frequently leads to asymmetrical fetal growth[61,62]. These FGR infants are typically born with larger head:body mass ratios[7], and compromised maturation of fetal organs – including the brain[68] – which likely manifests in its compromised function later in life.

The specific signaling pathways triggered in the fetus upon amino acid restriction which serve to link amino acid deprivation to decreased fetal growth in the FGR fetus remain largely elusive. Fetogenesis is a dynamic and molecularly complex phenomenon that involves the interaction of a variety of signaling pathways, abrogation of any of which may lead to deterred fetal growth and development. By elucidating the molecular

mechanisms linking amino acid deprivation to restricted fetal growth, we will further our understanding of FGR pathogenesis, which is quintessential in the future development of targeted therapies and diagnosis against the disorder that are currently lacking.

1.2 The Insulin-like Growth Factors (IGFs) in Fetal Growth and Development

To explore the mechanisms modulating fetal growth under amino acid restriction, we focused our attention on the key mediators of fetal growth and development. The Insulin-like Growth Factor (IGF)/IGF-Binding Protein(IGFBP) axis is critically involved in fetal cell growth and proliferation. During human pregnancy, the two IGFs (IGF-I, IGF-II) are synthesized by most embryonic and fetal tissues and play important roles in fetal growth[69-71]. As gestation progresses, IGF production increases[72] and as the fetal liver develops, the majority of IGF is hepatically synthesized[73] and secreted into the fetal circulation[74,75] where it contributes to systemic fetal growth and development[76].

The two IGFs have unique and shared functions in fetogenesis. Both IGFs are synthesized by in early gestation[77] and are necessary for the migration of trophoblast cells in the maternal decidua[78] which is crucial to healthy placental and fetal development. Fetal liver secretes IGF-II protein whose mRNA is transcribed from the paternally imprinted allele[79]with mRNA detection as early as 12-18 days gestation. Deficits in IGF-II signaling during early embryo development typically result in irregular organogenesis and fetal demise[80]. For example, it has been shown that mice null for *IGF-II* displayed pathological placental and embryonic growth[37]. IGF-II exists in the fetal serum in far greater concentrations (3-10 fold) than IGF-I[72,81], and this imbalance persists post-natally in humans where IGF-II is typically present in a 2.5-fold greater concentration in serum compared to IGF-I.

However, as gestation progresses, fetal development becomes increasingly dependent on IGF-I[82]. Unlike IGF-II, which has been proposed to provide continuous stimulus for growth[81], IGF-I activity is more sensitive to physiological and environmental cues, such as nutritional stress[81,83]. Fetal IGF-I secretion begins early in gestation (~6 weeks)[72] by all

fetal tissues and at this time, functions primarily in an autocrine/paracrine manner[84]. IGF-I secretion rises dramatically around 16-20 weeks gestation[85] as the fetal liver matures, and continues to increase systemically throughout the remaining gestational period[72,75,85,86]. Both the endocrine and paracrine functions of IGF-I become increasingly critical to the development of fetal tissues as gestation progresses[87]. During this period, IGF-I levels in cord serum of healthy fetuses rise to 10-80 ug/L[85], and only begin to decrease post-natally[72]. IGF-I is the key mediator of overall fetal growth[88-91], particularly in the third trimester[68,92,93] when it is the dominant regulator of organ maturation[80,94]. Fetal circulating IGF-I levels have been consistently and positively associated with infant birth-weight[82,86]. In the fetal brain, IGF-I signaling is critically involved in neurogenesis, synaptogenesis, and myelination[66,67].

Wang et. al. have suggested that IGF-I is synthesized separately in the mother and fetus and that the mitogen does not cross the placenta[95]. In concordance, Davenport et. al. reported a limited ability of IGF-I to transverse the placental barrier[96]. However, Bassett et. al. showed that the placenta expresses the cognate receptor of IGF-I (IGF-1 Receptor) and that fetal IGF-I levels are radically modulated by the placenta in late gestation[97]. Further, maternal and fetal IGF-I feedback to acutely influence placental function, as indicated by the ability of IGF-I to influence amino acid allocation between the maternal and fetal compartments via the placenta[82,98,99]. Together, these studies emphasize the importance of available IGF-I in the fetal compartment in regulating fetal development and illustrate the dynamic interplay between placental nutrient sensingand IGF signaling in influencing fetal growth.

1.2.1 Structural basis of IGF-I function via its receptor (IGF-1R)

The IGFs are mitogenic proteins that exist as single polypeptide chains, are highly conserved among species and are structurally and functionally homologous to insulin[120-122]. The tertiary IGF-I molecule contains a hydrophobic core similar to insulin in addition to a 12-residue connecting peptide, and a carboxy-terminal extension[123]. The IGFs and insulin share common ancestral history[124,125]; however, their functions have largely diverged over the course of evolution[124,125]. Unlike insulin, whose primary function is

metabolic regulation, the key function of the IGFs is to modulate cell growth and proliferation[126]. The IGFs participate directly in cell growth, metabolism, and proliferation[126,127] in both pre- and post-natal life by binding to their ubiquitinously-expressed cell-surface receptor, IGF-1 Receptor (IGF-1R)[122,126].

IGF-1R is structurally homologous to the insulin receptor (IR) (58% sequence identity)[128]. However, unlike the IRwhich participates primarily in glucose and lipid metabolism, IGF-1R activity is primarily associated with cell growth and differentiation[129]. IGF-1R is a heterotetrameric protein that contains two α subunits containing the extracellular ligand-binding domain, and two disulfide bond-liked β subunits with tyrosine kinase activity[130]. The extra-cellular domain maintains IGF-1R in an inactive state until ligand binding[131], upon which a conformational change induces IGF-1R autophosphorylation at the two β subunits (pTyr1135), thereby activating the receptor[131]. IGF-1R β phosphorylation is necessary and sufficient for its activation[131] and subsequent initiation of secondary messenger cascades which stimulate downstream mitogenic responses such as increased protein translation, proliferation and anti-apoptotic activity[122,130,132].

IGF-1R is the primary receptor for IGF-I and its activity has been shown to be critically involved in fetal development[105,136,137]. Mice null for *IGF-1R* weigh 45% of control birth weight perinatally and experience a high mortality rate, supporting that IGF-1R activity is a key factor affecting fetal development[105]. To a lesser degree, IGF-I and IGF-II can also stimulate IR and IGF-I/insulinhybrid receptor (HR)[138]. IGF-II also binds to IGF-2R, which functions only to sequester IGF-II and attenuate its mitogenic effects. Expression of both receptors is regulated based on environmental stimuli and dependent on the immediate needs of the organism.

1.2.2 IGF-I in FGR

Although IGF-II is more abundant in fetal circulation[72,100-102], only fluctuations in IGF-I bioavailability have been consistently associated with FGR[101,102]. In an experimental model, *IGF-II* overexpression in mice was unable to rescue stunted growth induced by

IGF-I depletion[104]. Analysis of human fetal cord blood demonstrates that FGR fetuses display decreased circulating, bioavailable IGF-I[76,88,101-103]. Further, knockdown mice deficient in *IGF-I* display decreased fetal weight and increased neonatal death in addition to aberrant post-natal development and cognitive deficits reminiscent of FGR[91,105]. Growth-restricted neonates in ovine and murine models of FGR demonstrate decreased placental and fetal sensitivity to IGF-I[63,106,107]. One study has demonstrated extremely stunted growth in a severe FGR human patient with a deletion in the gene encoding *IGF-I*[115].

Placental *IGF-I* expression is reduced in mouse models of FGR[108] and in human FGR infants[109,110]. Further, IGF-I protein levels are decreased in the circulation of mothers delivering FGR babies compared to maternal plasma from healthy pregnancies[111,112]and in their FGR infants at term[113].Conversely, malnourished human mothers who produce significantly more small for gestational age (SGA) and FGR neonates secrete higher levels of *total* IGF-I (free and bound), suggesting an adaptive maternal response to *in utero* nutrient restriction[114]. IGF-I levels are highly sensitive to nutrient availability[83,97,116,117]; in particular, it has been suggested that nutritional status in the second half of gestation affects fetal circulating IGF-I concentrations[83,88] . Perigestational maternal under-nutrition leads to decreased IGF-I in the fetus in late gestation[117].IGF-I levels, in turn, are positively correlated with infant birth-weight[95,119].

Although total IGF-I levels rise dramatically as gestation progresses[72], its bioavailability is contingent on developmental period, subject to tissue specificity and is highly sensitive to nutrient status[83]. Modulation of IGF-I bioavailability rather than total endogenous levels is critical to the regulation of fetal growth. For example, increased methylation of the *IGF-I* gene correlated to decreased total plasma IGF-I, but this decrease in IGF-I expression was not correlated with the FGR phenotype in an ovine model[86]. Based on the evidence provided, it is ascertainable that reduced IGF-I bioactivity is a hallmark of FGR.

1.3 Regulation of IGF-I bioavailability by the IGF binding proteins (IGFBPs)

Total IGF is abundant in circulation; however, its bioavailability is tightly regulated by the six IGF binding proteins (IGFBP-1-6)[139,140]. IGFBPs are secretory proteins that modulate IGF bioactivity by binding the mitogenic proteins in various extracellular compartments[141]. The six IGFBPs serve to regulate the distribution of IGFs between the circulation, tissue fluids, and cell surface binding sites, thereby tightly controlling IGF bioavailablity[139]. The majority of IGFs (>90%) typically exist in circulation bound to IGFBPs[132,385,386]. Each of the six binding proteins differently regulates IGF bioactivity, as they bind and sequester IGFs from their receptors with varying affinities and with varying functions such as to prolong their half-lives, attenuate their bioactivity, maintain them in circulation, or to target them to specific tissues[142].

1.3.1 IGFBP-3 is the general transport protein for IGF-I

Most IGF protein exists in circulation bound to IGFBP-3 in a stable, ternary complex with acid labile subunit (ALS) glycoprotein[143,144]. IGFBP-3 actions are generally growth-promoting, functioning to prolong the half-lives of and effectively transport both IGF-I and IGF-II throughout the circulation. The IGFBP-3:IGF-I:ALS complex has a half-life of 12 hours, which is significantly extended compared to free unbound IGF-I ($t^{1/2}$ = 10 minutes) or IGFBP-3 ($t^{1/2}$ = 30-90 minutes)[143]. At any given time, IGFBP-3 is associated with approximately 75% of total IGF, maintaining a reservoir of IGFs within the circulation either free or bound to other IGFBPs, available for immediate metabolic demands[143].

IGFBP-3 is the predominant circulating IGFBP in post-natal life at approximately a 10-time higher concentration than other IGFBPs[145]**.** Accordingly, IGFBP-3 serves as the major IGF transport protein while other IGFBPs are involved in more specific, acute regulation of IGF bioactivity[145]**.** Although IGFBP-3 sequesters the majority of total IGF-I, the role of this binding protein is generic and generally stable in circulation. Fluctuations in IGFBP-3 levels have not been found to be consistently associated with

disease states. In contrast, the roles of other IGFBPs are more specific. IGFBP-1 plays a crucial role in mitogen signaling in ovarian, endometrial, trophoblast and fetal and placental tissues[146] and is the key circulating IGFBP during gestation[75].

1.3.2 IGFBP-1 regulation of IGF-I during pregnancy

Hepatically secreted IGFBP-1 is a potent inhibitor of the IGFs both *in vitro*[147] and *in vivo*[148-150,186,187]. However, IGFBP-1 has a much greater affinity for IGF-I compared to IGF-II[151], supporting its role as a potent, specific inhibitorof IGF-I bioavailability. IGFBP-1 regulates free serum IGF-I bioavailability[154] by binding and sequestering IGF-I from IGF-1R, preventing it from transducing its growth-promoting effects[155-159]. IGFBP-1 has been suggested to bind to IGF-I and block its receptor-binding site[160].

IGFBP-1 is the predominant circulating IGFBP in fetal circulation, fetal liver, placenta and amniotic fluid during pregnancy[69,74,103,149,152,153]. IGFBP-1 is significantly elevated in pregnant versus non-pregnant women (approx. 2-fold) and is present at even higher concentrations in amniotic fluid[161]. During pregnancy, decidual cells secrete IGFBP-1 into the placenta[162,163] where it functions primarily to inhibit trophoblast invasion at the placental barrier, which is involved in embryo implantation[164]. In the fetal compartment, IGF-I bioavailability is tightly regulating by fetal hepatic IGFBP-1 which is widely acknowledged as a critical factor in altered human fetal development[12, 54, 66, 67, 175, 193]. Fetal liver is the primary source of fetal IGFBP-1[175] and fetal hepatic IGFBP-1 is the primary regulator of fetal IGF-I bioactivity[76].

Fetal IGFBP-1 levels rise dramatically around approximately 16-20 weeks gestation and rapidly reach their peak[72]. IGFBP-1/IGF-I binary complexes increase in tandem with fetal IGFBP-1 production at 16 weeks gestation[72]. Once the concentration of IGFBP-1 peaks, it remains at a steady concentration for the remaining gestational period in both the fetus[72]and maternal plasma[176] in normal, non-growth restricted pregnancies. Langford et. al. determined a steady concentration of 300 +/- 25.1 ug/L IGFBP-1 in healthy fetuses, which remained consistent throughout gestation[85]. In the fetus, IGFBP-1 levels fall after 33 weeks gestation and continue decreasing post-natally[95].

1.3.3 IGFBP-1 structural elements

The *Igfbp1* gene is located adjacent to *Igfbp3* on chromosome 7p12-p14[177].The *IGFBP-1* promoter contains the TATA element in addition to a cAMP response element (CRE), glucocorticoid response elements 1 (GRE1) and 2 (GRE2), and binding sites for hepatic nuclear factor 1(HNF1) and 3 (HNF3), the latter also known as the insulin-response element (IRE)[154]. Binding to the HNF1 motif on the *IGFBP-1* promoter contributes to tissue-specific expression of IGFBP-1 in the decidua, ovary, liver and kidney[154]. Systemically, insulin is the primary regulator of IGF-I transcription via binding to the IRE and subsequent inhibition of *IGFBP-1* transcription, whereas glucocorticoids and cAMP stimulate *IGFBP-1* expression[154].

The six IGFBPs possess several structural and functional similarities particularly in their highly conserved, cysteine-rich, amino- and carboxy-terminal domains[178]. The primary IGFBP sequence contains a string of 12 N-terminal and six C-terminal cysteines, which have been demonstrated to be necessary for optimal IGF binding and are conserved among all IGFBPs in mammalian systems[179]. The di-sulfide bonds in these highly conserved N- and C- terminal cysteine residues form a highly specific IGF-binding pocket[178,180]. Conversely, the linker region between the two terminal domains is mobile and not well conserved among the IGFBPs. The linker region contains sites for post-translational modification, protein/protein interactions, and ubiquitination that are unique to each IGFBP[139,156,181,182]. This linker region is prone to phosphorylation in IGFBP-1, -3, and 5[140], however, only phosphorylation of IGFBP-1 in this region enhances its affinity for IGF-I[166,183,184]and its ability to inhibit IGF bioactivity[155-158,185].

1.3.4 IGFBP-1 phosphorylation

Phosphorylation of IGFBP-1 dramatically increases its affinity for IGF-I[73,151] and potentiates its bioinhibitory effect on the mitogen[73,139,151,155]. IGFBP-1 phosphorylation is associated with the inhibition of IGF-I-mediated cell proliferation, amino acid transport and apoptosis[155]. Phosphorylated IGFBP-1 has also been shown to inhibit

IGF-I-stimulated DNA synthesis in experimental animals[156], smooth muscle cells[185] and fetal skin fibroblasts[157].

Previously, our team has demonstrated that IGFBP-1 hyperphosphorylation is associated with decreased IGF-I bioavailability in amniotic fluid from FGR pregnancies[171]and with decreased IGF-I bioactivity *in vitro*[172,173]. Our lab has also previously demonstrated that IGFBP-1 hyperphosphorylation is indicative of decreased IGF-I bioavailability *in vitro* by demonstrating a link between increased IGFBP-1 phosphorylation and decreased IGF-1R autophosphorylation in HepG2 cells[172]. Phosphorylated IGFBP-1 secreted by HepG2 cells displays a 6-fold higher affinity for IGF-I compared to non-phosphorylated IGFBP-1[73], and hyperphosphorylated IGFBP-1 species derived from human plasma displays approximately 10-fold greater binding affinity for IGF-I[151]. Interestingly, IGFBP-1 phosphorylation does not affect its propensity for IGF-II binding[153].

Sites of IGFBP-1 phosphorylation

Mass spectrometry has helped identify five phosphorylation sites on IGFBP-1 (Ser95, Ser98, Ser101, Ser119, Ser169)[158]. The phosphorylation status of IGFBP-1 at Ser101, Ser119 and Ser169 has been associated with altered IGF-I affinity[73,190,191]and IGF-I bioactivity *in vitro*[172].Jones et. al. demonstrated that site-directed mutagenesis of Ser101 to un-phosphorylatable Ala101 decreases IGFBP-1 affinity for IGF-I 3-fold[183]. By mutating phospho-acceptor residues to un-phosphorylatable Ala residues in HepG2 cells, our team has demonstrated that phosphorylation of IGFBP-1at Ser101, Ser119 and Ser169 variably affects IGF-I bioavailability, with most pronounced effects when IGFBP-1 was mutated to Ser101Ala and Ser119Ala[172]. Ser101, Ser119 and Ser169 are sensitive to phosphorylation *in vitro* in response to leucine restriction and are associated with potent increases in IGF-I affinity (up to 30-fold) upon phosphorylation[190].Our lab has previously demonstrated an increase in pSer101, pSer119 and pSer169 in amniotic fluid[171] and umbilical cord plasma[173] from FGR babies as well as in fetal hepatocytes from a baboon model of FGR[173].

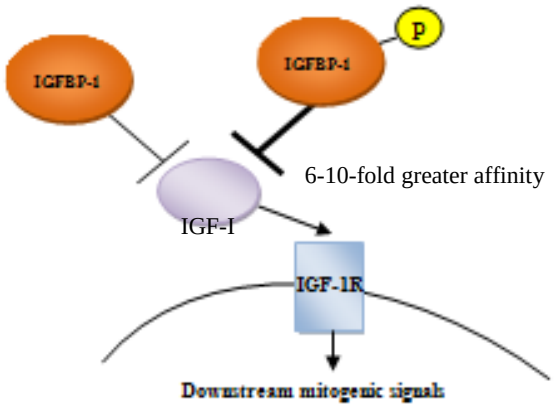


Figure 1.1.IGFBP-1 sequesters IGF-I from its cell surface receptor. Phosphorylated IGFBP-1 sequesters IGF-I with greater affinity.

1.3.5 Total and phosphorylated IGFBP-1 in normal and growth-restricted pregnancies

Total IGFBP-1 and fetal growth

Several groups have linked FGR with increased fetal IGFBP-1[56,93,95,103,112,160,192,194-196] FGR infants have been shown to have increased perigestational cord blood[93,95,160,194] and post-natal circulating[195,196] IGFBP-1. Elevated IGFBP-1 in maternal plasma[95,197] and placental tissues[112,113] are also inversely related to infant birth weight. A study on discordant twins (one FGR and one AGA infant) demonstrated increased IGFBP-1 in FGR infants compared to their AGA siblings, a difference that was not observed in twin pairs who experienced concordant growth[56]. This finding suggests that altered IGFBP-1 levels in FGR are likely due to environmental, rather than genetic, influences. Additionally, transgenic over-expression of *IGFBP-1* in murine fetuses decreased fetal birth weight by 18%[175], suggesting a causative relationship between IGFBP-1 and restricted fetal growth. HepG2 cells cultured in leucine concentrations observed in nutrient-restricted rats had significantly induced IGFBP-1 mRNA and protein expression[198]. Further, Bajoria et. al. reported that fetal IGFBP-1 production was elevated in FGR infants where amino acid supply was reduced[56]. The negative correlation between

fetal amino acid uptake and IGFBP-1 production suggests that increased IGFBP-1 synthesis may be a fetal response to decreased amino acid availability.

IGFBP-1 phosphorylation and fetal growth

Fetal liver contains an abudance of phosphorylated IGFBP-1 throughout the gestational period[162]. Conversely, IGFBP-1 phosphorylation in the maternal compartment appears to be temporally regulated. Amniotic fluid IGFBP-1 circulates primarily unphosphorylated in early pregnancy with singly and multiply phosphorylated IGFBP-1 present in decreasing concentrations[158], although the exact proportions of total to unphosphorylated IGFBP-1 have not been classified. However, as pregnancy progresses, the relative abundance of highly phosphorylated IGFBP-1 species increases in maternal plasma[162], placenta[162] and amniotic fluid[168].

The partially and highly phosphorylated IGFBP-1 isolated from cord plasma likely originates in the fetal liver[169]. Fetal IGF-I levels continue to rise throughout pregnancy while IGFBP-1 levels, once peaked, typically remain consistent for the remaining gestational period[72]. It is likely that IGFBP-1 phophorylation is a cellular mechanism employed to fine-tune IGF-I bioavailability, and that increased phosphorylation of IGFBP-1 rather than its total secreted levels is the principal regulator of IGF-I bioavailability in latter gestation.

Altered IGFBP-1 phosphorylation is associated with fetal abnormalities[165,171,173,174,189,199]. Phosphorylated IGFBP-1 is elevated in cord plasma of growth restricted babies relative to babies who are appropriate for gestational age (AGA)[165], with an accompanied decreased proportion of non-phosphorylated IGFBP-1 in these infants. Further, amniotic fluid from FGR pregnancies also displays an altered phospho-isoform profile for IGFBP-1compared to amniotic fluid from healthy pregnancies[191]. IGFBP-1 hyperphosphorylation at specific phosphosites (Ser101, Ser119 and Ser169) was previously detected by our team in amniotic fluid[171] and umbilical cord plasma[173] from FGR pregnancies. Our team also previously reported that hyperphosphorylation of IGFBP-1 at pSer101 and pSer169 in amniotic fluid is most dramatically increased in FGR[171], but that hyperphosphorylation at Ser119 most potently induces IGFBP-1 affinity for IGF-I[191].

In addition, we recently reported IGFBP-1 hyperphosphorylation at pSer101, pSer119, and pSer169 in umbilical cord plasma of FGR babies as well as fetal baboon hepatocytes from nutrient-restricted mothers[173]. In contrast, in human amniotic fluid, a decreased ratio of highly phosphorylated to lowly phosphorylated IGFBP-1 isoforms is linked with stimulated fetal growth[200].

In summary, the literature suggests that diminished amino acid supply to the fetus during gestation is a key factor in FGR onset, and that IGFBP-1 hyperphosphorylation leads to decreased IGF-I bioavailability. However, the signaling mechanisms involved in signaling amino acid deficiency to increased fetal hepatic IGFBP-1 phosphorylation are presently unknown. Herein, we explore candidate nutrient-sensing signaling pathways for their potential role in modulating hepatic IGFBP-1 secretion and phosphorylation in amino acid deprivation.

1.4 Nutrient-sensing signaling pathways

Nutrient deprivation is an environmental stressor, requiring an adaptive cellular response to optimize organismal outcomes. Upon nutritional stress, the organism typically defends overall homeostasis by reducing cell growth and survival and promoting energy conservation[201]. The mechanistic target of rapamycin (mTOR) and Amino Acid Response (AAR) are key sensors of nutrient (amino acid) availability in several cell types[202,203] including the liver[203]. Here, we highlight the functions of these two signaling pathways in the context of their possible roles in modulating amino acid deprivation-induced IGFBP-1 hyperphosphorylation in HepG2 cells, a model for fetal hepatocytes[204-208].

1.4.1 Mechanistic Target of Rapamycin (mTOR)

1.4.1.1 The two mTOR complexes

mTOR exists in two, ubiquitinously expressed[209], functional complexes, mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). The two complexes are composed of both unique and shared proteins. mTOR is the catalytic component common to both complexes[210], which also share DEP domain-contain mTOR-interacting protein

(DEPTOR), mammalian lethal with Sec13 protein 8 (mLST8), and Tti1–Tel2[211-213]. DEPTOR is an endogenous inhibitor of both complexes[213] and Ttil-Tel2 regulates complex formation for both mTORC1 and mTORC2[211]. mLST8 is critical to mTORC2 function[214]. Further, each complex is associated with a unique, key functional protein that responds to external stimuli and accordingly phosphorylates downstream effectors[210]. mTORC1 is associated with regulatory-associated protein of mammalian target of rapamycin (raptor)[215] and mTORC2 with rapamycin-insensitive companion of mammalian target of rapamycin (rictor)[214]. Raptor has been implicated in amino acid sensing and sub-cellular localization of mTORC1[216]. Similarly, rictor phosphorylates downstream mTORC2 effectors[210], recruits substrates[214], and confers structural stability to mTORC2[217].

The two complexes are differently regulated with independent and overlapping cellular functions[210]. Both complexes are sensitive to inhibition by rapamycin, though mTORC2 requires higher treatment doses and durations[173,214]. mTORC1 is endogenously inhibited by tuberous sclerosis 1 and 2 (TSC1-TSC2 complex) in response to a variety of stress-stimuli. When activated, mTORC1 phosphorylates and activates eukaryotic translation initiation factor 4E (4E-BP1) and p70-ribosomal S6 Kinase 1 (p70-S6K), which proceed to modulate cell growth and proliferation[210]. mTORC2 phosphorylates downstream effectors protein kinase C-α (PKCα) and AKT and has been typically implicated in maintaining the actin cytoskeleton, although recently has also been demonstrated to play a role in cell proliferation, survival, and morphology[217] and in the regulation of lipid homeostatis[218].

1.4.1.2 mTOR as a cellular nutrient sensor

mTOR signaling is sensitive to fluctuations in nutritional status[210,219], although the mechanisms by which mTOR senses nutrient availability have not been completely elucidated. In particular, mTOR has been proposed to respond to fluctuations in nutrient status via mTORC1, whereas mTORC2 is proposed to not be directly involved in cellular energy sensing[217]. Various factors have been discovered to be necessary for amino acid sensing by mTOR, suggesting that nutrient sensing by the signaling complex is highly

dynamic. Sancak et. al. demonstrated that sufficient amino acid availability is absolutely necessary for mTOR activity and involves the Rag family of proteins[216]. Amino acids promote RagA/B complex association with GTP[216]. These GTP-bound complexes subsequently associate with raptor and translocate to the lysosomal membrane where they interact with Ragulator, an essential component for amino acid sensing by mTORC1[209]. Further, Hardie et. al. suggested that cellular energy sensing by mTORC1 occurs via AMP-dependant kinase (AMPK)[220]. Adequate nutrient availability is sensed by AMPK, which subsequently phosphorylates and deactivates TSC1-TSC2 complexes and activates mTORC1[220]. Kim et. al. proposed that nutrient deprivation increases the stability of the mTORC1:raptor complex, sequestering mTORC1 in an inactive complex and inhibiting its kinase ability[221]. It is likely that all these components interplay in a dynamic mechanism to modulate mTOR activity under various nutritional cues.

Amino acid insufficiency leads to decreased phosphorylation of downstream mTOR effectors[222]. mTOR activity is especially sensitive to circulating BCAAs. BCAAs stimulate mTOR activity in multiple cell types[223] including murine hepatocytes as indicated via increased phosphorylation of p70-S6K and 4E-BP1[224,225]. mTORC1 activation is critically dependant on sufficient amino acid availability, especially on cellular leucine status[226]. mTOR signaling is a key component of leucine-stimulated protein synthesis in catabolic conditions[227]. Although mTORC2 has been proposed to be insensitive to amino acid status[228], the complete regulation and functions of mTORC2 have not been extensively classified[210]. Inhibition of mTOR activity by amino acid deprivation is reversed by the re-introduction of amino acids into the culture media[229]. Finally, mTOR nutrient sensing in the placenta has been implicated in FGR onset[230-232].

1.4.1.3 mTOR signaling as a potential link between nutrient deprivation and decreased fetal growth in FGR

Placental mTOR nutrient sensing has been implicated in FGR onset[230,231] and is particularly sensitive to activation by amino acids[233]. mTOR has been proposed as a key regulator of the placental expression of amino acid transporters that are critical to EAA transfer to the fetus[32,230,231]. Further, mTOR signaling is diminished in placentas from

maternal nutrient restricted (MNR) rats[32], MNR baboons[234] and in human FGR pregnancies[230,235]. Kavitha et. al. recently demonstrated, in MNR baboons, that mTOR and IGF-I activity inhibition led to a decrease in placental nutrient transporters and decreased offspring growth[234]. The role of mTOR signaling in the fetal compartment in FGR has been not as well established. Promisingly, our team has recently reported decreased mTOR activity in baboon hepatocytes from MNR pregnancies[173]. Further, Teodoro et. al. reported that BCAA supplementation reversed growth restriction in an induced murine model of FGR by activating the fetal hepatic mTOR signaling pathway[45].

In addition, mTOR signaling is necessary and sufficient for insulin-mediated inhibition of *Igfbp1* gene expression[236-238]. Conditioned cell media from mTORC1- and mTORC2-silenced trophoblast cells induced IGFBP-1 secretion and phosphorylation in cultured HepG2 cells[239], and our lab has recently linked decreased mTOR activity with increased IGFBP-1 protein secretion and phosphorylation[173]. However, whether mTOR is the mechanistic link between amino acid deprivation and hepatic IGFBP-1 hyperphosphorylation has yet to be determined.

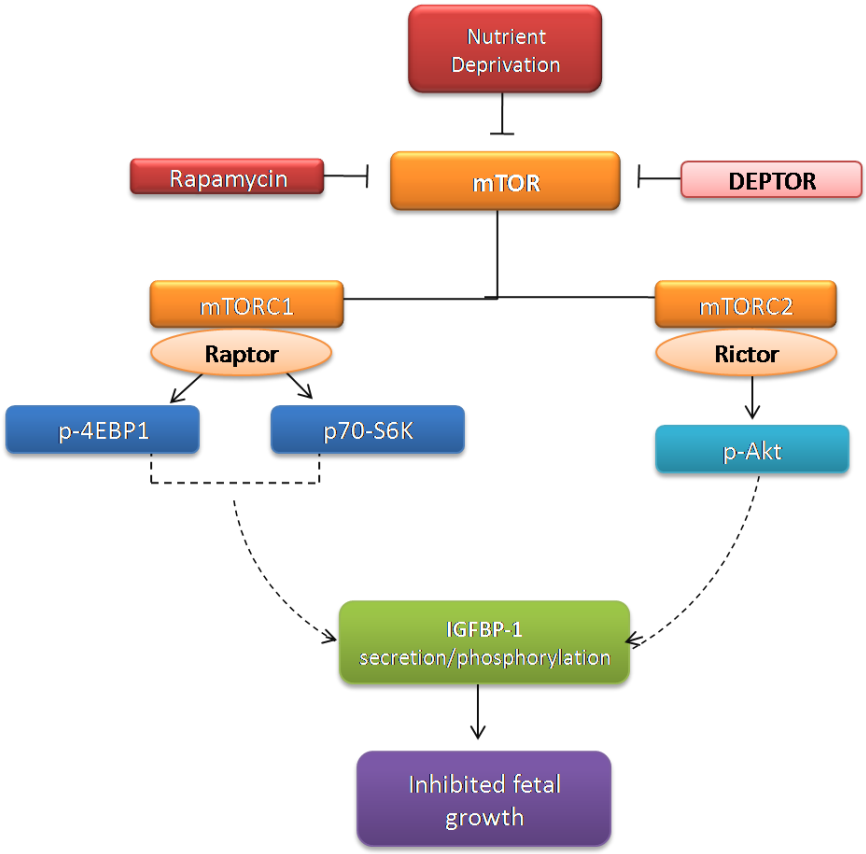


Figure 1.2. Model for IGFBP-1 phosphorylation by various components of the mTOR signaling components.

1.4.2 The Amino Acid Response (AAR)

Amino acid deprivation in mammalian cells activates a collection of intracellular signaling responses, collectively known as the Amino Acid Response (AAR), which lead to the induction of a stress-responsive transcriptional program[240]. The AAR is an evolutionarily conserved, adaptive cellular response to decreased circulating amino acids and is part of the Integrated Stress Response (ISR), which is initiated by multiple stress-sensing kinases upon distinct environmental stimuli. General control non-derepressible 2 (GCN2) is the specific sensor for the AAR. GCN2 functions exclusively to initiate an amino acid restriction-specific stress response and its activity is initiated upon reduced intake of essential amino acids or diminished intracellular synthesis of non-essential amino acidsvia sensing excess intracellular uncharged tRNAs[241]. Subsequent

GCN2phosphorylation of eukaryotic initiation factor 2 (eIF2a) initiates the AAR, leading to reduced global protein translationand a concurrent increase in the translation of pre-existing stress-responsive mRNAs, most notably Activating Transcription Factor 4 (ATF4). ATF4 is a transcription factor that then proceeds to up-regulate the transcription of several stress-responsive proteins. Overall protein translation is curtailed in favour of the up-regulation of a host of stress-responsive genes that serve to restore cellular homeostatis[242].

1.4.2.1 General control non-derepressible 2 (GCN2) is the AAR nutrient sensor

GCN2 is a serine/threonine kinase and a universally conserved sensor of cellular nutrient status from yeast (GCN4 homologue)[243] to mammalian systems. Among the four ISR kinases, GCN2 is uniquely sensitive to fluctuations in amino acid availability[244]. Within the AAR, GCN2 is the only identified sensor of amino acid depletion[245]. Deprivation of even a single amino acid is sufficient to induce GCN2 activity[246]. Activated GCN2 subsequently initiates stress-responsive downstream signaling pathways via phosphorylation of eIF2α (Ser51)[203,241,247-249].

Structurally, GCN2 contains a catalytic eukaryotic kinase domain that is well-conserved among all eukaryotic kinases[250,251]. Of the twelve sub-domains, catalytic subunits IV and V are the least conserved among eukaryotes[250,251]. However, there is significant sequence homology in the V domains between the eIF2α kinases, conferring substrate specificity to this kinase family[252]. GCN2 also contains C-terminal ribosome binding/dimerization, pseudo-kinase(regulatory), histidyl-tRNA (HisRS)-related, and N-terminal RWD domains that are not structurally conserved among the eIF2α kinases.

GCN2 typically circulates in its inactive form[246] in loose association with 80S ribosomes or actively translating polysomes[253]. Uncharged tRNAs, which accumulate as a result of reduced essential amino acid intake or non-essential amino acid synthesis, preferentially bind the HisRS domain over charged tRNAs[245]. tRNA binding leads to GCN2 dimerization[254] and subsequent autophosphorylation at Thr898[255], causing kinase activation. tRNA binding is facilitated by the binding of a GCN1/GCN20 protein

complex to the RWD domain[256,257] and by certain C-terminal lysine residues on GCN2[256], conferring additional sensitivity to the nutrient sensor. There are two reports of GCN2 binding to viral RNA[258,259], however, eIF2α is the only currently established target for GCN2 phosphorylation in mammalian systems.

The role of GCN2 in amino acid sensing is widely acknowledged. GCN2+/GCN2+ mice displayed decreased hepatic and adipose mass in response to leucine starvation, whereas GCN2-/GCN2- mice livers were not affected in size but rather displayed pronounced steatosis[260]. GCN2-/GCN2- mice do not avoid essential amino acid-deprived diets unlike their wild-type counterparts[261,262] and have decreased rates of survival[263]. GCN2 is also sensitive to glucose deficiency[264,265].

GCN2 phosphorylation of eIF2α occurs in a MEK/ERK dependant manner[266] (discussed further in section 1.4.2.3), which subsequently proceeds to initiate a stress-responsive transcriptional program via the increased translation of pre-existing ATF4 mRNAs and concurrent decrease in overall protein translation[241]. Increased GCN2 and eIF2α (pSer51) phosphorylation and ATF4 expression are therefore indicators of AAR pathway activation.

1.4.2.2 AAR propagation via eukaryotic initiation factor 2 alpha (eIF2α) phosphorylation and ATF4 expression

eIF2 is a heterotrimer of α, β, and γ subunits[248,257] and participates in mRNA translation by mediating Met-tRNA binding to the ribosome[227,267]. The process of translation initiation by eIF2 is GTP dependant [268,269]. eIF2γ is the main docking site for GTP/GDP, and eIF2β catalyzes eIF2-GDP conversion to eIF2-GTP. eIF2α is the enzyme's regulatory subunit[268,269]. Phosphorylation of eiF2α at Ser51 increases its affinity for eIF2β[251], thereby sequestering eIF2β-GDP in its inactive complex. Consequently, eIF2β is unable to catalyze the nucleotide exchange, reducing the functional capacity of the translation factor[270].

eiF2α is phosphorylated by four distinct stress-responsive kinases, rendering it the convergence point for the ISR[242,271]. eIF2α is exclusively phosphorylated by the four ISR

kinases *in vivo*, and sequence determinants remote from Ser51 have been proposed to confer this specificity[268]. The phospho-acceptor site (Ser51) on the alpha subunit is buried within a hydrophobic pocket preventing its phosphorylation by other *in vivo* kinases[268,269]. Kinase binding to eIF2α induces a conformational change that projects Ser51 into the active site, allowing its phosphorylation[268,269].

In mammalian cells, in addition to GCN2, eIF2α is also phosphorylated by the three other ISR kinases depending on the particular stressor: heme-regulated inhibitor kinase (HRI), double-stranded RNA-activated protein kinase (PKR), and PKR-like endoplasmic reticulum (ER) kinase (PERK). The transcriptional program elicited by eIF2α phosphorylation reduces global translation in favor of up-regulating gene products involved in cellular stress-management[244]. In a study by Dang et. al., 2.5% of total murine hepatic mRNA was down-regulated by eIF2α phosphorylation[272].

PERK is a component of the Unfolded Protein Response (UPR) that is activated under endoplasmic reticular (ER) stress[242,271] and has been most commonly investigated alongside GCN2 in regards to the transcriptional program that is elicited upon cellular stress. ATF4 induction, characteristic of eIF2α phosphorylation, which is intitiated by PERK elicits an overlapping transcriptional program with that of up-regulated ATF4 by GCN2 activation, suggesting that ATF4 is a critical signaling convergence point from cellular stress derived from amino acid insufficiency and from oxidative stress[273]. A comprehensive analysis of the gene profiles elicited by GCN2 and PERK in murine hepatocytes indicates that although some overlap exists in the programs elicited by GCN2 and PERK, each cellular stressor has a vastly distinct overall affect on the hepatic transcriptome[272]. The stress response elicited by AAR activation is therefore likely affected by a multitude of factors downstream of GCN2 and is not specific to ATF4. Specific attenuation of the AAR is therefore best accomplished via direct manipulation of GCN2.

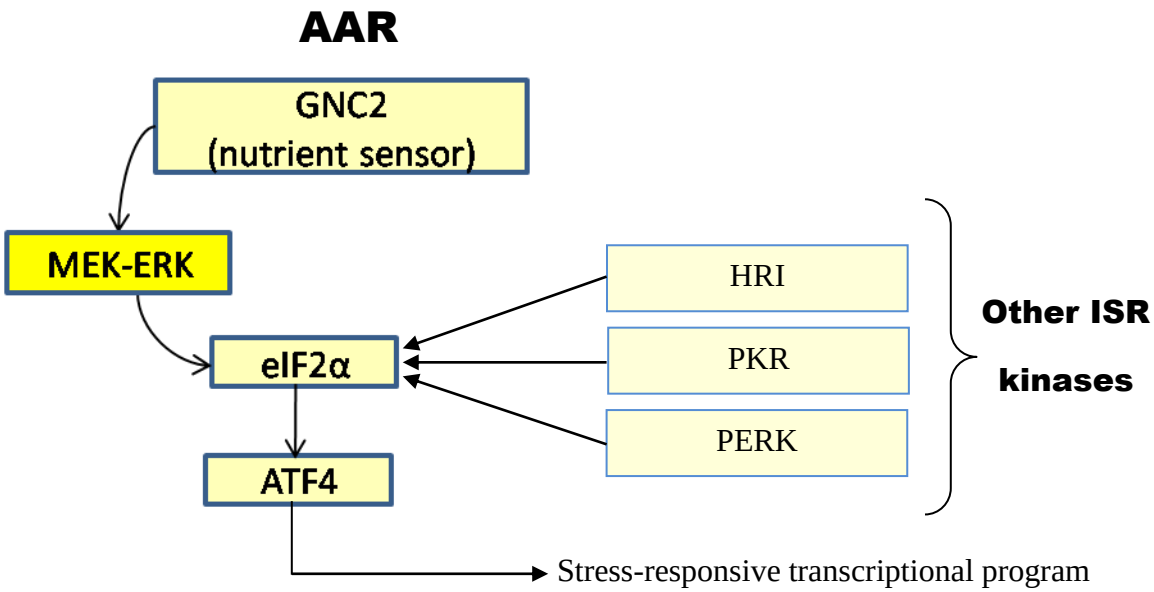


Figure 1.3. Overview of signaling components of the Amino Acid Response (AAR) as a component of the Integrated Stress Response (ISR).

1.4.2.3 Involvement of MAPKs in the AAR

The Mitogen-Activated Protein Kinase (MAPK) family of serine/threonine kinases is widely conserved among eukaryotic species, and encompasses a three-tier system of kinases. MAPKs are phosphorylated upstream by MAPK kinases (MKKs), which in turn are phosphorylated by MEK Kinases (MKKKs)[274]. MKKs are dual specificity kinases that phosphorylate Thr-X-Tyr motifs on their respective MAPKswhich in turn phosphorylate their own downstream targets, many of which are transcription factors[275]. There are four subfamilies of the MAPK signaling cascades in mammalian cells, each of which is involved in unique and overlapping intracellular functions[274]. Each subfamily contains several MAPKs, MEKs, and MKKs, serving as multiple integration points for various intracellular signals. One signaling arm ($MEK^{1/2}/ERK^{1/2}$) in particular is critically involved in cell growth and proliferation; the MAPKs in this cascade are known as ERK1 and ERK2, and this signaling arm will herein be referred to as MEK/ERK. An overview of the various components of the MEK/ERK signaling cascade is illustrated in Figure 1.3.

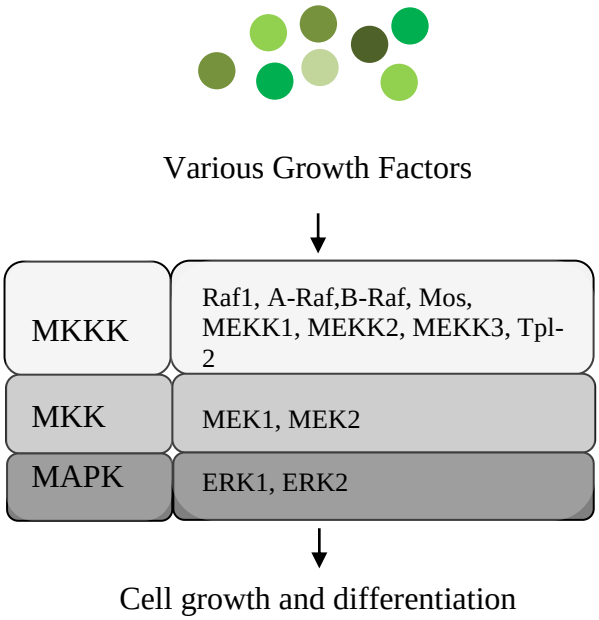


Figure 1.4. Schematic representation of the mitogenic (MEK/ERK) pathway including all potential MKKKS, MKKs, and MAPKs

The literature purports that MEK/ERK signaling is necessary for AAR propagation[245,266]. Inhibiting MEK/ERK signaling is therefore a strategy for attenuating the AAR. However, the role of MEK/ERK in cell growth and proliferation[274] suggests it may independently modulate IGFBP-1 secretion and phosphorylation. Inhibition of MEK/ERK signaling has been shown to decrease IGFBP-1 secretion in the human endometrium[276] and secretion of both IGF-I and IGFBP-1 in rat hepatocytes [277,278]. MEK/ERK signaling has also been implicated downstream of IGFBP-1 in modulating mitogenesis in the liver[279]. Various studiesreport differential involvements of MEK/ERK signaling in IGFBP-1 regulation[280-282]. However, its explicit role in the secretion and phosphorylation of IGFBP-1 in nutrient restriction, and whether these effects occur via the AAR, are unknown.

1.4.2.4 Potential role of the AAR in IGFBP-1 regulation under nutrient restriction in FGR

Decreased transfer of EAAs from the mother to fetus is observed in FGR[22,283,284], and the extent to which fetal amino acid transfer is restricted is correlated with FGR severity[283]. An important study by Strakovsy et. al. demonstrated that the AAR is activated in

placentas of rats fed nutrient-restricted diets during gestation, which also led to stunted growth of neonates[285]. More recently, increased eIF2α phosphorylation has been detected in placentas from human FGR pregnancies[235]. Considering that the AAR is an adaptive cellular response to restricted amino acids, and that FGR results from a maladaptive physiological response to decreased nutrient availability, it is extremely likely the AAR is implicated in FGR onset.

The amino acid-specific, and AAR-mediated, regulation of IGFBP-1 has been demonstrated at the transcriptional and translational levels. *IGFBP-1* expression is induced downstream of ATF4 in murine hepatocytes[286]. Further, *in vitro* [287]and i*n vivo*[288] studies support this observation by demonstrating that dietary amino acid restriction in rats leads to induced hepatic *IGFBP-1* mRNA expression[288]. Conversely, murine *IGF-I* gene expression is down-regulated by amino acid restriction[289]. Amino acid restriction-induced hepatic *IGFBP-1* mRNA is dependent on specific regions in the *IGFBP-1* promoter (IRE, GRE)[290], subsequently named the Amino Acid Response Unit (AARU)[291]. Increased *IGFBP-1* species in response to amino acid deprivation is accomplished in part by mRNA stabilization[287] in addition to increased transcription.

Upstream stimulatory factors 1 and 2 (USF-1, USF-2) have been identified as potential transcription factors in protein restriction-induced *IGFBP-1* transcription [291]. USF-1 and -2 are ubiquitously expressed and up-regulated in protein restricted conditions[291], suggesting that regulation of total IGFBP-1 output may be under the control of the AAR via stress-induced up-regulation of various downstream transcriptional factors. Interestingly, Averous et. al. demonstrated that primary hepatocytes derived from mice deficient for GCN2 were still able to induce *IGFBP-1* transcription under leucine restriction[287]. Together, the literature suggests that the AAR is partly involved in modulating*IGFBP-1* expression; however, the involvement of the AAR in the translation and post-translational regulation of IGFBP-1 has not been classified.

1.5 Kinases involved in the regulation of IGFBP-1 phosphorylation

Phosphorylation of IGFBP-1[73], IGFBP-3[292] and IGFBP-5[184] has been unequivocally demonstrated, while other IGFBPs display potential phosphorylation sites[140]. IGFBP-1 is likely phosphorylated by intracellular kinase(s) prior to its secretion[293]. Consensus sequence analysis and *in vitro* studies indicate that IGFBP-1 is a potential substrate for protein kinase CK2, protein kinase C (PKC) and protein kinase A (PKA)[140,168,188,293]. Here, we review these three potential kinases for their potential involvement in IGFBP-1 phosphorylation under leucine deprivation.

1.5.1 Protein Kinase CK2

1.5.1.1 Structure and Function

CK2 is a constitutively active, ubiquitous serine/threonine kinase with over 300 potential targets[294,295] including IGFBP-1[293]. Ubiquitinously expressed in mammalian cells[296] , CK2 exists in a tetrametric form with two identical regulatory (β) subunits[297,298] and two catalytic subunits (α and/or α')[298-302]. CK2 characteristically phosphorylates Ser or Thr residues located in close proximity to acidic amino acids[140,297,303] (consensus sequence: S-X-X-D/E) (Table 1.1). However, this minimal consensus sequence has been shown to be neither necessary[304] nor sufficient[305] for phosphorylation by CK2, suggesting that other structural determinants are involved in potentiating phosphorylation by CK2.

Differing only in their C-terminal domains, CK2a and CK2a' catalytic domains are 90% identical[306] and exhibit highly similar *in vitro* enzymatic properties[307]. CK2β is highly conserved among species with identical sequence homology in birds and mammals[296,308,309]. CK2 has been found to be localized in the cytoplasm as well as multiple intracellular organelles[310], functioning as both an endokinase[310] and ecktokinase[310,311]. CK2 subunits each target different substrates[312,313] and are individually implicated in robust regulatory cellular functions[296]. CK2 activity is responsive to various stress stimuli in mammalian cells[314-318].

1.5.1.2 CK2 phosphorylation of IGFBP-1

CK2 phosphorylates IGFBP-1 in HepG2[293] and endometrial stromal cells[188]. IGFBP-1 phosphorylation sites (Ser101, Ser119, Ser169) exist in proximity to acidic amino acids, characteristic of the CK2 consensus recognition motif [319,140](Table 1.1). However, whether or not IGFBP-1 is a true CK2 substrate has not been conclusively demonstrated. Previously in our lab, inhibition of CK2 activity via combined siRNA silencing of CK2α, CK2α' and CK2β or selective CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) decreased IGFBP-1 phosphorylation in HepG2 cells[173]. Additionally, our team has shown that CK2 activity and IGFBP-1 phosphorylation are concomitantly elevated in growth-restricted fetal baboon hepatocytes from nutrient-restricted mothers[173]. Previous work from our lab also showed that CK2 is a key regulator of IGFBP-1 phosphorylation (pSer101, pSer119, pSer169) downstream of mechanistic target of rapamycin (mTOR) signaling[173]. Whether CK2 mediates IGFBP-1 phosphorylation in amino acid deprivation remains to be established.

1.5.2 Protein Kinase C (PKC)

1.5.2.1 Structural and functional basis for inhibition

PKC is another indiscriminate protein kinase and is activated intracellularly by second messenger diacylgycerol[320]. The role of PKC in IGFBP-1 phosphorylation has not been established. Although PKC is a potential kinase for IGFBP-1 phosphorylation[140], based on the PKC consensus sequencewhich requires surrounding basic residues to the phospho-acceptor site[140], Ser101, Ser119 and Ser169 on IGFBP-1 are not likely sites for direct phosphorylation by PKC. However, PKC has been shown to be pivotal in the regulation of IGF-I bioactivity through uncharacterized mechanisms[321-327].

PKC is widely expressed in mammalian tissues. PKC consists of a regulatory N-domain and catalytic C-domain, and 4 conserved and 5 variable regions in its primary structure. 11 PKC isoforms (α, βI, βII , γ, δ, ε, θ , η, ζ , √, λ) have been identified with minor structural and functional differences[328]. PKC isoforms α, βI, βII and γ are considered

conventional isotypes (cPKCs) and share conserved (C1 and C2) modules in their regulatory domains[328]. Novel PKCs (nPKCs) consist of the δ, ε, θ and η isotypes and lack the C2 subunit, whereas atypical PKCs (aPKCs - ζ , √ and λ) lack both C2 and integral structural components of C1 rendering them insensitive to activation by diacylglycerols (DAGs) and phorbol esters[328]. The isoforms are variably regulated by intra- and extra-cellular stimuli[329] and the expression of certain isoforms is restricted to specific cell types: γ to the central nervous system, θ to the skeletal muscle and hematopoietic cells, and β to the pancreatic, adrenal and neuronal tissue[329]. There is also evidence for differential sub-cellular localization of the various PKC isoforms[329]. The PKC isoform profile of HepG2 cells has not been exclusively classified, although certain PKC isoforms have been specifically implicated in various stress-related responses in HepG2 cells[330,331]. PKC has been implicated in multiple diseases phenotypes, and the specific roles of individual isoforms are emerging in the literature[330].The development of pharmacological tools to elucidate the stimuli- and cell-specific roles of the various PKC isoforms is a current topic of investigation[328].

Staurosporine is the most widely used chemical inhibitor against PKC. Although its effects are potent, it has demonstrated cross-reactivity with other kinases including protein kinase A (PKA)[332]. On the other hand, a less potent derivative of staurosporine, Bisindolylmaleimide (BIS), is a very specific PKC inhibitor for all PKC isoforms[332].

1.5.2.2 PKC as a nutrient-sensitive kinase and its possible role in FGR

PKC activity is sensitive to fluctuations in nutrient availability. PKC integrates signals downstream of mTOR in an amino acid-sensing pathway and certain PKC subunits (α,δ,ε) are regulated by mTOR in amino acid-dependant manner. PKC functions downstream of mTOR to stimulate mitogenic protein synthesis[333] including leucine-induced DNA synthesis as assessed in cultured chicken hepatocytes[334]. Nishitani et. al. demonstrated that leucine-mediated glucose uptake occurs via PKC signaling[335]. However, leucine supplementation in rats stimulates hepatic PKC activity as well as redistribution of the kinase from the hepatocellular membrane to the cytosol through mTOR-independent mechanisms[336], suggesting that PKC is also independently

sensitive to nutrient supply. Further, PKC signaling is abrogated in certain nutritional disorders. For example, hepatic PKCα signaling participates in nutrient transport and its function is implicated in obesity and diabetes mellitus II[337]. PKC activity was attenuated in the pancreas[338] and skeletal muscle[339] of rats subjected to reduced protein intake during gestation.

Finally, there is some evidence for the involvement of PKC in FGR onset. One study demonstrates an altered expression profile of the various PKC isoforms in placentas from murine FGR pregnancies during the third trimester[340]. Artificially-induced hypoxia potentiated PKCα activity in human umbilical venous endothelial cells from normal pregnancies to comparable levels seen in the same cells derived from FGR pregnancies[341] and hepatic PKCζ expression is increased in FGR rats[342]. Together, these studies implicate a possible role for altered PKC signaling in FGR onset.

1.5.2.3 Interactions between PKCand the IGF/IGFBP signaling axis

Various isoforms of PKC have been implicated in modulating the cellular actions of IGF-I in a variety of tissues. While PKCα up-regulates IGF-I bioactivity in microtubes[321], PKCζ modulates IGF-I-stimulated macrophage differentiation[322] and along with PKCβ, PKCη, and PKCε, plays an essential role in IGF-I-mediated migration of vascular smooth muscle cells, DNA synthesis and gene expression[323]. PKCζ signaling modulates IGF-I bioactivity in vascular smooth muscle cells[324] and PKCθ has been shown to be critical in IGF-1R mediated oncogenic cell proliferation[325]. PKCζ modulates the effect of growth-inducing hormones (e.g. insulin) on downstream mitogenic protein synthesis and cell cycle progression[327]. Inhibition of pan-PKC with BIS inhibited both IGF-I-stimulated early cell differentiation and later cell proliferation in messenchymal cells[326]. Induction of pan-PKC activity stimulates HepG2 cell proliferation[343].

There is evidence for PKC regulation of the IGFBPs. Multiple PKC isoforms have been implicated in regulating IGFBP-2 and IGFBP-3 secretion by thyroid cells[344-346]. PKCα phosphorylates IGFBP-3, inducing its degradation[347]. Further, pan-PKC inhibition induces IGFBP-5 synthesis[348] and secretion[349,350] and IGFBP-4 secretion[349] in multiple

cell types. There is also some support for the role of PKC in the regulation of IGFBP-1output. For example, a study by Kachra et. al. in rat hepatocytes revealed that glucagon inhibited *IGFBP-1* mRNA expression via activation of PKC signaling[351]. Further, PKC plays a role in both the up- and down- regulation of IGFBP-1 secretion *in vitro* at the protein level, depending on the parameters of the exposure to the inhibitors[352,353].

The role of PKC in post-translational regulation of IGFBP-1 has not been classified. Given the nutritional sensitivity of PKC, its role in the regulation of IGF-I bioactivity and IGFBP-1 expression, and its dynamic interactions with members of the IGFBP family of proteins, we considered it a valuable kinase to assess for its role in the elusive signal transduction pathway modulating IGFBP-1 phosphorylation under leucine deprivation.

1.5.3 Protein Kinase A (PKA)

1.5.3.1 Structural and functional basis for inhibition

PKA is a multifunctional, ubiquitously expressed, intracellular kinase with a wide variety of protein targets[320,354], and is activated by secondary messenger cyclic AMP (cAMP)[320,354]. PKA is a heterotetrameric kinase, with two catalytic subunits containing dimerization and regulatory subunit-binding domains as well as binding sites for the kinase's substrate and for ATP, the kinase's phosphate source. cAMP binding to the homodimeric regulatory subunits causes its dissociation and consequent activation of the catalytic subunits. PKI (5-24) is a highly potent, competitive inhibitor of PKA. PKA residues Tyr235 and Phe239 on the regulatory subunits structurally integrate with Phe10 of PKI, preventing substrate (cAMP) binding and subsequent activation of PKA[355].

1.5.3.2 PKA as a nutrient-sensitive kinase

Hepatic PKA activity is sensitive to fluctuations in nutrient availability[356-359]. PKA signaling is potently decreased in murine livers after offspringwere fed low-protein diets[357,358]. Further, murine pancreatic PKA expression is reduced by nutrient restriction[360]. Decidualization of human endometrial stromal cells during human pregnancy occurs in a PKA-dependant mechanism[361] and the increased IGFBP-1 production characteristic of this process is also dependant on PKA signaling[361-363].

Finally, altered PKA signaling has been implicated in fetal brain development in FGR[364,365].

1.5.3.3 Interactions between PKA and the IGF/IGFBP signaling axis

PKA has been implicated in modulating systemic IGF-I mitogenic actions in multiple cell types[366-368]. For example, PKA regulates IGF-I-stimulated osteoclast-like cell formation[369], and PKA inhibition prevents IGF-I stimulated early chondrocyte cell proliferation and differentiation[326]. PKA kinase activity stimulates the *IGF-I* promoter[370].

The majority of studies linking PKA and the IGFBPs focus on PKA-regulation of IGFBP expression and synthesis in multiple cell types. PKA activation prevents chondrocytic IGFBP-3 and IGFBP-4 expression and secretion[371] as well as antagonizes IGFBP-4 and IGFBP-5 secretion in the murine ovaries[348]. Conversely, PKA stimulation induces IGFBP-5 expression[350]. Importantly, induction of PKA activity stimulates IGFBP-1 promoter activity[372], mRNA[372] expression and protein[372,373]levels in HepG2 cells. PKA activation modulates glucagon-stimulated IGFBP-1 expression in rat hepatocytes[351]. Conversely, PKA inhibition attenuates cAMP-stimulated IGFBP-1 synthesis. Promisingly, one study[188] links PKA to IGFBP-1 phosphorylation in endometrial stromal cells.

Table 1.1. Surrounding peptide sequence of the three IGFBP-1 phosphosites and consensus sites for CK2, PKC and PKA

Peptide	**Surrounding peptide sequence**[a]
IGFBP-1(E97-**S101**-L110)	ESPE**pS**TEITEEELL
IGFBP-1(D111-**S119**-E121)	DNFHLMAP**pS**EE
IGFBP-1(A165-**S169**-K175)	A QET**pS**GEEISK
Kinase	**Consensus site**[b]
CK2	**S/T**-X-X-D/E
PKC	**S/T**-X-R/K
PKA	R/K-R/K-X-**S/T**

[a] Abu Shehab M et. al. Site specific phosphorylation of insulin-like growth factor binding protein-1 (IGFBP-1) for evaluating clinical relevancy in fetal growth restriction. *J Proteome Res*. 2009;8(11):5325-5335.
[b] Coverley JA, Baxter RC. Phosphorylation of insulin-like growth factor binding proteins. *Mol Cell Endocrinol*. 1997;128(1-2):1-5

1.5.4 IGFBP-1 de-phosphorylation

Westwood et. al. proposed that IGFBP-1 dephosphorylation is another regulatory mechanism by which cells can stimulate IGF-I bioactivity by releasing the growth factor from sequestration[151,166]. Alkaline phosphatase (ALP) was recently identified as the phosphatase which de-phosphorylates IGFBP-1 *in vitro*[167] and *in vivo*[374]to decrease its propensity for IGF-I binding[167]. During pregnancy, placental ALP de-phosphorylates IGFBP-1 in order to increase IGF-I bioavailability[374]. Therefore, dephosphorylation of IGFBP-1 may be an additional mechanism by which cells modulate IGF-I bioavailability in FGR.

1.6 Experimental models

1.6.1 Leucine deprivation as a model for nutrient restriction

BCAAs are the key stimulators of global protein synthesis[227]. Supplementation of murine hepatocytes with EAAs, but particularly the BCAAs, maximally induces plasma protein synthesis, as indicated by induction of albumin and tranferrin secretion[375]. BCAA repletion alone is sufficient to maximally induce protein synthesis in skeletal muscle[376]. Leucine, along with isoleucine and valine, is a BCAA and one of the nine EAAs in humans. Leucine accounts for more than 20% of human dietary protein [223]. Administration of BCAAs, but specifically leucine, has mitogenic effects in the liver, such as the synthesis of hepatocyte growth factor, as well as in hepatocellular regeneration[377]. In addition to its structural role in protein synthesis, leucine has one of the most potent roles in intracellular signaling compared to other essential amino acids[223].

Studies of placental nutrient transfer in FGR have focused on trans-placental leucine transport, which is potently and consistently decreased in FGR[22,46,47,378]. Assessment of venous umbilical samples indicates that leucine and phenylalanine most rapidly cross the placenta from the mother to the fetus[47], and that leucine transport is particular heightened after 20 weeks gestation in human pregnancies[48].Oral administration of leucine augments protein synthesis in multiple tissue and cell types[379,380], however, does not affect global protein synthesis in the liver[379,381]. Rather, hepatic leucine administration induces the expression of specific mRNAs and significantly activates mTOR signaling[263,381].

There is strong evidence in the literature suggesting that leucine is unique in its ability to stimulate protein synthesis to the maximal level comparable to a mixture of all EAAs[227,376]*in vivo*. In previous *in vitro* studies in our lab, leucine deprivation reliably triggered IGFBP-1 hyperphosphorylation at Ser101, Ser119 and Ser169 in HepG2 cells[190].

1.6.2 HepG2 cells as a model for fetal hepatocytes

As described previously (Section 1.3.2), fetal liver is the primary source of fetal circulating IGFBP-1. *In vitro*, HepG2 cells are an ideal, widely-used model for human fetal hepatocytes [204-208]. They express a transcriptome and secretome[206,382,383] most similar to human fetal hepatocytes. HepG2 cells induce *IGFBP-1* mRNA expression when limited for leucine without affecting the expression of *IGF-I*[198]. Previous work in our lab demonstrated that, in a HepG2 cell culture model, leucine deprivation-induced phosphorylation of IGFBP-1 led to up a 21-fold increase in affinity for IGF-I *in vitro*[190]. Further, our lab has recently demonstrated that changes in IGFBP-1 secretion and phosphorylation in HepG2 cells are reflected in primary baboon hepatocytes[173], which have served a successful, physiologically relevant model in the study of regulation of IGFBP-1 phosphorylation in FGR[173,384]. Therefore, it is justified to use HepG2 cells to study the mechanisms underlying leucine deprivation-induced fetal hepatic IGFBP-1 phosphorylation.

1.7 Scope of Thesis

The fetal response to decreased nutrient (amino acid) supply likely involves multiple signaling pathways in an effort to optimize fetal health. A maladaptive molecular response to amino acid restriction is detrimental to fetal outcomes[53]. The signaling mechanisms linking amino acid deficiency to decreased cell growth and proliferation in FGR have not been elucidated. As described previously, the bioavailability of IGF-I is a key determinant of its ability to stimulate fetal cell growth and proliferation; IGFBP-1 phosphorylation (Ser101, Ser119 and Ser169) is an indicator of decreased IGF-I bioavailability and is increased in FGR. The mechanisms regulating IGFBP-1 secretion and phosphorylation under amino acid deprivation, which is a keycontributing factorto FGR onset, remain to beestablished. The overall **objective** of this study is to shed light on the molecular mechanisms by which fetal growth is regulated under amino acid deprivation by studying the signaling pathways modulating leucine deprivation-induced IGFBP-1 phosphorylation *in vitro*. The central **hypothesis** is that leucine deprivation-induced IGFBP-1 phosphorylation is modulated via down-regulation of the mTOR

signaling cascade and up-regulation of the AAR pathway. Further, we predict that IGFBP-1 phosphorylation under leucine restriction is modulated by multiple protein kinases (CK2, PKC, PKA). The specific aims encompassed in this thesis are three-fold:

Aim 1: Determine the role of **mTOR signaling** in mediating leucine deprivation-induced IGFBP-1 secretion and phosphorylation (pSer101, pSer119 and pSer169) (Chapter 2).

Aim 2: Establish whether the **AAR** is involved in modulating leucine deprivation-induced IGFBP-1 secretion and phosphorylation (pSer101, pSer119 and pSer169) and whether this occurs in a **MEK/ERK-dependant manner** (Chapter 2).

Aim 3:Survey**potential kinases** that may be involved in regulating IGFBP-1 phosphorylation (pSer101, pSer119 and pSer169) under leucine deprivation (Chapter 3).

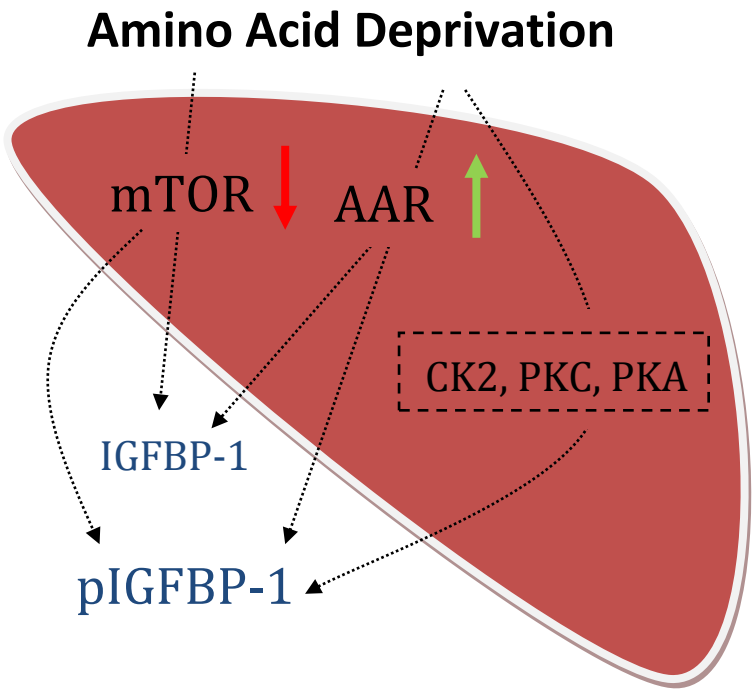


Figure 1.5. Proposed model for total and phospho-IGFBP-1 regulation by mTOR, AAR, and the kinases CK2, PKC and PKA under fetal amino acid deprivation

In Chapters 2 and 3, we analyze changes in IGFBP-1 secretion and phosphorylation in HepG2 cell media after treatments which have manipulated various molecular components of the mTOR and AAR signaling pathways or protein kinases CK2, PKC and PKA in both regular (leucine plus) or leucine minus conditions. Changes in IGFBP-1 secretion and phosphorylation are linked to concomitant changes in the activity of downstream pathway components. Identifying signaling pathways involved in leucine deprivation-mediated IGFBP-1 phosphorylation *in vitro* will provide the foundation for future *in vivo* studies aimed at contributing insight into the pathophysiology of FGR.

1.8 References

1. Pallotto EK, Kilbride HW. Perinatal outcome and later implications of intrauterine growth restriction. *Clin Obstet Gynecol*. 2006;49(2):257-269.

2. Barker DJ. Adult consequences of fetal growth restriction. *Clin Obstet Gynecol*. 2006;49(2):270-283.

3. Garite TJ, Clark R, Thorp JA. Intrauterine growth restriction increases morbidity and mortality among premature neonates. *Am J Obstet Gynecol*. 2004;191(2):481-487.

4. Cetin I, Corbetta C, Sereni LP, et al. Umbilical amino acid concentrations in normal and growth-retarded fetuses sampled in utero by cordocentesis. *Am J Obstet Gynecol*. 1990;162(1):253-261.

5. Zhang J, Mikolajczyk R, Grewal J, Neta G, Klebanoff M. Prenatal application of the individualized fetal growth reference. *Am J Epidemiol*. 2011;173(5):539-543.

6. Manning FA, Harman CR, Morrison I, Menticoglou SM, Lange IR, Johnson JM. Fetal assessment based on fetal biophysical profile scoring. IV. an analysis of perinatal morbidity and mortality. *Am J Obstet Gynecol*. 1990;162(3):703-709.

7. Divon MY, Guidetti DA, Braverman JJ, Oberlander E, Langer O, Merkatz IR. Intrauterine growth retardation--a prospective study of the diagnostic value of real-time sonography combined with umbilical artery flow velocimetry. *Obstet Gynecol*. 1988;72(4):611-614.

8. Chamberlain PF, Manning FA, Morrison I, Harman CR, Lange IR. Ultrasound evaluation of amniotic fluid volume. I. the relationship of marginal and decreased

amniotic fluid volumes to perinatal outcome. *Am J Obstet Gynecol*. 1984;150(3):245-249.

9. Barkehall-Thomas A, Wilson C, Baker L, ni Bhuinneain M, Wallace EM. Uterine artery doppler velocimetry for the detection of adverse obstetric outcomes in patients with elevated mid-trimester beta-human chorionic gonadotrophin. *Acta Obstet Gynecol Scand*. 2005;84(8):743-747.

10. Pardi G, Marconi AM, Cetin I. Placental-fetal interrelationship in IUGR fetuses--a review. *Placenta*. 2002;23 Suppl A:S136-41.

11. Manning FA, Hill LM, Platt LD. Qualitative amniotic fluid volume determination by ultrasound: Antepartum detection of intrauterine growth retardation. *Am J Obstet Gynecol*. 1981;139(3):254-258.

12. Harding JE, Bauer MK, Kimble RM. Antenatal therapy for intrauterine growth retardation. *Acta Paediatr Suppl*. 1997;423:196-200; discussion 201.

13. Diaz P, Powell TL, Jansson T. The role of placental nutrient sensing in maternal-fetal resource allocation. *Biol Reprod*. 2014;91(4):82.

14. Gluckman PD, Hanson MA, Mitchell MD. Developmental origins of health and disease: Reducing the burden of chronic disease in the next generation. *Genome Med*. 2010;2(2):14.

15. Cosmi E, Fanelli T, Visentin S, Trevisanuto D, Zanardo V. Consequences in infants that were intrauterine growth restricted. *J Pregnancy*. 2011;2011:364381.

16. Gluckman PD, Harding JE. Fetal growth retardation: Underlying endocrine mechanisms and postnatal consequences. *Acta Paediatr Suppl*. 1997;422:69-72.

17. Gluckman PD, Harding JE. Fetal growth retardation: Underlying endocrine mechanisms and postnatal consequences. *Acta Paediatr Suppl*. 1997;422:69-72.

18. Eriksson JG, Forsen T, Tuomilehto J, Winter PD, Osmond C, Barker DJ. Catch-up growth in childhood and death from coronary heart disease: Longitudinal study. *BMJ*. 1999;318(7181):427-431.

19. Resnik R. Intrauterine growth restriction. *Obstet Gynecol*. 2002;99(3):490-496.

20. 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*. 2005;20(3):301-322.

21. Hendrix N, Berghella V. Non-placental causes of intrauterine growth restriction. *Semin Perinatol*. 2008;32(3):161-165.

22. Jansson T, Scholtbach V, Powell TL. Placental transport of leucine and lysine is reduced in intrauterine growth restriction. *Pediatr Res*. 1998;44(4):532-537.

23. Economides DL, Proudler A, Nicolaides KH. Plasma insulin in appropriate- and small-for-gestational-age fetuses. *Am J Obstet Gynecol*. 1989;160(5 Pt 1):1091-1094.

24. Heinonen S, Taipale P, Saarikoski S. Weights of placentae from small-for-gestational age infants revisited. *Placenta*. 2001;22(5):399-404.

25. Barut F, Barut A, Gun BD, et al. Intrauterine growth restriction and placental angiogenesis. *Diagn Pathol*. 2010;5:24-1596-5-24.

26. Pijnenborg R, Bland JM, Robertson WB, Brosens I. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta*. 1983;4(4):397-413.

27. Sibley CP, Brownbill P, Dilworth M, Glazier JD. Review: Adaptation in placental nutrient supply to meet fetal growth demand: Implications for programming. *Placenta*. 2010;31 Suppl:S70-4.

28. Horgan RP, Broadhurst DI, Dunn WB, et al. Changes in the metabolic footprint of placental explant-conditioned medium cultured in different oxygen tensions from placentas of small for gestational age and normal pregnancies. *Placenta*. 2010;31(10):893-901.

29. Belkacemi L, Nelson DM, Desai M, Ross MG. Maternal undernutrition influences placental-fetal development. *Biol Reprod*. 2010;83(3):325-331.

30. Jansson N, Pettersson J, Haafiz A, et al. Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet. *J Physiol*. 2006;576(Pt 3):935-946.

31. Malandro MS, Beveridge MJ, Kilberg MS, Novak DA. Effect of low-protein diet-induced intrauterine growth retardation on rat placental amino acid transport. *Am J Physiol*. 1996;271(1 Pt 1):C295-303.

32. Rosario FJ, Jansson N, Kanai Y, Prasad PD, Powell TL, Jansson T. Maternal protein restriction in the rat inhibits placental insulin, mTOR, and STAT3 signaling and down-regulates placental amino acid transporters. *Endocrinology*. 2011;152(3):1119-1129.

33. Zhu MJ, Du M, Hess BW, Nathanielsz PW, Ford SP. Periconceptional nutrient restriction in the ewe alters MAPK/ERK1/2 and PI3K/Akt growth signaling pathways and vascularity in the placentome. *Placenta*. 2007;28(11-12):1192-1199.

34. Zhu MJ, Ma Y, Long NM, Du M, Ford SP. Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood triglycerides at midgestation in the ewe. *Am J Physiol Regul Integr Comp Physiol*. 2010;299(5):R1224-31.

35. Angiolini E, Coan PM, Sandovici I, et al. Developmental adaptations to increased fetal nutrient demand in mouse genetic models of Igf2-mediated overgrowth. *FASEB J*. 2011;25(5):1737-1745.

36. Constancia M, Angiolini E, Sandovici I, et al. Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. *Proc Natl Acad Sci U S A*. 2005;102(52):19219-19224.

37. Constancia M, Hemberger M, Hughes J, et al. Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature*. 2002;417(6892):945-948.

38. Sferruzzi-Perri AN, Vaughan OR, Haro M, et al. An obesogenic diet during mouse pregnancy modifies maternal nutrient partitioning and the fetal growth trajectory. *FASEB J*. 2013;27(10):3928-3937.

39. Jansson T, Powell TL. Role of placental nutrient sensing in developmental programming. *Clin Obstet Gynecol*. 2013;56(3):591-601.

40. Miller J, Turan S, Baschat AA. Fetal growth restriction. *Semin Perinatol*. 2008;32(4):274-280. doi: 10.1053/j.semperi.2008.04.010; 10.1053/j.semperi.2008.04.010.

41. Maulik D. Fetal growth restriction: The etiology. *Clin Obstet Gynecol*. 2006;49(2):228-235.

42. Cetin I, Ronzoni S, Marconi AM, et al. Maternal concentrations and fetal-maternal concentration differences of plasma amino acids in normal and intrauterine growth-restricted pregnancies. *Am J Obstet Gynecol*. 1996;174(5):1575-1583.

43. Bloomfield FH, van Zijl PL, Bauer MK, Harding JE. Effects of intrauterine growth restriction and intraamniotic insulin-like growth factor-I treatment on blood and amniotic fluid concentrations and on fetal gut uptake of amino acids in late-gestation ovine fetuses. *J Pediatr Gastroenterol Nutr*. 2002;35(3):287-297.

44. Di Giulio AM, Carelli S, Castoldi RE, Gorio A, Taricco E, Cetin I. Plasma amino acid concentrations throughout normal pregnancy and early stages of intrauterine growth restricted pregnancy. *J Matern Fetal Neonatal Med*. 2004;15(6):356-362.

45. Teodoro GF, Vianna D, Torres-Leal FL, et al. Leucine is essential for attenuating fetal growth restriction caused by a protein-restricted diet in rats. *J Nutr*. 2012;142(5):924-930.

46. Marconi AM, Paolini CL, Stramare L, et al. Steady state maternal-fetal leucine enrichments in normal and intrauterine growth-restricted pregnancies. *Pediatr Res*. 1999;46(1):114-119.

47. Paolini CL, Marconi AM, Ronzoni S, et al. Placental transport of leucine, phenylalanine, glycine, and proline in intrauterine growth-restricted pregnancies. *J Clin Endocrinol Metab*. 2001;86(11):5427-5432.

48. Cetin I, Marconi AM, Baggiani AM, et al. In vivo placental transport of glycine and leucine in human pregnancies. *Pediatr Res*. 1995;37(5):571-575.

49. Dicke JM, Henderson GI. Placental amino acid uptake in normal and complicated pregnancies. *Am J Med Sci*. 1988;295(3):223-227.

50. Glazier JD, Cetin I, Perugino G, et al. Association between the activity of the system A amino acid transporter in the microvillous plasma membrane of the human placenta and severity of fetal compromise in intrauterine growth restriction. *Pediatr Res*. 1997;42(4):514-519.

51. Mahendran D, Donnai P, Glazier JD, D'Souza SW, Boyd RD, Sibley CP. Amino acid (system A) transporter activity in microvillous membrane vesicles from the placentas of appropriate and small for gestational age babies. *Pediatr Res*. 1993;34(5):661-665.

52. Norberg S, Powell TL, Jansson T. Intrauterine growth restriction is associated with a reduced activity of placental taurine transporters. *Pediatr Res*. 1998;44(2):233-238.

53. Jang DG, Jo YS, Lee SJ, Kim N, Lee GS. Perinatal outcomes and maternal clinical characteristics in IUGR with absent or reversed end-diastolic flow velocity in the umbilical artery. *Arch Gynecol Obstet*. 2011;284(1):73-78.

54. Sifakis S, Akolekar R, Kappou D, Mantas N, Nicolaides KH. Maternal serum insulin-like growth factor (IGF-I) and binding proteins IGFBP-1 and IGFBP-3 at 11-13 weeks' gestation in pregnancies delivering small for gestational age neonates. *Eur J Obstet Gynecol Reprod Biol*. 2012;161(1):30-33.

55. Regnault TR, de Vrijer B, Galan HL, Wilkening RB, Battaglia FC, Meschia G. Umbilical uptakes and transplacental concentration ratios of amino acids in severe fetal growth restriction. *Pediatr Res*. 2013;73(5):602-611. doi: 10.1038/pr.2013.30; 10.1038/pr.2013.30.

56. Bajoria R, Sooranna SR, Ward S, Hancock M. Placenta as a link between amino acids, insulin-IGF axis, and low birth weight: Evidence from twin studies. *J Clin Endocrinol Metab*. 2002;87(1):308-315.

57. Girard JR, Ferre P, Gilbert M, Kervran A, Assan R, Marliss EB. Fetal metabolic response to maternal fasting in the rat. *Am J Physiol*. 1977;232(5):E456-63.

58. Morris NH, Burston D, Ramsay B, Sooranna SR. Free amino acid concentrations in normal and abnormal third trimester placental villi. *Eur J Clin Invest*. 1995;25(10):796-798.

59. Favretto D, Cosmi E, Ragazzi E, et al. Cord blood metabolomic profiling in intrauterine growth restriction. *Anal Bioanal Chem*. 2012;402(3):1109-1121.

60. Bhasin KK, van Nas A, Martin LJ, Davis RC, Devaskar SU, Lusis AJ. Maternal low-protein diet or hypercholesterolemia reduces circulating essential amino acids and leads to intrauterine growth restriction. *Diabetes*. 2009;58(3):559-566.

61. Uerpairojkit B, Chan L, Reece AE, Martinez E, Mari G. Cerebellar doppler velocimetry in the appropriate- and small-for-gestational-age fetus. *Obstet Gynecol*. 1996;87(6):989-993.

62. Bahado-Singh RO, Kovanci E, Jeffres A, et al. The doppler cerebroplacental ratio and perinatal outcome in intrauterine growth restriction. *Am J Obstet Gynecol*. 1999;180(3 Pt 1):750-756.

63. Jensen EC, van Zijl P, Evans PC, Harding JE. Effect of IGF-I on serine metabolism in fetal sheep. *J Endocrinol*. 2000;165(2):261-269.

64. Rudolph AM. The fetal circulation and its response to stress. *J Dev Physiol*. 1984;6(1):11-19.

65. Williams CE, Mallard C, Tan W, Gluckman PD. Pathophysiology of perinatal asphyxia. *Clin Perinatol*. 1993;20(2):305-325.

66. Rabin O, Lefauconnier JM, Chanez C, Bernard G, Bourre JM. Developmental effects of intrauterine growth retardation on cerebral amino acid transport. *Pediatr Res*. 1994;35(6):640-648.

67. Freedman LS, Samuels S, Fish I, et al. Sparing of the brain in neonatal undernutrition: Amino acid transport and incorporation into brain and muscle. *Science*. 1980;207(4433):902-904.

68. Gluckman PD, Pinal CS. Regulation of fetal growth by the somatotrophic axis. *J Nutr*. 2003;133(5 Suppl 2):1741S-1746S.

69. Han VK, Matsell DG, Delhanty PJ, Hill DJ, Shimasaki S, Nygard K. IGF-binding protein mRNAs in the human fetus: Tissue and cellular distribution of developmental expression. *Horm Res*. 1996;45(3-5):160-166.

70. Schoen TJ, Beebe DC, Clemmons DR, Chader GJ, Waldbillig RJ. Local synthesis and developmental regulation of avian vitreal insulin-like growth factor-binding proteins: A model for independent regulation in extravascular and vascular compartments. *Endocrinology*. 1992;131(6):2846-2854.

71. Matsell DG, Delhanty PJ, Stepaniuk O, Goodyear C, Han VK. Expression of insulin-like growth factor and binding protein genes during nephrogenesis. *Kidney Int*. 1994;46(4):1031-1042.

72. Skjaerbaek C, Frystyk J, Orskov H, Flyvbjerg A. Free IGF-I, IGFBP-1, and the binary complex of IGFBP-1 and IGF-I are increased during human pregnancy. *Horm Res*. 2004;62(5):215-220.

73. Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: Effects on affinity for IGF-I. *Proc Natl Acad Sci U S A*. 1991;88(17):7481-7485.

74. Tapanainen PJ, Bang P, Wilson K, Unterman TG, Vreman HJ, Rosenfeld RG. Maternal hypoxia as a model for intrauterine growth retardation: Effects on insulin-like growth factors and their binding proteins. *Pediatr Res*. 1994;36(2):152-158.

75. Han VK, Bassett N, Walton J, Challis JR. The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: Evidence for IGF-IGFBP interactions at the feto-maternal interface. *J Clin Endocrinol Metab*. 1996;81(7):2680-2693.

76. Chard T. Insulin-like growth factors and their binding proteins in normal and abnormal human fetal growth. *Growth Regul*. 1994;4(3):91-100.

77. Rappolee DA, Sturm KS, Behrendtsen O, Schultz GA, Pedersen RA, Werb Z. Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. *Genes Dev*. 1992;6(6):939-952.

78. Lacey H, Haigh T, Westwood M, Aplin JD. Mesenchymally-derived insulin-like growth factor 1 provides a paracrine stimulus for trophoblast migration. *BMC Dev Biol*. 2002;2:5.

79. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*. 1991;64(4):849-859.

80. D'Ercole AJ, Applewhite GT, Underwood LE. Evidence that somatomedin is synthesized by multiple tissues in the fetus. *Dev Biol*. 1980;75(2):315-328.

81. Fowden AL. The insulin-like growth factors and feto-placental growth. *Placenta*. 2003;24(8-9):803-812.

82. Bauer MK, Harding JE, Bassett NS, et al. Fetal growth and placental function. *Mol Cell Endocrinol*. 1998;140(1-2):115-120.

83. Kaaks R. Nutrition, insulin, IGF-1 metabolism and cancer risk: A summary of epidemiological evidence. *Novartis Found Symp*. 2004;262:247-60; discussion 260-68.

84. D'Ercole AJ, Hill DJ, Strain AJ, Underwood LE. Tissue and plasma somatomedin-C/insulin-like growth factor I concentrations in the human fetus during the first half of gestation. *Pediatr Res*. 1986;20(3):253-255.

85. Langford K, Nicolaides K, Miell JP. Maternal and fetal insulin-like growth factors and their binding proteins in the second and third trimesters of human pregnancy. *Hum Reprod*. 1998;13(5):1389-1393.

86. Carr JM, Owens JA, Grant PA, Walton PE, Owens PC, Wallace JC. Circulating insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs) and tissue mRNA levels of IGFBP-2 and IGFBP-4 in the ovine fetus. *J Endocrinol*. 1995;145(3):545-557.

87. Gluckman PD, Butler AA. Endocrine/paracrine interactions following administration of insulin-like growth factor I. *Acta Paediatr Suppl*. 1994;399:180-182.

88. Leger J, Oury JF, Noel M, et al. Growth factors and intrauterine growth retardation. I. serum growth hormone, insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein 3 levels in normally grown and growth-retarded human fetuses during the second half of gestation. *Pediatr Res*. 1996;40(1):94-100. doi: 10.1203/00006450-199607000-00017.

89. Parks JS. The ontogeny of growth hormone sensitivity. *Horm Res*. 2001;55 Suppl 2:27-31.

90. Funakoshi T, Ueda Y, Kobayashi A, Morikawa H, Mochizuki M. Studies on insulin-like growth factors (IGF-I, -II) and there binding proteins in normal human pregnancy. *Nihon Naibunpi Gakkai Zasshi*. 1990;66(7):688-699.

91. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (igf-1) and type 1 IGF receptor (Igf1r). *Cell*. 1993;75(1):59-72.

92. D'Ercole AJ, Calikoglu AS. Editorial review: The case of local versus endocrine IGF-I actions: The jury is still out. *Growth Horm IGF Res*. 2001;11(5):261-265.

93. Spencer JA, Chang TC, Jones J, Robson SC, Preece MA. Third trimester fetal growth and umbilical venous blood concentrations of IGF-1, IGFBP-1, and growth hormone at term. *Arch Dis Child Fetal Neonatal Ed*. 1995;73(2):F87-90.

94. Lok F, Owens JA, Mundy L, Robinson JS, Owens PC. Insulin-like growth factor I promotes growth selectively in fetal sheep in late gestation. *Am J Physiol*. 1996;270(5 Pt 2):R1148-55.

95. Wang HS, Lim J, English J, Irvine L, Chard T. The concentration of insulin-like growth factor-I and insulin-like growth factor-binding protein-1 in human umbilical cord serum at delivery: Relation to fetal weight. *J Endocrinol*. 1991;129(3):459-464.

96. Davenport ML, Clemmons DR, Miles MV, Camacho-Hubner C, D'Ercole AJ, Underwood LE. Regulation of serum insulin-like growth factor-I (IGF-I) and IGF binding proteins during rat pregnancy. *Endocrinology*. 1990;127(3):1278-1286.

97. Bassett NS, Breier BH, Hodgkinson SC, Davis SR, Henderson HV, Gluckman PD. Plasma clearance of radiolabelled IGF-1 in the late gestation ovine fetus. *J Dev Physiol*. 1990;14(2):73-79.

98. Harding JE, Liu L, Evans PC, Gluckman PD. Insulin-like growth factor 1 alters feto-placental protein and carbohydrate metabolism in fetal sheep. *Endocrinology*. 1994;134(3):1509-1514.

99. Liu L, Harding JE, Evans PC, Gluckman PD. Maternal insulin-like growth factor-I infusion alters feto-placental carbohydrate and protein metabolism in pregnant sheep. *Endocrinology*. 1994;135(3):895-900.

100. Bennett A, Wilson DM, Liu F, Nagashima R, Rosenfeld RG, Hintz RL. Levels of insulin-like growth factors I and II in human cord blood. *J Clin Endocrinol Metab*. 1983;57(3):609-612.

101. Lassarre C, Hardouin S, Daffos F, Forestier F, Frankenne F, Binoux M. Serum insulin-like growth factors and insulin-like growth factor binding proteins in the human fetus. Relationships with growth in normal subjects and in subjects with intrauterine growth retardation. *Pediatr Res*. 1991;29(3):219-225.

102. Cianfarani S, Germani D, Rossi P, et al. Intrauterine growth retardation: Evidence for the activation of the insulin-like growth factor (IGF)-related growth-promoting machinery and the presence of a cation-independent IGF binding protein-3 proteolytic activity by two months of life. *Pediatr Res*. 1998;44(3):374-380.

103. Giudice LC, de Zegher F, Gargosky SE, et al. Insulin-like growth factors and their binding proteins in the term and preterm human fetus and neonate with normal and extremes of intrauterine growth. *J Clin Endocrinol Metab*. 1995;80(5):1548-1555.

104. Moerth C, Schneider MR, Renner-Mueller I, et al. Postnatally elevated levels of insulin-like growth factor (IGF)-II fail to rescue the dwarfism of IGF-I-deficient mice except kidney weight. *Endocrinology*. 2007;148(1):441-451.

105. Baker J, Liu JP, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*. 1993;75(1):73-82.

106. Frampton RJ, Jonas HA, MacMahon RA, Larkins RG. Failure of IGF-1 to affect protein turnover in muscle from growth-retarded neonatal rats. *J Dev Physiol*. 1990;13(3):125-133.

107. Simmons RA, Flozak AS, Ogata ES. The effect of insulin and insulin-like growth factor-I on glucose transport in normal and small for gestational age fetal rats. *Endocrinology*. 1993;133(3):1361-1368.

108. Habli M, Jones H, Aronow B, Omar K, Crombleholme TM. Recapitulation of characteristics of human placental vascular insufficiency in a novel mouse model. *Placenta*. 2013;34(12):1150-1158.

109. Mirlesse V, Frankenne F, Alsat E, Poncelet M, Hennen G, Evain-Brion D. Placental growth hormone levels in normal pregnancy and in pregnancies with intrauterine growth retardation. *Pediatr Res*. 1993;34(4):439-442.

110. Verhaeghe J, Van Bree R, Van Herck E, Laureys J, Bouillon R, Van Assche FA. C-peptide, insulin-like growth factors I and II, and insulin-like growth factor binding protein-1 in umbilical cord serum: Correlations with birth weight. *Am J Obstet Gynecol*. 1993;169(1):89-97.

111. Schiessl B, Strasburger CJ, Bidlingmaier M, et al. Role of placental growth hormone in the alteration of maternal arterial resistance in pregnancy. *J Reprod Med*. 2007;52(4):313-316.

112. Holmes R, Montemagno R, Jones J, Preece M, Rodeck C, Soothill P. Fetal and maternal plasma insulin-like growth factors and binding proteins in pregnancies with appropriate or retarded fetal growth. *Early Hum Dev*. 1997;49(1):7-17.

113. Holmes RP, Holly JM, Soothill PW. Maternal insulin-like growth factor binding protein-1, body mass index, and fetal growth. *Arch Dis Child Fetal Neonatal Ed*. 2000;82(2):F113-7.

114. Mahajan SD, Singh S, Shah P, Gupta N, Kochupillai N. Effect of maternal malnutrition and anemia on the endocrine regulation of fetal growth. *Endocr Res*. 2004;30(2):189-203.

115. Woods KA, Camacho-Hubner C, Barter D, Clark AJ, Savage MO. Insulin-like growth factor I gene deletion causing intrauterine growth retardation and severe short stature. *Acta Paediatr Suppl*. 1997;423:39-45.

116. Underwood LE, Clemmons DR, Maes M, D'Ercole AJ, Ketelslegers JM. Regulation of somatomedin-C/insulin-like growth factor I by nutrients. *Horm Res*. 1986;24(2-3):166-176.

117. Gallaher BW, Oliver MH, Eichhorn K, et al. Circulating insulin-like growth factor II/mannose-6-phosphate receptor and insulin-like growth factor binding proteins in fetal sheep plasma are regulated by glucose and insulin. *Eur J Endocrinol*. 1994;131(4):398-404.

118. Gluckman PD. Editorial: Nutrition, glucocorticoids, birth size, and adult disease. *Endocrinology*. 2001;142(5):1689-1691.

119. Gluckman PD, Brinsmead MW. Somatomedin in cord blood: Relationship to gestational age and birth size. *J Clin Endocrinol Metab*. 1976;43(6):1378-1381.

120. Humbel RE. Insulin-like growth factors I and II. *Eur J Biochem*. 1990;190(3):445-462.

121. Herington AC. Insulin-like growth factors: Biochemistry and physiology. *Baillieres Clin Endocrinol Metab*. 1991;5(4):531-551.

122. Fant ME, Weisoly D. Insulin and insulin-like growth factors in human development: Implications for the perinatal period. *Semin Perinatol*. 2001;25(6):426-435.

123. Blundell TL, Bedarkar S, Rinderknecht E, Humbel RE. Insulin-like growth factor: A model for tertiary structure accounting for immunoreactivity and receptor binding. *Proc Natl Acad Sci U S A*. 1978;75(1):180-184.

124. Rinderknecht E, Humbel RE. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem*. 1978;253(8):2769-2776.

125. Alarcon C, Morales AV, Pimentel B, Serna J, de Pablo F. (Pro)insulin and insulin-like growth factor I complementary expression and roles in early development. *Comp Biochem Physiol B Biochem Mol Biol*. 1998;121(1):13-17.

126. Werner H, Weinstein D, Bentov I. Similarities and differences between insulin and IGF-I: Structures, receptors, and signalling pathways. *Arch Physiol Biochem*. 2008;114(1):17-22.

127. Murphy VE, Smith R, Giles WB, Clifton VL. Endocrine regulation of human fetal growth: The role of the mother, placenta, and fetus. *Endocr Rev*. 2006;27(2):141-169.

128. Siddle K. Signalling by insulin and IGF receptors: Supporting acts and new players. *J Mol Endocrinol*. 2011;47(1):R1-10.

129. Siddle K. Molecular basis of signaling specificity of insulin and IGF receptors: Neglected corners and recent advances. *Front Endocrinol (Lausanne)*. 2012;3:34.

130. Rechler MM, Nissley SP. Insulin-like growth factor (IGF)/somatomedin receptor subtypes: Structure, function, and relationships to insulin receptors and IGF carrier proteins. *Horm Res*. 1986;24(2-3):152-159.

131. Kavran JM, McCabe JM, Byrne PO, et al. How IGF-1 activates its receptor. *Elife*. 2014;3:10.7554/eLife.03772.

132. Baxter RC. Insulin-like growth factor (IGF)-binding proteins: Interactions with IGFs and intrinsic bioactivities. *Am J Physiol Endocrinol Metab*. 2000;278(6):E967-76.

133. Sun XJ, Rothenberg P, Kahn CR, et al. Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature*. 1991;352(6330):73-77.

134. Ozes ON, Akca H, Mayo LD, et al. A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proc Natl Acad Sci U S A*. 2001;98(8):4640-4645.

135. Bard-Chapeau EA, Hevener AL, Long S, Zhang EE, Olefsky JM, Feng GS. Deletion of Gab1 in the liver leads to enhanced glucose tolerance and improved hepatic insulin action. *Nat Med*. 2005;11(5):567-571.

136. Epaud R, Aubey F, Xu J, et al. Knockout of insulin-like growth factor-1 receptor impairs distal lung morphogenesis. *PLoS One*. 2012;7(11):e48071.

137. Kent LN, Ohboshi S, Soares MJ. Akt1 and insulin-like growth factor 2 (Igf2) regulate placentation and fetal/postnatal development. *Int J Dev Biol*. 2012;56(4):255-261.

138. Back K, Brannmark C, Stralfors P, Arnqvist HJ. Differential effects of IGF-I, IGF-II and insulin in human preadipocytes and adipocytes--role of insulin and IGF-I receptors. *Mol Cell Endocrinol*. 2011;339(1-2):130-135.

139. Clemmons DR, Busby WH, Arai T, et al. Role of insulin-like growth factor binding proteins in the control of IGF actions. *Prog Growth Factor Res*. 1995;6(2-4):357-366.

140. Coverley JA, Baxter RC. Phosphorylation of insulin-like growth factor binding proteins. *Mol Cell Endocrinol*. 1997;128(1-2):1-5.

141. Jones JI, Doerr ME, Clemmons DR. Cell migration: Interactions among integrins, IGFs and IGFBPs. *Prog Growth Factor Res*. 1995;6(2-4):319-327.

142. Collett-Solberg PF, Cohen P. Genetics, chemistry, and function of the IGF/IGFBP system. *Endocrine*. 2000;12(2):121-136.

143. Hasegawa T, Cohen P, Hasegawa Y, Fielder PJ, Rosenfeld RG. Characterization of the insulin-like growth factors (IGF) axis in a cultured mouse leydig cell line (TM-3). *Growth Regul*. 1995;5(3):151-159.

144. Lewitt MS, Saunders H, Baxter RC. Bioavailability of insulin-like growth factors (IGFs) in rats determined by the molecular distribution of human IGF-binding protein-3. *Endocrinology*. 1993;133(4):1797-1802.

145. Baxter RC. Circulating binding proteins for the insulinlike growth factors. *Trends Endocrinol Metab*. 1993;4(3):91-96.

146. Fowler DJ, Nicolaides KH, Miell JP. Insulin-like growth factor binding protein-1 (IGFBP-1): A multifunctional role in the human female reproductive tract. *Hum Reprod Update*. 2000;6(5):495-504.

147. Burch WM, Correa J, Shively JE, Powell DR. The 25-kilodalton insulin-like growth factor (IGF)-binding protein inhibits both basal and IGF-I-mediated growth of chick embryo pelvic cartilage in vitro. *J Clin Endocrinol Metab*. 1990;70(1):173-180.

148. Cox GN, McDermott MJ, Merkel E, et al. Recombinant human insulin-like growth factor (IGF)-binding protein-1 inhibits somatic growth stimulated by IGF-I and growth hormone in hypophysectomized rats. *Endocrinology*. 1994;135(5):1913-1920.

149. Rutanen EM. Insulin-like growth factors and insulin-like growth factor binding proteins in the endometrium. Effect of intrauterine levonorgestrel delivery. *Hum Reprod*. 2000;15 Suppl 3:173-181.

150. Rutanen EM, Pekonen F, Nyman T, Wahlstrom T. Insulin-like growth factors and their binding proteins in benign and malignant uterine diseases. *Growth Regul*. 1993;3(1):74-77.

151. Westwood M, Gibson JM, White A. Purification and characterization of the insulin-like growth factor-binding protein-1 phosphoform found in normal plasma. *Endocrinology*. 1997;138(3):1130-1136.

152. Hill DJ. Peptide growth factor interactions in embryonic and fetal growth. *Horm Res*. 1992;38(5-6):197-202.

153. Westwood M, Gibson JM, Davies AJ, Young RJ, White A. The phosphorylation pattern of insulin-like growth factor-binding protein-1 in normal plasma is different from that in amniotic fluid and changes during pregnancy. *J Clin Endocrinol Metab*. 1994;79(6):1735-1741.

154. Lee PD, Giudice LC, Conover CA, Powell DR. Insulin-like growth factor binding protein-1: Recent findings and new directions. *Proc Soc Exp Biol Med*. 1997;216(3):319-357.

155. Yu J, Iwashita M, Kudo Y, Takeda Y. Phosphorylated insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) inhibits while non-phosphorylated IGFBP-1 stimulates IGF-I-induced amino acid uptake by cultured trophoblast cells. *Growth Horm IGF Res*. 1998;8(1):65-70.

156. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev*. 2002;23(6):824-854.

157. Koistinen R, Itkonen O, Selenius P, Seppala M. Insulin-like growth factor-binding protein-1 inhibits binding of IGF-I on fetal skin fibroblasts but stimulates their DNA synthesis. *Biochem Biophys Res Commun*. 1990;173(1):408-415.

158. Dolcini L, Sala A, Campagnoli M, et al. Identification of the amniotic fluid insulin-like growth factor binding protein-1 phosphorylation sites and propensity to proteolysis of the isoforms. *FEBS J*. 2009;276(20):6033-6046.

159. Gibson JM, Westwood M, Lauszus FF, Klebe JG, Flyvbjerg A, White A. Phosphorylated insulin-like growth factor binding protein 1 is increased in pregnant diabetic subjects. *Diabetes*. 1999;48(2):321-326.

160. Hills FA, English J, Chard T. Circulating levels of IGF-I and IGF-binding protein-1 throughout pregnancy: Relation to birthweight and maternal weight. *J Endocrinol*. 1996;148(2):303-309.

161. Wathen NC, Egembah S, Campbell DJ, Farkas A, Chard T. Levels of insulin-like growth factor-binding protein-1 increase rapidly in amniotic fluid from 11 to 16 weeks of pregnancy. *J Endocrinol*. 1993;137(2):R1-4.

162. Martina NA, Kim E, Chitkara U, Wathen NC, Chard T, Giudice LC. Gestational age-dependent expression of insulin-like growth factor-binding protein-1 (IGFBP-1) phosphoisoforms in human extraembryonic cavities, maternal serum, and decidua suggests decidua as the primary source of IGFBP-1 in these fluids during early pregnancy. *J Clin Endocrinol Metab*. 1997;82(6):1894-1898.

163. Fang Q, Wang YX, Zhou Y. Insulin-like growth factor binding protein 1 and human embryonic development during 6 - 10 gestational weeks. *Chin Med J (Engl)*. 2004;117(4):488-491.

164. Pekonen F, Nyman T, Lahteenmaki P, Haukkamaa M, Rutanen EM. Intrauterine progestin induces continuous insulin-like growth factor-binding protein-1 production in the human endometrium. *J Clin Endocrinol Metab*. 1992;75(2):660-664.

165. Iwashita M, Sakai K, Kudo Y, Takeda Y. Phosphoisoforms of insulin-like growth factor binding protein-1 in appropriate-for-gestational-age and small-for-gestational-age fetuses. *Growth Horm IGF Res*. 1998;8(6):487-493.

166. Westwood M. Role of insulin-like growth factor binding protein 1 in human pregnancy. *Rev Reprod*. 1999;4(3):160-167.

167. Gibson JM, Aplin JD, White A, Westwood M. Regulation of IGF bioavailability in pregnancy. *Mol Hum Reprod*. 2001;7(1):79-87.

168. Koistinen R, Angervo M, Leinonen P, Hakala T, Seppala M. Phosphorylation of insulin-like growth factor-binding protein-1 increases in human amniotic fluid and decidua from early to late pregnancy. *Clin Chim Acta*. 1993;215(2):189-199.

169. Fowler D, Albaiges G, Lees C, Jones J, Nicolaides K, Miell J. The role of insulin-like growth factor binding protein-1 phosphoisoforms in pregnancies with impaired placental function identified by doppler ultrasound. *Hum Reprod*. 1999;14(11):2881-2885.

170. Abu Shehab M, Inoue S, Han VK, Gupta MB. Site specific phosphorylation of insulin-like growth factor binding protein-1 (IGFBP-1) for evaluating clinical relevancy in fetal growth restriction. *J Proteome Res*. 2009;8(11):5325-5335.

171. Abu Shehab M, Khosravi J, Han VK, Shilton BH, Gupta MB. Site-specific IGFBP-1 hyper-phosphorylation in fetal growth restriction: Clinical and functional relevance. *J Proteome Res*. 2010;9(4):1873-1881.

172. Abu Shehab M, Iosef C, Wildgruber R, Sardana G, Gupta MB. Phosphorylation of IGFBP-1 at discrete sites elicits variable effects on IGF-I receptor autophosphorylation. *Endocrinology*. 2013;154(3):1130-1143. doi: 10.1210/en.2012-1962; 10.1210/en.2012-1962.

173. Abu Shehab M, Damerill I, Shen T, et al. Liver mTOR controls IGF-I bioavailability by regulation of protein kinase CK2 and IGFBP-1 phosphorylation in fetal growth restriction. *Endocrinology*. 2014;155(4):1327-1339. doi: 10.1210/en.2013-1759; 10.1210/en.2013-1759.

174. Bhatia S, Faessen GH, Carland G, et al. A longitudinal analysis of maternal serum insulin-like growth factor I (IGF-I) and total and nonphosphorylated IGF-binding protein-1 in human pregnancies complicated by intrauterine growth restriction. *J Clin Endocrinol Metab*. 2002;87(4):1864-1870.

175. Watson CS, Bialek P, Anzo M, Khosravi J, Yee SP, Han VK. Elevated circulating insulin-like growth factor binding protein-1 is sufficient to cause fetal growth restriction. *Endocrinology*. 2006;147(3):1175-1186. doi: 10.1210/en.2005-0606.

176. Anim-Nyame N, Hills FA, Sooranna SR, Steer PJ, Johnson MR. A longitudinal study of maternal plasma insulin-like growth factor binding protein-1 concentrations during normal pregnancy and pregnancies complicated by pre-eclampsia. *Hum Reprod*. 2000;15(10):2215-2219.

177. Allander SV, Bajalica S, Larsson C, et al. Structure and chromosomal localization of human insulin-like growth factor-binding protein genes. *Growth Regul*. 1993;3(1):3-5.

178. Sitar T, Popowicz GM, Siwanowicz I, Huber R, Holak TA. Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins. *Proc Natl Acad Sci U S A*. 2006;103(35):13028-13033.

179. Lee PD, Conover CA, Powell DR. Regulation and function of insulin-like growth factor-binding protein-1. *Proc Soc Exp Biol Med*. 1993;204(1):4-29.

180. Chelius D, Baldwin MA, Lu X, Spencer EM. Expression, purification and characterization of the structure and disulfide linkages of insulin-like growth factor binding protein-4. *J Endocrinol*. 2001;168(2):283-296.

181. Xu Q, Yan B, Li S, Duan C. Fibronectin binds insulin-like growth factor-binding protein 5 and abolishes its ligand-dependent action on cell migration. *J Biol Chem*. 2004;279(6):4269-4277.

182. Bunn RC, Fowlkes JL. Insulin-like growth factor binding protein proteolysis. *Trends Endocrinol Metab*. 2003;14(4):176-181.

183. Jones JI, Busby WH,Jr, Wright G, Smith CE, Kimack NM, Clemmons DR. Identification of the sites of phosphorylation in insulin-like growth factor binding protein-1. regulation of its affinity by phosphorylation of serine 101. *J Biol Chem*. 1993;268(2):1125-1131.

184. Graham ME, Kilby DM, Firth SM, Robinson PJ, Baxter RC. The in vivo phosphorylation and glycosylation of human insulin-like growth factor-binding protein-5. *Mol Cell Proteomics*. 2007;6(8):1392-1405.

185. Busby WH,Jr, Klapper DG, Clemmons DR. Purification of a 31,000-dalton insulin-like growth factor binding protein from human amniotic fluid. isolation of two forms with different biologic actions. *J Biol Chem*. 1988;263(28):14203-14210.

186. Ritvos O, Ranta T, Jalkanen J, et al. Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells. *Endocrinology*. 1988;122(5):2150-2157.

187. Elgin RG, Busby WH,Jr, Clemmons DR. An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. *Proc Natl Acad Sci U S A*. 1987;84(10):3254-3258.

188. Frost RA, Tseng L. Insulin-like growth factor-binding protein-1 is phosphorylated by cultured human endometrial stromal cells and multiple protein kinases in vitro. *J Biol Chem*. 1991;266(27):18082-18088.

189. Frost RA, Bereket A, Wilson TA, Wojnar MM, Lang CH, Gelato MC. Phosphorylation of insulin-like growth factor binding protein-1 in patients with insulin-dependent diabetes mellitus and severe trauma. *J Clin Endocrinol Metab*. 1994;78(6):1533-1535.

190. Seferovic MD, Ali R, Kamei H, et al. Hypoxia and leucine deprivation induce human insulin-like growth factor binding protein-1 hyperphosphorylation and increase its biological activity. *Endocrinology*. 2009;150(1):220-231. doi: 10.1210/en.2008-0657; 10.1210/en.2008-0657.

191. Nissum M, Abu Shehab M, Sukop U, et al. Functional and complementary phosphorylation state attributes of human insulin-like growth factor-binding protein-1 (IGFBP-1) isoforms resolved by free flow electrophoresis. *Mol Cell Proteomics*. 2009;8(6):1424-1435.

192. Olausson H, Lof M, Brismar K, Forsum E, Sohlstrom A. Maternal serum concentrations of insulin-like growth factor (IGF)-I and IGF binding protein-1 before and during pregnancy in relation to maternal body weight and composition and infant birth weight. *Br J Nutr*. 2010;104(6):842-848.

193. Ong K, Kratzsch J, Kiess W, Costello M, Scott C, Dunger D. Size at birth and cord blood levels of insulin, insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-1 (IGFBP-1), IGFBP-3, and the soluble IGF-II/mannose-6-phosphate receptor in term human infants. the ALSPAC study team. avon longitudinal study of pregnancy and childhood. *J Clin Endocrinol Metab*. 2000;85(11):4266-4269.

194. Street ME, Seghini P, Fieni S, et al. Changes in interleukin-6 and IGF system and their relationships in placenta and cord blood in newborns with fetal growth restriction compared with controls. *Eur J Endocrinol*. 2006;155(4):567-574.

195. Wuarin L, Guertin DM, Ishii DN. Early reduction in insulin-like growth factor gene expression in diabetic nerve. *Exp Neurol*. 1994;130(1):106-114.

196. Jacob RJ, Sherwin RS, Bowen L, et al. Metabolic effects of IGF-I and insulin in spontaneously diabetic BB/w rats. *Am J Physiol*. 1991;260(2 Pt 1):E262-8.

197. Nonoshita LD, Wathen NC, Dsupin BA, Chard T, Giudice LC. Insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), and proteolyzed IGFBP-3 in embryonic cavities in early human pregnancy: Their potential relevance to maternal-embryonic and fetal interactions. *J Clin Endocrinol Metab*. 1994;79(5):1249-1255.

198. Jousse C, Bruhat A, Ferrara M, Fafournoux P. Physiological concentration of amino acids regulates insulin-like-growth-factor-binding protein 1 expression. *Biochem J*. 1998;334 (Pt 1)(Pt 1):147-153.

199. Bankowski E, Sobolewski K, Palka J, Jaworski S. Decreased expression of the insulin-like growth factor-I-binding protein-1 (IGFBP-1) phosphoisoform in pre-eclamptic wharton's jelly and its role in the regulation of collagen biosynthesis. *Clin Chem Lab Med*. 2004;42(2):175-181.

200. Loukovaara M, Leinonen P, Teramo K, Nurminen E, Andersson S, Rutanen EM. Effect of maternal diabetes on phosphorylation of insulin-like growth factor binding protein-1 in cord serum. *Diabet Med*. 2005;22(4):434-439. doi: 10.1111/j.1464-5491.2005.01430.x.

201. Sikalidis AK. Cellular and animal indispensable amino acid limitation responses and health promotion. can the two be linked? A critical review. *Int J Food Sci Nutr*. 2013;64(3):300-311.

202. Boukhettala N, Claeyssens S, Bensifi M, et al. Effects of essential amino acids or glutamine deprivation on intestinal permeability and protein synthesis in HCT-8 cells: Involvement of GCN2 and mTOR pathways. *Amino Acids*. 2012;42(1):375-383. doi: 10.1007/s00726-010-0814-x; 10.1007/s00726-010-0814-x.

203. Chotechuang N, Azzout-Marniche D, Bos C, et al. mTOR, AMPK, and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to protein intake in the rat. *Am J Physiol Endocrinol Metab*. 2009;297(6):E1313-23. doi: 10.1152/ajpendo.91000.2008; 10.1152/ajpendo.91000.2008.

204. Kelly JH, Darlington GJ. Modulation of the liver specific phenotype in the human hepatoblastoma line hep G2. *In Vitro Cell Dev Biol*. 1989;25(2):217-222.

205. Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab Dispos*. 2003;31(8):1035-1042.

206. Hart SN, Li Y, Nakamoto K, Subileau EA, Steen D, Zhong XB. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metab Dispos*. 2010;38(6):988-994.

207. Pal R, Mamidi MK, Das AK, Gupta PK, Bhonde R. A simple and economical route to generate functional hepatocyte-like cells from hESCs and their application in evaluating alcohol induced liver damage. *J Cell Biochem*. 2012;113(1):19-30.

208. Maruyama M, Matsunaga T, Harada E, Ohmori S. Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biol Pharm Bull*. 2007;30(11):2091-2097.

209. Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. *Science*. 2011;334(6056):678-683.

210. Cuyas E, Corominas-Faja B, Joven J, Menendez JA. Cell cycle regulation by the nutrient-sensing mammalian target of rapamycin (mTOR) pathway. *Methods Mol Biol*. 2014;1170:113-144.

211. Kaizuka T, Hara T, Oshiro N, et al. Tti1 and Tel2 are critical factors in mammalian target of rapamycin complex assembly. *J Biol Chem*. 2010;285(26):20109-20116.

212. Kim SG, Hoffman GR, Poulogiannis G, et al. Metabolic stress controls mTORC1 lysosomal localization and dimerization by regulating the TTT-RUVBL1/2 complex. *Mol Cell*. 2013;49(1):172-185.

213. Peterson TR, Laplante M, Thoreen CC, et al. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell*. 2009;137(5):873-886.

214. Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol*. 2004;14(14):1296-1302.

215. Oshiro N, Yoshino K, Hidayat S, et al. Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. *Genes Cells*. 2004;9(4):359-366.

216. Sancak Y, Peterson TR, Shaul YD, et al. The rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*. 2008;320(5882):1496-1501.

217. Jacinto E, Facchinetti V, Liu D, et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates akt phosphorylation and substrate specificity. *Cell*. 2006;127(1):125-137.

218. Powell JD, Delgoffe GM. The mammalian target of rapamycin: Linking T cell differentiation, function, and metabolism. *Immunity*. 2010;33(3):301-311.

219. Aramburu J, Ortells MC, Tejedor S, Buxade M, Lopez-Rodriguez C. Transcriptional regulation of the stress response by mTOR. *Sci Signal*. 2014;7(332):re2.

220. Hardie DG, Ross FA, Hawley SA. AMPK: A nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol*. 2012;13(4):251-262.

221. Kim DH, Sarbassov DD, Ali SM, et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell*. 2002;110(2):163-175.

222. Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem*. 1998;273(23):14484-14494.

223. Li F, Yin Y, Tan B, Kong X, Wu G. Leucine nutrition in animals and humans: MTOR signaling and beyond. *Amino Acids*. 2011;41(5):1185-1193. doi: 10.1007/s00726-011-0983-2; 10.1007/s00726-011-0983-2.

224. 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*. 2003;303(1):59-64.

225. Matsumura T, Morinaga Y, Fujitani S, Takehana K, Nishitani S, Sonaka I. Oral administration of branched-chain amino acids activates the mTOR signal in cirrhotic rat liver. *Hepatol Res*. 2005;33(1):27-32.

226. Nicklin P, Bergman P, Zhang B, et al. Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell*. 2009;136(3):521-534.

227. Kimball SR, Jefferson LS. Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. *J Nutr*. 2006;136(1 Suppl):227S-31S.

228. Garcia-Martinez JM, Alessi DR. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochem J*. 2008;416(3):375-385.

229. Fox HL, Kimball SR, Jefferson LS, Lynch CJ. Amino acids stimulate phosphorylation of p70S6k and organization of rat adipocytes into multicellular clusters. *Am J Physiol*. 1998;274(1 Pt 1):C206-13.

230. Roos S, Jansson N, Palmberg I, Saljo K, Powell TL, Jansson T. Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. *J Physiol*. 2007;582(Pt 1):449-459. doi: 10.1113/jphysiol.2007.129676.

231. Roos S, Powell TL, Jansson T. Placental mTOR links maternal nutrient availability to fetal growth. *Biochem Soc Trans*. 2009;37(Pt 1):295-298. doi: 10.1042/BST0370295; 10.1042/BST0370295.

232. Jansson T, Aye IL, Goberdhan DC. The emerging role of mTORC1 signaling in placental nutrient-sensing. *Placenta*. 2012;33 Suppl 2:e23-9.

233. Jefferson LS, Kimball SR. Amino acids as regulators of gene expression at the level of mRNA translation. *J Nutr*. 2003;133(6 Suppl 1):2046S-2051S.

234. Kavitha JV, Rosario FJ, Nijland MJ, et al. Down-regulation of placental mTOR, insulin/IGF-I signaling, and nutrient transporters in response to maternal nutrient restriction in the baboon. *FASEB J*. 2014;28(3):1294-1305.

235. Yung HW, Calabrese S, Hynx D, et al. Evidence of placental translation inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction. *Am J Pathol*. 2008;173(2):451-462.

236. Mounier C, Dumas V, Posner BI. Regulation of hepatic insulin-like growth factor-binding protein-1 gene expression by insulin: Central role for mammalian target of rapamycin independent of forkhead box O proteins. *Endocrinology*. 2006;147(5):2383-2391. doi: 10.1210/en.2005-0902.

237. Patel S, Lochhead PA, Rena G, et al. Insulin regulation of insulin-like growth factor-binding protein-1 gene expression is dependent on the mammalian target of rapamycin, but independent of ribosomal S6 kinase activity. *J Biol Chem*. 2002;277(12):9889-9895. doi: 10.1074/jbc.M109870200.

238. Patel S, Lipina C, Sutherland C. Different mechanisms are used by insulin to repress three genes that contain a homologous thymine-rich insulin response element. *FEBS Lett*. 2003;549(1-3):72-76.

239. Jansson, T., Eliasson, L., Rosario, F.J., Powell, T.L., Gupta, M.G. Remote control of fetal metabolism by placental mTOR signaling. *Reprod Sci*. 2012;19:151A [Abstract].

240. Kilberg MS, Balasubramanian M, Fu L, Shan J. The transcription factor network associated with the amino acid response in mammalian cells. *Adv Nutr*. 2012;3(3):295-306.

241. Gallinetti J, Harputlugil E, Mitchell JR. Amino acid sensing in dietary-restriction-mediated longevity: Roles of signal-transducing kinases GCN2 and TOR. *Biochem J*. 2013;449(1):1-10. doi: 10.1042/BJ20121098; 10.1042/BJ20121098.

242. Donnelly N, Gorman AM, Gupta S, Samali A. The eIF2alpha kinases: Their structures and functions. *Cell Mol Life Sci*. 2013;70(19):3493-3511.

243. Wilson WA, Roach PJ. Nutrient-regulated protein kinases in budding yeast. *Cell*. 2002;111(2):155-158.

244. Wek RC, Jiang HY, Anthony TG. Coping with stress: EIF2 kinases and translational control. *Biochem Soc Trans*. 2006;34(Pt 1):7-11.

245. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: How mammalian cells respond to amino acid limitation. *Annu Rev Nutr*. 2005;25:59-85. doi: 10.1146/annurev.nutr.24.012003.132145.

246. Castilho BA, Shanmugam R, Silva RC, Ramesh R, Himme BM, Sattlegger E. Keeping the eIF2 alpha kinase Gcn2 in check. *Biochim Biophys Acta*. 2014;1843(9):1948-1968.

247. Zhang P, McGrath BC, Reinert J, et al. The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Mol Cell Biol*. 2002;22(19):6681-6688.

248. Hinnebusch AG. Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. *J Biol Chem*. 1997;272(35):21661-21664.

249. Dong J, Qiu H, Garcia-Barrio M, Anderson J, Hinnebusch AG. Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. *Mol Cell*. 2000;6(2):269-279.

250. Hanks SK, Hunter T. Protein kinases 6. the eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. *FASEB J*. 1995;9(8):576-596.

251. de Haro C, Mendez R, Santoyo J. The eIF-2alpha kinases and the control of protein synthesis. *FASEB J*. 1996;10(12):1378-1387.

252. Schmedt C, Green SR, Manche L, Taylor DR, Ma Y, Mathews MB. Functional characterization of the RNA-binding domain and motif of the double-stranded RNA-dependent protein kinase DAI (PKR). *J Mol Biol*. 1995;249(1):29-44.

253. Ramirez M, Wek RC, Vazquez de Aldana CR, Jackson BM, Freeman B, Hinnebusch AG. Mutations activating the yeast eIF-2 alpha kinase GCN2: Isolation of alleles altering the domain related to histidyl-tRNA synthetases. *Mol Cell Biol*. 1992;12(12):5801-5815.

254. Narasimhan J, Staschke KA, Wek RC. Dimerization is required for activation of eIF2 kinase Gcn2 in response to diverse environmental stress conditions. *J Biol Chem*. 2004;279(22):22820-22832.

255. Romano PR, Garcia-Barrio MT, Zhang X, et al. Autophosphorylation in the activation loop is required for full kinase activity in vivo of human and yeast eukaryotic initiation factor 2alpha kinases PKR and GCN2. *Mol Cell Biol*. 1998;18(4):2282-2297.

256. Fernandez BO, Lorkovic IM, Ford PC. Mechanisms of ferriheme reduction by nitric oxide: Nitrite and general base catalysis. *Inorg Chem*. 2004;43(17):5393-5402.

257. Hinnebusch AG, Natarajan K. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot Cell*. 2002;1(1):22-32.

258. Berlanga JJ, Ventoso I, Harding HP, et al. Antiviral effect of the mammalian translation initiation factor 2alpha kinase GCN2 against RNA viruses. *EMBO J*. 2006;25(8):1730-1740.

259. Krishnamoorthy J, Mounir Z, Raven JF, Koromilas AE. The eIF2alpha kinases inhibit vesicular stomatitis virus replication independently of eIF2alpha phosphorylation. *Cell Cycle*. 2008;7(15):2346-2351.

260. Guo F, Cavener DR. The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. *Cell Metab*. 2007;5(2):103-114.

261. Hao S, Sharp JW, Ross-Inta CM, et al. Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science*. 2005;307(5716):1776-1778.

262. Maurin AC, Jousse C, Averous J, et al. The GCN2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores. *Cell Metab*. 2005;1(4):273-277.

263. Anthony TG, McDaniel BJ, Byerley RL, 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;279(35):36553-36561. doi: 10.1074/jbc.M404559200.

264. Ye J, Kumanova M, Hart LS, et al. The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. *EMBO J*. 2010;29(12):2082-2096.

265. Yang J, Chi Y, Burkhardt BR, Guan Y, Wolf BA. Leucine metabolism in regulation of insulin secretion from pancreatic beta cells. *Nutr Rev*. 2010;68(5):270-279. doi: 10.1111/j.1753-4887.2010.00282.x; 10.1111/j.1753-4887.2010.00282.x.

266. Thiaville MM, Pan YX, Gjymishka A, Zhong C, Kaufman RJ, Kilberg MS. MEK signaling is required for phosphorylation of eIF2alpha following amino acid limitation of HepG2 human hepatoma cells. *J Biol Chem*. 2008;283(16):10848-10857.

267. Kimball SR. Regulation of translation initiation by amino acids in eukaryotic cells. *Prog Mol Subcell Biol*. 2001;26:155-184.

268. Dar AC, Dever TE, Sicheri F. Higher-order substrate recognition of eIF2alpha by the RNA-dependent protein kinase PKR. *Cell*. 2005;122(6):887-900.

269. Dey M, Velyvis A, Li JJ, et al. Requirement for kinase-induced conformational change in eukaryotic initiation factor 2alpha (eIF2alpha) restricts phosphorylation of Ser51. *Proc Natl Acad Sci U S A*. 2011;108(11):4316-4321.

270. Hinnebusch AG. The eIF-2 alpha kinases: Regulators of protein synthesis in starvation and stress. *Semin Cell Biol*. 1994;5(6):417-426.

271. Dalton LE, Healey E, Irving J, Marciniak SJ. Phosphoproteins in stress-induced disease. *Prog Mol Biol Transl Sci*. 2012;106:189-221.

272. Dang Do AN, Kimball SR, Cavener DR, Jefferson LS. eIF2alpha kinases GCN2 and PERK modulate transcription and translation of distinct sets of mRNAs in mouse liver. *Physiol Genomics*. 2009;38(3):328-341.

273. Harding HP, Zhang Y, Zeng H, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell*. 2003;11(3):619-633.

274. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. *Physiol Rev*. 1999;79(1):143-180.

275. Siow YL, Kalmar GB, Sanghera JS, Tai G, Oh SS, Pelech SL. Identification of two essential phosphorylated threonine residues in the catalytic domain of Mekk1. indirect activation by Pak3 and protein kinase C. *J Biol Chem*. 1997;272(12):7586-7594.

276. Lathi RB, Hess AP, Tulac S, Nayak NR, Conti M, Giudice LC. Dose-dependent insulin regulation of insulin-like growth factor binding protein-1 in human endometrial stromal cells is mediated by distinct signaling pathways. *J Clin Endocrinol Metab*. 2005;90(3):1599-1606. doi: 10.1210/jc.2004-1676.

277. Lee MS, Kim MS, Park SY, Kang CW. Effects of betaine on ethanol-stimulated secretion of IGF-I and IGFBP-1 in rat primary hepatocytes: Involvement of p42/44 MAPK activation. *World J Gastroenterol*. 2006;12(11):1718-1722.

278. Lee SM, Alam R, Ho CJ, et al. Involvement of p42/44 MAPK in the effects of ethanol on secretion of insulin-like growth factor (IGF)-I and insulin-like growth factor binding protein (IGFBP)-1 in primary cultured rat hepatocytes. *Int J Neurosci*. 2007;117(2):187-201.

279. Leu JI, Crissey MA, Craig LE, Taub R. Impaired hepatocyte DNA synthetic response posthepatectomy in insulin-like growth factor binding protein 1-deficient mice with defects in C/EBP beta and mitogen-activated protein kinase/extracellular signal-regulated kinase regulation. *Mol Cell Biol*. 2003;23(4):1251-1259.

280. Band CJ, Posner BI. Phosphatidylinositol 3'-kinase and p70s6k are required for insulin but not bisperoxovanadium 1,10-phenanthroline (bpV(phen)) inhibition of insulin-like growth factor binding protein gene expression. evidence for MEK-independent activation of mitogen-activated protein kinase by bpV(phen). *J Biol Chem*. 1997;272(1):138-145.

281. Gan L, Han Y, Bastianetto S, Dumont Y, Unterman TG, Quirion R. FoxO-dependent and -independent mechanisms mediate SirT1 effects on IGFBP-1 gene expression. *Biochem Biophys Res Commun*. 2005;337(4):1092-1096.

282. Gan L, Pan H, Unterman TG. Insulin response sequence-dependent and -independent mechanisms mediate effects of insulin on glucocorticoid-stimulated insulin-like growth factor binding protein-1 promoter activity. *Endocrinology*. 2005;146(10):4274-4280.

283. Brown LD, Green AS, Limesand SW, Rozance PJ. Maternal amino acid supplementation for intrauterine growth restriction. *Front Biosci (Schol Ed)*. 2011;3:428-444.

284. Ross JC, Fennessey PV, Wilkening RB, Battaglia FC, Meschia G. Placental transport and fetal utilization of leucine in a model of fetal growth retardation. *Am J Physiol*. 1996;270(3 Pt 1):E491-503.

285. Strakovsky RS, Zhou D, Pan YX. A low-protein diet during gestation in rats activates the placental mammalian amino acid response pathway and programs the growth capacity of offspring. *J Nutr*. 2010;140(12):2116-2120. doi: 10.3945/jn.110.127803; 10.3945/jn.110.127803.

286. Marchand A, Tomkiewicz C, Magne L, Barouki R, Garlatti M. Endoplasmic reticulum stress induction of insulin-like growth factor-binding protein-1 involves ATF4. *J Biol Chem*. 2006;281(28):19124-19133.

287. Averous J, Maurin AC, Bruhat A, Jousse C, Arliguie C, Fafournoux P. Induction of IGFBP-1 expression by amino acid deprivation of HepG2 human hepatoma cells involves both a transcriptional activation and an mRNA stabilization due to its 3'UTR. *FEBS Lett*. 2005;579(12):2609-2614.

288. Straus DS, Burke EJ, Marten NW. Induction of insulin-like growth factor binding protein-1 gene expression in liver of protein-restricted rats and in rat hepatoma cells limited for a single amino acid. *Endocrinology*. 1993;132(3):1090-1100.

289. Kanamoto R, Yokota T, Hayashi SI. Expressions of c-myc and insulin-like growth factor-1 mRNA in the liver of growing rats vary reciprocally in response to changes in dietary protein. *J Nutr*. 1994;124(12):2329-2334.

290. Takenaka A, Komori K, Morishita T, Takahashi SI, Hidaka T, Noguchi T. Amino acid regulation of gene transcription of rat insulin-like growth factor-binding protein-1. *J Endocrinol*. 2000;164(3):R11-6.

291. Matsukawa T, Inoue Y, Oishi Y, Kato H, Noguchi T. Up-regulation of upstream stimulatory factors by protein malnutrition and its possible role in regulation of the IGF-binding protein-1 gene. *Endocrinology*. 2001;142(11):4643-4651.

292. Hoeck WG, Mukku VR. Identification of the major sites of phosphorylation in IGF binding protein-3. *J Cell Biochem*. 1994;56(2):262-273.

293. Ankrapp DP, Jones JI, Clemmons DR. Characterization of insulin-like growth factor binding protein-1 kinases from human hepatoma cells. *J Cell Biochem*. 1996;60(3):387-399.

294. Meggio F, Pinna LA. One-thousand-and-one substrates of protein kinase CK2? *FASEB J*. 2003;17(3):349-368.

295. St-Denis NA, Litchfield DW. Protein kinase CK2 in health and disease: From birth to death: The role of protein kinase CK2 in the regulation of cell proliferation and survival. *Cell Mol Life Sci*. 2009;66(11-12):1817-1829.

296. Litchfield DW. Protein kinase CK2: Structure, regulation and role in cellular decisions of life and death. *Biochem J*. 2003;369(Pt 1):1-15.

297. Pinna LA, Meggio F. Protein kinase CK2 ("casein kinase-2") and its implication in cell division and proliferation. *Prog Cell Cycle Res*. 1997;3:77-97.

298. Glover CV,3rd. On the physiological role of casein kinase II in saccharomyces cerevisiae. *Prog Nucleic Acid Res Mol Biol*. 1998;59:95-133.

299. Lozeman FJ, Litchfield DW, Piening C, Takio K, Walsh KA, Krebs EG. Isolation and characterization of human cDNA clones encoding the alpha and the alpha' subunits of casein kinase II. *Biochemistry*. 1990;29(36):8436-8447.

300. Litchfield DW, Lozeman FJ, Piening C, et al. Subunit structure of casein kinase II from bovine testis. demonstration that the alpha and alpha' subunits are distinct polypeptides. *J Biol Chem*. 1990;265(13):7638-7644.

301. Maridor G, Park W, Krek W, Nigg EA. Casein kinase II. cDNA sequences, developmental expression, and tissue distribution of mRNAs for alpha, alpha', and beta subunits of the chicken enzyme. *J Biol Chem*. 1991;266(4):2362-2368.

302. Gietz RD, Graham KC, Litchfield DW. Interactions between the subunits of casein kinase II. *J Biol Chem*. 1995;270(22):13017-13021.

303. Duncan JS, Turowec JP, Vilk G, Li SS, Gloor GB, Litchfield DW. Regulation of cell proliferation and survival: Convergence of protein kinases and caspases. *Biochim Biophys Acta*. 2010;1804(3):505-510.

304. Meek DW, Simon S, Kikkawa U, Eckhart W. The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II. *EMBO J*. 1990;9(10):3253-3260.

305. Meggio F, Marin O, Pinna LA. Substrate specificity of protein kinase CK2. *Cell Mol Biol Res*. 1994;40(5-6):401-409.

306. Litchfield DW, Luscher B. Casein kinase II in signal transduction and cell cycle regulation. *Mol Cell Biochem*. 1993;127-128:187-199.

307. Bodenbach L, Fauss J, Robitzki A, et al. Recombinant human casein kinase II. A study with the complete set of subunits (alpha, alpha' and beta), site-directed autophosphorylation mutants and a bicistronically expressed holoenzyme. *Eur J Biochem*. 1994;220(1):263-273.

308. Heller-Harrison RA, Meisner H, Czech MP. Cloning and characterization of a cDNA encoding the beta subunit of human casein kinase II. *Biochemistry*. 1989;28(23):9053-9058.

309. Boldyreff B, Piontek K, Schmidt-Spaniol I, Issinger OG. The beta subunit of casein kinase II: Cloning of cDNAs from murine and porcine origin and expression of the porcine sequence as a fusion protein. *Biochim Biophys Acta*. 1991;1088(3):439-441.

310. Faust M, Montenarh M. Subcellular localization of protein kinase CK2. A key to its function? *Cell Tissue Res*. 2000;301(3):329-340.

311. Bohana-Kashtan O, Pinna LA, Fishelson Z. Extracellular phosphorylation of C9 by protein kinase CK2 regulates complement-mediated lysis. *Eur J Immunol*. 2005;35(6):1939-1948.

312. Janeczko M, Maslyk M, Szyszka R, Baier A. Interactions between subunits of protein kinase CK2 and their protein substrates influences its sensitivity to specific inhibitors. *Mol Cell Biochem*. 2011;356(1-2):121-126.

313. Olsten ME, Litchfield DW. Order or chaos? an evaluation of the regulation of protein kinase CK2. *Biochem Cell Biol*. 2004;82(6):681-693.

314. Ahmed K, Gerber DA, Cochet C. Joining the cell survival squad: An emerging role for protein kinase CK2. *Trends Cell Biol*. 2002;12(5):226-230.

315. Davis AT, Wang H, Zhang P, Ahmed K. Heat shock mediated modulation of protein kinase CK2 in the nuclear matrix. *J Cell Biochem*. 2002;85(3):583-591.

316. Gerber DA, Souquere-Besse S, Puvion F, Dubois MF, Bensaude O, Cochet C. Heat-induced relocalization of protein kinase CK2. implication of CK2 in the context of cellular stress. *J Biol Chem*. 2000;275(31):23919-23926.

317. Keller DM, Zeng X, Wang Y, et al. A DNA damage-induced p53 serine 392 kinase complex contains CK2, hSpt16, and SSRP1. *Mol Cell*. 2001;7(2):283-292.

318. Sayed M, Kim SO, Salh BS, Issinger OG, Pelech SL. Stress-induced activation of protein kinase CK2 by direct interaction with p38 mitogen-activated protein kinase. *J Biol Chem*. 2000;275(22):16569-16573.

319. Marin O, Meggio F, Draetta G, Pinna LA. The consensus sequences for cdc2 kinase and for casein kinase-2 are mutually incompatible. A study with peptides derived from the beta-subunit of casein kinase-2. *FEBS Lett*. 1992;301(1):111-114.

320. Cohen P. Signal integration at the level of protein kinases, protein phosphatases and their substrates. *Trends Biochem Sci*. 1992;17(10):408-413.

321. Russell ST, Eley H, Tisdale MJ. Mechanism of attenuation of angiotensin-II-induced protein degradation by insulin-like growth factor-I (IGF-I). *Cell Signal*. 2007;19(7):1583-1595.

322. Liu Q, Ning W, Dantzer R, Freund GG, Kelley KW. Activation of protein kinase C-zeta and phosphatidylinositol 3'-kinase and promotion of macrophage differentiation by insulin-like growth factor-I. *J Immunol*. 1998;160(3):1393-1401.

323. Yano K, Bauchat JR, Liimatta MB, Clemmons DR, Duan C. Down-regulation of protein kinase C inhibits insulin-like growth factor I-induced vascular smooth muscle cell proliferation, migration, and gene expression. *Endocrinology*. 1999;140(10):4622-4632.

324. Xi G, Shen X, Maile LA, Wai C, Gollahon K, Clemmons DR. Hyperglycemia enhances IGF-I-stimulated src activation via increasing Nox4-derived reactive oxygen species in a PKCzeta-dependent manner in vascular smooth muscle cells. *Diabetes*. 2012;61(1):104-113.

325. Takahashi T, Uehara H, Ogawa H, Umemoto H, Bando Y, Izumi K. Inhibition of EP2/EP4 signaling abrogates IGF-1R-mediated cancer cell growth: Involvement of protein kinase C-theta activation. *Oncotarget*. 2015;6(7):4829-4844.

326. Ciarmatori S, Kiepe D, Haarmann A, Huegel U, Tonshoff B. Signaling mechanisms leading to regulation of proliferation and differentiation of the mesenchymal chondrogenic cell line RCJ3.1C5.18 in response to IGF-I. *J Mol Endocrinol*. 2007;38(4):493-508.

327. Mendez R, Kollmorgen G, White MF, Rhoads RE. Requirement of protein kinase C zeta for stimulation of protein synthesis by insulin. *Mol Cell Biol*. 1997;17(9):5184-5192.

328. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiol Rev*. 2008;88(4):1341-1378.

329. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*. 1992;258(5082):607-614.

330. Marino M, Distefano E, Caporali S, Ceracchi G, Pallottini V, Trentalance A. Beta-estradiol stimulation of DNA synthesis requires different PKC isoforms in HepG2 and MCF7 cells. *J Cell Physiol*. 2001;188(2):170-177.

331. Xia Q, Li CG, Sun AM, Zhang XL. PKC isoform selectivity and radiation-induced apoptosis of HepG2 cells. *Nan Fang Yi Ke Da Xue Xue Bao*. 2010;30(6):1376-1378.

332. Way KJ, Chou E, King GL. Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci*. 2000;21(5):181-187.

333. Facchinetti V, Ouyang W, Wei H, et al. The mammalian target of rapamycin complex 2 controls folding and stability of akt and protein kinase C. *EMBO J*. 2008;27(14):1932-1943.

334. Lee MY, Jo SD, Lee JH, Han HJ. L-leucine increases [3H]-thymidine incorporation in chicken hepatocytes: Involvement of the PKC, PI3K/Akt, ERK1/2, and mTOR signaling pathways. *J Cell Biochem*. 2008;105(6):1410-1419.

335. Nishitani S, Matsumura T, Fujitani S, Sonaka I, Miura Y, Yagasaki K. Leucine promotes glucose uptake in skeletal muscles of rats. *Biochem Biophys Res Commun*. 2002;299(5):693-696.

336. Vary TC, Goodman S, Kilpatrick LE, Lynch CJ. Nutrient regulation of PKCepsilon is mediated by leucine, not insulin, in skeletal muscle. *Am J Physiol Endocrinol Metab*. 2005;289(4):E684-94.

337. Farese RV, Sajan MP, Standaert ML. Insulin-sensitive protein kinases (atypical protein kinase C and protein kinase B/Akt): Actions and defects in obesity and type II diabetes. *Exp Biol Med (Maywood)*. 2005;230(9):593-605.

338. Ignacio-Souza LM, Reis SR, Arantes VC, et al. Protein restriction in early life is associated with changes in insulin sensitivity and pancreatic beta-cell function during pregnancy. *Br J Nutr*. 2013;109(2):236-247.

339. Ozanne SE, Olsen GS, Hansen LL, et al. Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. *J Endocrinol*. 2003;177(2):235-241.

340. Sugden MC, Langdown ML. Possible involvement of PKC isoforms in signalling placental apoptosis in intrauterine growth retardation. *Mol Cell Endocrinol*. 2001;185(1-2):119-126.

341. Casanello P, Krause B, Torres E, et al. Reduced l-arginine transport and nitric oxide synthesis in human umbilical vein endothelial cells from intrauterine growth restriction pregnancies is not further altered by hypoxia. *Placenta*. 2009;30(7):625-633.

342. Thompson NM, Norman AM, Donkin SS, et al. Prenatal and postnatal pathways to obesity: Different underlying mechanisms, different metabolic outcomes. *Endocrinology*. 2007;148(5):2345-2354.

343. Tang B, Zhang Y, Liang R, et al. Activation of the delta-opioid receptor inhibits serum deprivation-induced apoptosis of human liver cells via the activation of PKC and the mitochondrial pathway. *Int J Mol Med*. 2011;28(6):1077-1085.

344. Eggo MC, Sheppard MC, Evans FJ, Lord JM. Phorbol esters showing selective activation of PKC isozymes in vitro regulate thyroid function and insulin-like growth factor binding protein secretion. *Cell Signal*. 1994;6(4):439-448.

345. Wang JF, Hill DJ, Becks GP. Role of 3', 5' cyclic adenosine monophosphate and protein kinase C in the regulation of insulin-like growth factor-binding protein secretion by thyroid-stimulating hormone in isolated ovine thyroid cells. *J Endocrinol*. 1994;141(2):231-242.

346. Giannini S, Maggi M, Cresci B, et al. Platelet-activating factor enhances production of insulin-like growth factor binding proteins in a human adenocarcinoma cell line (HEC-1A). *Gynecol Oncol*. 1996;61(3):333-340.

347. Oh SH, Whang YM, Min HY, et al. Histone deacetylase inhibitors enhance the apoptotic activity of insulin-like growth factor binding protein-3 by blocking PKC-induced IGFBP-3 degradation. *Int J Cancer*. 2012;131(10):2253-2263.

348. Chamoun D, Choi D, Tavares AB, et al. Regulation of granulosa cell-derived insulin-like growth factor binding proteins (IGFBPs): Role for protein kinase-C in the pre- and posttranslational modulation of IGFBP-4 and IGFBP-5. *Biol Reprod*. 2002;67(3):1003-1012.

349. He H, Herington AC, Roupas P. Effects of phorbol ester and staurosporine on the actions of insulin-like growth factor-I on rat ovarian granulosa cells. *Endocrine*. 1995;3(2):159-167.

350. Erclik MS, Mitchell J. The role of protein kinase C-delta in PTH stimulation of IGF-binding protein-5 mRNA in UMR-106-01 cells. *Am J Physiol Endocrinol Metab*. 2002;282(3):E534-41.

351. Kachra Z, Yang CR, Murphy LJ, Posner BI. The regulation of insulin-like growth factor-binding protein 1 messenger ribonucleic acid in cultured rat hepatocytes: The roles of glucagon and growth hormone. *Endocrinology*. 1994;135(5):1722-1728.

352. Gong Y, Ballejo G, Alkhalaf B, Molnar P, Murphy LC, Murphy LJ. Phorbol esters differentially regulate the expression of insulin-like growth factor-binding proteins in endometrial carcinoma cells. *Endocrinology*. 1992;131(6):2747-2754.

353. Lee PD, Abdel-Maguid LS, Snuggs MB. Role of protein kinase-C in regulation of insulin-like growth factor-binding protein-1 production by HepG2 cells. *J Clin Endocrinol Metab*. 1992;75(2):459-464.

354. Cohen P. The role of protein phosphorylation in the hormonal control of enzyme activity. *Eur J Biochem*. 1985;151(3):439-448.

355. Lum H, Jaffe HA, Schulz IT, Masood A, RayChaudhury A, Green RD. Expression of PKA inhibitor (PKI) gene abolishes cAMP-mediated protection to endothelial barrier dysfunction. *Am J Physiol*. 1999;277(3 Pt 1):C580-8.

356. O'Brien LJ, Levac KD, Nagy LE. Moderate dietary protein and energy restriction modulate cAMP-dependent protein kinase activity in rat liver. *J Nutr*. 1998;128(6):927-933.

357. Stephen LL, Nagy LE. Very low protein diets induce a rapid decrease in hepatic cAMP-dependent protein kinase followed by a lower increase in adenylyl cyclase activity in rats. *J Nutr*. 1996;126(7):1799-1807.

358. Rozwadowski M, Stephen LL, Goss PM, Bray TM, Nagy LE. Activity of cAMP-dependent protein kinase is reduced in protein-energy malnourished rats. *J Nutr*. 1995;125(3):401-409.

359. Goss PM, Bray TM, Nagy LE. Regulation of hepatocyte glutathione by amino acid precursors and cAMP in protein-energy malnourished rats. *J Nutr*. 1994;124(3):323-330.

360. Milanski M, Arantes VC, Ferreira F, et al. Low-protein diets reduce PKAalpha expression in islets from pregnant rats. *J Nutr*. 2005;135(8):1873-1878.

361. Tierney EP, Tulac S, Huang ST, Giudice LC. Activation of the protein kinase A pathway in human endometrial stromal cells reveals sequential categorical gene regulation. *Physiol Genomics*. 2003;16(1):47-66.

362. Tang M, Mazella J, Zhu HH, Tseng L. Ligand activated relaxin receptor increases the transcription of IGFBP-1 and prolactin in human decidual and endometrial stromal cells. *Mol Hum Reprod*. 2005;11(4):237-243.

363. Kusama K, Yoshie M, Tamura K, et al. Regulation of decidualization in human endometrial stromal cells through exchange protein directly activated by cyclic AMP (epac). *Placenta*. 2013;34(3):212-221.

364. Chen H, Li J, Liu J, Liu L, Liu N, Song YZ. Effects of prenatal taurine on mRNA expression of PKA CREB signal pathway and glial cell line derived neurotrophic factor

in fetal rat brains of intrauterine growth restriction. *Zhongguo Dang Dai Er Ke Za Zhi*. 2009;11(11):923-926.

365. Liu J, Liu Y, Wang XF, Chen H, Yang N. Antenatal taurine supplementation improves cerebral neurogenesis in fetal rats with intrauterine growth restriction through the PKA-CREB signal pathway. *Nutr Neurosci*. 2013;16(6):282-287.

366. Li G, Cong L, Gasser J, Zhao J, Chen K, Li F. Mechanisms underlying the anti-proliferative actions of adiponectin in human breast cancer cells, MCF7-dependency on the cAMP/protein kinase-A pathway. *Nutr Cancer*. 2011;63(1):80-88.

367. Dremier S, Coulonval K, Perpete S, et al. The role of cyclic AMP and its effect on protein kinase A in the mitogenic action of thyrotropin on the thyroid cell. *Ann N Y Acad Sci*. 2002;968:106-121.

368. Subramaniam S, Shahani N, Strelau J, et al. Insulin-like growth factor 1 inhibits extracellular signal-regulated kinase to promote neuronal survival via the phosphatidylinositol 3-kinase/protein kinase A/c-raf pathway. *J Neurosci*. 2005;25(11):2838-2852.

369. Kaji H, Sugimoto T, Kanatani M, Nishiyama K, Nasu M, Chihara K. Insulin-like growth factor-I mediates osteoclast-like cell formation stimulated by parathyroid hormone. *J Cell Physiol*. 1997;172(1):55-62.

370. Fernandez S, Genis L, Torres-Aleman I. A phosphatase-independent gain-of-function mutation in PTEN triggers aberrant cell growth in astrocytes through an autocrine IGF-1 loop. *Oncogene*. 2014;33(32):4114-4122.

371. DiBattista JA, Dore S, Morin N, Abribat T. Prostaglandin E2 up-regulates insulin-like growth factor binding protein-3 expression and synthesis in human articular chondrocytes by a c-AMP-independent pathway: Role of calcium and protein kinase A and C. *J Cell Biochem*. 1996;63(3):320-333.

372. Suwanichkul A, DePaolis LA, Lee PD, Powell DR. Identification of a promoter element which participates in cAMP-stimulated expression of human insulin-like growth factor-binding protein-1. *J Biol Chem*. 1993;268(13):9730-9736.

373. Frost RA, Nystrom GJ, Lang CH. Stimulation of insulin-like growth factor binding protein-1 synthesis by interleukin-1beta: Requirement of the mitogen-activated protein kinase pathway. *Endocrinology*. 2000;141(9):3156-3164. doi: 10.1210/endo.141.9.7641.

374. Solomon AL, Siddals KW, Baker PN, Gibson JM, Aplin JD, Westwood M. Placental alkaline phosphatase de-phosphorylates insulin-like growth factor (IGF)-binding protein-1. *Placenta*. 2014;35(7):520-522.

375. Montoya A, Gomez-Lechon MJ, Castell JV. Influence of branched-chain amino acid composition of culture media on the synthesis of plasma proteins by serum-free cultured rat hepatocytes. *In Vitro Cell Dev Biol*. 1989;25(4):358-364.

376. Fulks RM, Li JB, Goldberg AL. Effects of insulin, glucose, and amino acids on protein turnover in rat diaphragm. *J Biol Chem*. 1975;250(1):290-298.

377. Tomiya T, Omata M, Fujiwara K. Significance of branched chain amino acids as possible stimulators of hepatocyte growth factor. *Biochem Biophys Res Commun*. 2004;313(2):411-416.

378. Geddie G, Moores R, Meschia G, Fennessey P, Wilkening R, Battaglia FC. Comparison of leucine, serine and glycine transport across the ovine placenta. *Placenta*. 1996;17(8):619-627.

379. Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC. Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats. *Am J Physiol Endocrinol Metab*. 2002;283(4):E824-35.

380. Lynch CJ, Patson BJ, Anthony J, Vaval A, Jefferson LS, Vary TC. Leucine is a direct-acting nutrient signal that regulates protein synthesis in adipose tissue. *Am J Physiol Endocrinol Metab*. 2002;283(3):E503-13.

381. Reiter AK, Anthony TG, Anthony JC, Jefferson LS, Kimball SR. The mTOR signaling pathway mediates control of ribosomal protein mRNA translation in rat liver. *Int J Biochem Cell Biol*. 2004;36(11):2169-2179.

382. Tyakht AV, Ilina EN, Alexeev DG, et al. RNA-seq gene expression profiling of HepG2 cells: The influence of experimental factors and comparison with liver tissue. *BMC Genomics*. 2014;15:1108-2164-15-1108.

383. Costantini S, Di Bernardo G, Cammarota M, Castello G, Colonna G. Gene expression signature of human HepG2 cell line. *Gene*. 2013;518(2):335-345.

384. Li C, Shu ZJ, Lee S, et al. Effects of maternal nutrient restriction, intrauterine growth restriction, and glucocorticoid exposure on phosphoenolpyruvate carboxykinase-1 expression in fetal baboon hepatocytes in vitro. *J Med Primatol*. 2013;42(4):211-219.

385. Endogenous Hormones and Breast Cancer Collaborative Group, Key TJ, Appleby PN, Reeves GK, Roddam AW. Insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and breast cancer risk: Pooled individual data analysis of 17 prospective studies. *Lancet Oncol*. 2010;11(6):530-542.

386. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer*. 2008;8(12):915-928

387. Barker DJ. In utero programming of chronic disease. *Clin Sci (Lond).* 1998;95(2):115-128.

388. Godfrey KM, Barker DJ. Fetal nutrition and adult disease. *Am J Clin Nutr*. 2000;71(5 Suppl):1344S-52S.

389. Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. *Lancet*. 1993;341(8850):938-941.

Chapter 2

Total and phosphorylated IGFBP-1 in leucine deprivation: roles for the mTOR and AAR signal transduction pathways

A version of this chapter has been accepted for publication and reproduced with permission from Elsevier:

Malkani N, Jansson T, and Gupta MB. IGFBP-1 phosphorylation in response to leucine deprivation is mediated by the AAR pathway. *Mol Cell Endocrinol.* 2015.

2.1 Introduction

Fetal Growth Restriction (FGR) arises from a heterogeneous group of factors and although its etiology is multi-factorial, FGR is primarily a result of placental insufficiency, i.e. the inability of the placenta to effectively deliver nutrients and oxygen to the fetus[1,2]. FGR infants fail to achieve their full growth potential after suffering nutritional deprivation[3] and are at an increased risk for perinatal mortality[4]. FGR affects ~5 to 7% of all pregnancies[5]. FGR babies born under the 10th percentile for estimated birth weight are predisposed to greater risks of childhood and adult metabolic, cardiovascular, and neurological complications[4,6,7]. The mechanisms by which the fetus signals nutrient deficiency to attenuating fetal growth are not well understood.

Insulin-like growth factor I (IGF-I), synthesized mainly by the fetal liver, is a key regulator of fetal growth. Fetal serum IGF-I levels are significantly reduced in the growth-restricted fetuses[8]. IGF binding protein-1 (IGFBP-1) primarily secreted by the fetal liver[9] is the key fetal circulating IGFBP during gestation[10]. Elevated fetal circulating IGFBP-1 and decreased IGF-I levels are strongly correlated with FGR onset[11-13]. IGFBP-1 functions by binding IGF-I and sequestering it from its receptor, IGF-1R, consequently preventing it from transducing mitogenic signals[14-16]. Phosphorylation of IGFBP-1 increases the binding affinity of IGFBP-1 for IGF-I[17] and sequesters IGF-I thereby resulting in its decreased bioavailability[16,18-20].

Human FGR fetuses often have decreased fetal circulating levels of essential amino acids, such as leucine[21-24]. In our laboratory, we have previously demonstrated that leucine deprivation triggered hyperphosphorylation of IGFBP-1 in HepG2 cells at discrete sites, which markedly increased the affinity of IGFBP-1 for IGF-I, and inhibited IGF-I-dependent cell growth[25]. Although modest increases in IGFBP-1 phosphorylation were found in HepG2 cells cultured under lower leucine concentrations (70 and 140 μM leucine), leucine deprivation (0 μM leucine) distinctly increased IGFBP-1 phosphorylation compared to HepG2 cells cultured with leucine (450 μM leucine)[25]. Furthermore, we have recently demonstrated that IGFBP-1 phosphorylation was increased at three different sites (Ser101, Ser119, and Ser169) in human umbilical cord

plasma from FGR pregnancies and in liver from baboon FGR fetuses[26]. These data indicate that increased phosphorylation of IGFBP-1 at specific sites plays an important role in FGR pathogenesis.

FGR is characterized by decreased amino acid availability[2,27], which activates the Amino Acid Response (AAR)[28] and inhibits mechanistic target of rapamycin (mTOR) signaling[29,30]. The AAR signaling pathway is highly responsive to changes in amino acid availability[28]. Amino acids, oxygen and growth factor signaling activate mTOR signaling[30-33]. mTOR integrates nutrient and mitogenic signals to regulate cell growth and cell division[34]. mTOR exists in two complexes, mTOR Complex 1 (mTORC1) and 2, with the protein raptor associated to mTORC1 and rictor associated to mTORC2[35,36]. Activated mTORC1 phosphorylates 4E-BP1 and p70-S6K and promotes protein translation[34,37]. mTORC2 phosphorylates Akt and PKCα and regulates cell metabolism and survival[36,37]. Oxygen, growth factor and amino acids, particularly leucine and arginine, activate mTORC1 signaling[32,38-41]. mTORC1, in particular, is inhibited by the binding of rapamycin although longer treatments and higher doses of rapamycin have also been shown to inhibit mTORC2[26,37].

We have previously shown using HepG2 cells and baboon fetal hepatocytes *in vitro*that inhibition of mTOR signaling resulted in increased IGFBP-1 phosphorylation at the three specific sites[26]. In addition using a baboon model of FGR in the same study we also identified that increased site-specific IGFBP-1 phosphorylation in FGR is linked with an inhibition of the mTOR and stimulation of protein kinase CK2. However, the mechanisms underlying IGFBP-1 hyperphosphorylation specifically in conditions of amino acid deprivation remain to be established.

The AAR pathway is activated under conditions of cellular nutrient stress[28,42-47]. General control non-derepressible 2 (GCN2) is the key sensor of cellular nutrient status, which is activated upon sensing excess uncharged cytoplasmic tRNAs[45,46]. Leucine deprivation activates and phosphorylates GCN2 at pThr898 which subsequently phosphorylates eukaryotic initiation factor 2 (eiF2) at pSer51 of the alpha subunit (eiF2α)[47]. Phosphorylated eiF2α (pSer51), which is increased in abundance in FGR[48], proceeds to

inhibit eIF2B activity and therefore overall global protein synthesis while concurrently promoting the translation of certain stress-responsive mRNAs, including activating transcription factor 4 (ATF4)[45,47]. ATF4 is a critical stress-responsive transcription factor, which, when synthesized, promotes the transcription of several growth-arresting genes[49]. eiF2α (pSer51) phosphorylation and total ATF4 expression levels are therefore functional readouts of AAR activity. The role of the AAR signal transduction pathway in regulating IGFBP-1 phosphorylation has, to our knowledge, not previously been investigated.

In this study, we hypothesized that inhibition of mTOR signaling and AAR activation increase IGFBP-1 secretion and phosphorylation at specific sites in response to amino acid deprivation. We used HepG2 cells as a model for human fetal hepatocytes[50-54] to investigate the mechanisms linking mTOR and AAR signaling with IGFBP-1 phosphorylation under leucine deprivation. We studied the secretion and phosphorylation of IGFBP-1 in HepG2 cells in response to mTOR inhibition (rapamycin) or AAR inhibition (U0126). Alternatively, cells were transfected with siRNA targeting raptor+rictor or DEP domain-containing mTOR-interacting protein (DEPTOR) (to inhibit or activate mTORC1 and C2, respectively)[37], and ERK1/2 and/or GCN2 (to inhibit ERK-mediated AAR)[45,55] in cells cultured with or without leucine. Finally, we verified that changes in IGFBP-1 phosphorylation under leucine deprivation altered IGF-I bioactivity by employing our previously established IGF-1R autophosphorylation assay[26,56] which supported the functional significance of our findings.

2.2 Methods

2.2.1 Cell culture

Human hepatocellular carcinoma cells (HepG2), purchased from ATCC (Mananassas, VA), were cultured in Dulbecco's modified Eagle medium with nutrient mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA) at 37°C in 20% O_2 and 5% CO_2 as we described previously[25,26].

2.2.2 Leucine deprivation

HepG2 cells were treated in specialized DMEM/F12 selectively deprived and restored of specific amino acids and were incubated in the specialized media either deprived of (0 μM) or supplemented with (450 μM) leucine as we described in our previous study[25].

Cells were further incubated in leucine plus or leucine deprived media during rapamycin (100 nM), U0126 (10 μM), or TBB (1 μM) treatments or following transfection with siRNA. Cell media and cell lysate were collected following 24 hour (chemical treatments) or 72 hour (siRNA treatments) exposure to the specialized media.

2.2.3 Inhibitor treatments

HepG2 cells were plated in 12-well culture dishes until cultures reached 75% confluence then starved for 6 hours in 2% FBS (DMEM/F12) prior to treatments with chemical inhibitors. Based on previous dose-dependency data, HepG2 cells were treated post-6 hour starvation for 24 hours using 100 nM rapamycin, 1 μM TBB as we reported previously[26] or treated with 10 μM U0126 after assessment via dose-dependency experiments. Following treatments, cell media and cell lysate were prepared as we described[26] and stored at -80°C.

2.2.4 RNA interference silencing

HepG2 cells were plated at 65% confluence in 12-well culture plates. Silencing using siRNA against raptor+rictor, DEPTOR, GCN2 (Sigma-Aldrich, St Louis, MO, USA) or ERK (Cell Signaling Technologies, Beverly, MA, USA) in HepG2 cells was achieved using transfection with 100 nM siRNA and 5 μL Dharmafect transfection reagent 4 (Thermo Scientific, Rockford, IL, USA) in regular, serum free DMEM/F12. To simultaneously ensure maximal silencing and maximize cell survival, the transfected cell media was replaced after 24 hours with specialized leucine plus or leucine deprived media and studied after 72 hours (96 hours following transfection). Western immunoblot analysis was used to determine the efficiency of target silencing.

2.2.5 Cell viability assay

We tested the effect of leucine deprivation, TBB and U0126 treatments on cell viability using the Trypan Blue exclusion assay to ensure these treatments did not sacrifice cell viability. Following leucine deprivation and U0126 treatments, cells were trypsinized and re-suspended in 10% FBS media. Cell suspensions were diluted 1:1 with 0.4% Trypan blue and counted with the Countess Automated Cell Counter (Life Technologies, Carlsbad, CA). A measure of live/total cells was used as an indicator of cell survival.

2.2.6 SDS-PAGE and Western Blotting

Equal amounts of cell lysate protein (35-50 μg) were separated by SDS-PAGE to determine total expression and phosphorylation of p70-S6K (Thr389), Akt (Ser473), ERK (Thr202/pTyr204), eiF2α (Ser51), GCN2 (Thr898), and IGF-1Rβ (Tyr1135), as well as total expression levels of siRNA target proteins, ATF4 and β-actin (Appendix A; Supplementary Table 1). IGFBP-1 secretion and phosphorylation (Ser101, Ser119 and Ser169) by HepG2 cells were determined using equal volume of cell media (30-40 μL).

Nitrocellulose membranes from immunoblot analyses were shaken with 5% skim milk or 5% BSA diluted in Tris-buffered saline (TBS) plus 0.1 % Tween-20 and blocked for 1 hour. All primary antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA) with the exception of monoclonal antihuman IGFBP-1 (mAb 6303) (Medix Biochemica, Kauniainen, Finland), total and phospho GCN2 (Abcam, Cambridge, MA, USA)pre-validatedcustom phosphosite-specific IGFBP-1 polyclonal antibodies targeting pSer101, pSer119, and pSer169 (generated at YenZyme Antibodies LLC, San Francisco, CA, USA). Primary antibodies were all used at a dilution of 1:1000, and peroxidase-labelled goat-anti mouse or goat-anti rabbit antibodies (1:10000, BioRad Laboratories Inc.) were used as secondary antibodies. Band intensities were determined using densitometry in Image Lab (Beta 3) software (BioRad).

2.2.7 Validation of custom phosphosite-specific IGFBP-1 antibodies

In our previous study[56], four IGFBP-1 mutants with single amino acid substitutions (Ser98Ala, Ser101Ala, Ser119Ala and Ser169Ala) were generated that disrupted IGFBP-1 phosphorylation. We used these well-characterized mutants and wild-type (positive control) IGFBP-1 and performed western blots using our custom phosphosite-specific IGFBP-1 antibodies (pSer101, 119 and pSer169) as primary antibodies in order to validate their specificity. The data shown in the Figure 3[56] using pSer101 and pSer169-specific antibodies clearly validate that the pS101 antibody only recognized IGFBP-1 when Ser101 was phosphorylated and pSer169 antibody when Ser169 was phosphorylated. The validity of the IGFBP-1 antibody specific to pSer119 was similarly reported in a subsequent study in Supplementary Figure 7[26]. The Ser119Ala mutant only reacted with IGFBP-1 on immunoblot when phosphorylated at Ser119, validating the specificity of respective antibody. In addition, we used human amniotic fluid (AF) as an additional positive control. We had previously confirmed for IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169 residues in amniotic fluid[56-58]. To include appropriate negative controls, we utilized alkaline phosphatase (ALP)-treated amniotic fluid, wild-type and mutant IGFBP-1 samples to demonstrate diminished reactivity with IGFBP-1 following ALP treatments[56-59]. These data unequivocally demonstrate that our custom phosphosite-specific IGFBP-1 antibodies are highly specific to only phosphorylated forms of IGFBP-1 at their respective sites.

2.2.8 IGF-I receptor activation assay

P6 cells (a gift from Dr. R. Baserga, Thomas Jefferson University, Philadelphia, PA), are immortalized mouse embryonic fibroblast cells (a BALB/c3T3 cell line) that over-express human IGF-1R but do not express IGF-I[60]. Therefore, they are a widely established model for the assessment of IGF-1R activation under IGF-I addition[61,62]. We have tested the validity of P6 cells under conditions of variable IGFBP-1 phosphorylation previously in our laboratory[26,56]. P6 cells were cultured in DMEM/F12 with sodium pyruvate supplemented with 10% FBS.

The P6 cell bioassay was performed in FBS-free conditions as we described previously[56]. Aliquots of HepG2 cell media from various treatments containing equal concentrations of IGFBP-1 were buffer-exchanged to P6 cell media using Amicon Ultra-0.5 mL Centrifugal Filter Units (Millipore, Darmstadt, Germany) per manufacturer instructions. Samples were subsequently incubated with rhIGF-I (25 ng/mL) for two hours at room temperature. P6 cells were treated for 10 minutes with the P6 media containing IGFBP-1/IGF-I complexes. Cells were subsequently lysed and samples were separated using SDS-PAGE gels and immunoblot analysis was performed to asses IGF-1R autophosphorylation using phosphosite-specific IGF-1Rβ (Tyr1135) primary antibody.

2.2.9 Data presentation and statistics

Data was analyzed using GraphPad Prism 5 (Graph Pad Software Inc., CA). Three replicates were analyzed for each treatment condition, including the control treatment.

For each quantified protein, the mean density of the control sample bands was assigned an arbitrary value of 1, and averaged densitometry values for each treatment were expressed relative to this mean. We employed One-way analysis of variance with Dunnet's Multiple Comparison Post-Test and expressed results as the mean ± Standard Error of Measurement (SEM). Significance was accepted at $*p<0.05$. n=3.

2.3 Results

2.3.1 Rapamycin and/or leucine deprivation inhibit mTOR signaling

First, we assessed changes in mTORC1 and C2 signaling in response to leucine deprivation and rapamycin as a pharmacological mTOR inhibitor. Based on our previous dose dependencydatafor leucine deprivation in HepG2 cells[25], we cultured HepG2 cells in media with leucine (450 μM) or deprived of leucine (0 μM). Cells were cultured with or without rapamycin (100 nM). We assessed changes in mTORC1 and C2 signaling activity in response to leucine deprivation and/or rapamycin by investigating changes in

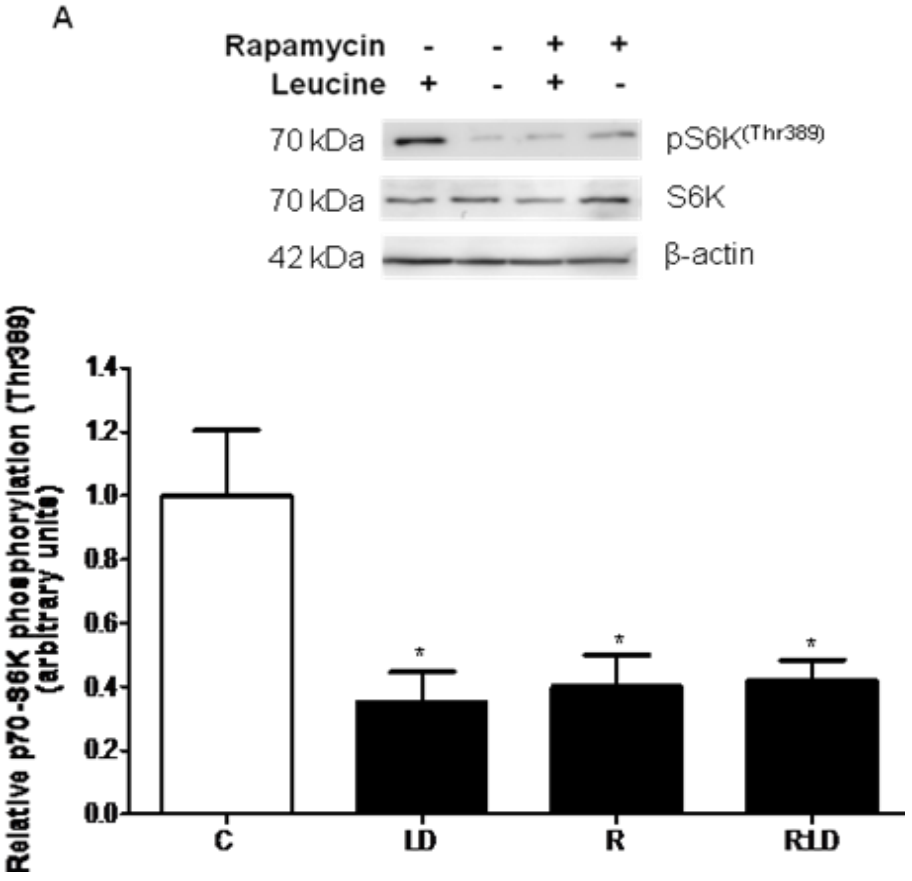
phosphorylation of downstream effectors p70-S6K (Thr389) and Akt (Ser473) as functional readouts of mTORC1 and C2 activity, respectively.

As evidenced in Figure 2.1A-B, we noted a significant decrease in mTORC1 and C2 activity by leucine deprivation, rapamycin treatment, and leucine deprivation and rapamycin combined, indicated by decreased phosphorylation (-60%) of p70-S6K (Thr389) and Akt (Ser473) (-65-70%) under these three treatments. These data demonstrate that both mTORC1 and C2 activity are significantly inhibited by rapamycin. In addition, leucine deprivation inhibited mTORC1 and C2 activity to the same extent as rapamycin with no additive effect when the two treatments were combined (Figure 2.1A-B), supporting that the degrees to which mTORC1 and C2 were inhibited were not significantly different between treatments.

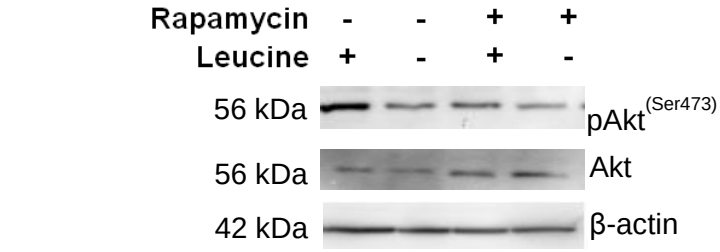
2.3.2 mTOR inhibition increases IGFBP-1 secretion but not IGFBP-1 phosphorylation in leucine deprivation

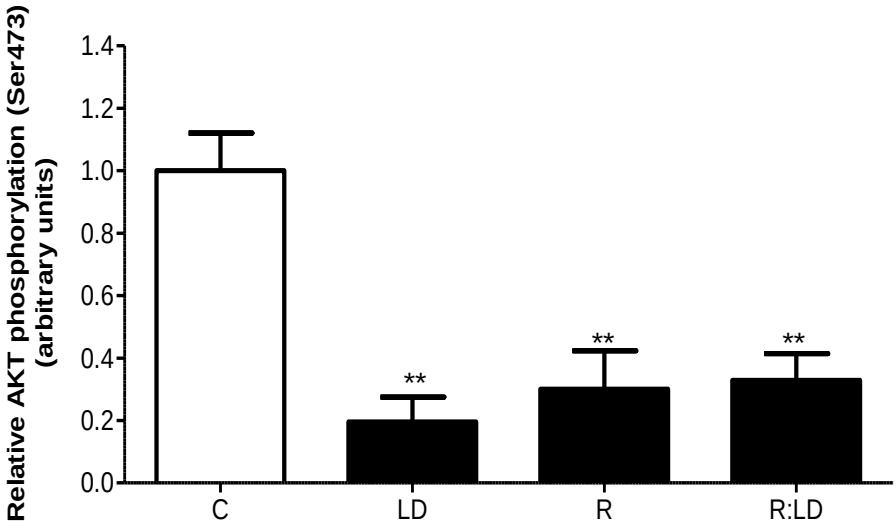
To test our hypothesis that mTOR signaling is responsible for changes in IGFBP-1 secretion and phosphorylation in amino acid limitation, we first investigated the effects of rapamycin and leucine deprivation on IGFBP-1 secretion and phosphorylation in HepG2 cells singly and combined. To examine these changes, we resolved equal volumes of HepG2 cell media from rapamycin-treated cells with leucine (450 μM) or in leucine deprived (0 μM) conditions (Figure 2.1C-F). As demonstrated in Figure 2.1C, IGFBP-1 secretion was increased (+400%) both in leucine deprivation and rapamycin and these effects were not additive when both treatments were combined. To investigate whether this effect was consistent with IGFBP-1 phosphorylation, we used custom phosphosite-specific IGFBP-1 antibodies against pSer101, pSer119 and pSer169 (Figure 2.1D-F). We demonstrated that while rapamycin consistently induced IGFBP-1 phosphorylation (Ser101, +400%, Ser119, +200% and Ser169, +400%), leucine deprivation increased IGFBP-1 phosphorylation beyond that seen by rapamycin treatment alone (Ser101, +1000%, Ser119, +500% and Ser169, +1200%) (Figure 2.1D-F). Further, there was no additive effect on IGFBP-1 phosphorylation at any of the three sites in combined leucine deprivation and rapamycin treatment compared to leucine deprivation alone (Figure 2.1D-F), suggesting that mTOR signaling does not induce IGFBP-1 phosphorylation in a

parallel mechanism, but rather contributes partially to IGFBP-1 phosphorylation induced by leucine deprivation. Together, these findings strongly suggest that while leucine deprivation inhibited mTOR signaling to the same extent as rapamycin, changes in IGFBP-1 phosphorylation under nutrient deprivation are only in part regulated by mTOR signaling. While mTOR inhibition was sufficient to induce total IGFBP-1 secretion to the same extent as leucine deprivation, IGFBP-1 phosphorylation due to leucine deprivation is only partially regulated by mTOR signaling. These data suggest that mTOR signaling and leucine deprivation function in a common mechanism to induce total IGFBP-1 secretion, additional mechanisms are involved in regulation of IGFBP-1 phosphorylation.

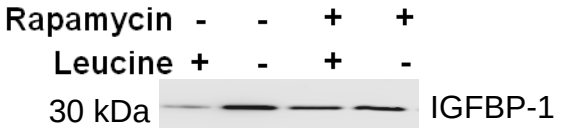


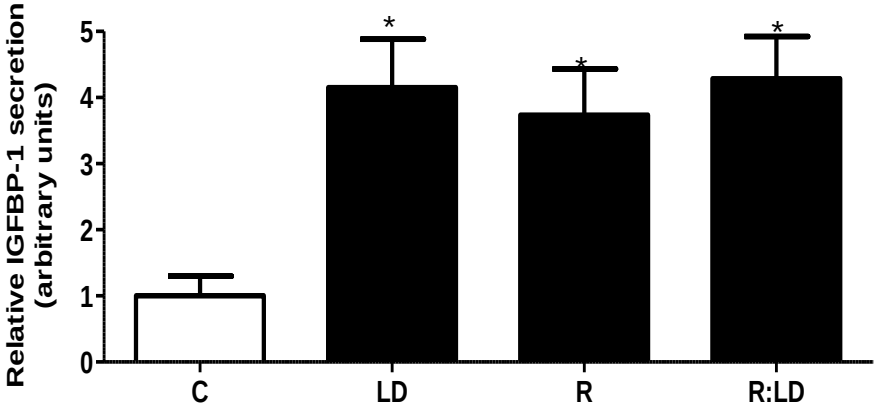
B


Rapamycin - - + +
Leucine + - + -
56 kDa
pAkt(Ser473)
56 kDa
Akt
42 kDa
β-actin

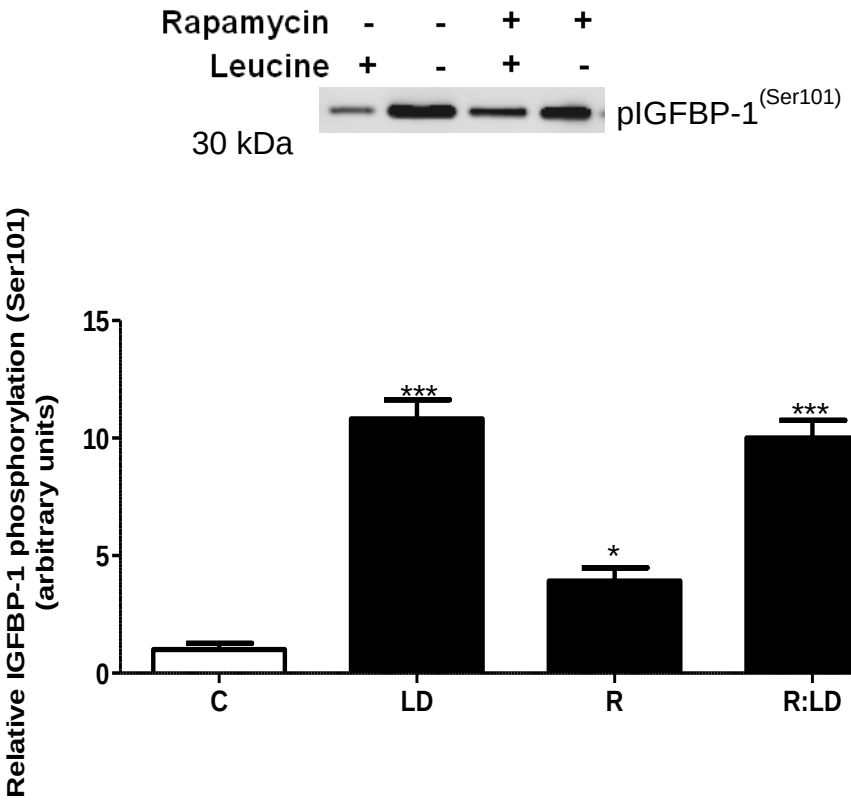

Relative AKT phosphorylation (Ser473) (arbitrary units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
C
LD
R
R:LD

C

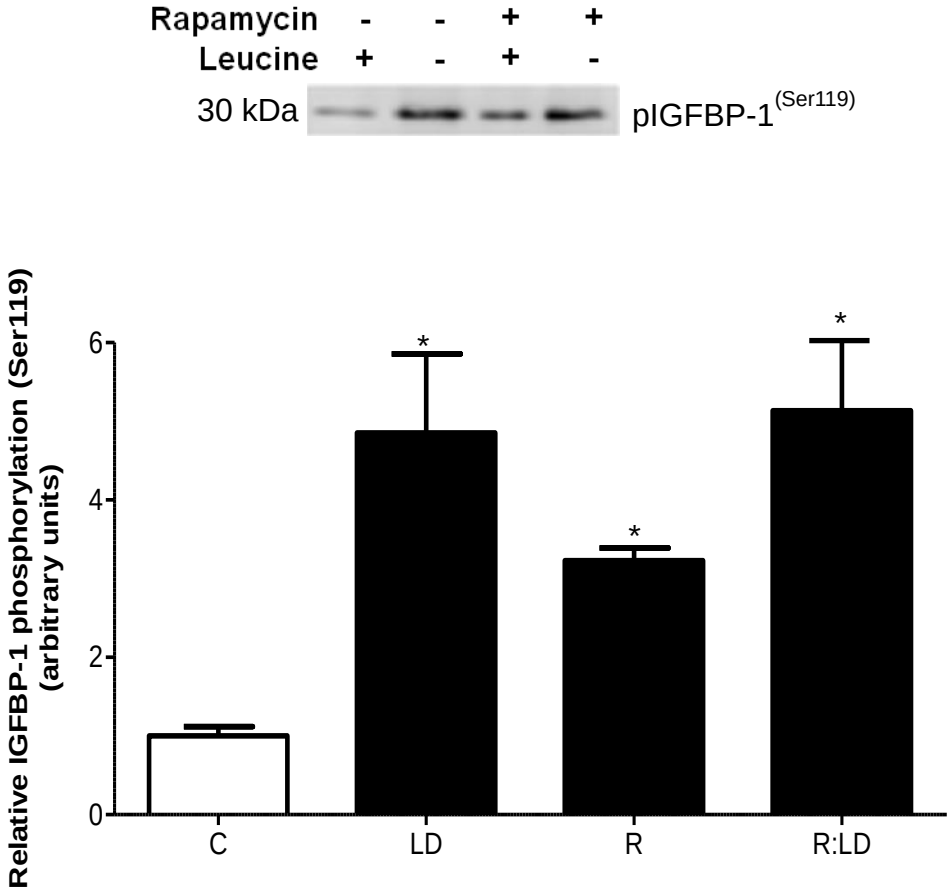

Rapamycin - - + +
Leucine + - + -
30 kDa
IGFBP-1


Relative IGFBP-1 secretion (arbitrary units)
5
4
3
2
1
0
*
*
*
C
LD
R
R:LD

D


Rapamycin - - + +
Leucine + - + -
30 kDa
pIGFBP-1(Ser101)
Relative IGFBP-1 phosphorylation (Ser101) (arbitrary units)
15
10
5
0
***
*
***
C
LD
R
R:LD

E


Rapamycin - - + +
Leucine + - + -
30 kDa
pIGFBP-1(Ser119)
Relative IGFBP-1 phosphorylation (Ser119) (arbitrary units)
6
4
2
0
*
*
*
C
LD
R
R:LD

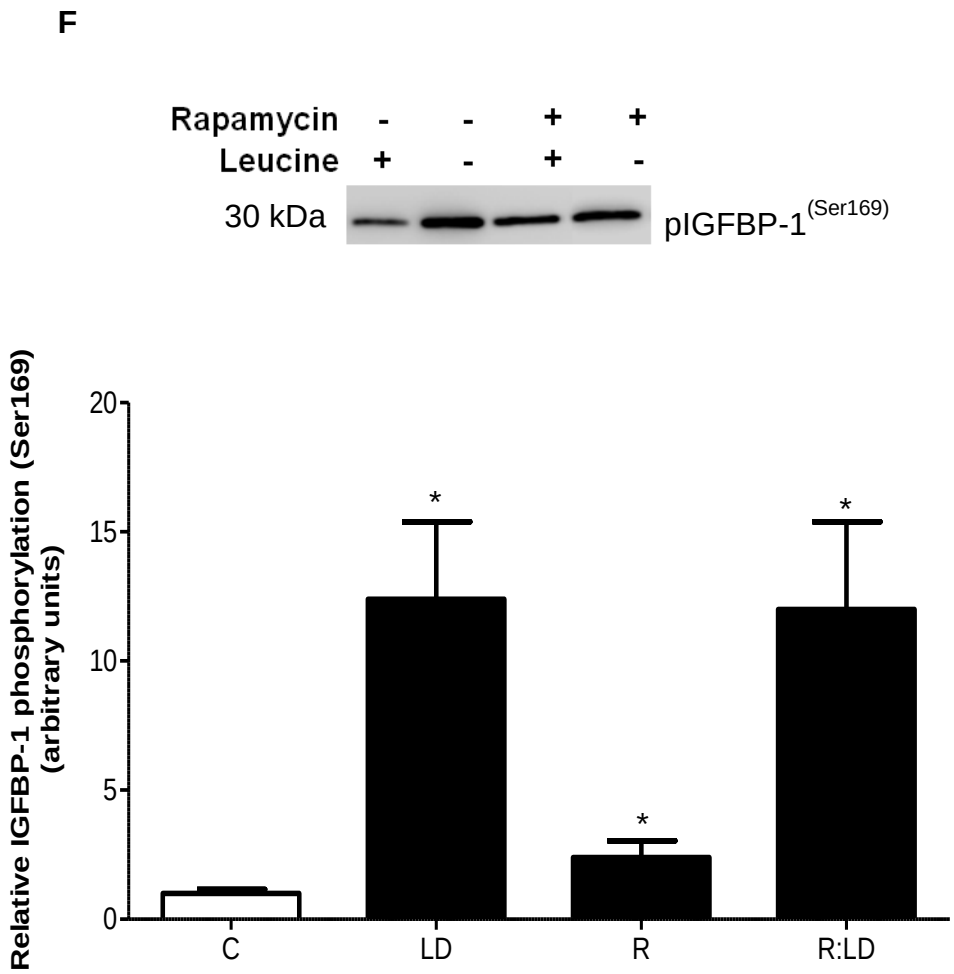


Figure 2.1. The effect of leucine deprivation and rapamycin on mTORC1+C2 activity and IGFBP-1 secretion and phosphorylation.A representative immunoblot of HepG2 cell lysate (35 μg per lane) assayed for **A.** S6K (Thr389) phosphorylation and **B.** Akt (Ser 473) phosphorylation. A representative western immunoblot of HepG2 cell media (40 μL per well) displaying **C.** total IGFBP-1 secretion and **D-F.** IGFBP-1 phosphorylated at Ser101, Ser119, and Ser169 in control, leucine deprivation, rapamycin, and combined leucine deprivation+rapamycin treatments. Values are displayed as mean + SEM. *p<0.05, **p=0.001-0.05, ***p <0.001 versus control; One-way analysis of variance, Dunnet's Post-Test. n=3. C: Control, 450 μM leucine. LD: Leucine deprivation, 0 μM leucine. R: Rapamycin (100 nM), 450 μM leucine. R:LD: Rapamycin (100 nM), 0 μM leucine.

2.3.3 Inhibition of mTOR signaling by raptor and rictor silencing confirms that mTOR induces IGFBP-1 secretion, but not phosphorylation during leucine deprivation

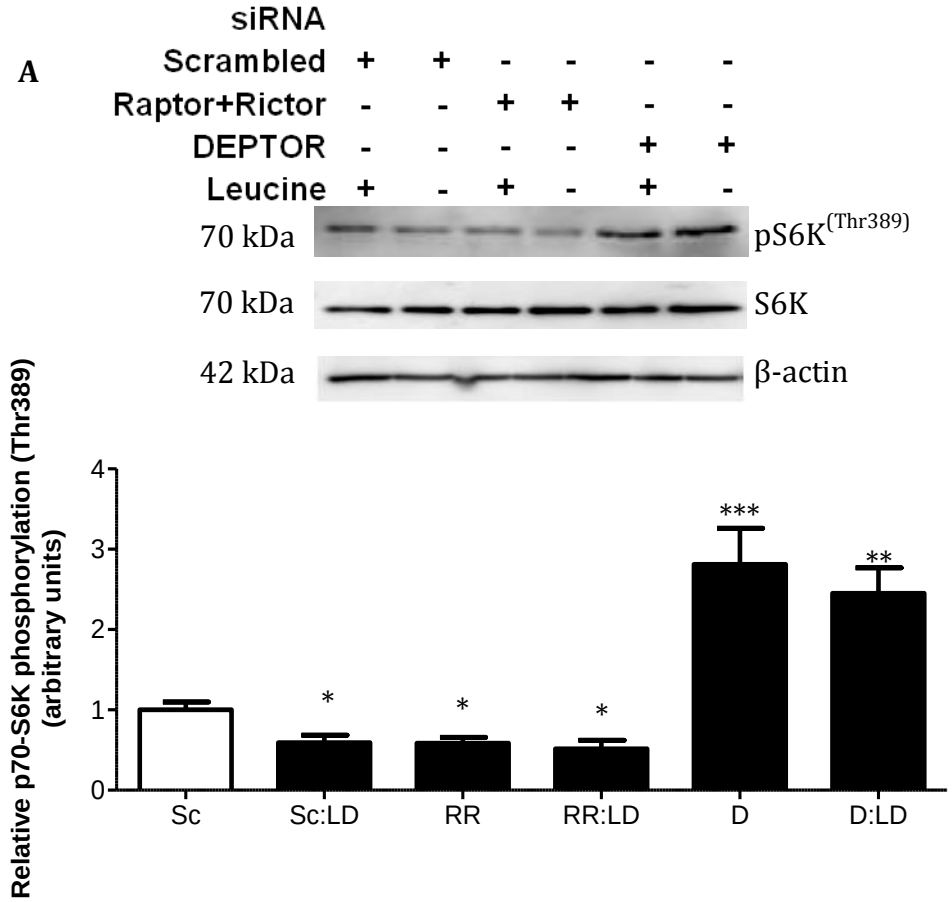
For selective inhibition of mTOR complexes, we employed an RNAi strategy to silence both raptor and rictor in leucine or leucine deprived conditions with HepG2 cells. Primarily, we confirmed efficient silencing of both raptor (-45 to 50%) and rictor (-50%) (Appendix A; Supplementary Figure2.1A-B). Raptor+rictor silencing successfully inhibited mTORC1 activity as seen by reduced (-50%) phosphorylation of p70-S6K (Thr389) to a similar extent as leucine deprivation or combined leucine deprivation and raptor+rictor silencing (Figure 2.2A). Similarly, mTORC2 activity was reduced to a similar extent in leucine deprivation, raptor+rictor silencing and combined leucine deprivation and raptor+rictor silencing as assessed by a reduction (-50%) in phosphorylation of Akt (Ser473) (Figure 2.2B). We therefore confirmed the ability of raptor+rictor silencing to effectively inhibit mTORC1 and C2 signaling.

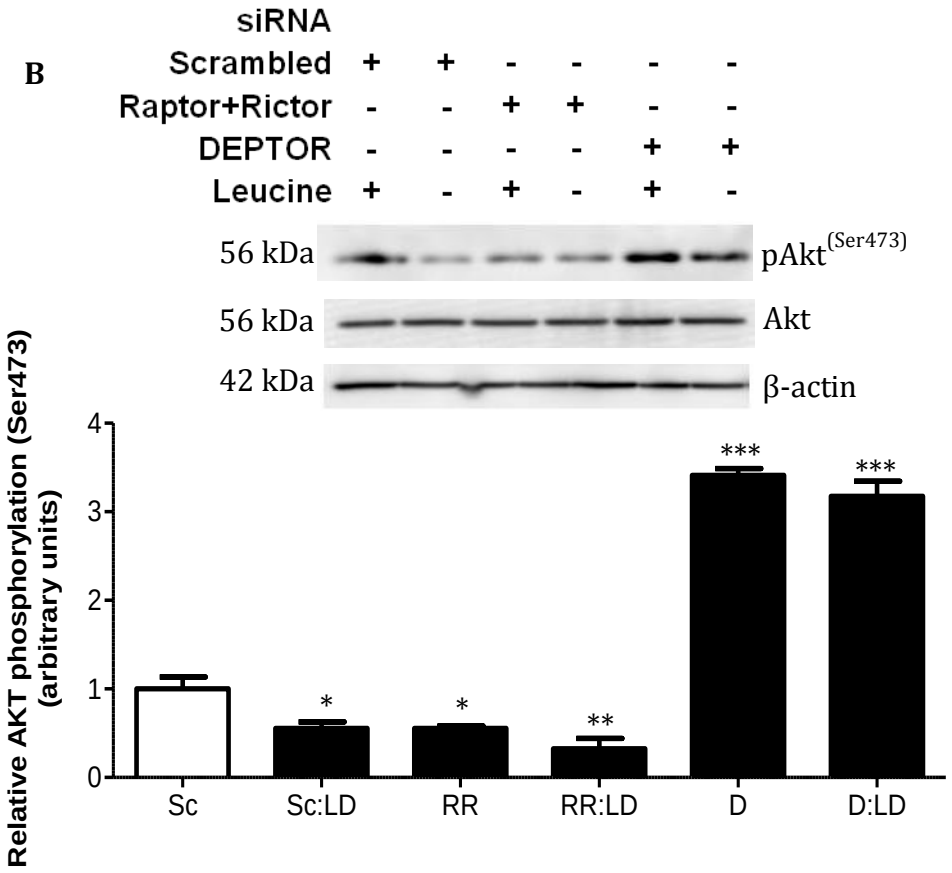
Next, we sought to assess whether raptor+rictor silencing translated to changes in IGFBP-1 secretion and phosphorylation. We demonstrated that raptor+rictor silencing induced total IGFBP-1 secretion (+350%) to levels comparable to leucine deprivation with or without raptor+rictor silencing (+400%), and that combined raptor+rictor silencing and leucine deprivation had no additive effect on IGFBP-1 secretion compared to leucine deprivation alone (Figure 2.2C). As expected, raptor+rictor silencing induced IGFBP-1 phosphorylation at all three sites (Ser101, +400%, Ser119, +200% and Ser169, +400%) but was unable to achieve the phosphorylation induced in the presence of leucine deprivation (Ser101, +2000%, Ser119, +1100% and Ser169, +2300%) (Figure 2.2D-F).

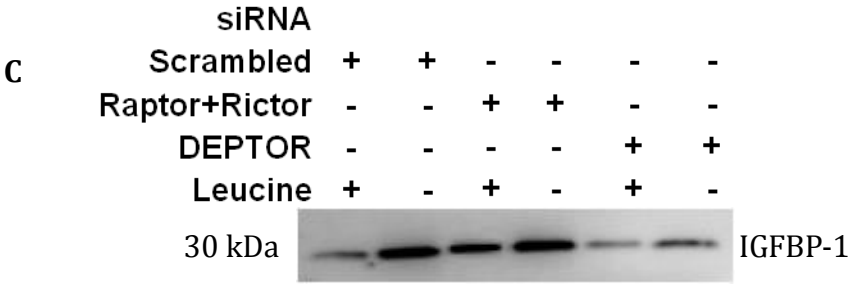
2.3.4 Activation of mTORC1 and C2 signaling by DEPTOR silencing attenuates leucine deprivation-induced IGFBP-1 secretion but not phosphorylation

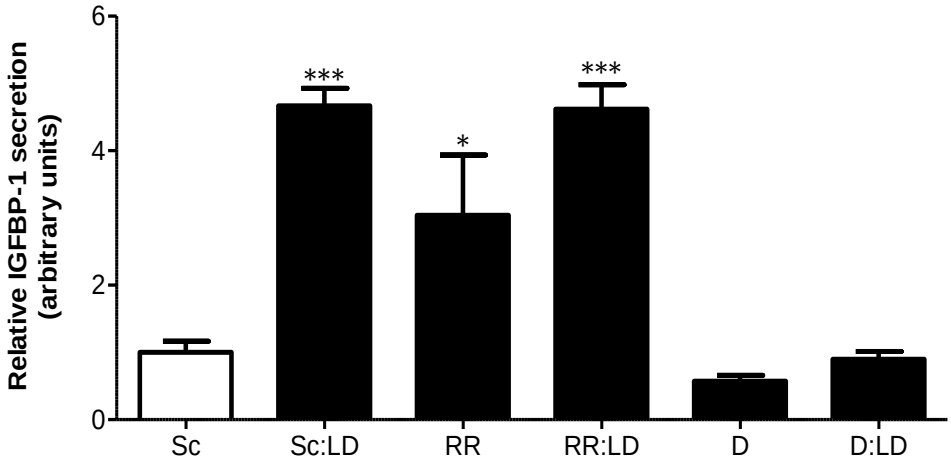
To further study changes in IGFBP-1 secretion and phosphorylation under mTOR signaling, we selectively activated the mTOR pathway using siRNA against DEPTOR, an endogenous inhibitor against mTORC1 and C2 activity. To maximize cell viability, serum free DMEM/F12 media containing transfection reagent was aspirated immediately following the 24 hour transfection period. Cell media were subsequently replaced with DMEM with (450 μM) or without (0 μM) leucine for an additional 72 hours. We first confirmed that our RNAi approach efficiently reduced (-50%) DEPTOR expression (Appendix A; Supplementary Figure 2.1C). Analysis of cell lysates was used to examine changes in phosphorylation of downstream mTOR effectors (Figure 2.2A-B). DEPTOR silencing successfully induced p70-S6K (pThr389) phosphorylation (+250%) (Figure 2.2A) and Akt (pSer473) phosphorylation (+300%) (Figure 2.2B) regardless of leucine status, supporting that constitutive activation of the mTOR pathway induces mTORC1 and C2 signaling downstream of leucine deprivation.

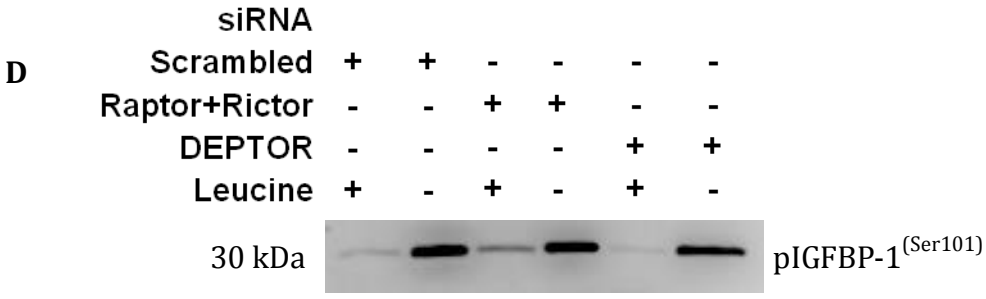
We proceeded to test whether mTOR activation was able to prevent changes in IGFBP-1 secretion and phosphorylation caused by leucine deprivation. Activating mTOR signaling by DEPTOR silencing successfully attenuated leucine deprivation-induced IGFBP-1 secretion (+400%) (Figure 2.2C) but was unable to prevent leucine deprivation-induced phosphorylation of IGFBP-1 at all three sites (Figure 2.2D-F). Since mTORC1 and C2 inhibition induced total IGFBP-1 secretion only to the same extent as leucine deprivation, and since mTORC1 and C2 activation completely prevents this induction, we assert that mTOR signaling is the key mechanism implicated in IGFBP-1 secretion, but not phosphorylation, induced by leucine deprivation.

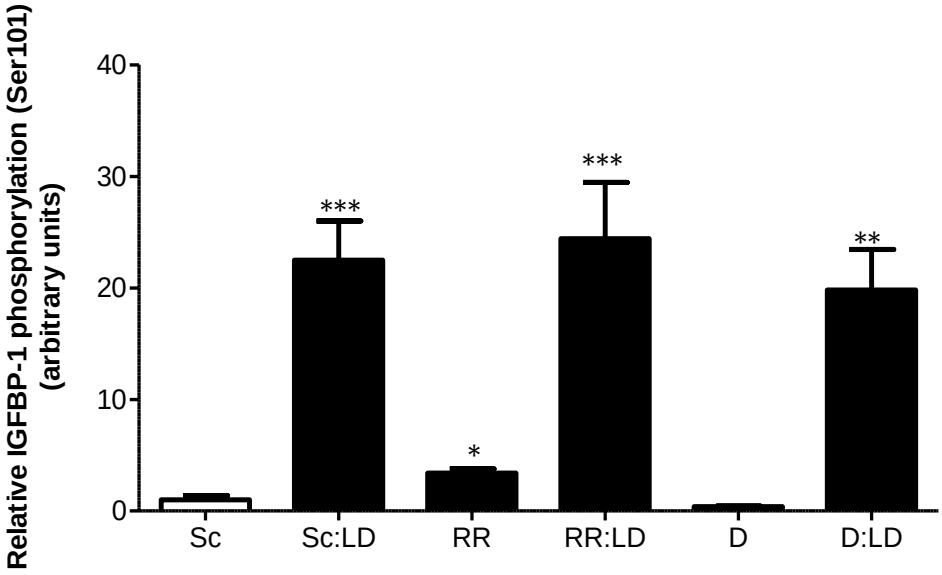

A
siRNA
Scrambled + + - - - -
Raptor+Rictor - - + + - -
DEPTOR - - - - + +
Leucine + - + - + -
70 kDa
pS6K(Thr389)
70 kDa
S6K
42 kDa
β-actin
Relative p70-S6K phosphorylation (Thr389) (arbitrary units)
0
1
2
3
4
*
*
*
***
**
Sc
Sc:LD
RR
RR:LD
D
D:LD


B
siRNA
Scrambled + + - - - -
Raptor+Rictor - - + + - -
DEPTOR - - - - + +
Leucine + - + - + -
56 kDa
pAkt(Ser473)
56 kDa
Akt
42 kDa
β-actin
Relative AKT phosphorylation (Ser473) (arbitrary units)
0
1
2
3
4
*
*
**
***
***
Sc
Sc:LD
RR
RR:LD
D
D:LD


C
siRNA
Scrambled + + - - - -
Raptor+Rictor - - + + - -
DEPTOR - - - - + +
Leucine + - + - + -
30 kDa
IGFBP-1


Relative IGFBP-1 secretion (arbitrary units)
6
4
2
0
***
*
***
Sc
Sc:LD
RR
RR:LD
D
D:LD


D
siRNA
Scrambled + + - - - -
Raptor+Rictor - - + + - -
DEPTOR - - - - + +
Leucine + - + - + -
30 kDa
pIGFBP-1(Ser101)


Relative IGFBP-1 phosphorylation (Ser101) (arbitrary units)
40
30
20
10
0
***
*
***
**
Sc
Sc:LD
RR
RR:LD
D
D:LD

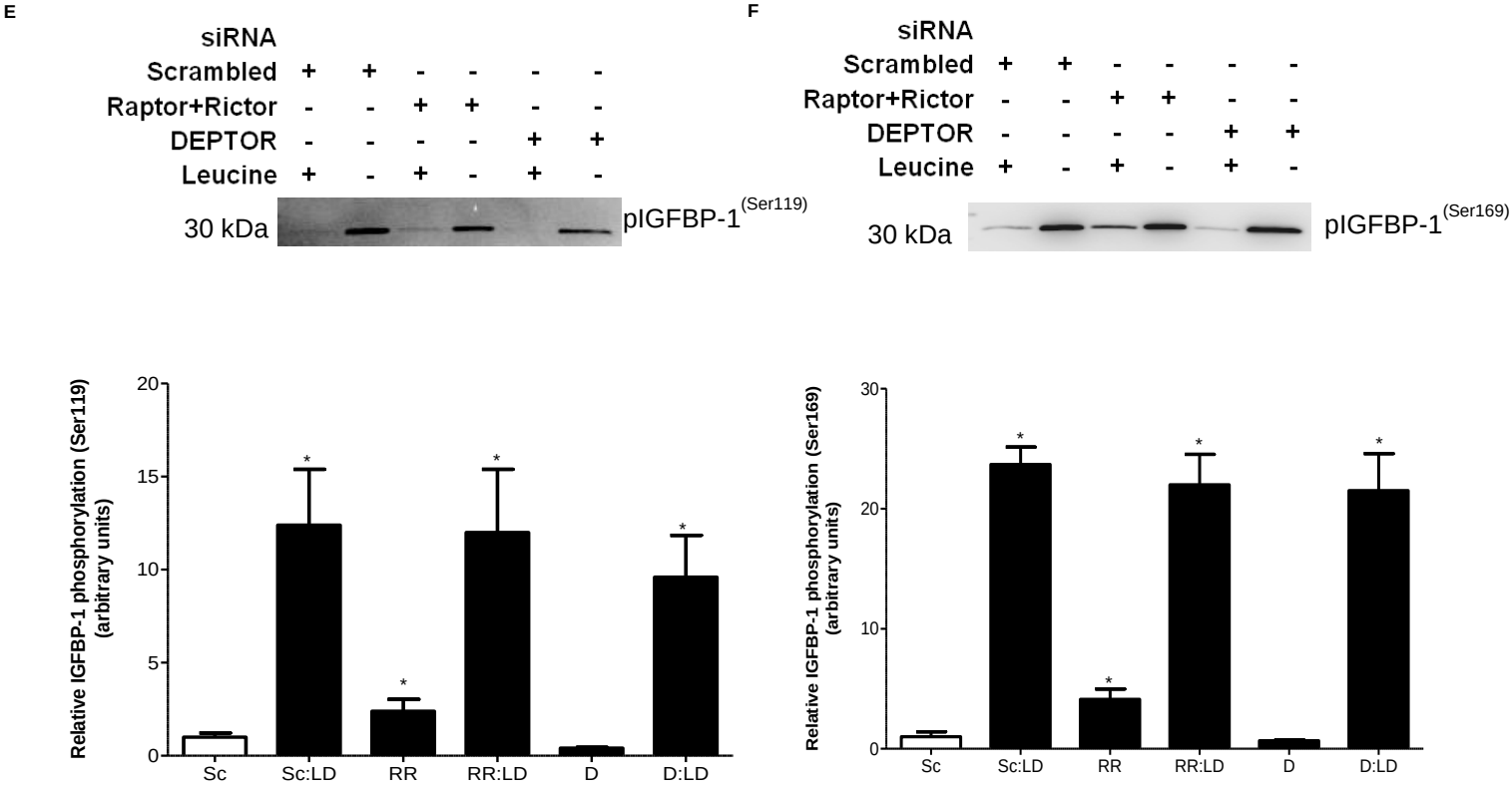


Figure 2.2. The effect of raptor+rictor or DEPTOR silencing on IGFBP-1 secretion and phosphorylation in leucine deprivation.A representative western immunoblot of HepG2 cell lysates (35 μg per lane) displaying A. S6K (Thr389) phosphorylation and B. Akt (Ser473) phosphorylation. A representative western immunoblot of C. total IGFBP-1 secretion and D-F. IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169 in equal amounts (40 μL per well)of cell media of scrambled, raptor+rictor, DEPTOR siRNA with and without leucine deprivation in HepG2 cells. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ****p* < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.Sc: Scrambled siRNA, 450 μM leucine. Sc:LD: Scrambled siRNA, 0 μM leucine (Leucine Deprivation). RR: Raptor+Rictor siRNA, 450 μM leucine. RR:LD: Raptor+Rictor siRNA, 0 μM leucine. D: DEPTOR siRNA, 450 μM leucine. D:LD: DEPTOR siRNA, 0 μM leucine.

2.3.5 Inhibition of AAR (MEK/ERK) signaling attenuates the amino acid response triggered by leucine deprivation

To investigate stress-responsive pathways other than mTOR that may be involved in regulating IGFBP-1 phosphorylation under leucine deprivation, we inhibited AAR signaling, which is activated under cellular amino acid deprivation[42,46,63]. To chemically inhibit AAR signaling, we used AAR (MEK1/2) inhibitor U0126 (10 μM) since MEK signaling is necessary for GCN2-mediated eiF2α phosphorylation (pSer51) and subsequent propagation of the AAR[55]. We tested the effects of U0126 on leucine-mediated IGFBP-1 secretion and phosphorylation.

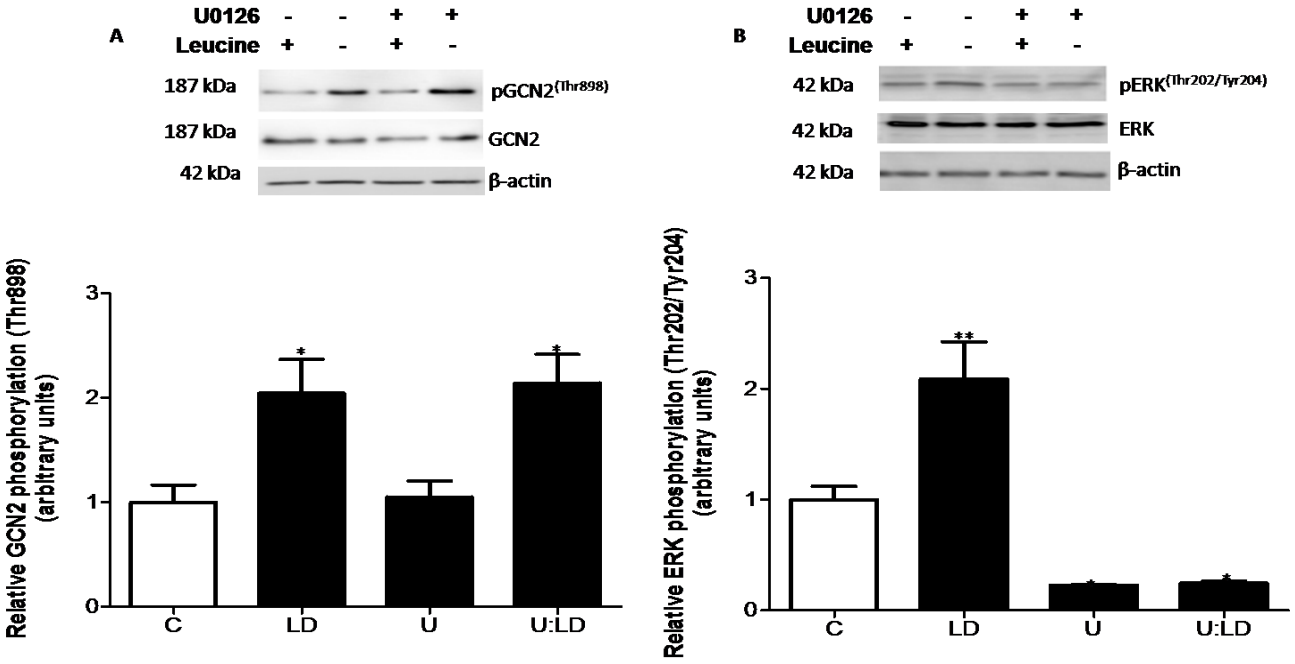
GCN2 was activated, as indicated by an increase in phosphorylation of GCN2 (Thr898), under leucine deprivation (+200%) regardless of MEK status (Figure 2.3A). Leucine deprivation also induced MEK activity proportionate to GCN2 as indicated by increased ERK phosphorylation (Thr202/Tyr204) (+200%), which was on the contrary decreased in the presence of U0126 (-50%) regardless of leucine status (Figure 2.3B).

Figures 2.3C-D indicate that leucine deprivation and subsequent GCN2 activation further stimulate the AAR as evidenced by an increase in eiF2α (Ser51) phosphorylation (+150%) and total ATF expression (+200%) in leucine deprived samples. However, AAR (MEK/ERK) inhibition with U0126 was successful in preventing leucine deprivation-induced AAR propagation downstream of GCN2. We concluded this because in the presence of the inhibitor, leucine deprivation was unable to induce eiF2α phosphorylation (Ser51) and ATF4 expression beyond control values (Figure 2.3C-D). Together, these data advocate the importance of MEK/ERK signaling in AAR propagation and confirm that chemical inhibition of AAR (MEK1/2) is sufficient in attenuating the AAR cascade.

2.3.6 AAR (MEK/ERK) inhibition prevents leucine deprivation-induced IGFBP-1 secretion and phosphorylation

We assessed whether attenuation of the AAR results in changes in downstream IGFBP-1 secretion and phosphorylation. Total IGFBP-1 secretion was induced (+200%) in leucine deprivation and reduced (-50%) in the presence of U0126, regardless of leucine status

(Figure 2.3E). IGFBP-1 phosphorylation was strongly induced (Ser101, +700%, Ser119, +250% and Ser169, +900%) under leucine deprivation and reduced (-50%) at all three sites regardless of leucine status when AAR (MEK/ERK) was inhibited (Figure 2.3F-H). Importantly, the presence of inhibitor did not allow leucine deprivation to induce IGFBP-1 secretion (Figure 2.3E) or phosphorylation at any of the three phosphosites (Figures 2.3F-H). To validate changes in IGFBP-1 secretion and phosphorylation, we performed the Trypan Blue exclusion assay and demonstrated no significant change in post-treatment cell viability between treatment conditions (Appendix A; Supplementary Figure 2).



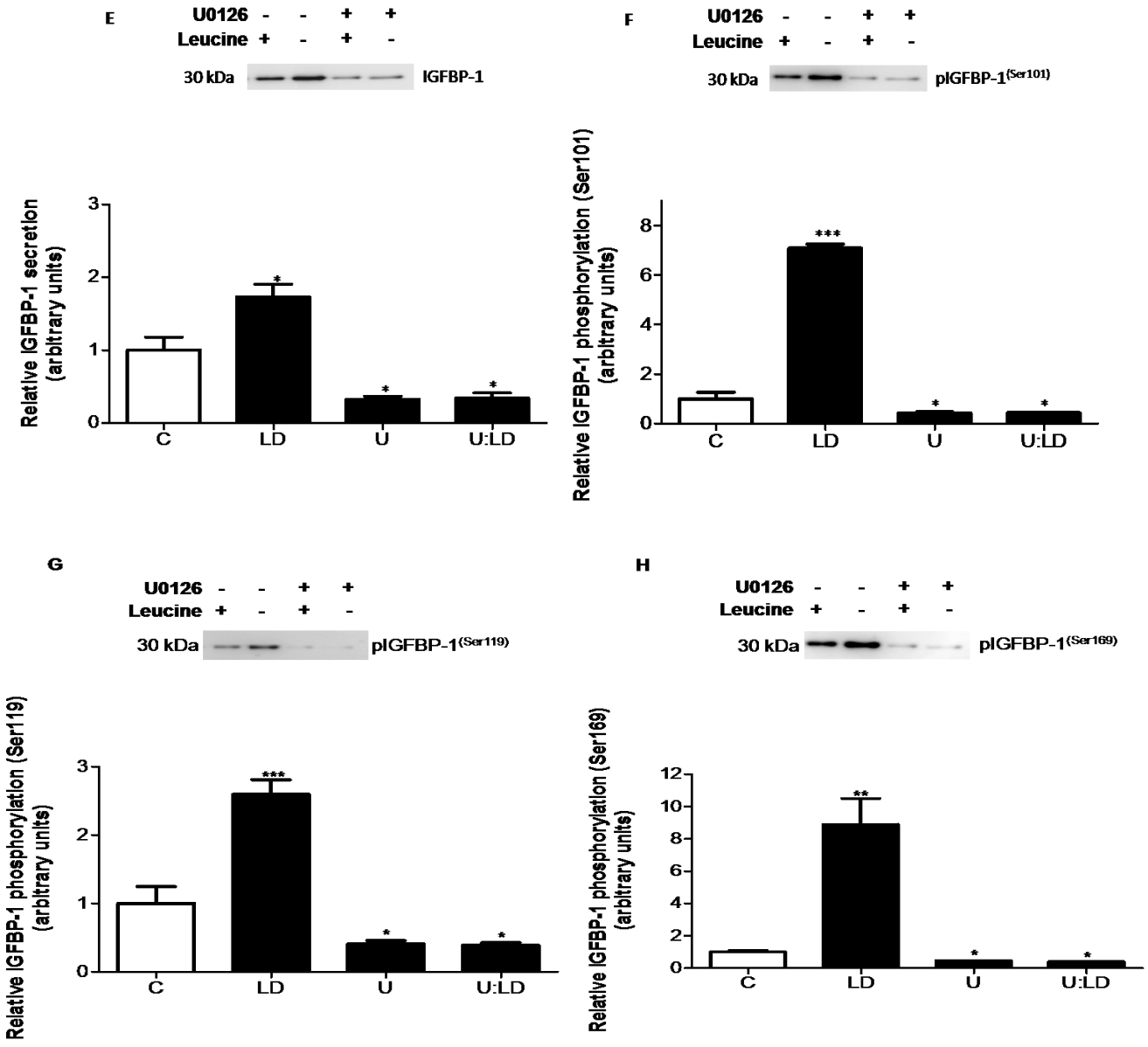


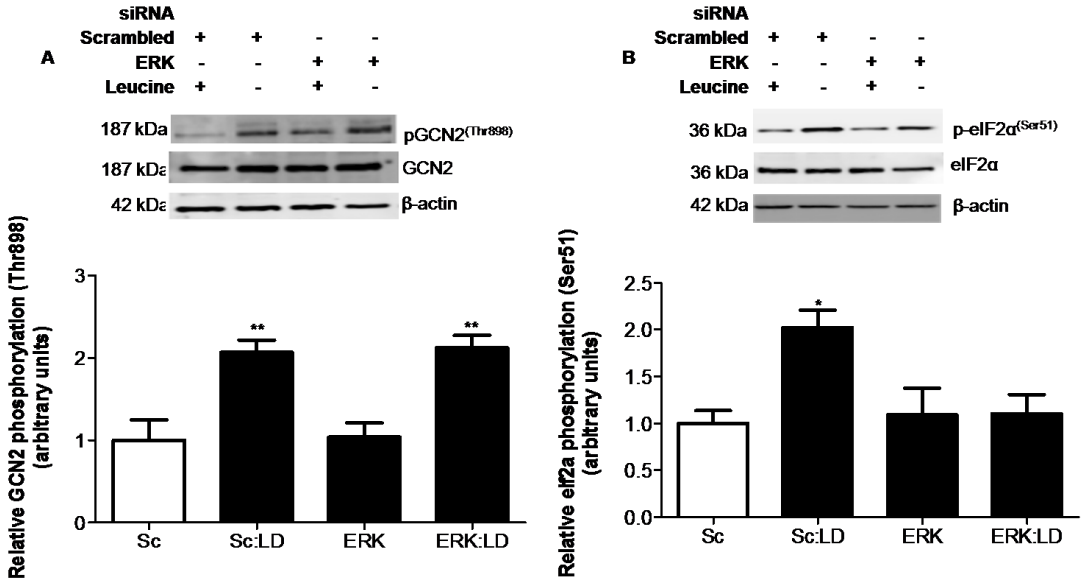
Figure 2.3. Effect of pharmacological AAR inhibitor U0126 (MEK1/2) on IGFBP-1 secretion and phosphorylation.A. Representative western immunoblot of HepG2 cell lysates (50 µg per lane) tested for GCN2 (Thr898) phosphorylation. A representative western immunoblot of HepG2 cell lysates (35 µg per lane) display B. ERK (Thr202/Tyr204) phosphorylation and C. eiF2α (Ser51) phosphorylation and in leucine deprivation and U0126 treatments. D. Representative western immunoblot of total ATF4 expression (50 µg per lane). Representative western immunoblots indicating E. total IGFBP-1 secretion and F-H. IGFBP-1 phosphorylation at Ser101, Ser119, and Ser169.in HepG2 cell media in control, leucine deprivation, U0126, and leucine deprivation+U0126 treatments. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p <

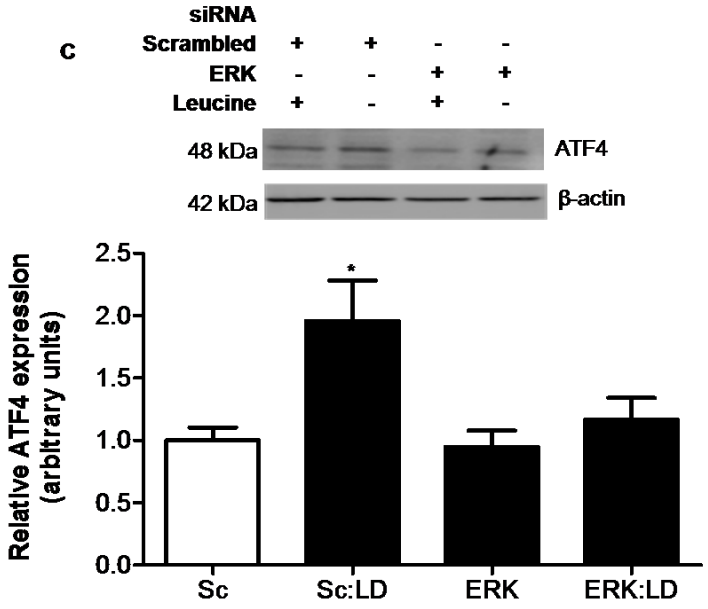
0.001 versus control; One-way analysis of variance; Dunnet'sMultipleComparisonTest; n=3.C: Control, 450 μM leucine. LD: Leucine deprivation, 0 μM leucine. U: U0126 (10 μM), 450 μM leucine. U:LD: U0126 (10 μM), 0 μM leucine.

2.3.7 siRNA silencing of ERK (to inhibit ERK-mediated AAR) prevents leucine deprivation-induced IGFBP-1 secretion and phosphorylation

To determine that changes in IGFBP-1 secretion and phosphorylation under leucine deprivation and inhibition by U0126 were specific and that effects were targeted, in a subset of experiments, cells were treated with siRNA against ERK to attenuate ERK-mediated AAR signaling. First, we validated ERK silencing efficiency by assessing cell lysates for total ERK expression (-45%) (Appendix A; Supplementary Figure 3). GCN2 phosphorylation (Thr898) was induced by leucine deprivation (+200%) regardless of ERK status (Figure 2.4A). Although leucine deprivation triggered AAR signaling, it was not stimulated downstream of GCN2 in cells where ERK was silenced as seen by a lack of induction in eiF2α phosphorylation (Ser51) and ATF4 expression, which were otherwise both triggered (+200%) in leucine deprivation (Figure 2.4B-C).

We examined whether ERK silencing (to inhibit ERK-mediated AAR) was able to attenuate IGFBP-1 secretion and phosphorylation in HepG2 cells. IGFBP-1 secretion and phosphorylation at all three sites (Ser101, Ser119, and Ser169) was not significantly different from control values in the presence or absence of leucine when ERK was silenced (Figures 2.4D-G), supporting our finding that IGFBP-1 secretion and phosphorylation under leucine deprivation is mediated by the AAR in a MEK/ERK dependant mechanism.


A
siRNA
Scrambled + + - -
ERK - - + +
Leucine + - + -
187 kDa
pGCN2(Thr898)
187 kDa
GCN2
42 kDa
β-actin
Relative GCN2 phosphorylation (Thr898) (arbitrary units)
3
2
1
0
**
**
Sc
Sc:LD
ERK
ERK:LD
B
siRNA
Scrambled + + - -
ERK - - + +
Leucine + - + -
36 kDa
p-eIF2α(Ser51)
36 kDa
eIF2α
42 kDa
β-actin
Relative elf2a phosphorylation (Ser51) (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
*
Sc
Sc:LD
ERK
ERK:LD


C
siRNA
Scrambled + + - -
ERK - - + +
Leucine + - + -
48 kDa
ATF4
42 kDa
β-actin
Relative ATF4 expression (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
*
Sc
Sc:LD
ERK
ERK:LD

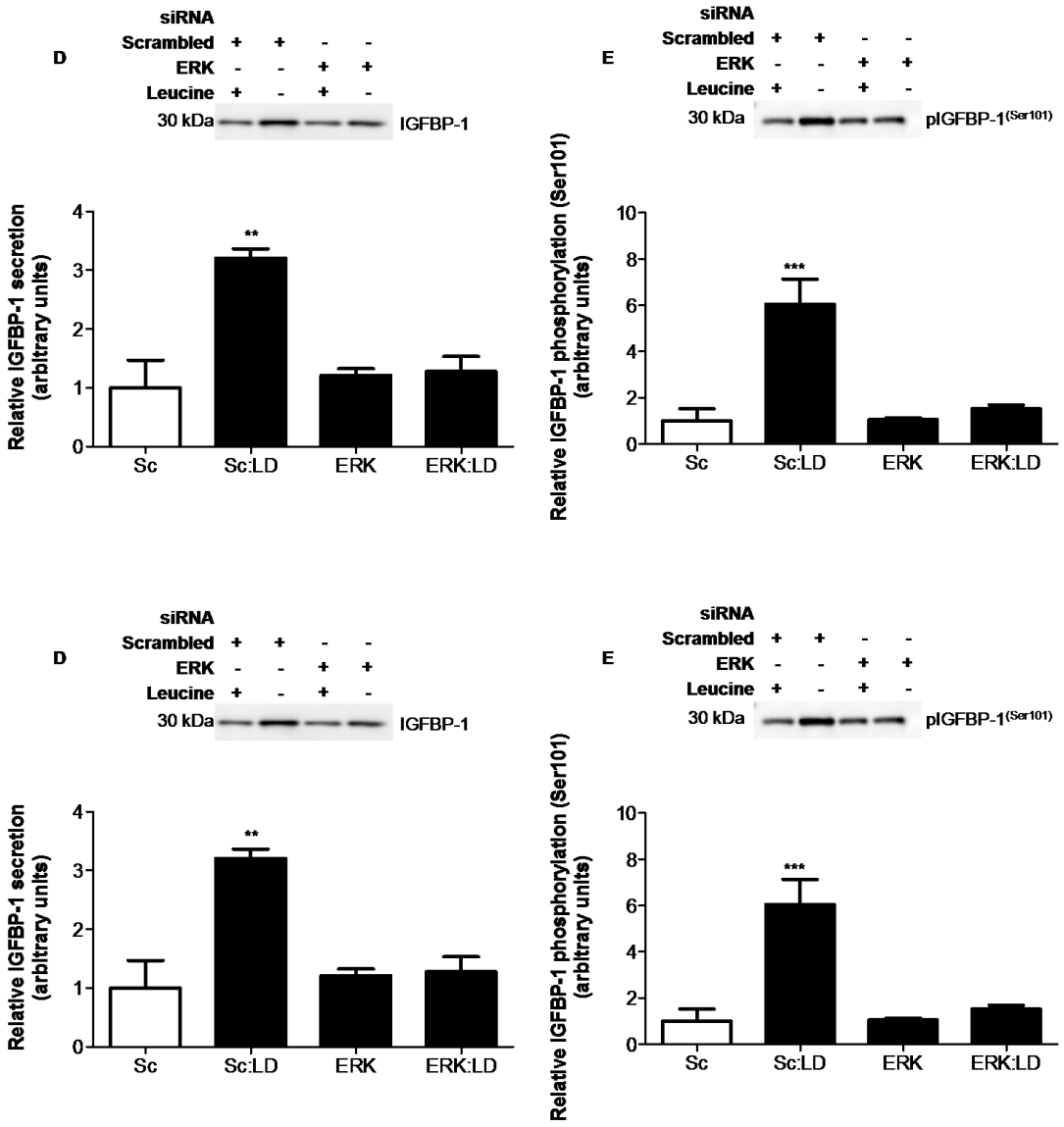
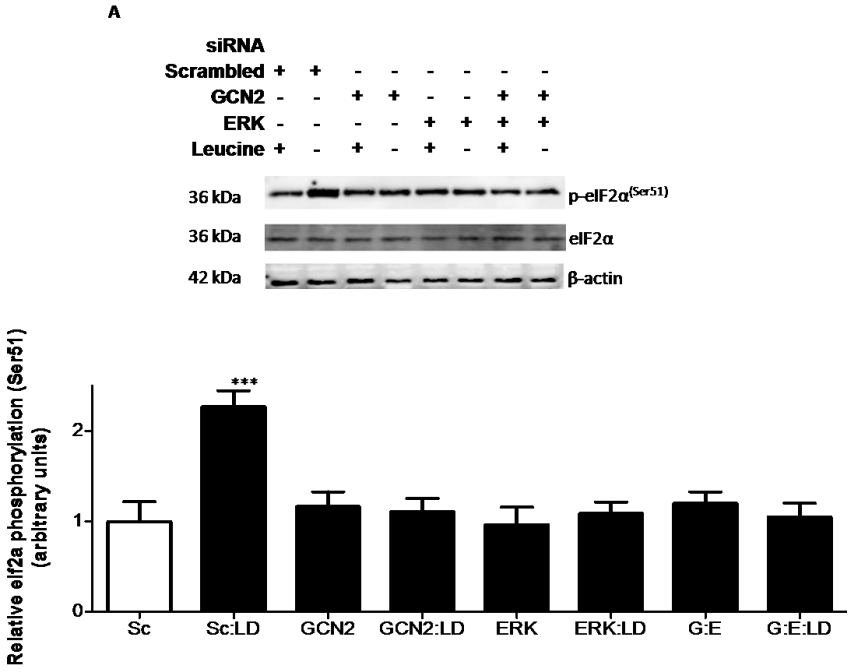


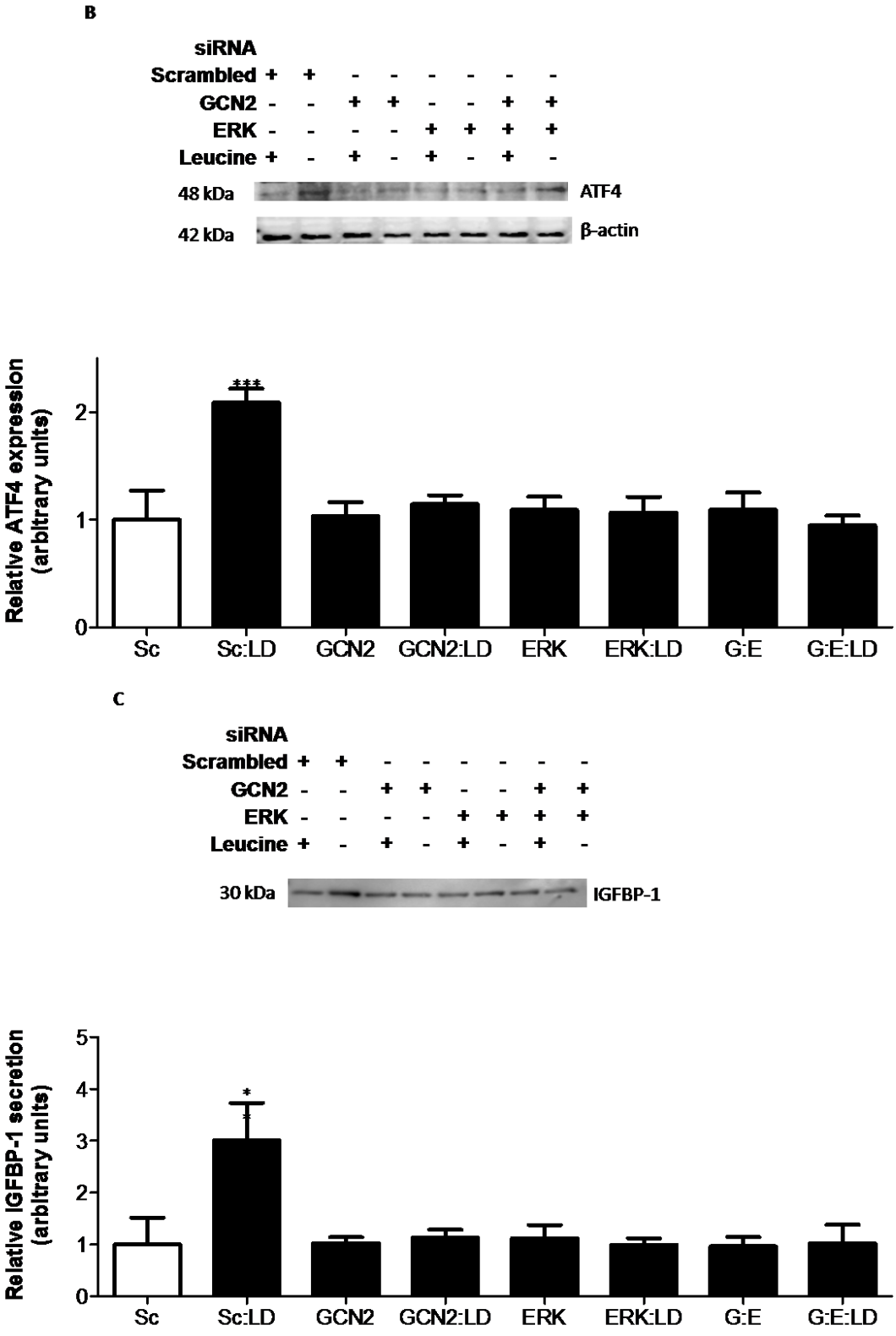
Figure 2.4. Effects of ERK siRNA on IGFBP-1 secretion and phosphorylation. Representative western immunoblots of HepG2 cell lysates (50 µg per lane) treated with scrambled or ERK siRNA with or without leucine deprivation displaying**A.** GCN2 (Thr898) phosphorylation,**B.**eiF2α (Ser51) phosphorylation and**C**. total ATF expression. A representative western immunoblot of **D.**total IGFBP-1 secretion and E-G. IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169 in equal amounts of cell media of HepG2 cell media treated with scrambled or ERK siRNA with and without leucine deprivation. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ****p* < 0.001 versus control; One-way analysis of variance; Dunnet'sMultipleComparisonTest; n=3.Sc: Scrambled siRNA, 450 µM leucine. Sc:LD: Scrambled siRNA, 0 µM leucine (Leucine

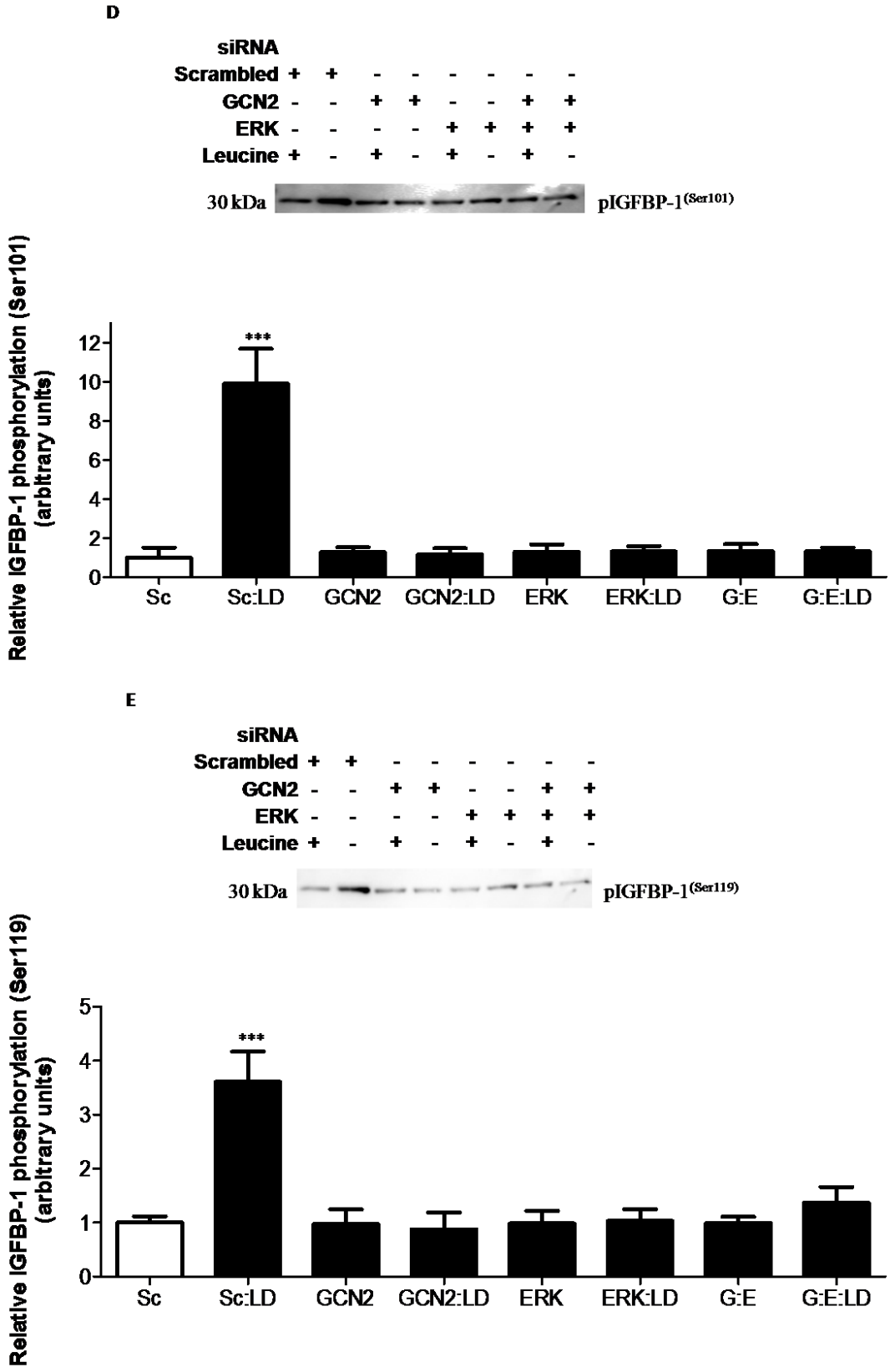
Deprivation). ERK: ERK1/2 siRNA, 450 μM leucine. ERK:LD: ERK1/2 siRNA, 0 μM leucine.

2.3.8 AAR inhibition via GCN2 silencing and ERK inhibition (to inhibit ERK-mediated AAR) act in a common mechanism to regulate IGFBP-1 secretion and phosphorylation in leucine deprivation

To investigate that MEK/ERK inhibition-mediated regulation of IGFBP-1 secretion and phosphorylation functions in accordance with the AAR, we inhibited AAR signaling via GCN2 silencing in tandem with MEK/ERK signaling and assessed changes in downstream AAR effectors and IGFBP-1 secretion and phosphorylation. We used siRNA to knockdown GCN2 to prevent AAR-sensing, separately and together with ERK siRNA. We first confirmed GCN2 (-50%) and ERK (-50%) knockdown efficiency using cell lysates (Appendix A; Supplementary Figure 4A-B). GCN2 and ERK silencing, separately and together, prevented AAR propagation downstream of GCN2 as seen by a lack of increase in eiF2α phosphorylation (Ser51) and ATF4 expression in leucine deprivation by cells silenced for GCN2 and/or ERK, both of which were otherwise induced (+200%) by leucine deprivation (Figure 2.5A-B). Silencing of either or both proteins also attenuated the induction of IGFBP-1 secretion and phosphorylation in leucine deprivation (Figure 2.5C-F). Interestingly, there was no additional reduction in IGFBP-1 secretion or phosphorylation in leucine plus or leucine deprived samples when both pathways were inhibited simultaneously (Figure 2.5C-F) suggesting that MEK/ERK signaling and the AAR function in a common mechanism to regulate both IGFBP-1 secretion and phosphorylation in leucine deprivation.




B
siRNA
Scrambled + + - - - - - -
GCN2 - - + + - - + +
ERK - - - - + + + +
Leucine + - + - + - + -
48 kDa
ATF4
42 kDa
β-actin
Relative ATF4 expression (arbitrary units)
***
0
1
2
Sc
Sc:LD
GCN2
GCN2:LD
ERK
ERK:LD
G:E
G:E:LD
C
siRNA
Scrambled + + - - - - - -
GCN2 - - + + - - + +
ERK - - - - + + + +
Leucine + - + - + - + -
30 kDa
IGFBP-1
Relative IGFBP-1 secretion (arbitrary units)
*
0
1
2
3
4
5
Sc
Sc:LD
GCN2
GCN2:LD
ERK
ERK:LD
G:E
G:E:LD


D
siRNA
Scrambled + + - - - - - -
GCN2 - - + + - - + +
ERK - - - - + + + +
Leucine + - + - + - + -
30 kDa
pIGFBP-1(Ser101)
Relative IGFBP-1 phosphorylation (Ser101) (arbitrary units)
***
12
10
8
6
4
2
0
Sc
Sc:LD
GCN2
GCN2:LD
ERK
ERK:LD
G:E
G:E:LD
E
siRNA
Scrambled + + - - - - - -
GCN2 - - + + - - + +
ERK - - - - + + + +
Leucine + - + - + - + -
30 kDa
pIGFBP-1(Ser119)
Relative IGFBP-1 phosphorylation (Ser119) (arbitrary units)
***
5
4
3
2
1
0
Sc
Sc:LD
GCN2
GCN2:LD
ERK
ERK:LD
G:E
G:E:LD

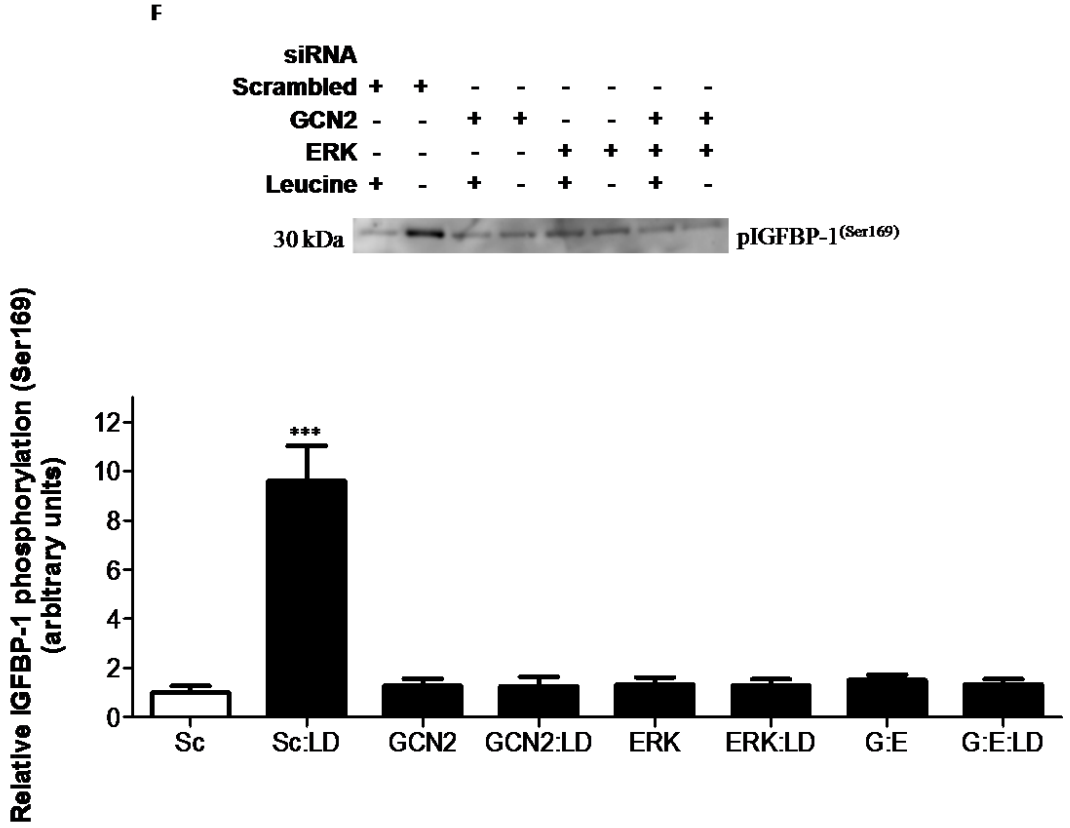


Figure 2.5. Effects of ERK and/or GCN2 silencing on IGFBP-1 secretion and phosphorylation. A. eiF2α (Ser51) phosphorylation and **B**. total ATF4 expression in HepG2 cell lysates silenced with GCN2 and/or ERK siRNA with or without leucine deprivation. A representative western immunoblot of **C.** total IGFBP-1 secretion and **D-F.** IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169 in equal amounts (40 µL per lan)e of cell media of HepG2 cells treated with scrambled, ERK, GCN2, or combined ERK+GCN2 siRNA with and without leucine deprivation.) treated with scrambled, ERK, GCN2, or ERK+GCN2 siRNA with or without leucine deprivation.Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ****p* < 0.001 versus control; One-way analysis of variance; Dunnet'sMultipleComparisonTest; n=3. Sc: Scrambled siRNA, 450 µM leucine. Sc:LD: Scrambled siRNA, 0 µM leucine (Leucine Deprivation). GCN2: GCN2 siRNA, 450 µM leucine. GCN2:LD: GCN2 siRNA, 0 µM leucine. ERK: ERK1/2 siRNA, 450 µM leucine. ERK:LD: ERK1/2 siRNA, 0 µM leucine. G:E: GCN2+ERK1/2 siRNA, 450 µM leucine. G:E:LD: GCN2+ERK1/2 siRNA, 0 µM leucine.

2.3.9 IGFBP-1 phosphorylation induced by leucine deprivation is mediated by CK2

We have hereby established that the AAR is responsible for mediating total IGFBP-1 secretion as well as IGFBP-1 phosphorylation in leucine deprivation, and that mTOR signaling partially regulates IGFBP-1 phosphorylation but is the key mechanistic link between nutrient deprivation and total IGFBP-1 secretion. Based on previous data from our lab[26] that CK2 mediates mTOR-induced IGFBP-1 phosphorylation, we investigated whether CK2 is also involved in leucine deprivation-mediated IGFBP-1 phosphorylation. We inhibited CK2 signaling using CK2 inhibitor TBB (1 μM) as previously[26] in leucine plus or leucine deprived media. Following treatments, evaluation of HepG2 cells indicated intact cellular morphology, and Trypan Blue exclusion assay demonstrated that TBB treatments did not significantly alter the vitality of HepG2 cells (Appendix A; Supplementary Figure S5), supporting that normal cell physiology was intact post-treatment.

Leucine deprivation significantly induced total IGFBP-1 secretion (+250 to 300%) regardless of whether they were treated with TBB, suggesting that CK2 is not involved in regulating total IGFBP-1 secretion during leucine deprivation (Figure 2.6A). However, similar to MEK/ERK- dependent AAR inhibition, TBB prevented the phosphorylation of IGFBP-1 at all three sites as seen by an overall reduction (-60%) of IGFBP-1 phosphorylation (Ser101, Ser119 and Ser169) in TBB-treated cells and no significant increase in phosphorylation in leucine deprived versus leucine plus samples when both are treated with TBB (Figure 2.6B-D). Therefore, since inhibition of CK2 activity attenuated leucine deprivation-induced IGFBP-1 phosphorylation without affecting total IGFBP-1 secretion, we assert that CK2 may potentially be the major kinase responsible for IGFBP-1 phosphorylation under conditions of leucine deprivation.

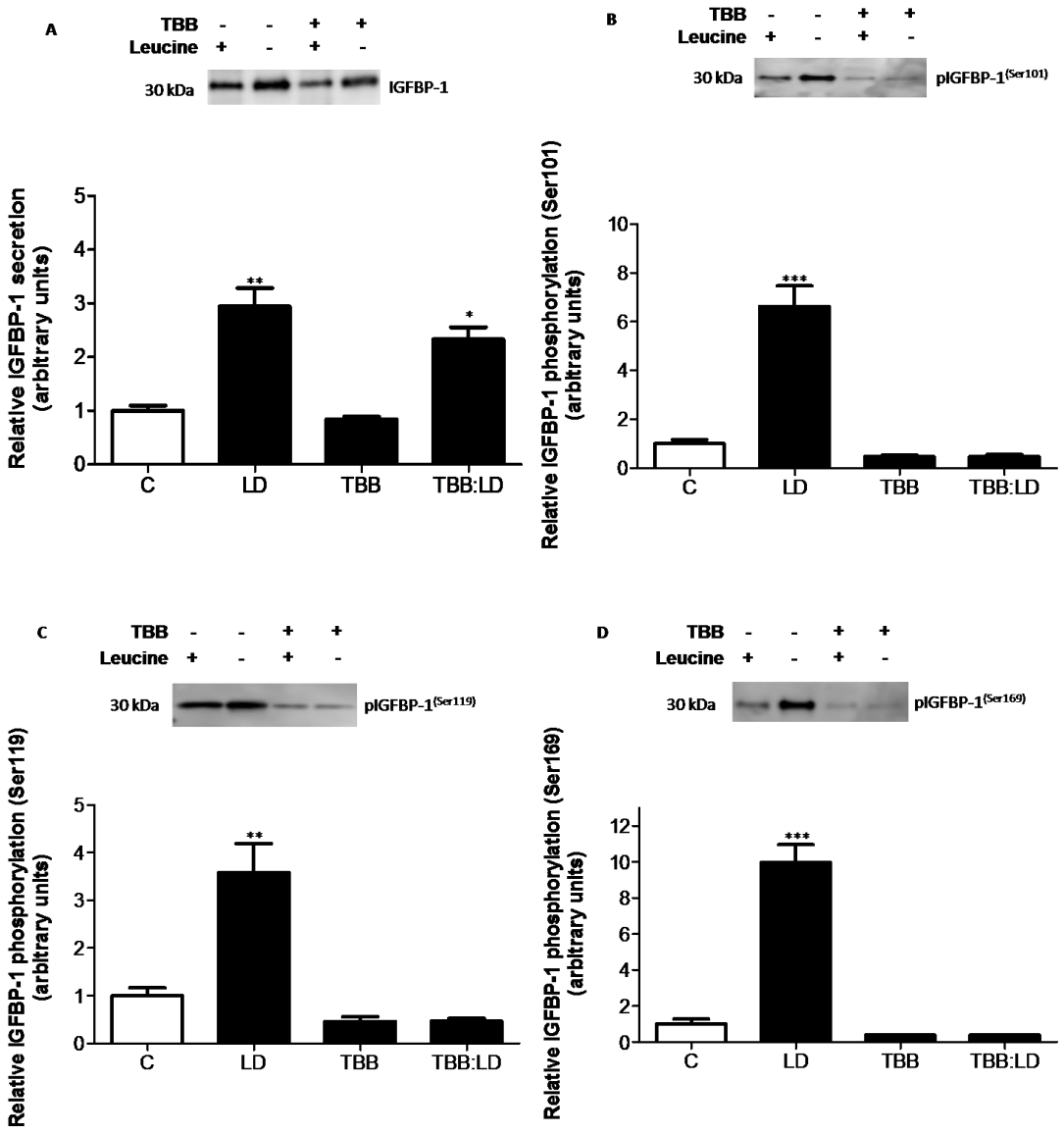


Figure 2.6. Effect of CK2 inhibition on IGFBP-1 secretion and phosphorylation. A. A representative blot of equal volumes of HepG2 cell media (40 µL per lane) treated with leucine plus (control), leucine deprivation, TBB (1 µM), or leucine deprivation+TBB**. B-D.** Representative western immunoblots of equal volumes of HepG2 cell media assessed for IGFBP-1 phosphorylation at Ser101, Ser119, and Ser169. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ****p* < 0.0001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. C: Control, 450 µM leucine. LD: Leucine deprivation, 0 µM leucine. TBB: TBB (1 µM), 450 µM leucine. TBB:LD: TBB (1 µM), 0 µM leucine.

2.3.10 Increases in IGFBP-1 phosphorylation due to leucine deprivation inhibit IGF-I bioactivity

To assess whether changes in IGFBP-1 phosphorylation under leucine deprivation effectively regulate IGF-I bioactivity, we employed an IGF-1 receptor (IGF-1Rβ) autophosphorylation assay in P6 cells to test for IGF-1Rβ (Tyr1135) autophosphorylation[26,56] as an indicator of IGF-I bioactivity during leucine deprivation. When P6 cells were incubated with 25 ng/mL IGF-I only (positive control), we observed a drastic increase in IGF-1R phosphorylation (Tyr1135) (+2500%) compared to P6 cells without IGF-I (negative control) (Figure 2.6), demonstrating the ability of IGF-I to stimulate IGF-1Rβ autophosphorylation in P6 cells. P6 cells were also treated with IGF-I plus post-treatment HepG2 cell media (leucine plus or leucine deprivation). The amount of leucine plus or leucine deprivation media used in the treatment was adjusted for total IGFBP-1 and buffer-exchanged to P6 cell media as described above (Section 2.3.6) to ensure that changes in IGF-1Rβ phosphorylation were not due to differences in media composition.

When P6 cells were incubated with IGFBP-1 from HepG2 cells with 450 µM leucine (basal) media+IGF-I (control), a significant reduction in IGF-1R autophosphorylation was observed (-40%) compared to P6 cells incubated with IGF-I only. This suggests that basal levels of IGFBP-1 secreted by HepG2 cells were able to sequester bioavailable IGF-I and subsequently reduce IGF-1R signaling. On the other hand IGF-I induced IGF-1Rβ phosphorylation was almost completely abolished (-90%) when HepG2 cell media from leucine deprivation (0 µM leucine) was used. These data suggest that IGF-1Rβ phosphorylation was inhibited due to the presence increase in IGFBP-1 phosphorylation due to leucine deprivation (Figure 2.6). Considering equal concentration of total IGFBP-1 from HepG2 cell media incubated with IGF-I was used to activate IGF-1R in P6 cells for each of the treatments, these data support that the hyperphosphorylation of IGFBP-1 in leucine deprivation effectively inhibits IGF-I bioactivity through reduced IGF-1Rβ autophosphorylation (pTyr1135).

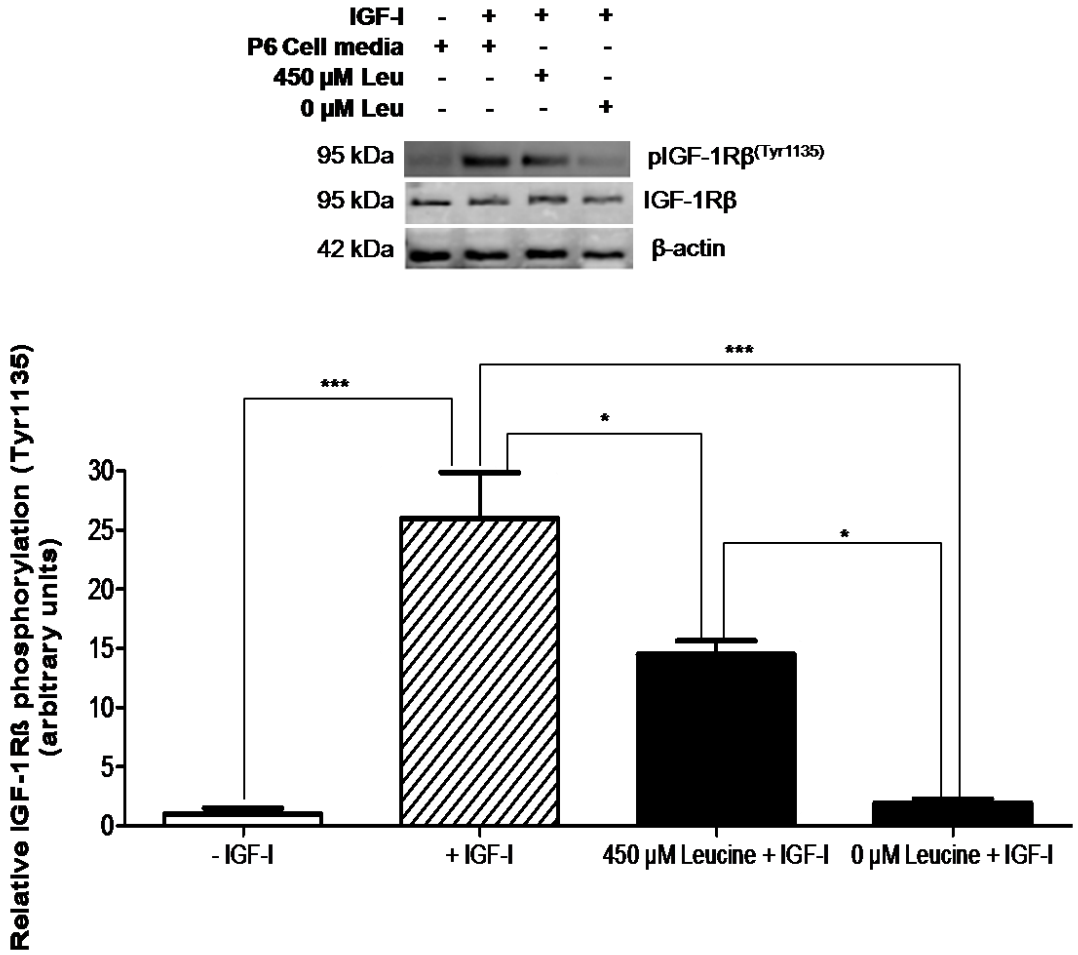


Figure 2.7. The effect of leucine deprivation-induced IGFBP-1 phosphorylation on IGF-1R autophosphorylation. HepG2 cells were treated in leucine plus (450 µM Leu) or leucine deprived (0 µM Leu) for 24 hours. Equal concentrations of IGFBP-1 in HepG2 cell media were mixed with P6 media (serum free) and human recombinant IGF-I (25 ng/mL) for 2 hours to allow IGFBP-1 to sequester IGF-I. Ten minute exposure to P6 cells allowed the induction of IGF-I-mediated IGF-1Rβ autophosphorylation (Tyr1135). A representative western immunoblot of cell lysates (50 µg per lane) from P6 cells over-expressing IGF-IR. Blots were assessed for IGF-IR autophosphorylation (Tyr1135). Increased IGFBP-1 phosphorylation due to leucine deprivation results in significantly decreased IGF-1R activation. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. – IGF-I: Negative control, no IGF-I, no IGFBP-1. +IGF-I: Positive control, 25 ng/mL IGF-I, no IGFBP-1. 450 µM leucine + IGF-I: IGFBP-1 from HepG2 cell media with leucine plus 25 ng/mL IGF-I. 0 µM leucine + IGF-I: IGFBP-1 from HepG2 cell media without leucine plus 25 ng/mL IGF-I.

2.4 Discussion

In this study, we use HepG2 cells, to show for the first time that the AAR regulates IGFBP-1 secretion and phosphorylation at Ser101, Ser119 and Ser169 in amino acid deprivation. Although mTOR inhibition induced IGFBP-1 secretion in leucine deprivation, it failed to increase IGFBP-1 phosphorylation to the levels induced by leucine deprivation alone. Activation of mTOR validated these findings, suggesting that mTOR regulates IGFBP-1 secretion but not IGFBP-1 phosphorylation in leucine deprivation. However, when the AAR was blocked, it prevented both IGFBP-1 secretion and phosphorylation in response to leucine deprivation. These findings are consistent with our hypothesis that mTOR inhibition and AAR activation increase IGFBP-1 secretion and phosphorylation independently in response to amino acid deprivation. This study provides a novel understanding of the mechanisms modulating IGF-I bioavailability and potentially fetal growth under reduced amino acid availability in FGR.

Nutrient deprivation is a leading cause of FGR[2,27], a perinatal disorder which increases the risks of both severe childhood and adult metabolic and neurological complications. IGF-I is the key regulator of fetal growth beginning at ~16-20-weeks gestation[64,65] and its altered circulating levels during gestation are correlated with fetal growth complications[66-68]. IGF-I bioavailability is strongly influenced by the phosphorylation status of IGFBP-1[69]. Previous literature reports [59,70,71] including ours[26,57,58] indicate strong association of IGFBP-1 phosphorylation with altered fetal growth. Mimicking hypoxia and leucine deprivation in HepG2 cells, the two key conditions associated with human FGR *in vivo*, we have previously demonstrated site-specific hyperphosphorylation of IGFBP-1 *in vitro*[25]. Furthermore, our previous data have also shown that increase in phosphorylation of IGFBP-1 at specific sites (Ser101, Ser119 and Ser169) in human amniotic fluid to be associated with increased binding affinity for IGF-I in FGR[57]. These data provided strong rationale to determine the mechanistic details of how IGFBP-1 phosphorylation is controlled in restriction of fetal growth such as due to lack of nutrient availability in FGR.

FGR is characterized by decreased amino acid availability[2,21,27,72] which is known to activate the AAR[44,63] and inhibit the mTOR signaling pathway[29,41]. We investigated the two regulatory pathways (AAR and mTOR), to determine the mechanism linking reduced nutrient availability to IGFBP-1 secretion and phosphorylation and consequently, reduced IGF-I bioavailability and downstream IGF-I bioactivity via assessing changes in IGF-1R autophosphorylation.

We utilized HepG2 cells in this study as a well-established model for human fetal hepatocytes[50-54]. The HepG2 and fetal hepatocyte transcriptomes[54,73,74] and secretomes[38,50,53,75] are highly similar. Moreover, we have shown that HepG2 cells are also highly responsive to hypoxia and leucine deprivation[25]. In addition we have tested the validity of our data with IGFBP1 using well-characterized[76] fetal primary baboon hepatocytes[26]

The mTOR pathway modulates cell growth and function in response to changes in the levels of growth factors, such as IGF-I and nutrients, and is down-regulated under reduced cellular energy states[29,30,37,40,41]. Our results with pharmacological inhibitor (rapamycin) and RNAi based inhibition of mTORC1 and C2 signaling have previously demonstrated induced IGFBP-1 secretion and phosphorylation in HepG2 cells[26]. In the present study, our new findings demonstrated that while mTOR signaling plays a vital role in regulating IGFBP-1 secretion, interestingly, leucine deprivation impinges on additional mechanisms to produce its downstream stress response on IGFBP-1 secretion and phosphorylation.

Dietary protein restriction significantly induces hepatic *IGFBP-1* mRNA expression in rats[77] and is consistently reduced in human circulation in conditions of reduced nutrient intake[78,79]. It has also been reported that leucine deprivation is sufficient in inducing maximal *IGFBP-1* mRNA expression compared to the individual or combined restriction of any other essential amino acid[80]. It is well established that the AAR signal transduction pathway is activated by limitation or imbalance of essential amino acids[40,42-47]. It has also been previously shown that deprivation of a single essential amino acid, leucine, is

sufficient to induce AAR activation via GCN2 phosphorylation[43]. To our knowledge the role of AAR in regulation of IGFBP-1 phosphorylation has however not yet been investigated.

In concordance with literature reports[55], we showed that leucine deprivation activated the AAR, as expected, and that this effect was attenuated downstream of GCN2 when MEK/ERK was inhibited. Attenuating the AAR via MEK/ERK and/or GCN2 inhibition/silencing clearly prevented induction of both IGFBP-1 secretion and phosphorylation due to leucine deprivation; this importantly shows that the mitogenic MEK/ERK cascade mediates leucine deprivation-induced IGFBP-1 secretion and phosphorylation via its interactions with the AAR pathway. This cross-talk is vital in transducing the AAR and elucidating downstream changes in IGFBP-1 secretion and phosphorylation, suggesting that MEK/ERK-dependant AAR signaling is the key mechanism involved in fetal hepatic IGFBP-1 phosphorylation, and consequently, regulation of IGF-I bioavailability. Since inhibition of the AAR was sufficient to attenuate this response, we assert that IGFBP-1 secretion and phosphorylation is regulated primarily by the AAR in a MEK-dependant manner while mTOR contributes a significant role in regulation of IGFBP-1 secretion. It is unclear whether the AAR and mTOR signaling function in common or parallel mechanisms to regulate IGF-I bioavailability and bioactivity. In light of our findings, we propose a model where lack of amino acid availability inhibits hepatic mTOR signaling while simultaneously inducing the AAR pathway, leading to an increase in IGFBP-1 phosphorylation and decreased fetal cell growth and proliferation (Figure 2.8).

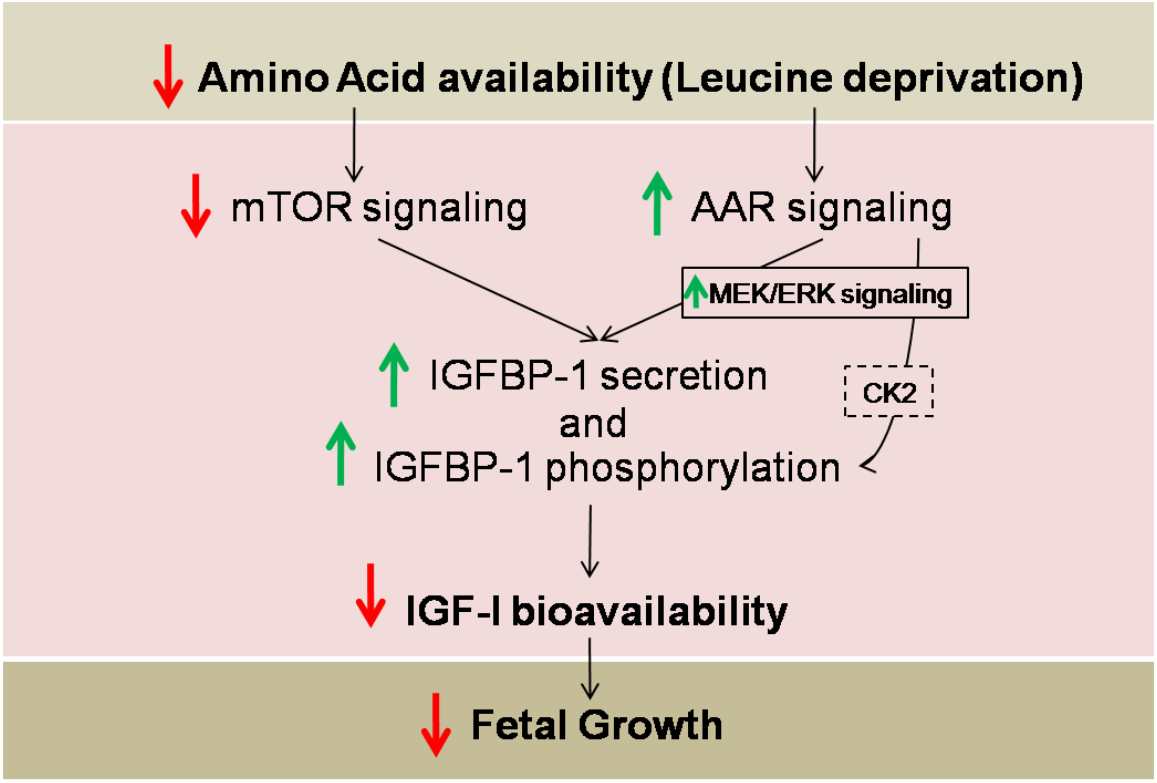


Figure 2.8. Proposed model of fetal growth regulation in FGR. Amino acid limitation causes a decrease in mTOR signaling which leads primarily to an increase in IGFBP-1 secretion. A concurrent induction in the AAR pathway signals an increase in IGFBP-1 secretion and phosphorylation, to regulate IGF-I bioavailability and decreased downstream fetal growth.

Although increased total IGFBP-1secretion is a strong indicator of reduced IGF-I bioavailability, we assert that the pathogenesis of FGR is due primarily to phosphosite-specific changes in IGFBP-1 phosphorylation, supported by our earlier data that show increases (30-300-fold) in IGF-1 affinity to be linked with marked increases in distinct site-specific IGFBP-1 phosphorylation (Ser101, Ser119, Ser169)[25].

Notably, not all serine residues in our study due to leucine deprivation appear to be equally prone to phosphorylation and this reflects an additional level of complexity of the regulation of IGF-I bioavailability. For example, it is possible that different types of

cellular stresses, such as leucine deprivation versus hypoxia, have distinct effects on IGF-I bioavailability mediated by different patterns of IGFBP-1 serine phosphorylation[25]. Although the true biological significance of the variable degree of phosphorylation at the different serine residues in response to leucine deprivation remains to be established, we speculate that induction of phosphorylation at Ser119 under leucine deprivation in this study possibly acts through synergistic interactions with doubly or multiply phosphorylated residues which may result in the high affinity of IGFBP-1 for IGF-I. These synergistic multi-site interactions were not tested in our previous study[56] or elsewhere in the literature. Such interactions would require further experimental evidence through structure-functional and quantitative mass spectrometry (MRM MS) studies. In addition, we expect that the relatively weaker induction of phosphorylation at Ser119 compared to Ser101 and Ser169 under leucine deprivation also reflects the functional characteristics and substrate preference of the protein kinases responsible for phosphorylating respective serine residues.

The three phosphorylated Ser residues in IGFBP-1 are surrounded by acidic amino acids, making them conducive to phosphorylation by CK2[69,81]. CK2 has been suggested to be a kinase that could phosphorylate IGFBP-1 *in vitro*[82]. However, we have recently demonstrated that inhibition of CK2 by pharmacological inhibitor TBB or targeting CK2 holoenzyme using RNAi prevented phosphorylation of IGFBP-1 in HepG2 cells in culture[26]. In the current study we further indicate that CK2 is involved in IGFBP-1 phosphorylation under leucine deprivation implicating CK2 as a potential kinase regulating increased IGFBP-1 phosphorylation under nutrient deprivation. Whether CK2 is the key kinase linking the AAR signaling pathway in fetal liver to IGFBP-1 phosphorylation under leucine deprivation is currently not known.

In conclusion, our findings in this study are consistent with the possibility that the AAR signaling is involved in pathogenesis of FGR. Levels of essential amino acids are reduced in FGR fetuses[83-86]. We have shown, for the first time, that activation of the AAR in the fetal liver may constitute a key mechanism linking decreased amino acid availability to decreased IGF-I bioavailability and reduced fetal growth in FGR. To test this hypothesis, animal models relevant to human FGR, such as our established baboon model of FGR[26],

will be useful. Because of the inaccessibility of the human fetus, implications for the diagnosis and treatment of FGR are not immediately apparent. However, the decidua constitutes the primary source of IGFBP-1 in the maternal circulation during pregnancy[87,88], and maternal IGFBP-1 phosphorylation is increased in FGR pregnancies[89]. If phosphorylation of decidual IGFBP-1 is regulated by the AAR and mTOR signaling pathways, our findings may be used in future to develop approaches for diagnosis and intervention in FGR.

2.5 References

1. Brown LD, Green AS, Limesand SW, Rozance PJ. Maternal amino acid supplementation for intrauterine growth restriction. *Front Biosci (Schol Ed)*. 2011;3:428-444.

2. Miller J, Turan S, Baschat AA. Fetal growth restriction. *Semin Perinatol*. 2008;32(4):274-280.

3. Jang DG, Jo YS, Lee SJ, Kim N, Lee GS. Perinatal outcomes and maternal clinical characteristics in IUGR with absent or reversed end-diastolic flow velocity in the umbilical artery. *Arch Gynecol Obstet*. 2011;284(1):73-78.

4. Pallotto EK, Kilbride HW. Perinatal outcome and later implications of intrauterine growth restriction. *Clin Obstet Gynecol*. 2006;49(2):257-269.

5. Romo A, Carceller R, Tobajas J. Intrauterine growth retardation (IUGR): Epidemiology and etiology. *Pediatr Endocrinol Rev*. 2009;6 Suppl 3:332-336.

6. Baschat AA. Fetal responses to placental insufficiency: An update. *BJOG*. 2004;111(10):1031-1041.

7. Barker DJ. Adult consequences of fetal growth restriction. *Clin Obstet Gynecol*. 2006;49(2):270-283.

8. Leger J, Oury JF, Noel M, et al. Growth factors and intrauterine growth retardation. I. serum growth hormone, insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein 3 levels in normally grown and growth-retarded human fetuses during the second half of gestation. *Pediatr Res*. 1996;40(1):94-100.

9. Lee PD, Conover CA, Powell DR. Regulation and function of insulin-like growth factor-binding protein-1. *Proc Soc Exp Biol Med*. 1993;204(1):4-29.

10. Han VK, Matsell DG, Delhanty PJ, Hill DJ, Shimasaki S, Nygard K. IGF-binding protein mRNAs in the human fetus: Tissue and cellular distribution of developmental expression. *Horm Res*. 1996;45(3-5):160-166.

11. Watson CS, Bialek P, Anzo M, Khosravi J, Yee SP, Han VK. Elevated circulating insulin-like growth factor binding protein-1 is sufficient to cause fetal growth restriction. *Endocrinology*. 2006;147(3):1175-1186.

12. Larsson A, Palm M, Basu S, Axelsson O. Insulin-like growth factor binding protein-1 (IGFBP-1) during normal pregnancy. *Gynecol Endocrinol*. 2013;29(2):129-132.

13. Reece EA, Wiznitzer A, Le E, Homko CJ, Behrman H, Spencer EM. The relation between human fetal growth and fetal blood levels of insulin-like growth factors I and II, their binding proteins, and receptors. *Obstet Gynecol*. 1994;84(1):88-95.

14. Valentinis B, Baserga R. IGF-I receptor signalling in transformation and differentiation. *Mol Pathol*. 2001;54(3):133-137.

15. Clemmons DR, Busby WH, Arai T, et al. Role of insulin-like growth factor binding proteins in the control of IGF actions. *Prog Growth Factor Res*. 1995;6(2-4):357-366.

16. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev*. 2002;23(6):824-854.

17. Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: Effects on affinity for IGF-I. *Proc Natl Acad Sci U S A*. 1991;88(17):7481-7485.

18. Yu J, Iwashita M, Kudo Y, Takeda Y. Phosphorylated insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) inhibits while non-phosphorylated IGFBP-1 stimulates IGF-I-induced amino acid uptake by cultured trophoblast cells. *Growth Horm IGF Res*. 1998;8(1):65-70.

19. Wong MS, Fong CC, Yang M. Biosensor measurement of the interaction kinetics between insulin-like growth factors and their binding proteins. *Biochim Biophys Acta*. 1999;1432(2):293-301.

20. Koistinen R, Itkonen O, Selenius P, Seppala M. Insulin-like growth factor-binding protein-1 inhibits binding of IGF-I on fetal skin fibroblasts but stimulates their DNA synthesis. *Biochem Biophys Res Commun*. 1990;173(1):408-415.

21. Regnault TR, de Vrijer B, Galan HL, Wilkening RB, Battaglia FC, Meschia G. Umbilical uptakes and transplacental concentration ratios of amino acids in severe fetal growth restriction. *Pediatr Res*. 2013;73(5):602-611.

22. Sathishkumar K, Elkins R, Chinnathambi V, Gao H, Hankins GD, Yallampalli C. Prenatal testosterone-induced fetal growth restriction is associated with down-regulation of rat placental amino acid transport. *Reprod Biol Endocrinol*. 2011;9:110-7827-9-110.

23. Teodoro GF, Vianna D, Torres-Leal FL, et al. Leucine is essential for attenuating fetal growth restriction caused by a protein-restricted diet in rats. *J Nutr*. 2012;142(5):924-930.

24. Wu G. Amino acids: Metabolism, functions, and nutrition. *Amino Acids*. 2009;37(1):1-17.

25. Seferovic MD, Ali R, Kamei H, et al. Hypoxia and leucine deprivation induce human insulin-like growth factor binding protein-1 hyperphosphorylation and increase its biological activity. *Endocrinology*. 2009;150(1):220-231.

26. Abu Shehab M, Damerill I, Shen T, et al. Liver mTOR controls IGF-I bioavailability by regulation of protein kinase CK2 and IGFBP-1 phosphorylation in fetal growth restriction. *Endocrinology*. 2014;155(4):1327-1339.

27. Maulik D. Fetal growth restriction: The etiology. *Clin Obstet Gynecol*. 2006;49(2):228-235.

28. Strakovsky RS, Zhou D, Pan YX. A low-protein diet during gestation in rats activates the placental mammalian amino acid response pathway and programs the growth capacity of offspring. *J Nutr*. 2010;140(12):2116-2120.

29. Roos S, Jansson N, Palmberg I, Saljo K, Powell TL, Jansson T. Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. *J Physiol*. 2007;582(Pt 1):449-459.

30. Roos S, Powell TL, Jansson T. Placental mTOR links maternal nutrient availability to fetal growth. *Biochem Soc Trans*. 2009;37(Pt 1):295-298.

31. Martin PM, Sutherland AE. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. *Dev Biol*. 2001;240(1):182-193.

32. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell*. 2006;124(3):471-484.

33. Arsham AM, Howell JJ, Simon MC. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J Biol Chem*. 2003;278(32):29655-29660.

34. Wang X, Proud CG. mTORC1 signaling: What we still don't know. *J Mol Cell Biol*. 2011;3(4):206-220.

35. Oshiro N, Yoshino K, Hidayat S, et al. Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. *Genes Cells*. 2004;9(4):359-366.

36. Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol*. 2004;14(14):1296-1302.

37. Foster KG, Fingar DC. Mammalian target of rapamycin (mTOR): Conducting the cellular signaling symphony. *J Biol Chem*. 2010;285(19):14071-14077.

38. Matsumura T, Morinaga Y, Fujitani S, Takehana K, Nishitani S, Sonaka I. Oral administration of branched-chain amino acids activates the mTOR signal in cirrhotic rat liver. *Hepatol Res*. 2005;33(1):27-32.

39. Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem*. 1998;273(23):14484-14494.

40. Chotechuang N, Azzout-Marniche D, Bos C, et al. mTOR, AMPK, and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to protein intake in the rat. *Am J Physiol Endocrinol Metab*. 2009;297(6):E1313-23.

41. Li F, Yin Y, Tan B, Kong X, Wu G. Leucine nutrition in animals and humans: MTOR signaling and beyond. *Amino Acids*. 2011;41(5):1185-1193.

42. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: How mammalian cells respond to amino acid limitation. *Annu Rev Nutr*. 2005;25:59-85.

43. Thiaville MM, Dudenhausen EE, Zhong C, Pan YX, Kilberg MS. Deprivation of protein or amino acid induces C/EBPbeta synthesis and binding to amino acid response elements, but its action is not an absolute requirement for enhanced transcription. *Biochem J*. 2008;410(3):473-484.

44. Zhou D, Pan YX. Gestational low protein diet selectively induces the amino acid response pathway target genes in the liver of offspring rats through transcription factor binding and histone modifications. *Biochim Biophys Acta*. 2011;1809(10):549-556.

45. Hinnebusch AG. Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. *J Biol Chem*. 1997;272(35):21661-21664.

46. Dong J, Qiu H, Garcia-Barrio M, Anderson J, Hinnebusch AG. Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. *Mol Cell*. 2000;6(2):269-279.

47. Deng J, Harding HP, Raught B, et al. Activation of GCN2 in UV-irradiated cells inhibits translation. *Curr Biol*. 2002;12(15):1279-1286.

48. Yung HW, Calabrese S, Hynx D, et al. Evidence of placental translation inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction. *Am J Pathol*. 2008;173(2):451-462.

49. Kilberg MS, Shan J, Su N. ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol Metab*. 2009;20(9):436-443.

50. Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab Dispos*. 2003;31(8):1035-1042.

51. Kelly JH, Darlington GJ. Modulation of the liver specific phenotype in the human hepatoblastoma line hep G2. *In Vitro Cell Dev Biol*. 1989;25(2):217-222.

52. Maruyama M, Matsunaga T, Harada E, Ohmori S. Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biol Pharm Bull*. 2007;30(11):2091-2097.

53. Pal R, Mamidi MK, Das AK, Gupta PK, Bhonde R. A simple and economical route to generate functional hepatocyte-like cells from hESCs and their application in evaluating alcohol induced liver damage. *J Cell Biochem*. 2012;113(1):19-30.

54. Hart SN, Li Y, Nakamoto K, Subileau EA, Steen D, Zhong XB. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metab Dispos*. 2010;38(6):988-994.

55. Thiaville MM, Pan YX, Gjymishka A, Zhong C, Kaufman RJ, Kilberg MS. MEK signaling is required for phosphorylation of eIF2alpha following amino acid limitation of HepG2 human hepatoma cells. *J Biol Chem*. 2008;283(16):10848-10857.

56. Abu Shehab M, Iosef C, Wildgruber R, Sardana G, Gupta MB. Phosphorylation of IGFBP-1 at discrete sites elicits variable effects on IGF-I receptor autophosphorylation. *Endocrinology*. 2013;154(3):1130-1143.

57. Abu Shehab M, Khosravi J, Han VK, Shilton BH, Gupta MB. Site-specific IGFBP-1 hyper-phosphorylation in fetal growth restriction: Clinical and functional relevance. *J Proteome Res*. 2010;9(4):1873-1881.

58. Nissum M, Abu Shehab M, Sukop U, et al. Functional and complementary phosphorylation state attributes of human insulin-like growth factor-binding protein-1 (IGFBP-1) isoforms resolved by free flow electrophoresis. *Mol Cell Proteomics*. 2009;8(6):1424-1435.

59. Westwood M, Gibson JM, Davies AJ, Young RJ, White A. The phosphorylation pattern of insulin-like growth factor-binding protein-1 in normal plasma is different from that in amniotic fluid and changes during pregnancy. *J Clin Endocrinol Metab*. 1994;79(6):1735-1741.

60. Pietrzkowski Z, Sell C, Lammers R, Ullrich A, Baserga R. Roles of insulinlike growth factor 1 (IGF-1) and the IGF-1 receptor in epidermal growth factor-stimulated growth of 3T3 cells. *Mol Cell Biol*. 1992;12(9):3883-3889.

61. Resnicoff M, Sell C, Ambrose D, Baserga R, Rubin R. Ethanol inhibits the autophosphorylation of the insulin-like growth factor 1 (IGF-1) receptor and IGF-1-mediated proliferation of 3T3 cells. *J Biol Chem*. 1993;268(29):21777-21782.

62. Vasilcanu R, Vasilcanu D, Sehat B, et al. Insulin-like growth factor type-I receptor-dependent phosphorylation of extracellular signal-regulated kinase 1/2 but not akt (protein kinase B) can be induced by picropodophyllin. *Mol Pharmacol*. 2008;73(3):930-939.

63. Kilberg MS, Balasubramanian M, Fu L, Shan J. The transcription factor network associated with the amino acid response in mammalian cells. *Adv Nutr*. 2012;3(3):295-306.

64. Chellakooty M, Vangsgaard K, Larsen T, et al. A longitudinal study of intrauterine growth and the placental growth hormone (GH)-insulin-like growth factor I axis in maternal circulation: Association between placental GH and fetal growth. *J Clin Endocrinol Metab*. 2004;89(1):384-391.

65. Skjaerbaek C, Frystyk J, Orskov H, Flyvbjerg A. Free IGF-I, IGFBP-1, and the binary complex of IGFBP-1 and IGF-I are increased during human pregnancy. *Horm Res*. 2004;62(5):215-220.

66. Koutsaki M, Sifakis S, Zaravinos A, Koutroulakis D, Koukoura O, Spandidos DA. Decreased placental expression of hPGH, IGF-I and IGFBP-1 in pregnancies complicated by fetal growth restriction. *Growth Horm IGF Res*. 2011;21(1):31-36.

67. McIntyre HD, Serek R, Crane DI, et al. Placental growth hormone (GH), GH-binding protein, and insulin-like growth factor axis in normal, growth-retarded, and diabetic pregnancies: Correlations with fetal growth. *J Clin Endocrinol Metab*. 2000;85(3):1143-1150.

68. Hayati AR, Cheah FC, Yong JF, Tan AE, Norizah WM. The role of serum insulin-like growth factor I (IGF-I) in neonatal outcome. *J Clin Pathol*. 2004;57(12):1299-1301.

69. Coverley JA, Baxter RC. Phosphorylation of insulin-like growth factor binding proteins. *Mol Cell Endocrinol*. 1997;128(1-2):1-5.

70. Iwashita M, Sakai K, Kudo Y, Takeda Y. Phosphoisoforms of insulin-like growth factor binding protein-1 in appropriate-for-gestational-age and small-for-gestational-age fetuses. *Growth Horm IGF Res*. 1998;8(6):487-493.

71. Westwood M, Gibson JM, White A. Purification and characterization of the insulin-like growth factor-binding protein-1 phosphoform found in normal plasma. *Endocrinology*. 1997;138(3):1130-1136.

72. Neerhof MG, Thaete LG. The fetal response to chronic placental insufficiency. *Semin Perinatol*. 2008;32(3):201-205.

73. Tyakht AV, Ilina EN, Alexeev DG, et al. RNA-seq gene expression profiling of HepG2 cells: The influence of experimental factors and comparison with liver tissue. *BMC Genomics*. 2014;15:1108-2164-15-1108.

74. Costantini S, Di Bernardo G, Cammarota M, Castello G, Colonna G. Gene expression signature of human HepG2 cell line. *Gene*. 2013;518(2):335-345.

75. Jacobson HI, Lemanski N, Narendran A, Agarwal A, Bennett JA, Andersen TT. Hormones of pregnancy, alpha-feto protein, and reduction of breast cancer risk. *Adv Exp Med Biol*. 2008;617:477-484.

76. Li C, Shu ZJ, Lee S, et al. Effects of maternal nutrient restriction, intrauterine growth restriction, and glucocorticoid exposure on phosphoenolpyruvate carboxykinase-1 expression in fetal baboon hepatocytes in vitro. *J Med Primatol*. 2013;42(4):211-219.

77. Straus DS, Burke EJ, Marten NW. Induction of insulin-like growth factor binding protein-1 gene expression in liver of protein-restricted rats and in rat hepatoma cells limited for a single amino acid. *Endocrinology*. 1993;132(3):1090-1100.

78. Grimble RF, Whitehead RG. Fasting serum-aminoacid patterns in kwashiorkor and after administration of different levels of protein. *Lancet*. 1970;1(7653):918-920.

79. Baertl JM, Placko RP, Graham GG. Serum proteins and plasma free amino acids in severe malnutrition. *Am J Clin Nutr*. 1974;27(7):733-742.

80. Jousse C, Bruhat A, Ferrara M, Fafournoux P. Physiological concentration of amino acids regulates insulin-like-growth-factor-binding protein 1 expression. *Biochem J*. 1998;334 (Pt 1)(Pt 1):147-153.

81. Litchfield DW. Protein kinase CK2: Structure, regulation and role in cellular decisions of life and death. *Biochem J*. 2003;369(Pt 1):1-15.

82. Ankrapp DP, Jones JI, Clemmons DR. Characterization of insulin-like growth factor binding protein-1 kinases from human hepatoma cells. *J Cell Biochem*. 1996;60(3):387-399.

83. Bhasin KK, van Nas A, Martin LJ, Davis RC, Devaskar SU, Lusis AJ. Maternal low-protein diet or hypercholesterolemia reduces circulating essential amino acids and leads to intrauterine growth restriction. *Diabetes*. 2009;58(3):559-566.

84. Bajoria R, Sooranna SR, Ward S, Hancock M. Placenta as a link between amino acids, insulin-IGF axis, and low birth weight: Evidence from twin studies. *J Clin Endocrinol Metab*. 2002;87(1):308-315.

85. Di Giulio AM, Carelli S, Castoldi RE, Gorio A, Taricco E, Cetin I. Plasma amino acid concentrations throughout normal pregnancy and early stages of intrauterine growth restricted pregnancy. *J Matern Fetal Neonatal Med*. 2004;15(6):356-362.

86. Favretto D, Cosmi E, Ragazzi E, et al. Cord blood metabolomic profiling in intrauterine growth restriction. *Anal Bioanal Chem*. 2012;402(3):1109-1121.

87. Martina NA, Kim E, Chitkara U, Wathen NC, Chard T, Giudice LC. Gestational age-dependent expression of insulin-like growth factor-binding protein-1 (IGFBP-1) phosphoisoforms in human extraembryonic cavities, maternal serum, and decidua

suggests decidua as the primary source of IGFBP-1 in these fluids during early pregnancy. *J Clin Endocrinol Metab*. 1997;82(6):1894-1898.

88. Fang Q, Wang YX, Zhou Y. Insulin-like growth factor binding protein 1 and human embryonic development during 6 - 10 gestational weeks. *Chin Med J (Engl)*. 2004;117(4):488-491.

89. Koistinen R, Angervo M, Leinonen P, Hakala T, Seppala M. Phosphorylation of insulin-like growth factor-binding protein-1 increases in human amniotic fluid and decidua from early to late pregnancy. *Clin Chim Acta*. 1993;215(2):189-199.

Chapter 3

Exploring the kinases involved in leucine deprivation-mediated IGFBP-1 phosphorylation

.

3.1 Introduction

Fetal Growth Restriction (FGR) predisposes infants to severe childhood and adult morbidities[1,2], making it an important area for investigation. Affecting 5-7% of pregnancies[3], FGR commonly results from insufficient *in utero* availability of nutrients, such as oxygen and essential amino acids, to the fetus due to either maternal malnutrition or inadequate placental nutrient transfer from mother to fetus[4-6]. The fetal response to perigestational nutrient deficiency can lead to restricted growth, although the molecular mechanisms by which this occurs are largely unknown. The insulin-like growth factors (IGF-I and IGF-II) are critical factors in fetal growth and development across species[7-10]. Knockout studies of *IGF-I* and *IGF-II* in mice demonstrate that both IGFs are crucial to fetal growth and development[11], and that deficits in *IGF-II* lead to pathological placental, embryonic, and organ development as well as fetal demise[12,13]. IGF-II provides a continuous stimulus for growth[14]and exists in the fetal serum in far greater concentrations (3-10 fold) than IGF-I[14,15]. However, as gestation progresses, fetal development becomes increasingly dependent on IGF-I[16], whose function is acutely sensitive to physiological and environmental cues, such as nutritional stress[14,17]. In human studies, fetal IGF-I levels in particular have been consistently positively associated with fetal size and birth weight[18-21], and its fetal circulating levels are decreased in growth restricted human fetuses[8,19,21-23]. IGF-Binding Protein 1 (IGFBP-1) secreted from the fetal liver[24], the predominant fetal circulating IGFBP in pre-natal life[25-28], is a potent inhibitor of IGF-I bioavailability *in vitro* and *in vivo*[29-33]and functions by sequestering IGF-I from its cell-surface cognate receptor (IGF-1R), preventing downstream cell growth and proliferation[34]. Phosphorylated IGFBP-1 isolated from HepG2 cell media, an *in vitro* model for fetal hepatocytes[35-39] as well as phosphorylated IGFBP-1 from human plasma demonstrates a 6-10 fold greater affinity for IGF-I compared to the non-phosphorylated isoform[29,40].The phosphorylation status of fetal IGFBP-1 during pregnancy has been associated with fetal growth abnormalities[41-44]. Importantly, our team has recently detected elevated levels of phosphorylated IGFBP-1 (pSer101, pSer119, pSer169) in umbilical cord plasma[45] from FGR babies.

Previous work in our lab has established that FGR is associated with fetal IGFBP-1 hyperphosphorylation[42,45], making it crucial to identify the molecular mechanisms which mediate amino acid deprivation-induced IGFBP-1 phosphorylation, which, to date, have not been extensively classified. Leucine depletion stimulates IGFBP-1 phosphorylation (pSer101, pSer119, pSer169) in HepG2 cells[46]which we recently demonstrated to be linked to the activation of the Amino Acid Response (AAR) (Chapter 2). The specific kinases which phosphorylate IGFBP-1 at Ser101, Ser119, and Ser169 in leucine deprivation, however, have not been reported.

Initialstudies of the kinases whichphosphorylate IGFBP-1 were conducted in human endometrial stromal cells derived from pregnant women and cultured *in vitro*[47]. Due to the proximity of IGFBP-1 phospho-serines to acidic residues conducive to phosphorylation by CK2, and based on the elevated presence of PKA substrate, cAMP, in stromal cells[47], IGFBP-1 was isolated from cell media from cultured stromal cells and incubated with purified protein kinase CK2 or protein kinase A (PKA). Both kinases induced phosphorylation of previously non-phosphorylated IGFBP-1 indicating that CK2 and PKA can phosphorylate IGFBP-1 *in vitro*[47]. However, the direct exposure of CK2 or PKA to IGFBP-1 when the protein and kinases are co-incubated is not reflective of intracellular conditions; thus, the kinases which phosphorylate IGFBP-1 when co-incubated with the substrate do not necessarily represent the kinases which are triggered intracellularly to phosphorylate IGFBP-1 with high affinity in either regular or nutrient restricted conditions. Additionally, the distinct IGFBP-1 residues that were phosphorylated by these kinases were not reported. The proximity of acidic amino acids (Aspartic Acid (D), Glutamic Acid (E)) to residues Ser101, Ser119, and Ser169 on IGFBP-1 make them conducive to direct phosphorylation by CK2 (Table 3.1). Ser101 and Ser169 are proximal to structured regions on the IGFBP-1 molecule that contain the IGF-I-binding domain, whereas Ser119 is contained within the unstructured linker region that is unique to each IGFBP[48]. A subsequent study by Ankrapp et. al. demonstrated that partially purified CK2 from HepG2 cell extracts phosphorylated recombinant human IGFBP-1 produced by CHO cells *in vitro* at Ser101 and Ser169[49]. The functional relevance of CK2 in phosphorylating IGFBP-1 within live cells, however, was not

established. CK2 is a pleiotropic kinase with over 300 potential substrates[50]; therefore, to establish CK2 as the key, specific kinase implicated in IGFBP-1 phosphorylation in live cells, it was necessary to specifically inhibit CK2 using both pharmacological and siRNA approaches and to measure subsequent IGFBP-1 phosphorylation, a strategy which was recently undertaken in our laboratory in HepG2 cells[45].

The recent demonstration by our team that pharmacological (TBB) and siRNA inhibition of CK2 reduces IGFBP-1 phosphorylation in live, cultured HepG2 cells in addition to the demonstration that hepatic CK2 activity is elevated in growth-restricted baboon offspring from mothers who received restricted diets during gestation[45], provides strong rationale for our investigation as to whether CK2 is a direct mechanistic link between amino acid depletion and IGFBP-1 phosphorylation.

PKA activity is also sensitive to fluctuations in nutrient availability[51-56] and reduction in its activity has been linked to total IGFBP-1 mRNA and protein expression in HepG2 cells[57,58]. Similarly, overall protein kinase C (PKC) activity is decreased in nutrient deficiency[59-63], and has been linked to elevated IGFBP-1 protein secretion in HepG2 cells[64]. However, whether PKA and PKC are linked to IGFBP-1 phosphorylation, in either regular leucine or leucine-deprived conditions, has not been reported. PKC exists in a variety of isotypes comprising of conventional PKCs (cPKCs: α, βI, βII and γ), novel PKCs (nPKCs: δ, ε, θ and η) and atypical PKCs (aPKCs: ζ, ν and λ)[65] all of which are dynamically regulated by a variety of intra- and extra-cellular stimuli[66]. The placental PKC isoform profile is altered in mice with induced FGR[67,68] although the exact function of each isoform is unknown. Neither PKA nor PKC contain consensus sequence sites for the direct phosphorylation of the IGFBP-1 protein at Ser101, Ser119 or Ser169 (Table 3.1) despite PKA being implicated as an IGFBP-1 kinase when the purified kinase was co-incubated with the substrate *in vitro*[47]. It is possible that PKA and PKC phosphorylate IGFBP-1 at additional Ser or Thr residues for which it shares consensus sequence sites (Table 3.1). IGFBP-1 was previously shown to be phosphorylated at Ser95 and Ser98, suggesting that additional IGFBP-1 residues in addition to Ser101, Ser119 and Ser169 which are hyperphosphorylated in FGR[45,69], may also be prone to phosphorylation[70]. PKC or PKA may indirectly modulate IGFBP-1 phosphorylation (Ser101, Ser119 and

Ser169) in HepG2 cells via phosphorylation at discrete IGFBP-1 residues for which it shares consensus sequences (Table 3.1), or via up-stream signaling networks which induce CK2 activity. For example, both kinases have been implicated down-stream of mechanistic target of rapamycin (mTOR signaling) and may function in a common signaling mechanism to modulate IGFBP-1 phosphorylation by mTOR, which was previously demonstrated to mediate IGFBP-1 phosphorylation via CK2[45].

In this study, we sought to elucidate the roles of CK2, PKC, and PKA in modulating IGFBP-1 phosphorylation (Ser101, Ser119 and Ser169) in leucine-deprived HepG2 cells, which have been validated as an *in vitro* model for human fetal hepatocytes[35-39]. We hypothesized that CK2 activity is induced by leucine deprivation and is directly linked to IGFBP-1 phosphorylation (Ser101, Ser119 and Ser169) in HepG2 cells. We focused on IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169 because these sites have been demonstrate to modulate IGFBP-1 affinity for IGF-I[42,46,71,72] and have been shown by our team to be hyperphosphorylated in FGR[45,69]. Further, based on the down-regulated activity of the PKCs and of PKA in nutrient restriction which has been demonstrated in multiple cell types *in vitro*[51-53,59-63], we hypothesized a possible link between PKC and PKA and IGFBP-1 phosphorylation in leucine deprivation. Due to the fact that Ser101, Ser119 and Ser169 do not fall in the consensus sequences for PKC or PKA, we predict that any identified links between PKC or PKA and IGFBP-1 phosphorylation will be indirect and inflicted via CK2.

To examine the mechanistic links between CK2, PKC and PKA and IGFBP-1 phosphorylation in leucine deprivation, we inhibited CK2, PKC or PKA in regular media (450 µM leucine, equivalent to standard DMEM/F12) and in leucine deprived (0 µM leucine, to ensure maximal IGFBP-1 phosphorylation[46] conditionsand studied changes in IGFBP-1 secretion and phosphorylation (pSer101, pSer119 and pSer169) by western immunoblot analyses of conditioned cell media. To down-regulate overall PKC signaling, we used non-isoform discriminate PKC inhibitor Bisindolylmaleimide (BIS) and pan-PKC siRNA, which targets all cPKCs and nPKCs in addition to aPKCζ, and aPKCν. We attribute functional significance to our findings using our established[45,71] IGF-1R autophosphorylation assay.Whereas direct exposure of purified IGFBP-1 to CK2 or PKA

demonstrated an ability for the two kinases to phosphorylate IGFBP-1*in vitro* in earlier studies[47,49], it was not previously studied whether the direct, intracellular inhibition of these kinases would alter IGFBP-1 phosphorylation in regular (leucine plus) or in leucinedeprived conditions. These previous investigations did not adequately demonstrate that CK2 or PKA were linked to IGFBP-1 phosphorylation in live cells; thus, the direct inhibition of candidate kinases (CK2, PKA, PKC) for IGFBP-1 phosphorylation in HepG2 cells in this study sought to confirm whether the kinases are essential to leucine deprivation-mediated IGFBP-1 phosphorylation *in vitro*. To our knowledge, we provide the first report of the relative effects of inhibiting CK2, PKA or PKC in modulating leucine deprivation-induced IGFBP-1 phosphorylation (pSer101, pSer119, and pSer169) in HepG2 cells, which conclusively illustrate the functional roles of the kinases in modulating IGFBP-1 phosphorylation in leucine restriction which have not been previously reported. Identifying the specific intracellular kinases that are linked to IGFBP-1 phosphorylation in leucine deprivation in cultured HepG2 cells is important to provide insight into the signaling mechanisms that mediate IGFBP-1 phosphorylation in leucine deprivation.

Table 3.1. IGFBP-1 peptide sequence (45-180) and possible phosphorylation sites for CK2, PKC and PKA.

IGFBP-1 peptide sequence (45-180)	^{45}ACGVA**p**T^{50}ARCARGL**p**S^{58}CRALPGEQQPLHALTRGQGACVQESDASAP HAAEAGSPESPE**pS**^{101}TEITEEELLDNFHLMAP**p**S^{119}EEDHSILWDAISTYDG SKALHVTNIKKWKEPCRIELYRVVESLAKAQET**p**S^{169}GEEISKFYLPN^{180} (CK2 sites: TEITEEEL; EEDH; GEEI)	
Kinase consensus sequences:	CK2**: pS/T**-X-X-D/E	**pS**^{101}TEITEEE **p**S^{119}EED **p**S^{169}GEE
	PKC: **pS/T**-X-R/K	**p**T^{50}AR **p**S^{58}CR
	PKA: R/K-R/K-X-**pS/T**	NIL

IGFBP-1 residues phosphorylated in HepG2 cells in response to leucine deprivation[46] and in FGR umbilical cord plasma[45] (Ser101, Ser119 and Ser169) are likely sites for phosphorylation by CK2. Conversely, PKC and PKA are not likely to directly phosphorylate IGFBP-1 at these sites.

3.2 Methods

3.2.1 Cell culture

Human hepatocellular carcinoma (HepG2) cells were purchased from ATCC (Mananassas, VA). HepG2 cells were cultured in DMEM/F-12 supplemented with 10% FBS (Invitrogen Corp., Carlsbad, CA). Cultures were incubated in 20% O_2 and 5% CO_2 and maintained at 37°C.

3.2.2 Leucine deprivation

HepG2 cell treatments were conducted in specialized DMEM/F12 previously deprived and restored of specific amino acids[46]. Cells were incubated in this specialized media supplemented with 450 μM leucine to mimic leucine concentrations in regular DMEM/F12 as a control (leucine plus) or in media that was not supplemented with leucine (0 μM leucine; leucine minus) to ensure maximum induction of IGFBP-1 phosphorylation as previously[46]. The concentrations of all other amino acids were consistent between the two sets of media.

3.2.3 Inhibitor treatments

HepG2 cells were plated in 12-well plates and grown to ~75% confluence. After starvation in 2% FBS (DMEM/F12) for 6 hours, cell media was replaced with specialized leucine plus or leucine minus media containing the various inhibitors. Concentrations for BIS (7.5 μM) and PKI 5-24 (PKI) (100 nM) treatments were determined based on dose-dependency treatments (Appendix C). Phosphorylation of CREB (Ser133) was used to assess changes in PKA activity. TBB was used at a concentration of 1 μM as reported previously[71]. HepG2 cells were incubated with the inhibitors for 24 hours, after which cell media and lysates were prepared as previously[71].

3.2.4 RNA interference (RNAi) silencing

HepG2 cells were plated in 12-well culture plates and grown to 60% confluence. 5 μL Dharmafect transfection reagent 4 (Thermo Scientific, Rockford, IL, USA) was used to transfect 100 nM siRNA against CK2α, CK2α', CK2β (SMARTpool, Thermo Scientific,

Rockford, IL, USA) or pan-PKC (against PKC isoforms α, β, βII, γ, δ, ε, η, θ, ζ, and ν) (Santa Cruz Biotechnology, Dallas, TX, USA) in serum free DMEM/F12 for 24 hours to ensure maximal silencing efficiency. Transfection media containing the transfection reagent was removed 24 hours post-transfection. HepG2 cells were subsequently incubated with specialized leucine plus or leucine minus media for an additional 72 hours. Western immunoblot analysis of CK2α, CK2α' and CK2β demonstrated effective silencing of CK2 subunits, whereas immunoblot analysis of PKCδ and PKCε together represented effective silencing of pan-PKC.

3.2.5 Cell viability assay

To ensure that leucine deprivation and/or chemical BIS did not compromise cell viability, we employed a trypan bBlue exclusion assay. This confirmed that decreases in IGFBP-1 phosphorylation were not attributable to compromised cell vitality due to exposure to the treatment stimuli. Following leucine deprivation and/or TBB or BIS treatments, cells were re-suspended in serum-free DMEM/F12. Cell suspensions were diluted 1:1 with 0.4% trypan blue and counted using the Countess Automated Cell Counter (Life Technologies, Carlsbad, CA). Cell survival was determined as a ratio of live/total cells.

3.2.6 SDS-PAGE and Western Blotting

Equal amounts of cell lysate protein (40-50 μg) were separated by SDS-polyacrylamide gel electrophoresis to determine total expression of CK2α, CK2α', CK2β, PKCδ, PKCε, CREB, IGF-1Rβ, and phosphorylation of CREB (pSer133), IGF-1Rβ (pTyr1135). IGFBP-1 secretion and phosphorylation at Ser101, Ser119 and Ser169 by HepG2 cells was determined by western immunoblot of equal volumes of cell media (40-50 μL). Cell lysates blots were probed with β-actin primary antibody and bands were used for normalization. Equal loading of conditioned cell media was verified using fibrinogen antibody (Appendix D).

To block non-specific protein binding on nitrocellulose membranes, we incubated blots with 5% skim milk diluted in Tris-buffered saline with 0.1 % Tween-20 (TBST) for 1 hour at room temperature. Alternatively, blots for monoclonal IGFBP-1 were blocked

with 5% Bovine Serum Albumin in TBST and blots for total CK2α, CK2α' or CK2β were blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Bad Homburg, Germany). Monoclonal anti-human IGFBP-1 (mAb 6303) was obtained from Medix Biochemica (Kauniainen, Finland) and custom IGFBP-1 polyclonal antibodies against pSer101, pSer119, and pSer169 were generated at YenZyme Antibodies LLC, San Francisco, CA, USA. The custom phosphosite-specific antibodies against pSer101 and pSer169 have been previously validated in the context of non-phosphorylatable IGFBP-1 mutants[71]. The specificity of phosphosite-specific pSer119 antibody was subsequently validated in the same manner[45]. Anti-fibrinogen primary antibody was purchased from Sigma Aldrich (St. Louis, MO, USA) and antibodies against CK2α, CK2α' and CK2β were a kind gift from Dr. D.Litchfield, Western University, London, ON. Remaining primary antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA). All primary antibodies were diluted to 1:1000 with the exception of β-actinwhich was diluted to 1:3000. Peroxidase-labeled goat-anti mouse or goat-anti rabbit secondary antibodies were obtained from BioRad Laboratories Inc., and used at a concentration of 1:10000. Densitometric analyses of bands were conducted using Image Lab (Beta 3) software.

3.2.7 IGF-1 receptor (IGF-1R) activation assay

P6 cells are an immortalized BALB-c3T3 mouse embryo fibroblast cell line derived to over-express IGF-1R[56]. We cultured P6 cells in DMEM/F12 with sodium pyruvate, supplemented with 10% FBS. Post-treatment conditioned HepG2 cell media containing variable concentrations of total and phosphorylated IGFBP-1 were aliquoted to contain equal amounts of total IGFBP-1. In addition to leucine plus and leucine minus conditioned HepG2 cell media which was previously used to assess IGF-1R autophosphorylation in P6 cells (Chapter 2), we used leucine plus and leucine minus conditioned media from HepG2 cells which had been incubated with TBB or BIS. In order to ensure that changes in IGF-1Rβ phosphorylation are caused by differential degrees of IGFBP-1 phosphorylation rather than total circulating IGFBP-1 or differences in media composition between treatments, media aliquots were buffer-exchanged to serum-free P6 cell media (DMEM/F12, with sodium pyruvate) using Amicon Ultra-

0.5 mL Centrifugal Filter Units (Millipore, Darmstadt, Germany) per manufacturer instructions. Buffer-exchanged conditioned media were subsequently incubated with rhIGF-I (25 ng/mL) for two hours at room temperature. P6 cells in 12-well plates were grown to 75% confluency. For treatment, P6 cells were incubated with prepared media aliquots for 10 minutes. Following the incubation period, treatment media was aspirated. Equal amounts of total protein from post-treatment P6 cell lysates were separated using SDS-polyacrylamide gel electrophoresis. Western immunoblots were used to assess changes in IGF-1R autophosphorylation (pTyr1135) using phosphosite-specific IGF-1Rβ (pTyr1135).

3.2.8 CK2 Activity Assay

CK2 activity was measured in HepG2 cell extracts from treatments with leucine deprivation with and without CK2 inhibitor (TBB) or PKC inhibitor (BIS). As previously[71,73], the synthetic peptide substrate DSD (RRRDDDSDDD)(100 µL) which was formerly described[50] was used to assess CK2 activity. Phosphorus-32 (P^{32}) was purchased from PerkinElmer (Waltham, MA, USA) and DSD peptide was a kind gift from Dr. David Litchfield.

3.2.9 Data presentation and statistics

GraphPad Prism 5 (Graph Pad Software Inc., CA) was used for all data analyses. In each independent experiment, the densitometric values for control bands were averaged, and this number was assigned an arbitrary value of 1. Densitometric values for each treatment were averaged among replicates and expressed relative to control. For assessment of statistical significance, we used one-way analysis of variance (ANOVA) with Dunnet's Multiple Comparison Post-Test and expressed results as the mean ± Standard Error of Measurement (SEM). Significance was accepted at *p<0.05, **p=0.01-0.05, ***p<0.01. n=3.

3.3 Results

3.3.1 Silencing of CK2α+α'+β subunits confirms that CK2 contributes to modulating IGFBP-1 phosphorylation but not secretion caused by leucine deprivation

Based on our previous datawhich indicate that pharmacological CK2 inhibitor, TBB, prevents leucine deprivation-induced IGFBP-1 phosphorylation (Chapter 2), we used a targeted approach (RNA*i*) to silence the CK2 holoenzyme by siRNA against the three CK2 subunits (CK2α+α'+β) and assessed changes in total and phosphorylated IGFBP-1 in leucine deprivation. The expression of CK2α and CK2α' were reduced 45-55% and CK2β expression was reduced 50-55% (Appendix B; Supplementary Figure 3.1). We assessed whether CK2 silencing affected leucine deprivation-induced IGFBP-1 secretion and phosphorylation. Leucine deprivation induced IGFBP-1 secretion (+350%) regardless of CK2 status (Figure 3.1A). While leucine deprivation induced IGFBP-1 phosphorylation (pSer101: +800%, pSer119: +300%, pSer169: +600%), leucine-deprived cells also silenced for the CK2 holoenzyme did not phosphorylate IGFBP-1 atany of the three sites. IGFBP-1 phosphorylation was not significantly elevated in HepG2 cells which were both CK2-silenced and leucine-starved compared to control (leucine plus, scrambled siRNA), confirming that leucine deprivation-induced phosphorylation, but not secretion, occurs in a CK2-dependent mechanism (Figure 3.1A-D).

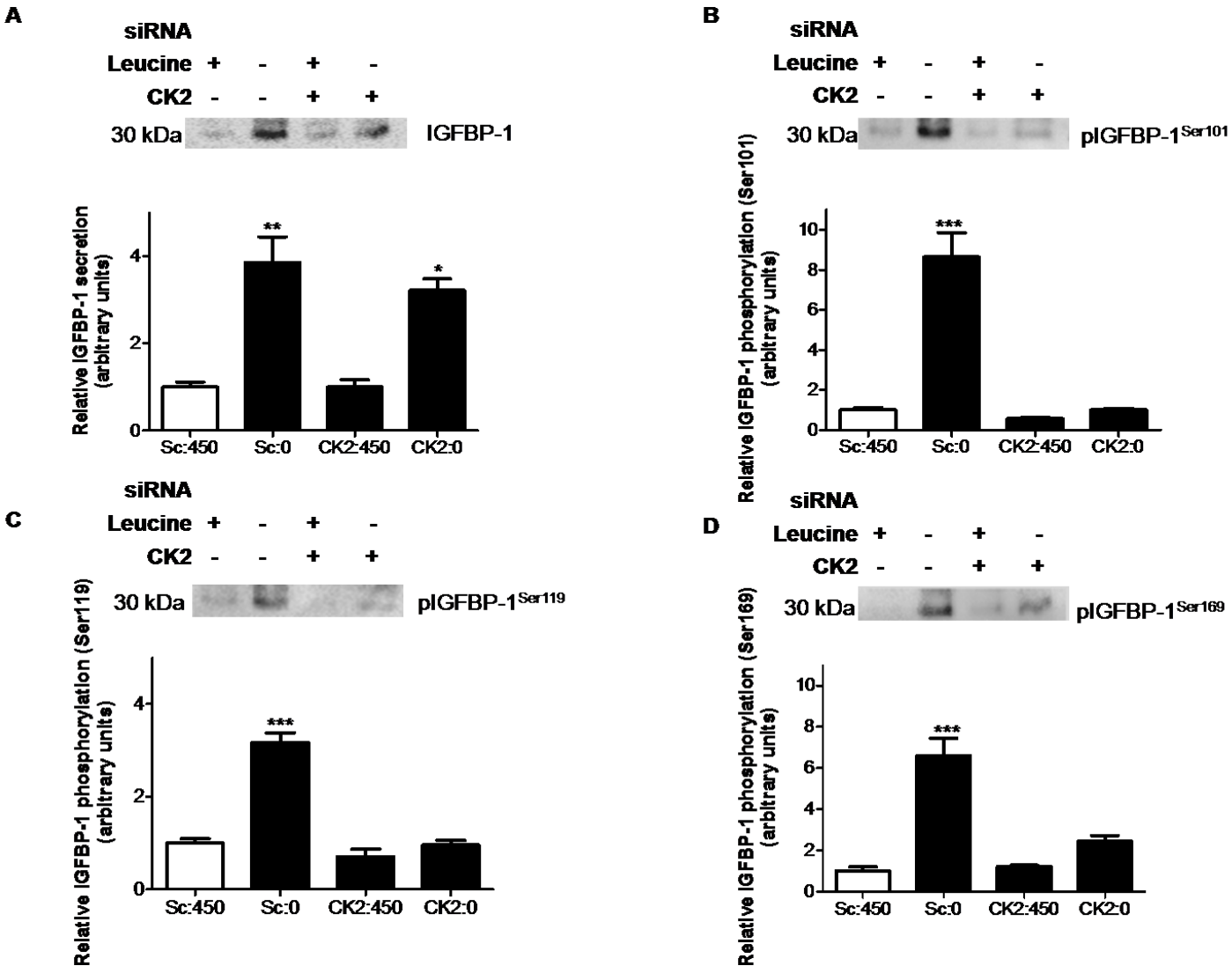


Figure 3.1. The effect of CK2 holoenzyme (α+α'+β) silencing on leucine deprivation-induced IGFBP-1 secretion and phosphorylation. Silencing of the CK2 holoenzyme attenuates IGFBP-1 phosphorylation induced by leucine deprivation. Representative western immunoblots of HepG2 cell media (50 µL per well) treated with scrambled siRNA, or siRNA against the CK2 holoenzyme (α+α'+β) in regular leucine plus or leucine deprived conditions as assessed for A. IGFBP-1 secretion and B. IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. Sc:450: Scrambled siRNA, 450 µM

leucine. Sc:0: Scrambled siRNA, 0 μM leucine. CK2:450 CK2 holoenzyme (α+α'+β) siRNA, 450 μM leucine. CK2:0: CK2 holoenzyme (α+α'+β) siRNA, 0 μM leucine.

3.3.2 Inhibition of PKC signaling with Bisindolylmalemide (BIS) supports that PKC contributes to the modulation of IGFBP-1 phosphorylation caused by leucine deprivation

We use pharmacological PKC inhibitor Bisindolylmalemide (BIS) (7.5 μM) in leucine plus or leucine minus conditions in HepG2 cells. A post-treatment Trypan Blue exclusion assay demonstrated that the vitality of HepG2 cells was not significantly affected by BIS treatments (Appendix B; Supplementary Figure 3.2), confirming that overt cell mortality did not contribute to observed changes in total and phosphorylated IGFBP-1 output by BIS. We assessed whether inhibition of PKC signaling translated to changes in IGFBP-1 secretion and phosphorylation under leucine restriction. We demonstrated that leucine deprivation induced total IGFBP-1 (+250%) regardless of the presence of BIS (Figure 3.2A). However, while leucine deprivation potently induced IGFBP-1 phosphorylation (pSer101: +800%, pSer119: +300%, pSer169: +600%) it was unable to achieve the same effect in the presence of the PKC inhibitor (Figure 3.2B-D). BIS-mediated inhibition of PKC signaling decreased IGFBP-1 phosphorylation at all three phospho-sites (pSer101: -30%, pSer119: -40%, pSer169: -50%), and leucine deprivation-induced changes in IGFBP-1 phosphorylation were not significantly different from control in the presence of BIS (Figures 3.2B-D). These data suggest that PKC signaling is involved in mediating IGFBP-1 phosphorylation under conditions of leucine deprivation.

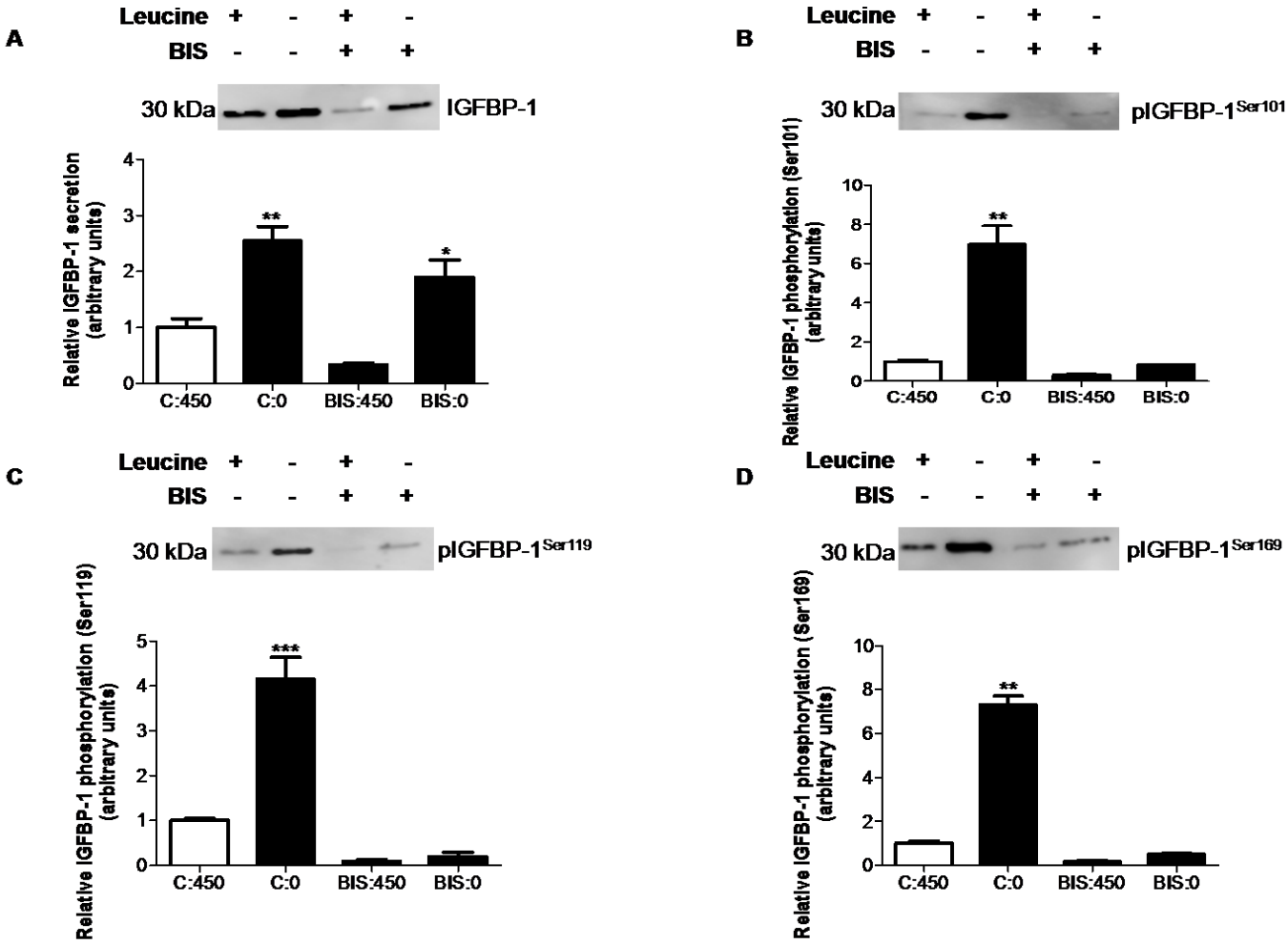


Figure 3.2. Effect of pharmacological pan-PKC inhibitor Bisindolylmaleimide (BIS) on IGFBP-1 secretion and phosphorylation.BIS prevents IGFBP-1 phosphorylation in leucine deprivation, demonstrated via representative western immunoblots of HepG2 cell media indicating A. total IGFBP-1 secretion and B. IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169 in control, leucine deprivation, BIS, and leucine deprivation+BIS treatments. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance;
Dunnet's Multiple Comparison Test; n=3. C:450 Control, 450 μM leucine. C:0: Leucine deprivation, 0 μM leucine. BIS:450: Bisindolylmaleimide (7.5 μM), 450 μM leucine. BIS:0: Bisindolylmaleimide (7.5 μM), 0 μM leucine.

3.3.3 Silencing of PKC confirms that PKC contributes to modulating IGFBP-1 phosphorylation caused by leucine deprivation

To confirm PKC involvement in mediating IGFBP-1 phosphorylation in leucine deprivation, we utilized RNA*i* to specifically knockdown total PKC expression in HepG2 cells. We utilized non-isoform specific siRNA against PKC (pan-PKC), which targets all conventional and novel PKC isoforms (cPKCs: α+βI+βII+γ and nPKCs: δ+ε+η+θ) as well as two out of three atypical PKC isoforms (aPKCs: ζ+ν). cPKCs and nPKCs contain slight structural differences but, unlike aPKCs, are both equally responsive to intracellular activation by diacylglycerols (DAGs) and phorbol esters[65]. We verified efficient PKC silencing via western immunoblot analysis of two representative PKC isoforms, nPKCδ and nPKCε, which are known to be down-regulated in nutrient deprivation[61] and with known prominent expression in HepG2 cells[74], as a distinct PKC isoforms profile has not been characterized. The expression of both PKCδ and PKCε were decreased 50% regardless of leucine status (Appendix B; Supplementary Figure 3.3).

We evaluated the effect of PKC silencing on leucine deprivation-induced IGFBP-1 secretion and phosphorylation. Leucine deprivation induced IGFBP-1 secretion (+450%) regardless of PKC status (Figure 3.3A). Conversely, PKC silencing strongly attenuated the ability of leucine deprivation to induce IGFBP-1 phosphorylation, which was otherwise potently increased (pSer101: +1000%, pSer119: +500%, pSer169: +800%) (Figure 3.3B-D). IGFBP-1 phosphorylation was only moderately induced by leucine deprivation when PKC was silenced (pSer101: +400%, pSer119: +250%, pSer169: +300%). Therefore, silencing of PKC reduces IGFBP-1 phosphorylation in leucine deprivation at all three sites (pSer101: -60%, pSer119: -50%, pSer169: -70%).

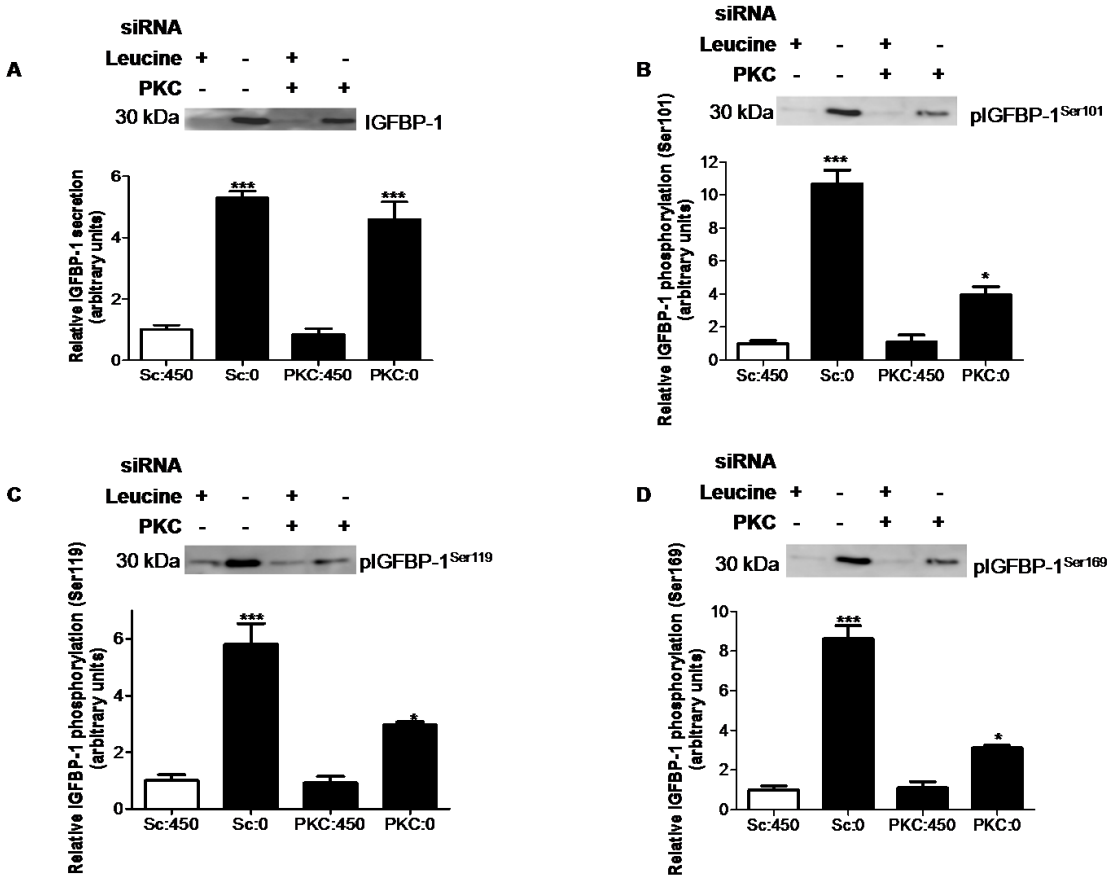
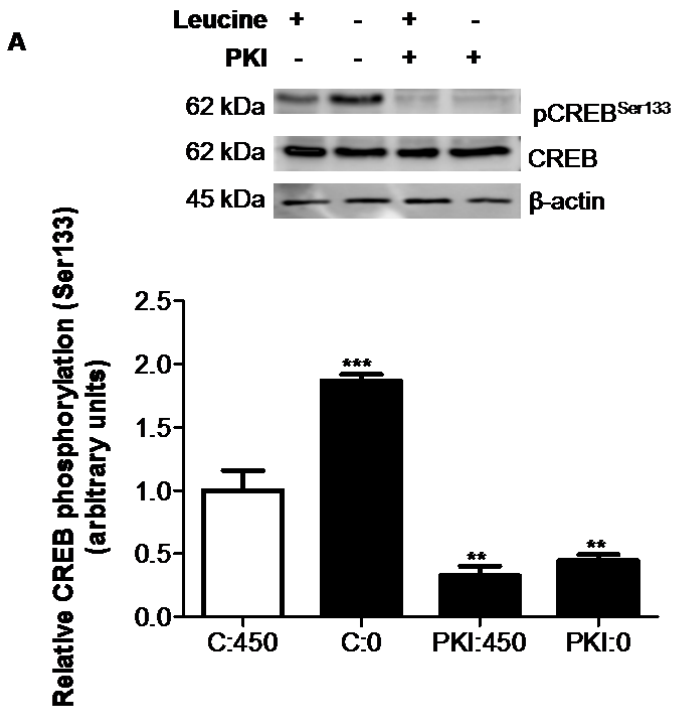


Figure 3.3. Effects of pan-PKC siRNA on IGFBP-1 secretion and phosphorylation in leucine deprivation.RNA*i*-mediated inhibition of PKC mitigates leucine deprivation-induced IGFBP-1 phosphorylation.**A.** A representative western immunoblot of total IGFBP-1 secretion in equal amounts (50 µL) of cell media treated with scrambled or ERK siRNA with and without leucine deprivation. **B-D**. Representative western immunoblots of HepG2 cell media (50 µL) treated with scrambled or pan-PKC siRNA in regular or leucine deprived and assayed for IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ***p< 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.Sc:450 Scrambled siRNA, 450 µM leucine. Sc:0: Scrambled siRNA, 0 µM leucine. PKC: PKC siRNA, 450 µM leucine. PKC:LD: PKC siRNA, 0 µM leucine.

3.3.4 Inhibition of PKA signaling does not affect IGFBP-1 phosphorylation in nutrient deprivation

To investigate whether PKA signaling is involved in regulating IGFBP-1 phosphorylation under leucine deprivation, we used selective PKA inhibitor, PKI (100 nM), in HepG2 cells in leucine plus or leucine minus conditions. We first verified that PKA signaling is sensitive to leucine deprivation by demonstrating an increase in Creb (Ser133) phosphorylation (+200%), which was subsequently reduced (-75%) in the presence of PKI (Figure 3.4A) regardless of leucine status. Figures 3.4B-E indicate that leucine deprivation increased IGFBP-1 secretion (+300%) and phosphorylation (pSer101: +1000%, pSer119: +500%, pSer169: +750%) whether or not PKA was inhibited. These data suggest that PKA inhibition is unable to attenuate IGFBP-1 phosphorylation (pSer101, pSer119, pSer169) induced by leucine deprivation, and that PKA does not modulate hepatic IGFBP-1 secretion phosphorylation under leucine deprivation.



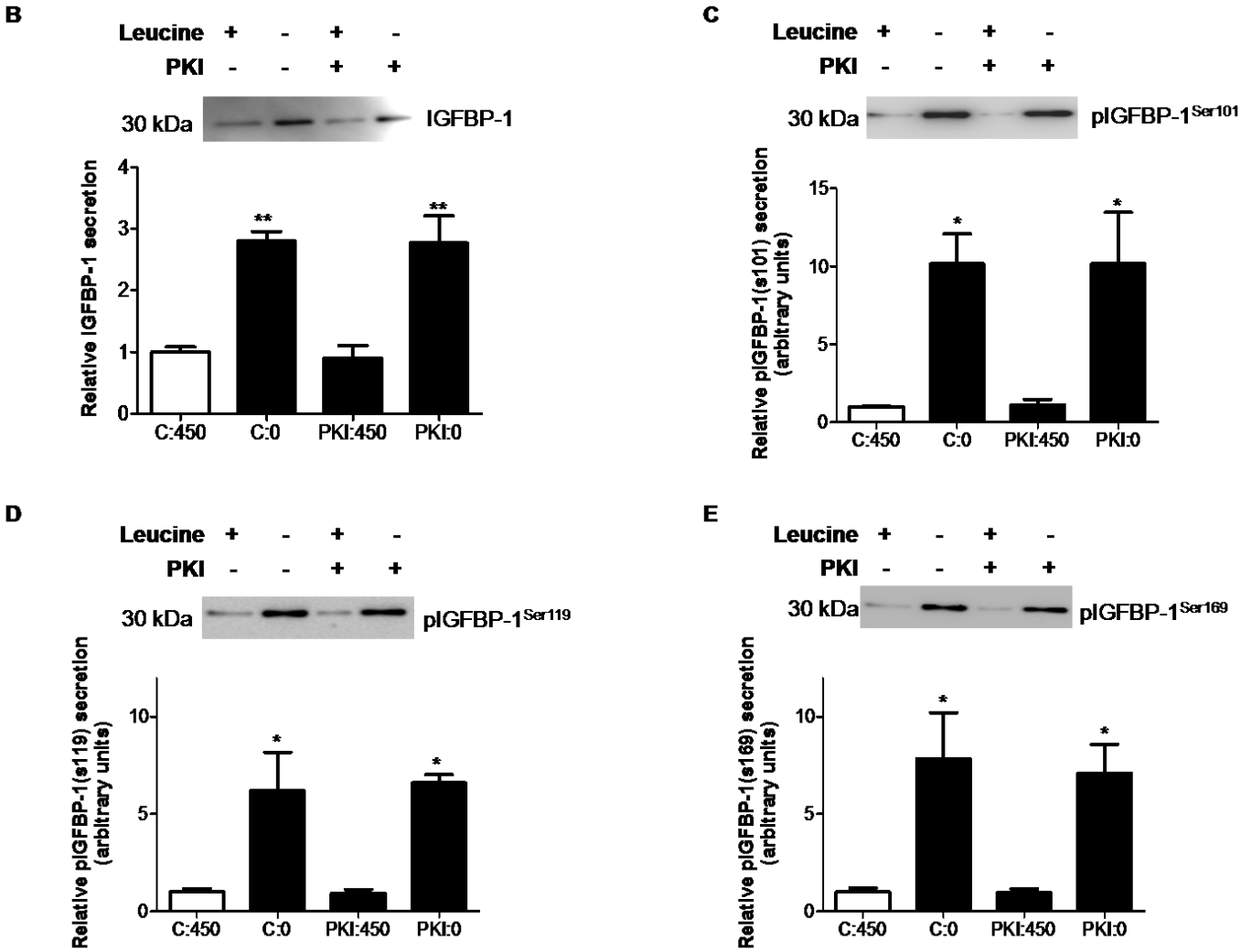


Figure 3.4. Effects of PKI (5-24) inhibition of PKA on leucine deprivation-induced IGFBP-1 secretion and phosphorylation. Leucine deprivation induced and PKI treatment reduced PKA activity, which was not associated with IGFBP-1 phosphorylation in leucine deprivation. Representative western immunoblots of HepG2 cell media indicating **A.** total and phosphorylated Creb (Ser133) as an indicator of PKA activity and **B-E.** IGFBP-1 secretion and phosphorylation at Ser101, Ser119 and Ser169 in control, leucine deprivation, PKI, and leucine deprivation+PKI treatments. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. C: Control, 450 μM leucine. LD: Leucine deprivation, 0 μM leucine. PKI: PKI (5-24) (100 nM), 450 μM leucine. PKI:LD: PKI (5-24) (100 nM), 0 μM leucine.

3.3.5 CK2 or PKC kinase inhibition prevents decrease in IGF-I bioactivity due to IGFBP-1 hyperphosphorylation

We predicted that prevention of leucine deprivation-induced IGFBP-1 phosphorylation by CK2 or PKC inhibition effectively restores IGF-I bioactivity. To asses this, we employed our IGF-1 receptor (IGF-1Rβ) autophosphorylation assay in P6 cells to test for IGF-1R (pTyr1135) autophosphorylation (Figure 3.5), which we used as a measure of IGF-I bioactivity. We assessed IGF-1Rβ autophopshorylatio (pTyr1135) after exposure of the P6 cells to conditioned HepG2 cell media from the various inhibitor treatments. P6 cells over-express IGF-1R but do not express IGF-I[56]. Addition of 25 ng/mL IGF-I only to P6 cell media (positive control) caused a drastic increase in IGF-1R (pTyr1135) phosphorylation (a; +2700%) compared to P6 cells incubated in media not supplemented with IGF-I (negative control) (Figure 3.5), proving that IGF-I addition successfully induces IGF-1R activity in P6 cells. We considered the IGF-1R autophosphorylation induced by the addition of IGF-I only (positive control) as 100% activation of the receptor.

P6 cells were also treated with IGF-I (25 ng/mL) in tandem with conditioned media from HepG2 cells. Firstly, basal phospho-IGFBP-1 levels (from treatments with 450 μM leucine and no inhibitors) was used as a control for the comparison of IGF-1R autophosphorylation to other treatments (lane 3). Next, we used HepG2 conditioned cell mediafrom cellsthat had been previously deprived of leucine and/or treated with chemical ihibitors against CK2 (TBB) or PKC (BIS). The varied volumes of conditioned cell media from each treatment used in the assay were adjusted to contain equal amounts of total IGFBP-1, ensuring that changes in IGF-1Rβ autophosphorylation in treated P6 cells are caused by changes in the degree of phosphorylated IGFBP-1.

As expected, basal phospho-IGFBP-1 levels (from treatments with 450 μM leucine) reduced IGF-1Rβ autophosphorylation (pTyr1135) (b; -45%), regardless of the presence of inhibitors, compared to when P6 cells were treated with IGF-I only (positive control) (Figure 3.5). The reduction in IGF-1R autophosphorylation by this treatment served as an additional positive control for the comparison to pIGFBP-1 levels from other treatments.

Further, the elevated phospho-IGFBP-1 in leucine-deprived HepG2 cell media almost completely abolished IGF-1Rβ autophosphorylation (pTyr1135) when no inhibitor was present in the conditioned cell media (c; -90%) (Figure 3.5). When IGFBP-1 phosphorylation had been prevented by CK2 inhibition (TBB) or PKC inhibition (BIS) in HepG2 cell and the conditioned cell media incubated with P6 cells, IGF-1R activity in P6 cells remained at basal levels.

Therefore, when either CK2 or PKC was inhibited (with TBB or BIS, respectively), leucine deprivation was unable to decrease IGF-I bioactivity (Figure 3.5). These data provide strong evidence that the hyperphosphorylation of IGFBP-1 in leucine deprivation attenuates IGF-I bioactivity via diminished IGF-1Rβ autophosphorylation (pTyr1135) (Figure 3.5), and that this effect is prevented by CK2 or PKC inhibition. These findings are summarized in Table 3.2.

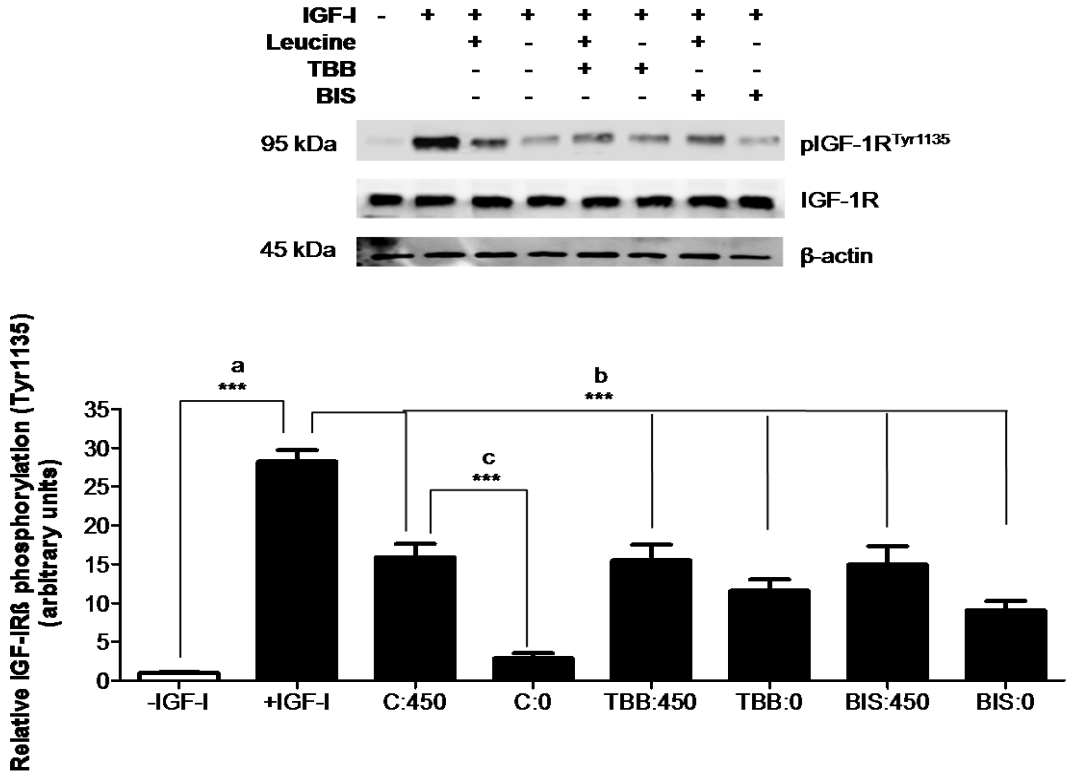


Figure 3.5. The effects of CK2 and PKC inhibition of IGFBP-1 phosphorylation on IGF-1R autophosphorylation.CK2 or PKC inhibition mitigates leucine deprivation-induced reduction of IGF-I bioactivity. HepG2 cell media samples were aliquoted to contain equal concentrations of IGFBP-1 and buffer-exchanged to serum-free P6 media (DMEM/F12 with pyruvate). Aliquots were then incubated with human recombinant IGF-I (25 ng/mL) for 2 hours to allow IGFBP-1 binding to IGF-I, followed by a ten minute exposure to P6 cells to allow induction of IGF-I-mediated IGF-1Rβ autophosphorylation (Tyr1135). The representative western immunoblot of post-treatment P6 cell lysates (50 µg per lane) assessed for IGF-IRβ autophosphorylation (Tyr1135) indicates that leucine deprivation-stimulated IGFBP-1 phosphorylation reduced IGF-1R activation, but was unable to elicit this effect in the presence of BIS or TBB. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. – IGF-I: Negative control, no IGF-I, no IGFBP-1. +IGF-I: Positive control, 25 ng/mL IGF-I, no IGFBP-1. C:450: Control, 450 µM leucine. C:0: Leucine deprivation, 0 µM leucine. TBB:450: TBB (1 µM), 450 µM leucine. TBB:0: TBB (1 µM), 0 µM leucine. BIS:450: Bisindolylmaleimide (7.5 µM), 450 µM leucine. BIS:0: Bisindolylmaleimide (7.5 µM), 0 µM leucine.

Table 3.2. Summary of various treatments on P6 cells and consequent changes in IGF-1Rβ autophosphorylation (Tyr1135) as a measure of IGF-I bioactivity.

Sample	Treatment	Relative IGF-1R (Tyr1135) autophosphorylation	Significance	Conclusion
1 -IGF-I	P6 CM only	Baseline IGF-1R phosphorylation arbitrarily set at 1 (negative control)		
2 +IGF-I	P6 CM + IGF-I	+2700% (positive control)	p<0.001 vs negative control	IGF-I induces IGF-1R autophosphorylation
3 C:450	*HepG2 CM from leucine plus (450 μM) treatment + IGF-I	+1500% (positive control)	p<0.001 vs positive control (lane 2)	Basal pIGFBP-1 levels reduce IGF-1R autophosphorylation compared to when there is no IGFBP-1 present (lane 2)
4 C:0	*HepG2 CM from leucine minus (0 μM) treatment + IGF-I	+250%	p<0.001 vs C:450 (positive control, lane 3)	Leucine deprivation reduces IGF-1R autophosphorylation compare to basal pIGFBP-1 levels
5 TBB:450	*HepG2 CM from leucine plus (450 μM) and TBB treatment + IGF-I	+1500%	p<0.001 vs positive control, n.s. vs C:450 (positive control, lane 3)	IGFBP-1 phosphorylation prevented by TBB did not cause a further reduction of IGF-1R autophosphorylation in leucine deprivation
6 TBB:0	*HepG2 CM from leucine minus (0 μM) and TBB treatment + IGF-I	+1200%	p<0.001 vs positive control, n.s. vs C:450 (positive control, lane 3)	
7 BIS:450	*HepG2 CM from leucine plus (450 μM) and BIS treatment + IGF-I	+1400%	p<0.001 vs positive control, n.s. vs C:450 (positive control, lane 3)	IGFBP-1 phosphorylation prevented by BIS did not cause a further reduction of IGF-1R autophosphorylation in leucine deprivation
8 BIS:0	*HepG2 CM from leucine minus (0 μM) and BIS treatment + IGF-I	+1000%	p<0.001 vs positive control, n.s. vs C:450 (positive control, lane 3)	

A tabular summary of various treatments on P6 cells and their effect on IGF-1R autophosphorylation (Tyr1135). Addition of IGF-I stimulated IGF-1R phosphorylation. All *HepG2 samples contained varied CM volumes that were aliquoted for equal total IGFBP-1. Leucine deprivation (4) reduced IGF-1R autophosphorylation to a greater extent than leucine plus samples (3) only when TBB or BIS were not present (6, 8). CM=conditioned media; *HepG2 CM were buffer-exchanged to P6 cell media prior to treatment. One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. n.s.=not significant.

3.3.6 Inhibition of CK2 (TBB) or PKC (BIS) signaling attenuates leucine deprivation-induced CK2 activity

Utilizing a well-established CK2 activity assay[75] as previously[45], we assessed the effect of leucine deprivation on CK2 activity in HepG2 cell lysates. Leucine deprivation increased CK2 activity (+300%) whereas CK2 inhibitor TBB, reduced CK2 activity (-50%) despite leucine status (Figure 3.6). As anticipated[76], PKC inhibitor (BIS) did not affect CK2 activity in basal conditions; however, BIS completely obstructed the ability of leucine deprivation to induce CK2 activity. We thereby demonstrated that leucine deprivation-mediated induction of CK2 activity is PKC-dependent. These data suggest that PKC contributes to IGFBP-1 phosphorylation via activating CK2 in leucine deprivation, implicating the two kinases in a common mechanism regulating IGFBP-1 phosphorylation in leucine deprivation.

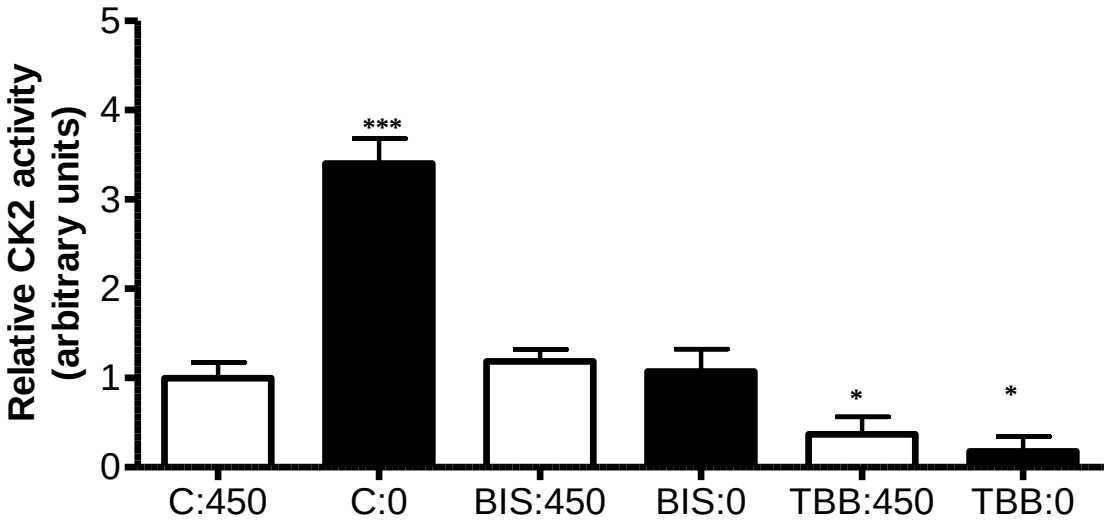


Figure 3.6. Effects of various inhibitor treatments on CK2 activity.A CK2 activity assay demonstrates that leucine deprivation induces CK2 activity, an effect that is attenuated by BIS. TBB decreases CK2 activity regardless of leucine status. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. C:450: Control, 450 μM leucine. C:0: Leucine deprivation, 0 μM leucine. TBB:450: TBB (1 μM), 450 μM leucine. TBB:0: TBB (1 μM), 0 μM leucine. BIS:450: Bisindolylmaleimide (7.5 μM), 450 μM leucine. BIS:0: Bisindolylmaleimide (7.5 μM), 0 μM leucine.

3.4 Discussion

In this study, we demonstrate that CK2 and PKC, but not PKA, are involved in mediating IGFBP-1 phosphorylation (Ser101, Ser119, Ser169) caused by leucine deprivation in HepG2 cells. Inhibition of CK2 or PKC attenuated IGFBP-1 phosphorylation (pSer101, pSer119, pSer169) elicited by leucine deprivation without affecting the induction of total IGFBP-1. Our findings are consistent with our hypothesis that CK2 and PKC modulate hepatic IGFBP-1 phosphorylation in response to leucine deprivation, but contradict our prediction that PKA is also implicated in this mechanism. Importantly, we demonstrate that inhibition of CK2 or PKC prevented leucine deprivation-induced decreases in IGF-I bioactivity via our established[40] IGF-1R autophosphorylation assay, illustrating roles for both CK2 and PKC in modulating IGF-I bioactivity under amino acid (leucine) restriction *in vitro.* Importantly, we demonstrated that PKC inhibition attenuated leucine deprivation-stimulated CK2 activity without affecting CK2 activity in basal conditions, suggesting that PKC likely modulates IGFBP-1 phosphorylation by diminishing CK2 activity in leucine deprivation (Figure 3.6, bar 2 vs. bar 4).

~~This study is the first, to our knowledge, to demonstrate roles for both CK2 and PKC in modulating fetal hepatic IGFBP-1 phosphorylation in response to leucine deprivation.~~ Since the majority of IGF exists in circulation bound to one of the IGFBPs[77], fluctuations in the relatively low amount of free circulating IGF-I can have dramatic effects on its capacity to transduce cell growth and proliferation. As the predominant circulating fetal IGFBP during gestation[28], the ability of IGFBP-1 to modulate IGF-I bioavailability is critical to fetal growth. *In utero* amino acid deprivation is a hallmark of FGR[5,78], and acutely influences IGFBP-1 phosphorylation[46] and IGF-I bioavailability[17,79]. IGFBP-1 phosphorylation is increased in growth-restricted fetuses[44], an occurrence specific to humans[44,80]. Reports from our laboratory have demonstrated that human FGR fetuses display elevated pIGFBP-1 (pSer101, pSer119 and pSer169) in cord serum[45] and in amniotic fluid[69]. Since IGFBP-1 hyperphosphorylation is correlated with reduced IGF-I bioactivity *in vitro* and *in vivo*, such as determined through an IGF-1R phosphorylation assay previously in our lab[71], the kinases and phosphatases which phosphorylate and de-

phosphorylate IGFBP-1, respectively, in leucine deprivation are likely critical in modulating IGF-I bioavailability, and potentially contribute to FGR pathogenesis *in vivo*.

In previous studies, CK2[47,49] and PKA[47] were proposed to phosphorylate IGFBP-1 when the kinases were directly incubated with the purified substrate. However, whether these kinases are mechanistically linked to IGFBP-1 phosphorylation within live cells cannot be extrapolated from these studies, since other kinases may be elicited under various stimuli, such as nutritional stress, to be functionally relevant and impinge on IGFBP-1 phosphorylation in these circumstances. By inhibiting our kinases of interest and measuring IGFBP-1 phosphorylation in conditioned cell media from the same cells, we identify direct links between these kinases and IGFBP-1 phosphorylation in live cells.

We recently illustrated that CK2 is mechanistically linked to IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169 by demonstrating decreased phosphorylated IGFBP-1 in conditioned cell media from HepG2 cells treated with TBB (CK2 inhibitor)[45]. Using a similar *in vitro* approach in the current study, we demonstrate that CK2 is a key mechanistic link between leucine deprivation and IGFBP-1 phosphorylation. Together with our previous[45] observations that CK2 activity is elevated in growth-restricted baboon hepatocytes *in vivo*, our findings implicate CK2 as the likely kinase modulating IGFBP-1 phosphorylation under amino acid deprivation in FGR. PKA, on the other hand, has not been mechanistically linked to IGFBP-1 phosphorylation in live cells. Additionally, PKC has not been studied for its explicit role in regulating IGFBP-1 phosphorylation despite possessing consensus sequence sites for multiple residues on the IGFBP-1 molecule (Table 3.1) and its altered expression in FGR placentas[68]. In addition to Ser101, Ser119 and Ser169 which have been demonstrated by our team to be hyperphosphorylated in FGR[42,45,69], phosphorylation of IGFBP-1 at additional sites such as Ser95 and Ser98 which have been previously identified by us and by others[46, 70,71] may be functionally relevant both independently and through synergistic interactions with Ser101, Ser119 and Ser169 in FGR.

The three phosphorylated residues in IGFBP-1 (Ser101, Ser119, Ser169) in FGR are were identified by LC-MS/MS previously in our lab[81], and are proximal to acidic amino acids

(Table 3.1) adjacent to structured IGF-I-binding (Ser101, Ser169) and in unstructured regulatory (Ser119) regions on the IGFBP-1 molecule[48], making them conducive to direct phosphorylation by CK2[50,82]. In particular, Ser119 and Ser169 contain exact consensus sequences for direct phosphorylation by CK2 (Table 3.1). Leucine deprivation induces CK2 activity, which likely leads to the kinase directly phosphorylating IGFBP-1 at Ser101, Ser119 and Ser169 in HepG2 cells, however, future CK2:IGFBP-1binding studies would clarify whether IGFBP-1 is a true CK2 substrate. On the other hand, IGFBP-1 is less likely to be directly phosphorylated by PKC at Ser101, Ser119 and Ser169, since direct phosphorylation by this kinase requires the proximity of basic amino acid residues to the phospho-acceptor site (consensus site: pS/T-X-R/K)[82] (Table 3.1). It is possible that PKC regulates IGFBP-1 phosphorylation via upstream modulation of CK2 activity, since it was demonstrated here that BIS, a PKC-specific inhibitor, prevents CK2 induction by leucine deprivation without affecting CK2 activity in basal conditions, in concurrence with previous literature reports[76](Figure 3.6). It is also possible that PKC phosphorylates IGFBP-1 at discrete sites, such as pT^{50}AR and pS^{58}CR (Table 3.1), with functional effects on IGFBP-1 activity, which can be investigated in future studies. This finding unequivocally links CK2 and PKC signaling in a common signaling network specifically in leucine deprivation. It is therefore likely that PKC-mediated IGFBP-1 phosphorylation occurs via downstream interactions with CK2 in leucine deprivation.

In our previous reports, phosphorylation at the various Ser residues in IGFB-1 elicited variable affects on IGFBP-1 affinity for IGF-I[46], and mutations which rendered any of the three sites un-phosphorylatable potentiated IGF-I bioactivity, as determined via IGF-1R autophosphorylation in HepG2 cells[71]. However, the exact conformational changes on the IGFBP-1 molecule elicited by phosphorylation at Ser101, Ser119 and Ser169 that affect its affinity for IGF-I are unknown. It is likely that phosphorylation at Ser101, Ser119 and Ser169 function concomitantly to induce conformational changes which increase IGFBP-1 affinity for IGF-I. To assess this hypothesis, future structural studies on CK2/IGFBP-1 binding will be valuable. Overall PKC activity is reduced and the expression patterns of the various isoforms are altered in cellular nutritional stress[59,60,83-85]. The isoform-specific distribution and functions of PKC are not well understood. Unlike the cPKCs, nPKCs do

not contain the C2 regulatory domain; however, the functional relevance of this structural difference has not been established[65]. Comparatively, aPKCs lack the structural components of cPKCs and nPKCs that are required for their activation by diacylglycerols and phorbal esters, suggesting atypical intracellular regulation of the aPKCs[65]. Assaying the down-regulated expression of all PKCs silenced by siRNA against pan-PKC was not realistic in this study. Therefore, we verified efficient PKC knockdown using the expression levels of nPKCδ and nPKCε as representatives as measures of the efficiency of pan-PKC siRNA, as these isotypes have been established to be predominantly expressed in HepG2 cells and have demonstrated to be sensitive to nutritional status[61,74]. Down-regulation of overall PKC activity has been linked to total IGFBP-1 mRNA and protein expression[64,86,87], although its effects on post-translational regulation of IGFBP-1 have not been classified. ~~We report for the first time that PKC is involved in modulating IGFBP-1 phosphorylation in leucine deprivation, and that PKC function is necessary for leucine deprivation to induce CK2 activity *in vitro*.~~ The placentas of rats with glucocorticoid-induced FGR display an altered pattern of PKC isoform expression[67]. This suggests that PKC signaling is dynamically implicated in FGR although the exact role of PKC function is FGR has not been established. If PKC modulates CK2 activity in growth-restricting conditions *in vivo*, it is possible that PKC and CK2 are mechanistically linked to IGFBP-1 phosphorylation in FGR.

~~Although PKA activity is associated with IGFBP-1 phosphorylation in endometrial stromal cells[44],~~ The inability of PKA inhibition to effectively prevent IGFBP-1 phosphorylation in HepG2 cells confers specificity to CK2 and PKC in the mediation[47] of leucine deprivation-stimulated phosphorylation of hepatic IGFBP-1. Therefore, CK2 and PKC, but not PKA, may be critical in modulating IGFBP-1 phosphorylation in the fetal compartment in FGR. Our results indicate that CK2 and PKC modulate leucine deprivation-induced IGFBP-1 hyperphosphorylation at all three sites (pSer101, pSer119 and pSer169). Whether phosphorylation of Ser101, Ser119 and Ser169 is independently regulated or a collection of co-dependent phosphorylation events is a topic for future exploration. Similar to our previous results (Chapter 2), we have demonstrated here that leucine deprivation most potently stimulates IGFBP-1 phosphorylation at Ser101 and

Ser169. During pregnancy, placental alkaline phosphatase de-phosphorylates IGFBP-1 in order to increase IGF-I bioavailability[88]. Therefore, de-phosphorylation of IGFBP-1 may be an additional mechanism by which cells modulate IGF-I bioavailability in FGR.

In conclusion, our present study identifies protein kinases CK2 and PKC as critical mediators of IGFBP-1 phosphorylation under nutrient (leucine) deprivation *in vitro*. Taken together with our previous studies[45], we speculate that CK2 is the key modulator of IGFBP-1 phosphorylation in response to amino acid restriction in FGR. Our novel demonstration that PKC is also linked to IGFBP-1 phosphorylation in leucine deprivation *in vitro*, and that PKC inhibition attenuates leucine deprivation-induced CK2 activity, illustrates a potential role for PKC signaling in a combined signaling mechanism with CK2 in modulating IGF-I bioavailability in FGR. Since leucine deprivation-induced decreases in IGF-I bioactivity are prevented by inhibition of CK2 or PKC, our findings bare physiological relevance. *In vivo*, the targeted inhibition of CK2 or PKC activity to circumvent amino acid deprivation-induced loss of IGF-I bioactivity may be valuable. By linking CK2 and PKC in a common mechanism modulating IGFBP-1 phosphorylation in leucine deprivation *in vitro*, we are one step closer to unraveling the molecular mechanisms underlying *in utero* FGR.

3.5 References

1. Pallotto EK, Kilbride HW. Perinatal outcome and later implications of intrauterine growth restriction. *Clin Obstet Gynecol*. 2006;49(2):257-269.

2. Barker DJ. Adult consequences of fetal growth restriction. *Clin Obstet Gynecol*. 2006;49(2):270-283.

3. Garite TJ, Clark R, Thorp JA. Intrauterine growth restriction increases morbidity and mortality among premature neonates. *Am J Obstet Gynecol*. 2004;191(2):481-487.

4. Bajoria R, Sooranna SR, Ward S, Hancock M. Placenta as a link between amino acids, insulin-IGF axis, and low birth weight: Evidence from twin studies. *J Clin Endocrinol Metab*. 2002;87(1):308-315.

5. Maulik D. Fetal growth restriction: The etiology. *Clin Obstet Gynecol*. 2006;49(2):228-235.

6. Regnault TR, de Vrijer B, Galan HL, Wilkening RB, Battaglia FC, Meschia G. Umbilical uptakes and transplacental concentration ratios of amino acids in severe fetal growth restriction. *Pediatr Res*. 2013;73(5):602-611. doi: 10.1038/pr.2013.30; 10.1038/pr.2013.30.

7. Murphy VE, Smith R, Giles WB, Clifton VL. Endocrine regulation of human fetal growth: The role of the mother, placenta, and fetus. *Endocr Rev*. 2006;27(2):141-169.

8. Leger J, Oury JF, Noel M, et al. Growth factors and intrauterine growth retardation. I. serum growth hormone, insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein 3 levels in normally grown and growth-retarded human fetuses during the second half of gestation. *Pediatr Res*. 1996;40(1):94-100. doi: 10.1203/00006450-199607000-00017.

9. Parks JS. The ontogeny of growth hormone sensitivity. *Horm Res*. 2001;55 Suppl 2:27-31.

10. Funakoshi T, Ueda Y, Kobayashi A, Morikawa H, Mochizuki M. Studies on insulin-like growth factors (IGF-I, -II) and there binding proteins in normal human pregnancy. *Nihon Naibunpi Gakkai Zasshi*. 1990;66(7):688-699.

11. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (igf-1) and type 1 IGF receptor (Igf1r). *Cell*. 1993;75(1):59-72.

12. D'Ercole AJ, Calikoglu AS. Editorial review: The case of local versus endocrine IGF-I actions: The jury is still out. *Growth Horm IGF Res*. 2001;11(5):261-265.

13. Constancia M, Angiolini E, Sandovici I, et al. Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. *Proc Natl Acad Sci U S A*. 2005;102(52):19219-19224.

14. Fowden AL. The insulin-like growth factors and feto-placental growth. *Placenta*. 2003;24(8-9):803-812.

15. Skjaerbaek C, Frystyk J, Orskov H, Flyvbjerg A. Free IGF-I, IGFBP-1, and the binary complex of IGFBP-1 and IGF-I are increased during human pregnancy. *Horm Res*. 2004;62(5):215-220.

16. Bauer MK, Harding JE, Bassett NS, et al. Fetal growth and placental function. *Mol Cell Endocrinol*. 1998;140(1-2):115-120.

17. Kaaks R. Nutrition, insulin, IGF-1 metabolism and cancer risk: A summary of epidemiological evidence. *Novartis Found Symp*. 2004;262:247-60; discussion 260-68.

18. Gluckman PD, Johnson-Barrett JJ, Butler JH, Edgar BW, Gunn TR. Studies of insulin-like growth factor -I and -II by specific radioligand assays in umbilical cord blood. *Clin Endocrinol (Oxf)*. 1983;19(3):405-413.

19. Lassarre C, Hardouin S, Daffos F, Forestier F, Frankenne F, Binoux M. Serum insulin-like growth factors and insulin-like growth factor binding proteins in the human fetus. relationships with growth in normal subjects and in subjects with intrauterine growth retardation. *Pediatr Res*. 1991;29(3):219-225.

20. Verhaeghe J, Van Bree R, Van Herck E, Laureys J, Bouillon R, Van Assche FA. C-peptide, insulin-like growth factors I and II, and insulin-like growth factor binding protein-1 in umbilical cord serum: Correlations with birth weight. *Am J Obstet Gynecol*. 1993;169(1):89-97.

21. Giudice LC, de Zegher F, Gargosky SE, et al. Insulin-like growth factors and their binding proteins in the term and preterm human fetus and neonate with normal and extremes of intrauterine growth. *J Clin Endocrinol Metab*. 1995;80(5):1548-1555.

22. Chard T. Insulin-like growth factors and their binding proteins in normal and abnormal human fetal growth. *Growth Regul*. 1994;4(3):91-100.

23. Cianfarani S, Germani D, Rossi P, et al. Intrauterine growth retardation: Evidence for the activation of the insulin-like growth factor (IGF)-related growth-promoting machinery and the presence of a cation-independent IGF binding protein-3 proteolytic activity by two months of life. *Pediatr Res*. 1998;44(3):374-380.

24. Han VK, Bassett N, Walton J, Challis JR. The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: Evidence for IGF-IGFBP interactions at the feto-maternal interface. *J Clin Endocrinol Metab*. 1996;81(7):2680-2693.

25. Watson CS, Bialek P, Anzo M, Khosravi J, Yee SP, Han VK. Elevated circulating insulin-like growth factor binding protein-1 is sufficient to cause fetal growth restriction. *Endocrinology*. 2006;147(3):1175-1186. doi: 10.1210/en.2005-0606.

26. Martina NA, Kim E, Chitkara U, Wathen NC, Chard T, Giudice LC. Gestational age-dependent expression of insulin-like growth factor-binding protein-1 (IGFBP-1) phosphoisoforms in human extraembryonic cavities, maternal serum, and decidua

suggests decidua as the primary source of IGFBP-1 in these fluids during early pregnancy. *J Clin Endocrinol Metab*. 1997;82(6):1894-1898.

27. Tapanainen PJ, Bang P, Wilson K, Unterman TG, Vreman HJ, Rosenfeld RG. Maternal hypoxia as a model for intrauterine growth retardation: Effects on insulin-like growth factors and their binding proteins. *Pediatr Res*. 1994;36(2):152-158.

28. Han VK, Matsell DG, Delhanty PJ, Hill DJ, Shimasaki S, Nygard K. IGF-binding protein mRNAs in the human fetus: Tissue and cellular distribution of developmental expression. *Horm Res*. 1996;45(3-5):160-166.

29. Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: Effects on affinity for IGF-I. *Proc Natl Acad Sci U S A*. 1991;88(17):7481-7485.

30. Burch WM, Correa J, Shively JE, Powell DR. The 25-kilodalton insulin-like growth factor (IGF)-binding protein inhibits both basal and IGF-I-mediated growth of chick embryo pelvic cartilage in vitro. *J Clin Endocrinol Metab*. 1990;70(1):173-180.

31. Cox GN, McDermott MJ, Merkel E, et al. Recombinant human insulin-like growth factor (IGF)-binding protein-1 inhibits somatic growth stimulated by IGF-I and growth hormone in hypophysectomized rats. *Endocrinology*. 1994;135(5):1913-1920.

32. Rutanen EM. Insulin-like growth factors and insulin-like growth factor binding proteins in the endometrium. effect of intrauterine levonorgestrel delivery. *Hum Reprod*. 2000;15 Suppl 3:173-181.

33. Rutanen EM, Pekonen F, Nyman T, Wahlstrom T. Insulin-like growth factors and their binding proteins in benign and malignant uterine diseases. *Growth Regul*. 1993;3(1):74-77.

34. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev*. 2002;23(6):824-854.

35. Kelly JH, Darlington GJ. Modulation of the liver specific phenotype in the human hepatoblastoma line hep G2. *In Vitro Cell Dev Biol*. 1989;25(2):217-222.

36. Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab Dispos*. 2003;31(8):1035-1042.

37. Hart SN, Li Y, Nakamoto K, Subileau EA, Steen D, Zhong XB. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metab Dispos*. 2010;38(6):988-994.

38. Pal R, Mamidi MK, Das AK, Gupta PK, Bhonde R. A simple and economical route to generate functional hepatocyte-like cells from hESCs and their application in evaluating alcohol induced liver damage. *J Cell Biochem*. 2012;113(1):19-30.

39. Maruyama M, Matsunaga T, Harada E, Ohmori S. Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biol Pharm Bull*. 2007;30(11):2091-2097.

40. Westwood M, Gibson JM, White A. Purification and characterization of the insulin-like growth factor-binding protein-1 phosphoform found in normal plasma. *Endocrinology*. 1997;138(3):1130-1136.

41. Bankowski E, Sobolewski K, Palka J, Jaworski S. Decreased expression of the insulin-like growth factor-I-binding protein-1 (IGFBP-1) phosphoisoform in pre-eclamptic wharton's jelly and its role in the regulation of collagen biosynthesis. *Clin Chem Lab Med*. 2004;42(2):175-181.

42. Nissum M, Abu Shehab M, Sukop U, et al. Functional and complementary phosphorylation state attributes of human insulin-like growth factor-binding protein-1 (IGFBP-1) isoforms resolved by free flow electrophoresis. *Mol Cell Proteomics*. 2009;8(6):1424-1435.

43. Frost RA, Bereket A, Wilson TA, Wojnar MM, Lang CH, Gelato MC. Phosphorylation of insulin-like growth factor binding protein-1 in patients with insulin-dependent diabetes mellitus and severe trauma. *J Clin Endocrinol Metab*. 1994;78(6):1533-1535.

44. Iwashita M, Sakai K, Kudo Y, Takeda Y. Phosphoisoforms of insulin-like growth factor binding protein-1 in appropriate-for-gestational-age and small-for-gestational-age fetuses. *Growth Horm IGF Res*. 1998;8(6):487-493.

45. Abu Shehab M, Damerill I, Shen T, et al. Liver mTOR controls IGF-I bioavailability by regulation of protein kinase CK2 and IGFBP-1 phosphorylation in fetal growth restriction. *Endocrinology*. 2014;155(4):1327-1339. doi: 10.1210/en.2013-1759; 10.1210/en.2013-1759.

46. Seferovic MD, Ali R, Kamei H, et al. Hypoxia and leucine deprivation induce human insulin-like growth factor binding protein-1 hyperphosphorylation and increase its

biological activity. *Endocrinology*. 2009;150(1):220-231. doi: 10.1210/en.2008-0657; 10.1210/en.2008-0657.

47. Frost RA, Tseng L. Insulin-like growth factor-binding protein-1 is phosphorylated by cultured human endometrial stromal cells and multiple protein kinases in vitro. *J Biol Chem*. 1991;266(27):18082-18088.

48. Sitar T, Popowicz GM, Siwanowicz I, Huber R, Holak TA. Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins. *Proc Natl Acad Sci U S A*. 2006;103(35):13028-13033.

49. Ankrapp DP, Jones JI, Clemmons DR. Characterization of insulin-like growth factor binding protein-1 kinases from human hepatoma cells. *J Cell Biochem*. 1996;60(3):387-399.

50. Litchfield DW. Protein kinase CK2: Structure, regulation and role in cellular decisions of life and death. *Biochem J*. 2003;369(Pt 1):1-15.

51. O'Brien LJ, Levac KD, Nagy LE. Moderate dietary protein and energy restriction modulate cAMP-dependent protein kinase activity in rat liver. *J Nutr*. 1998;128(6):927-933.

52. Milanski M, Arantes VC, Ferreira F, et al. Low-protein diets reduce PKAalpha expression in islets from pregnant rats. *J Nutr*. 2005;135(8):1873-1878.

53. Stephen LL, Nagy LE. Very low protein diets induce a rapid decrease in hepatic cAMP-dependent protein kinase followed by a lower increase in adenylyl cyclase activity in rats. *J Nutr*. 1996;126(7):1799-1807.

54. Rozwadowski M, Stephen LL, Goss PM, Bray TM, Nagy LE. Activity of cAMP-dependent protein kinase is reduced in protein-energy malnourished rats. *J Nutr*. 1995;125(3):401-409.

55. Goss PM, Bray TM, Nagy LE. Regulation of hepatocyte glutathione by amino acid precursors and cAMP in protein-energy malnourished rats. *J Nutr*. 1994;124(3):323-330.

56. Pietrzkowski Z, Sell C, Lammers R, Ullrich A, Baserga R. Roles of insulinlike growth factor 1 (IGF-1) and the IGF-1 receptor in epidermal growth factor-stimulated growth of 3T3 cells. *Mol Cell Biol*. 1992;12(9):3883-3889.

57. Suwanichkul A, DePaolis LA, Lee PD, Powell DR. Identification of a promoter element which participates in cAMP-stimulated expression of human insulin-like growth factor-binding protein-1. *J Biol Chem*. 1993;268(13):9730-9736.

58. Frost RA, Nystrom GJ, Lang CH. Stimulation of insulin-like growth factor binding protein-1 synthesis by interleukin-1beta: Requirement of the mitogen-activated protein kinase pathway. *Endocrinology*. 2000;141(9):3156-3164. doi: 10.1210/endo.141.9.7641.

59. Ozanne SE, Olsen GS, Hansen LL, et al. Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. *J Endocrinol*. 2003;177(2):235-241.

60. Lee MY, Jo SD, Lee JH, Han HJ. L-leucine increases [3H]-thymidine incorporation in chicken hepatocytes: Involvement of the PKC, PI3K/Akt, ERK1/2, and mTOR signaling pathways. *J Cell Biochem*. 2008;105(6):1410-1419.

61. Vary TC, Goodman S, Kilpatrick LE, Lynch CJ. Nutrient regulation of PKCepsilon is mediated by leucine, not insulin, in skeletal muscle. *Am J Physiol Endocrinol Metab*. 2005;289(4):E684-94.

62. da Silva Lippo BR, Batista TM, de Rezende LF, et al. Low-protein diet disrupts the crosstalk between the PKA and PKC signaling pathways in isolated pancreatic islets. *J Nutr Biochem*. 2015.

63. Tang B, Zhang Y, Liang R, et al. Activation of the delta-opioid receptor inhibits serum deprivation-induced apoptosis of human liver cells via the activation of PKC and the mitochondrial pathway. *Int J Mol Med*. 2011;28(6):1077-1085.

64. Lee PD, Abdel-Maguid LS, Snuggs MB. Role of protein kinase-C in regulation of insulin-like growth factor-binding protein-1 production by HepG2 cells. *J Clin Endocrinol Metab*. 1992;75(2):459-464.

65. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiol Rev*. 2008;88(4):1341-1378.

66. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*. 1992;258(5082):607-614.

67. Sugden MC, Langdown ML. Possible involvement of PKC isoforms in signalling placental apoptosis in intrauterine growth retardation. *Mol Cell Endocrinol*. 2001;185(1-2):119-126.

68. Thompson NM, Norman AM, Donkin SS, et al. Prenatal and postnatal pathways to obesity: Different underlying mechanisms, different metabolic outcomes. *Endocrinology*. 2007;148(5):2345-2354.

69. Abu Shehab M, Khosravi J, Han VK, Shilton BH, Gupta MB. Site-specific IGFBP-1 hyper-phosphorylation in fetal growth restriction: Clinical and functional relevance. *J Proteome Res*. 2010;9(4):1873-1881.

70. Dolcini L, Sala A, Campagnoli M, et al. Identification of the amniotic fluid insulin-like growth factor binding protein-1 phosphorylation sites and propensity to proteolysis of the isoforms. *FEBS J*. 2009;276(20):6033-6046.

71. Abu Shehab M, Iosef C, Wildgruber R, Sardana G, Gupta MB. Phosphorylation of IGFBP-1 at discrete sites elicits variable effects on IGF-I receptor autophosphorylation. *Endocrinology*. 2013;154(3):1130-1143. doi: 10.1210/en.2012-1962; 10.1210/en.2012-1962.

72. Jones JI, Busby WH,Jr, Wright G, Smith CE, Kimack NM, Clemmons DR. Identification of the sites of phosphorylation in insulin-like growth factor binding protein-1. regulation of its affinity by phosphorylation of serine 101. *J Biol Chem*. 1993;268(2):1125-1131.

73. Vilk G, Saulnier RB, St Pierre R, Litchfield DW. Inducible expression of protein kinase CK2 in mammalian cells. evidence for functional specialization of CK2 isoforms. *J Biol Chem*. 1999;274(20):14406-14414.

74. Rypka M, Cervenkova K, Uherkova L, Poczatkova H, Bogdanova K, Vesely J. Changes in mRNA levels of intracellular fatty acid metabolism regulators in human hepatoma HepG2 cells following their treatment with non-esterified fatty acids and dehydroepiandrosterone. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2005;149(2):251-256.

75. Litchfield DW, Lozeman FJ, Piening C, et al. Subunit structure of casein kinase II from bovine testis. demonstration that the alpha and alpha' subunits are distinct polypeptides. *J Biol Chem*. 1990;265(13):7638-7644.

76. Channavajhala P, Seldin DC. Functional interaction of protein kinase CK2 and c-myc in lymphomagenesis. *Oncogene*. 2002;21(34):5280-5288.

77. Fant ME, Weisoly D. Insulin and insulin-like growth factors in human development: Implications for the perinatal period. *Semin Perinatol*. 2001;25(6):426-435.

78. Miller J, Turan S, Baschat AA. Fetal growth restriction. *Semin Perinatol*. 2008;32(4):274-280. doi: 10.1053/j.semperi.2008.04.010; 10.1053/j.semperi.2008.04.010.

79. Bennett A, Wilson DM, Liu F, Nagashima R, Rosenfeld RG, Hintz RL. Levels of insulin-like growth factors I and II in human cord blood. *J Clin Endocrinol Metab*. 1983;57(3):609-612.

80. Peterkofsky B, Gosiewska A, Wilson S, Kim YR. Phosphorylation of rat insulin-like growth factor binding protein-1 does not affect its biological properties. *Arch Biochem Biophys*. 1998;357(1):101-110.

81. Abu Shehab M, Inoue S, Han VK, Gupta MB. Site specific phosphorylation of insulin-like growth factor binding protein-1 (IGFBP-1) for evaluating clinical relevancy in fetal growth restriction. *J Proteome Res*. 2009;8(11):5325-5335.

82. Coverley JA, Baxter RC. Phosphorylation of insulin-like growth factor binding proteins. *Mol Cell Endocrinol*. 1997;128(1-2):1-5.

83. Nishitani S, Matsumura T, Fujitani S, Sonaka I, Miura Y, Yagasaki K. Leucine promotes glucose uptake in skeletal muscles of rats. *Biochem Biophys Res Commun*. 2002;299(5):693-696.

84. Farese RV, Sajan MP, Standaert ML. Insulin-sensitive protein kinases (atypical protein kinase C and protein kinase B/Akt): Actions and defects in obesity and type II diabetes. *Exp Biol Med (Maywood)*. 2005;230(9):593-605.

85. Ignacio-Souza LM, Reis SR, Arantes VC, et al. Protein restriction in early life is associated with changes in insulin sensitivity and pancreatic beta-cell function during pregnancy. *Br J Nutr*. 2013;109(2):236-247.

86. Kachra Z, Yang CR, Murphy LJ, Posner BI. The regulation of insulin-like growth factor-binding protein 1 messenger ribonucleic acid in cultured rat hepatocytes: The roles of glucagon and growth hormone. *Endocrinology*. 1994;135(5):1722-1728.

87. Gong Y, Ballejo G, Alkhalaf B, Molnar P, Murphy LC, Murphy LJ. Phorbol esters differentially regulate the expression of insulin-like growth factor-binding proteins in endometrial carcinoma cells. *Endocrinology*. 1992;131(6):2747-2754.

88. Solomon AL, Siddals KW, Baker PN, Gibson JM, Aplin JD, Westwood M. Placental alkaline phosphatase de-phosphorylates insulin-like growth factor (IGF)-binding protein-1. *Placenta*. 2014;35(7):520-522.

Chapter 4

Summary and Conclusions

4.1 Summary of Findings

Amino acid (leucine) deprivation stimulated IGFBP-1 phosphorylation at Ser101, Ser119 and Ser169 in all experiments conducted in HepG2 cells (Chapters 2 and 3), as anticipated from previous reports[1]. However, the molecular mechanisms modulating hepatic IGFBP-1 phosphorylation in leucine deprivation had previously remained elusive. Through selective manipulation of the mTOR and AAR signaling cascades (Chapter 2) as well as protein kinases CK2, PKC and PKA (Chapter 3) in leucine deprived conditions, we have successfully shed light on the signaling pathways linking reduced leucine supply to stimulated IGFBP-1 phosphorylation *in vitro*. These findings contribute insight to how nutrient deprivation leads to decreased fetal growth, by way of increased IGFBP-1 phosphorylation, in FGR.

In Chapter 2, we show that manipulation of hepatic amino acid-sensing mTOR and AAR pathways variably affected the ability of leucine deprivation to elicit IGFBP-1 phosphorylation. Based on previous findings by our group that mTOR modulates IGFBP-1 phosphorylation in baboon fetal hepatocytes from a primate model for FGR[2], and considering that mTOR is critically involved in placental nutrient sensing and is highly sensitive to amino acid status[3], we had initially hypothesized that mTORC1+C2 signaling was the primary mechanism by which IGFBP-1 phosphorylation is induced under leucine deprivation. We demonstrated that the fetal hepatic (HepG2) mTOR pathway was indeed down-regulated by leucine restriction, as expected; however, this was linked to only a partial induction of the IGFBP-1 phosphorylation otherwise caused by leucine deprivation. We concluded that mTOR signaling only partially modulated IGFBP-1 phosphorylation under leucine deprivation because although leucine deprivation reduces mTORC1+C2 activity, it stimulates IGFBP-1 phosphorylation to a greater extent than mTORC1+C2 inhibition by itself. Importantly, although leucine deprivation and mTOR inhibition both separately induced IGFBP-1 phosphorylation to different extents, their effects were not cumulative when combined. This observation suggested that mTOR-mediated induction of IGFBP-1 phosphorylation occurred in a common mechanism with leucine deprivation.

We verified our findings via constitutive activation of mTORC1+C2 by DEPTOR silencing, which resulted in the inability of leucine deprivation to inhibit mTORC1+C2 activity. This prevented leucine deprivation from eliciting IGFBP-1 secretion; however, IGFBP-1 phosphorylation (Ser101, Ser119 and Ser169) was nevertheless induced by leucine deprivation. These observations confirmed that although mTOR is a key mediator of IGFBP-1 secretion under leucine restriction, there was a need to explore additional cellular mechanisms that the cell may rely on to induce IGFBP-1 phosphorylation under nutritional stress.

The observation that alternate mechanisms in addition to mTOR are elicited by leucine deprivation to induce IGFBP-1 phosphorylation prompted the investigation of the AAR. The AAR is a specific cellular response to restricted amino acid availability[4], and is known to be systemically involved in mediating the stress response to nutritional insufficiency to down-regulate overall cell growth and proliferation, making it a likely candidate for modulation of IGFBP-1 phosphorylation under leucine deprivation. Due to the commercial unavailability of specific pharmacological inhibitors against the AAR, our primary investigation of the AAR relied on its cross-talk with MEK/ERK signaling. MEK/ERK signaling is necessary for the propagation of the AAR by GCN2[5], the exclusive nutrient sensor to the AAR[4]. We chemically inhibited MEK/ERK signaling with pharmacological inhibitor U0126 in order to wholly mitigate the activity of this specific MKK/MAPK arm of the MKKK signaling cascade[6]. We successfully demonstrated that U0126 was able to attenuate the AAR downstream of GCN2 and that this fully obstructed the ability of leucine deprivation to induce IGFBP-1 phosphorylation (Ser101, Ser119 and Ser169). We confirmed and validated these findings with ERK1/2 siRNA, which was conducted separately and combined with GCN2 siRNA in order to conclusively determine whether leucine deprivation-mediated IGFBP-1 phosphorylation occurs via the AAR. This led to our experiment where we silenced ERK1/2 and GCN2 in tandem, and proved that the ability of MEK/ERK to modulate IGFBP-1 phosphorylation under leucine deprivation is, in fact, due to its participation in the AAR.

In Chapter 3, we surveyed various kinases speculated to modulate IGFBP-1 phosphorylation for their ability to mediate IGFBP-1 phosphorylation in leucine

deprivation.The molecular mechanisms regulating IGFBP-1 phosphorylation, especially under nutrient depletion, are largely unexplored in the literature. CK2, PKC, and PKA have been suggested to phosphorylate IGFBP-1[7]. Specifically, our sites of interest (pSer101, pSer119, pSer169) are likely to be directly phosphorylated by CK2[7] due to surrounding acidic amino acid residues to the phospho-acceptor sites. Recent success by our team in demonstrating that that CK2 modulates IGFBP-1 phosphorylation in HepG2 cells and primary baboon hepatocytes[2] motivated this investigation. We presupposed that inhibiting CK2, but not PKC or PKA, in leucine deprivation would attenuate leucine deprivation-induced IGFBP-1 phosphorylation and that this would extend our previous[2] observations that CK2 is the key kinase involved in IGFBP-1 phosphorylation in nutrient restricted conditions. First, using pharmacological inhibitor TBB against CK2, we demonstrated that CK2 inhibition obstructed the ability of leucine deprivation to induce IGFBP-1 phosphorylation. In these same samples, we verified that TBB reduced CK2 activity regardless of leucine status, which otherwise stimulated CK2 activity. The ability of TBB to attenuate IGFBP-1 phosphorylation was independent of overall IGFBP-1 secretion, since total IGFBP-1 output was elevated in leucine deprivation despite the presence of TBB. Follow-up siRNA experiments against CK2(α+α'+β) proved that CK2 is essential for leucine deprivation to stimulate IGFBP-1 phosphorylation at all three sites (Ser101, Ser119 and Ser169).

Chemical inhibition of PKA was unable to alter IGFBP-1 phosphorylation in HepG2 cells regardless of leucine status. However, PKC inhibition or silencing was demonstrated here, for the first time, to not only modulate hepatic IGFBP-1 phosphorylation but to also coordinate this event under leucine deprivation (Chapter 3). Unlikely to be a direct kinase for the phosphorylation of IGFBP-1 at our sites of interest (pSer101, pSer119 and pSer169)[7], we assessed whether the ability of PKC to modulate IGFBP-1 phosphorylation was due to mechanistic cross-talk with CK2. Our CK2 activity assay verified that PKC inhibition with BIS prevented the ability of leucine deprivation to induce CK2 activity, although BIS did not affect CK2 activity under basal conditions, as expected[8]. The finding that CK2 likely phosphorylates IGFBP-1 downstream of PKC in leucine deprivation is the first report of potential interactions between PKC and CK2 in HepG2

cells. Finally, to attribute functional relevance to our findings, we used our previously established[9] IGF-1R autophosphorylation assay to demonstrate that changes in phospho-IGFBP-1 content in conditioned media variably affected IGF-I bioactivity.

Table 4.1. Summary of various treatments on IGFBP-1 secretion and phosphorylation.

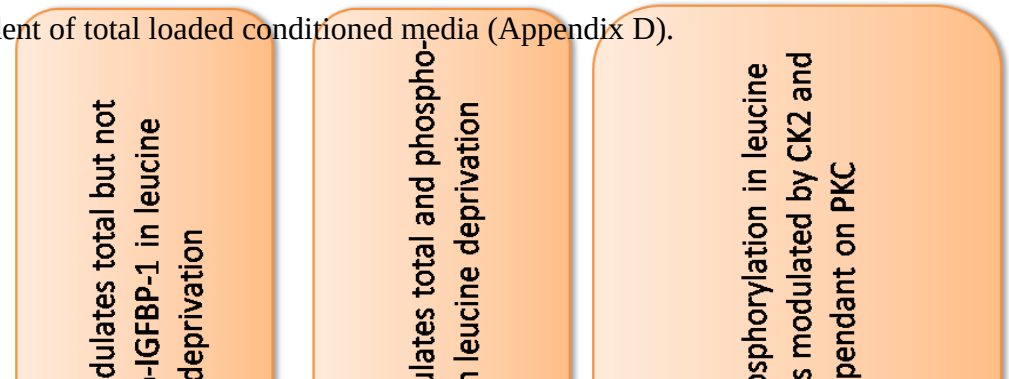
Treatment	Effect on intracellular signaling	Figure Reference	IGFBP-1 Secretion	IGFBP-1 Phosphorylation	Figure reference
mTOR pathway					
Rapamycin	mTORC1+C2 inhibition	Figures 2.1A-B	↑	No significant effect	Figures 2.1C-F
Raptor+Rictor siRNA		Figures 2.2A-B			Figures 2.2C-F
DEPTOR siRNA	mTORC1+C2 activation		↓		
AAR pathway					
U0126	AAR pathway inhibition	Figures 2.3A-D	↓	↓	Figures 2.3E-H
ERK siRNA		Figures 2.4A-C			Figures 2.4D-G
GCN2 siRNA		Figures 2.5A-B			Figures 2.5C-F
ERK+GCN2 siRNA					
Kinase Treatments					
TBB	CK2 inhibition	Figure 3.7	No significant effect	↓	Figures 3.1A-D
CK2α+α'+β siRNA		N/A			Figures 3.2A-D
BIS	PKC inhibition	N/A			Figures 3.3A-D
pan-PKC siRNA		N/A			Figures 3.4A-D
PKI (5-24)	PKA inhibition	Figure 3.5A	No significant effect		Figures 3.5B-E

mTOR signaling modulates IGFBP-1 secretion but not phosphorylation caused by leucine deprivation. The AAR regulates both IGFBP-1 secretion and phosphorylation in leucine deprivation. CK2 and PKC are key kinases involved in modulating IGFBP-1 phosphorylation in leucine deprivation.

Loading controls for secretory proteins

A recurring concern in this study, as well as in the literature, is a lack of a consistent loading control for western blots of secretory proteins in conditioned cell media. β-actin served as a reliable loading control for western blots of whole cell lysates throughout this study, however, treatment-dependent fluctuations in IGFBP-1 secretion and phosphorylation were determined with western blots using conditioned media from HepG2 cell cultures, which have not been previously demonstrated to secrete any protein in a consistent quantity irrespective of treatments. Reversible stains for total protein, such as Ponceau stains, are not well accepted tools for the detection of equal loading between lanes due to their lack of sensitivity and specificity of the bands being stained[10,11]. Proteins that are enriched in conditioned media compared to lysatesamples are specific to cell-type and may be used as quality controls for western blots[12]. To improve the validity of our western blots, we speculated as to which HepG2 secreted protein could serve as a reliable loading control. Based on the HepG2 secretome[13], which indicates that fibrinogen is synthesized and secreted in high quantities by HepG2 cells[13-15] we evaluated whether fibrinogen could serve as an effective loading control for western blots of our conditioned HepG2 cell media.

Due to the observation that fibrinogen secretion remained consistent among treatments regardless of the presence of inhibitor, fibrinogen output served as a valuable tool to ensure equal loading among lanes (Appendix D; Supplementary Figure 4.4). A representative western blot of conditioned media from leucine plus, leucine minus, and combined leucine plus/minus treatments with the various inhibitors that affect IGFBP-1 phosphorylation used in this study (TBB, BIS, PKI, U0126) was probed with primary antibody against pSer101 (IGFBP-1), total IGFBP-1, and fibrinogen. Fibrinogen secretion remained consistent among all treatments irrespective of inhibitor-induced fluctuations in IGFBP-1 secretion or phosphorylation. Therefore, we verified that the differences in IGFBP-1 secretion and phosphorylation elicited by inhibitor treatments are independent of total loaded conditioned media (Appendix D).



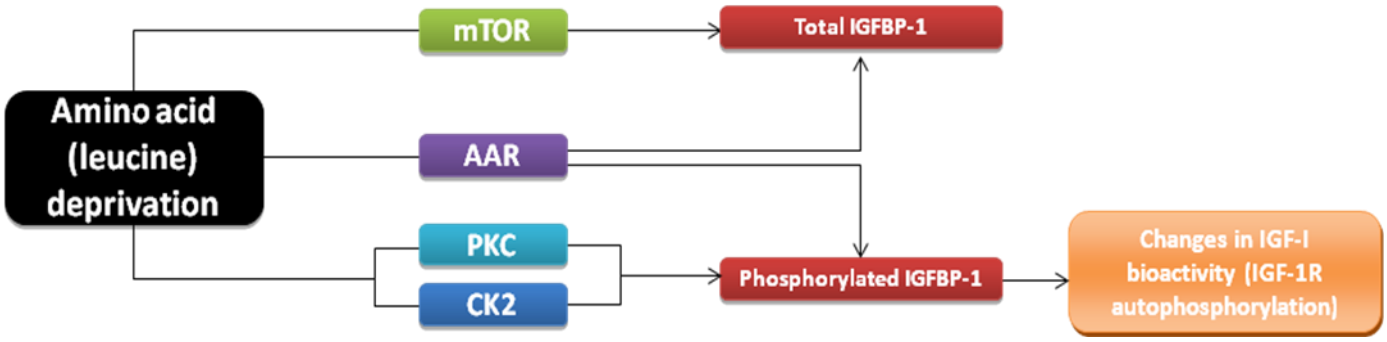


Figure 4.2. Schematic of established mechanistic links between leucine deprivation and total and phospho-IGFBP-1.

4.2 Extensions, Perspectives and Significance

The bioinhibitory capacity of IGFBP-1 on IGF-I is widely acknowledged. For example, IGFBP-1 impedes IGF-I-dependent amino acid transport[16] and DNA synthesis[17,18]. The capacity of phosphorylated IGFBP-1to bind and sequester IGF-I is greater than that of the un-phosphorylated isoform[19,20], suggesting that IGFBP-1 hyperphosphorylation bears significant functional relevance in the context of IGF-I bioactivity. The extent to which leucine deprivation induced IGFBP-1 phosphorylation (up to 2500%) in this study far superseded the extent to which leucine deprivation induced overall IGFBP-1 secretion (maximum observed 500% induction), demonstrating the impact leucine restriction has on mitigating IGF-I bioactivity through regulating its binding protein. This observation justifies why the identification of the signaling mechanisms which link leucine deprivation to IGFBP-1 hyper-phosphorylation is absolutely critical in understanding the regulation of IGF-I bioactivity under amino acid restriction.

The finding that mTOR mediates IGFBP-1 secretion but is only partially responsible for IGFBP-1 phosphorylation in leucine deprivation, combined with the observation that CK2 or PKC inhibition prevents IGFBP-1 phosphorylation without affecting its overall secretion in leucine deprivation, demonstrates that the molecular mechanisms elicited by leucine deprivation to regulate IGFBP-1 phosphorylation are independent of those which

modulate its total secretion. This clear illustration of a bipartite mode of regulation for IGFBP-1 secretion compared to its phosphorylation under leucine restriction suggests that IGFBP-1 phosphorylation occurs as an independent cellular response to nutritional stress. This finding has functional relevance in FGR, which our team has previouslyassociated with hyperphosphorylated IGFBP-1 in both maternal[22] and fetal[2] compartments. Linking CK2 and PKC – which were identified in this study to modulate IGFBP-1 phosphorylation in leucine deprivation – to the AAR will be valuable in elucidating the specific cellular response toamino acid deprivation to phosphorylate IGFBP-1*in vitro*. Identification of the signaling mechanisms modulating IGFBP-1 phosphorylation in this study lays the groundwork forfuture investigation on the roles of these specific signaling mechanisms in modulating IGFBP-1 phosphorylation in amino acid deprivation*in vivo*. If IGFBP-1 phosphorylation is modulated *in vivo* by the same molecular components identified in this study, these pathways may serve as candidatesfor future studies aimed at identifying targets for manipulation to offset IGFBP-1 phosphorylation in FGR.

The ultimate goal of the AAR is to arrest anabolic activity in favour of energy preservation upon amino acid restriction[23]. Induced ATF4 expression is not exclusive to amino acid deprivation – in fact, ATF4 is a conjunction point for multiple stress-responsive pathways such as the Unfolded Protein Response (UPR) and elicits stressor-specific gene programs. The gene expression profiles elicited by ATF4 from the AAR (via GCN2 sensing) and UPR are mutually exclusive, although there is a significant degree of overlap[23]. The mechanism by which ATF4 discriminates between multiple upstream signals is not understood. Thus, our rationale for silencing GCN2 rather than eIF2α or ATF4 is the specificity of GCN2 to the AAR. Although the AAR was demonstrated to be indispensable in modulating IGFBP-1 phosphorylation in leucine-deprived HepG2 cells (Chapter 2), it is likely that the AAR coordinates withadditional signaling mechanisms down-stream, including mTOR, in order to transduce its effects. It is possible that the AAR and mTOR signaling pathways impinge on each other downstream to modulate leucine deprivation-induced IGFBP-1 secretion and phosphorylation in a common mechanism. Indeed, the mTOR and AAR pathways exhibit

a knownconvergence point: DNA damage response 1 (REDD1). GCN2-mediated eiF2α phosphorylation induces REDD1[24], an essential component of the mTOR signaling cascade[25], thereby coordinating the two pathwaysdownstream. Another mode of convergence may, in fact, be CK2, which has shown to interact with ATF4[26,27], possibly linking the nutrient-responsive kinase with the AAR. Future studies to elucidate the interaction between the AAR and mTOR pathways *in vitro* will provide further insight into the mechanisms modulating IGFBP-1 phosphorylation.

Previous work by our lab[2] has linked mTOR signaling and CK2 kinase activity in the regulation of hepatic IGFBP-1 phosphorylation. The studies presented here demonstrate that additional mechanisms to mTOR and CK2 are implicated in IGFBP-1 phosphorylation in amino acid deprivation. The cellular response to nutritional stress is dynamic. For instance, the AAR (Chapter 2) and PKC (Chapter 3) were discovered, for the first time, to play pivotal roles in IGFBP-1 phosphorylation in leucine-restricted conditions *in vitro*. The ability of PKC to mediate IGFBP-1 phosphorylation at Ser101, Ser119, and Ser169 while simultaneously modulating CK2 activity in leucine deprivation illustrates a novel role for PKC in modulating IGFBP-1 phosphorylation. Together, our findings demonstrate that the regulation of IGF-I bioactivity via phosphorylation of its binding protein is dynamic and influenced by multiple signaling pathways.

Combined with previous *in vitro* studies demonstrating that CK2 directly phosphorylates IGFBP-1 in HepG2 cells[28] and past work in our lab which linked CK2 activity with IGFBP-1 phosphorylation in fetal hepatocytes from MNR baboon mothers[2], our findings suggest that CK2 is the key kinase responsible for directly phosphorylating fetal hepatic IGFBP-1 in amino acid deprivation. The contribution of PKC to IGFBP-1 phosphorylation had not been previously reported, although its role in regulating IGFBP-1 at the transcriptional and translational level has been studied[32-34]. PKC does not contain consensus sequence sites for phosphorylation at IGFBP-1 at Ser101, Ser119 and Ser169, although it does contain consensus sequence sites for IGFBP-1 phosphorylation at Ser50 and Ser58. It is possible that PKC-mediated phosphorylation at these discrete sites contributes to modulating IGFBP-1 affinity for IGF-I independently or through synergistic effects with Ser101, Ser119 and Ser169. Future studies employing mass

spectrometry will be valuable in assessing the phosphorylation of IGFBP-1 at these sites in leucine deprivation. Considering the greater likelihood that IGFBP-1 at Ser101, Ser119 and Ser169 is phosphorylated by CK2 due to surrounding amino acid residues on IGFBP-1, our finding that PKC modulates IGFBP-1 phosphorylation at these sites in leucine deprivation warranted exploration of the interaction between PKC and CK2 in leucine deprivation. Our CK2 activity assay which demonstrated that PKC inhibition attenuated leucine deprivation-induced CK2 activitysuggested that the effect of PKC on IGFBP-1 phosphorylation (Ser101, Ser119 and Ser169) is likely due to an upstream link in a common signaling mechanism with CK2 in leucine deprivation. PKC and CK2are both implicated downstream of mTOR signaling, however, whether PKC directly modulates CK2 or whether other molecular components are implicated is not known. It is possible that CK2 activity is regulated downstream of PKC in leucine deprivation, and is an attractive area for future *in vitro* research in HepG2 cells.

To reduce overall PKC signaling, we used pharmacological inhibitor BIS which inhibits all 12 PKC isoforms[31]or pan-PKC siRNA.It is possible that specific PKC isoforms, rather than overall PKC activity, are specifically implicated in CK2-mediated IGFBP-1 phosphorylation. Future *in vitro* can be conducted in HepG2 cells to assess isoform-specific roles of PKC in modulating IGFBP-1 in leucine deprivation using targeted RNA*i*against specific PKC isoforms.

The mechanism by which pSer101, pSer119 and pSer169 elicit conformational changes on the IGFBP-1 molecule to contribute to increased affinity of IGFBP-1 for IGF-I is presently unknown. The complete 3D structure of IGFBP-1 has yet to be resolved[35]. Ser101 and Ser169 are proximal to structured, IGF-I binding regions in the quaternary protein structure while Ser119 is contained within the protein's mobile linker region[45]. Covalent modifications at these sites likely cause conformational changes that influence IGFBP-1 interactions with IGF-I. It is notable that IGFBP-1 phosphorylation protects it from proteolytic cleavage[36],thus increasing the compound's half-life. The induction of phosphorylation at Ser101, Ser119 and Ser169 in leucine deprivation suggests that the collective contribution of phosphorylation at each of the three sites is essential in modulating IGF-I

bioavailability compared to phosphorylation at any site alone. Previously published data from our lab demonstrated that IGFBP-1 individually mutated at any of the three studied phospho-acceptor sites (Ser101Ala, Ser119Ala and Ser169Ala) affect the ability of IGFBP-1 to modulate IGF-I bioavailability[9], validating the functional relevance of phosphorylation at these three sites. The existence of multiple phosphorylated IGFBP-1 residues highlights the importance of potential synergistic effects between the various phosphorylated residues. The observation that phosphorylation at Ser101, Ser119 and Ser169 was prone to inhibition by all biological and chemical treatments in this study suggests that the regulation of phosphorylation at each of the residues is implicated in a common signaling mechanism. It is ascertainable that phosphorylation of a particular phospho-site can lead, either directly or indirectly, to the phosphorylation of the other residues. Finally, future molecular modeling studies of phosphorylated IGFBP-1 may be valuable to demonstrate how the individual and combined phosphorylation of Ser101, Ser119 and Ser169 elicits conformational changes to IGFBP-1 structure to directly contribute to IGF-I affinity.

Significance and Future Directions

By elucidating the relative contributions of the mTOR and AAR signaling pathways and CK2, PKC, and PKA kinases in regulating IGFBP-1 phosphorylation under leucine deprivation, this study has contributed vital pieces to the understanding of IGFBP-1 phosphorylation *in vitro.* Our findings provide justification for future investigation into the mechanistic links between the various molecular components shown here to modulate IGFBP-1 phosphorylation in leucine deprivation. For example, it is unknown whether PKC and CK2 mediated IGFBP-1 phosphorylation is coordinated downstream of the AAR in a common signaling mechanism. It is also yet to be determined the precise mechanism by which PKC modulates IGFBP-1 phosphorylation at three sites for which it does not share consensus sequences (Ser101, Ser119, Ser169). PKC may modulate IGFBP-1 phosphorylation through synergistic effects of phosphorylation at descrete residues or via direct modulation of CK2 activity. The next step would be to replicate

these findings in *in vivo* models, such as our lab's established primary fetal baboon hepatocytes, in order to further attribute functional significance to our findings.

Due to the variety of factors which contribute to fetal development[43,44], the appropriate functioning of intracellular signaling mechanisms which modulate fetal growth is paramount to healthy fetal development. Several intracellular signaling pathways impinge on one another, and abrogation in any component of this molecular web can lead to altered fetal growth. Decoding the signaling pathways involved in IGFBP-1 phosphorylation in amino acid-restricted conditions *in vitro* will provide clues as to the signaling mechanisms implicated in the pathogenesis of FGR *in utero*. The idea that adult morbidity has "developmental origins" has strong implications on public health and disease prevention[37], and we hope that the findings from this study will set the groundwork for future studies aimed at elucidating the pathogenesis of FGR.

4.3 References

1. Seferovic MD, Ali R, Kamei H, et al. Hypoxia and leucine deprivation induce human insulin-like growth factor binding protein-1 hyperphosphorylation and increase its biological activity. *Endocrinology*. 2009;150(1):220-231. doi: 10.1210/en.2008-0657; 10.1210/en.2008-0657.

2. Abu Shehab M, Damerill I, Shen T, et al. Liver mTOR controls IGF-I bioavailability by regulation of protein kinase CK2 and IGFBP-1 phosphorylation in fetal growth restriction. *Endocrinology*. 2014;155(4):1327-1339. doi: 10.1210/en.2013-1759; 10.1210/en.2013-1759.

3. Jansson T, Aye IL, Goberdhan DC. The emerging role of mTORC1 signaling in placental nutrient-sensing. *Placenta*. 2012;33 Suppl 2:e23-9.

4. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: How mammalian cells respond to amino acid limitation. *Annu Rev Nutr*. 2005;25:59-85. doi: 10.1146/annurev.nutr.24.012003.132145.

5. Thiaville MM, Pan YX, Gjymishka A, Zhong C, Kaufman RJ, Kilberg MS. MEK signaling is required for phosphorylation of eIF2alpha following amino acid limitation of HepG2 human hepatoma cells. *J Biol Chem*. 2008;283(16):10848-10857.

6. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. *Physiol Rev*. 1999;79(1):143-180.

7. Coverley JA, Baxter RC. Phosphorylation of insulin-like growth factor binding proteins. *Mol Cell Endocrinol*. 1997;128(1-2):1-5.

8. Channavajhala P, Seldin DC. Functional interaction of protein kinase CK2 and c-myc in lymphomagenesis. *Oncogene*. 2002;21(34):5280-5288.

9. Abu Shehab M, Iosef C, Wildgruber R, Sardana G, Gupta MB. Phosphorylation of IGFBP-1 at discrete sites elicits variable effects on IGF-I receptor autophosphorylation. *Endocrinology*. 2013;154(3):1130-1143. doi: 10.1210/en.2012-1962; 10.1210/en.2012-1962.

10. Yonan CR, Duong PT, Chang FN. High-efficiency staining of proteins on different blot membranes. *Anal Biochem*. 2005;338(1):159-161.

11. Harper S, Speicher DW. Detection of proteins on blot membranes. *Curr Protoc Protein Sci*. 2001;Chapter 10:Unit 10.8.

12. Chen Y, Gu B, Wu S, et al. Using enrichment index for quality control of secretory protein sample and identification of secretory proteins. *J Mass Spectrom*. 2009;44(3):397-403.

13. Mangrum JB, Martin EJ, Brophy DF, Hawkridge AM. Intact stable isotope labeled plasma proteins from the SILAC-labeled HepG2 secretome. *Proteomics*. 2014.

14. Matsumoto M, Matsuura T, Aoki K, et al. An efficient system for secretory production of fibrinogen using a hepatocellular carcinoma cell line. *Hepatol Res*. 2015;45(3):315-325.

15. Redman CM, Xia H. Fibrinogen biosynthesis. assembly, intracellular degradation, and association with lipid synthesis and secretion. *Ann N Y Acad Sci*. 2001;936:480-495.

16. Ritvos O, Ranta T, Jalkanen J, et al. Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells. *Endocrinology*. 1988;122(5):2150-2157.

17. Koistinen R, Itkonen O, Selenius P, Seppala M. Insulin-like growth factor-binding protein-1 inhibits binding of IGF-I on fetal skin fibroblasts but stimulates their DNA synthesis. *Biochem Biophys Res Commun*. 1990;173(1):408-415.

18. Elgin RG, Busby WH,Jr, Clemmons DR. An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. *Proc Natl Acad Sci U S A*. 1987;84(10):3254-3258.

19. Westwood M. Role of insulin-like growth factor binding protein 1 in human pregnancy. *Rev Reprod*. 1999;4(3):160-167.

20. Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: Effects on affinity for IGF-I. *Proc Natl Acad Sci U S A*. 1991;88(17):7481-7485.

21. Abu Shehab M, Inoue S, Han VK, Gupta MB. Site specific phosphorylation of insulin-like growth factor binding protein-1 (IGFBP-1) for evaluating clinical relevancy in fetal growth restriction. *J Proteome Res*. 2009;8(11):5325-5335.

22. Abu Shehab M, Khosravi J, Han VK, Shilton BH, Gupta MB. Site-specific IGFBP-1 hyper-phosphorylation in fetal growth restriction: Clinical and functional relevance. *J Proteome Res*. 2010;9(4):1873-1881.

23. Wek RC, Jiang HY, Anthony TG. Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans*. 2006;34(Pt 1):7-11.

24. Whitney ML, Jefferson LS, Kimball SR. ATF4 is necessary and sufficient for ER stress-induced upregulation of REDD1 expression. *Biochem Biophys Res Commun*. 2009;379(2):451-455.

25. Morel M, Couturier J, Pontcharraud R, et al. Evidence of molecular links between PKR and mTOR signalling pathways in abeta neurotoxicity: Role of p53, Redd1 and TSC2. *Neurobiol Dis*. 2009;36(1):151-161.

26. Schneider CC, Ampofo E, Montenarh M. CK2 regulates ATF4 and CHOP transcription within the cellular stress response signalling pathway. *Cell Signal*. 2012;24(9):1797-1802.

27. Ampofo E, Sokolowsky T, Gotz C, Montenarh M. Functional interaction of protein kinase CK2 and activating transcription factor 4 (ATF4), a key player in the cellular stress response. *Biochim Biophys Acta*. 2013;1833(3):439-451.

28. Ankrapp DP, Jones JI, Clemmons DR. Characterization of insulin-like growth factor binding protein-1 kinases from human hepatoma cells. *J Cell Biochem*. 1996;60(3):387-399.

29. Bren GD, Pennington KN, Paya CV. PKC-zeta-associated CK2 participates in the turnover of free IkappaBalpha. *J Mol Biol*. 2000;297(5):1245-1258.

30. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiol Rev*. 2008;88(4):1341-1378.

31. Way KJ, Chou E, King GL. Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci*. 2000;21(5):181-187.

32. Kachra Z, Yang CR, Murphy LJ, Posner BI. The regulation of insulin-like growth factor-binding protein 1 messenger ribonucleic acid in cultured rat hepatocytes: The roles of glucagon and growth hormone. *Endocrinology*. 1994;135(5):1722-1728.

33. Gong Y, Ballejo G, Alkhalaf B, Molnar P, Murphy LC, Murphy LJ. Phorbol esters differentially regulate the expression of insulin-like growth factor-binding proteins in endometrial carcinoma cells. *Endocrinology*. 1992;131(6):2747-2754.

34. Lee PD, Abdel-Maguid LS, Snuggs MB. Role of protein kinase-C in regulation of insulin-like growth factor-binding protein-1 production by HepG2 cells. *J Clin Endocrinol Metab*. 1992;75(2):459-464.

35. Forbes BE, McCarthy P, Norton RS. Insulin-like growth factor binding proteins: A structural perspective. *Front Endocrinol (Lausanne)*. 2012;3:38.

36. Gibson JM, Aplin JD, White A, Westwood M. Regulation of IGF bioavailability in pregnancy. *Mol Hum Reprod*. 2001;7(1):79-87.

37. Gluckman PD, Hanson MA, Mitchell MD. Developmental origins of health and disease: Reducing the burden of chronic disease in the next generation. *Genome Med*. 2010;2(2):14.

38. Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for siRNA therapeutics. *Nat Mater*. 2013;12(11):967-977.

39. Deng Y, Wang CC, Choy KW, et al. Therapeutic potentials of gene silencing by RNA interference: Principles, challenges, and new strategies. *Gene*. 2014;538(2):217-227.

40. Ronzoni S, Marconi AM, Paolini CL, Teng C, Pardi G, Battaglia FC. The effect of a maternal infusion of amino acids on umbilical uptake in pregnancies complicated by intrauterine growth restriction. *Am J Obstet Gynecol*. 2002;187(3):741-746.

41. Kim WK, Ryu YH, Seo DS, Lee CY, Ko Y. Effects of oral administration of insulin-like growth factor-I on circulating concentration of insulin-like growth factor-I and growth of internal organs in weanling mice. *Biol Neonate*. 2006;89(3):199-204.

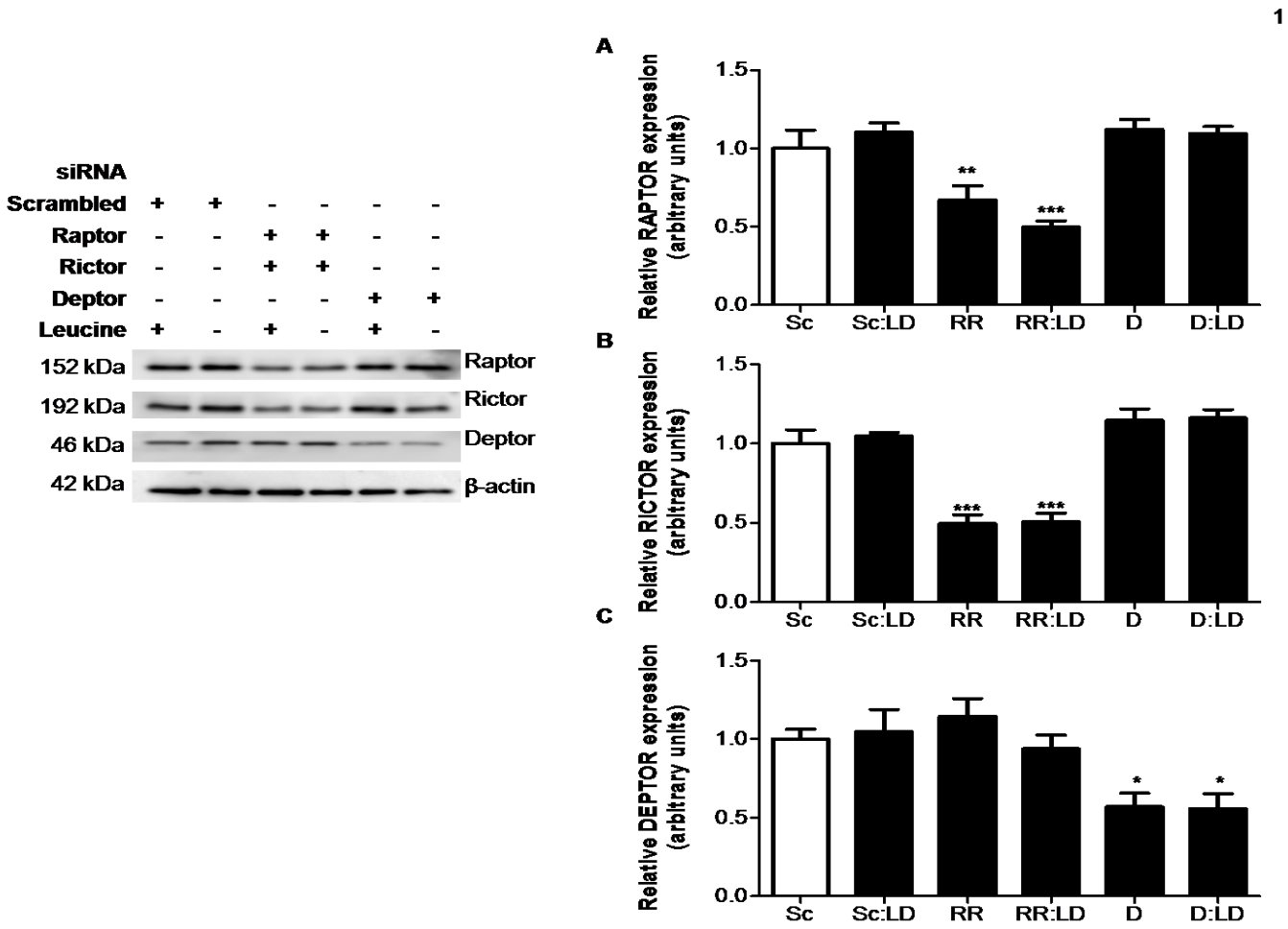
42. Fullwood MJ, Zhou W, Shenolikar S. Targeting phosphorylation of eukaryotic initiation factor-2alpha to treat human disease. *Prog Mol Biol Transl Sci*. 2012;106:75-106.

43. Miller J, Turan S, Baschat AA. Fetal growth restriction. *Semin Perinatol*. 2008;32(4):274-280. doi: 10.1053/j.semperi.2008.04.010; 10.1053/j.semperi.2008.04.010.

44. Maulik D. Fetal growth restriction: The etiology. *Clin Obstet Gynecol*. 2006;49(2):228-235.

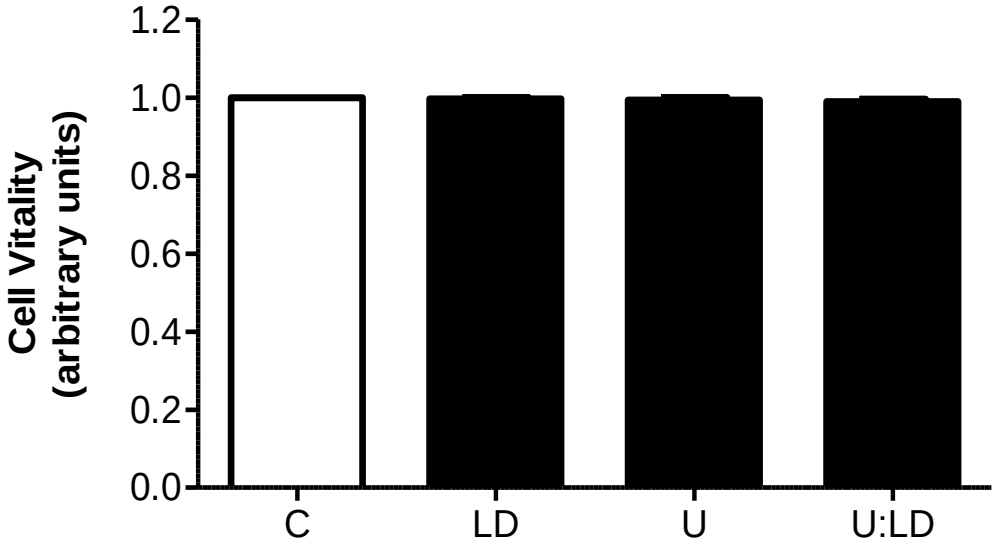
45. Sitar T, Popowicz GM, Siwanowicz I, Huber R, Holak TA. Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins. *Proc Natl Acad Sci U S A*. 2006;103(35):13028-13033.

Appendix A



Supplementary Figure 2. 1. Efficiency of raptor+rictor and DEPTOR silencing. A representative immunoblot of HepG2 cell lysates (50 µg per lane) assayed for raptor, rictor, or DEPTOR expression following siRNA silencing. **A-B**. Raptor+rictor silencing significantly reduced the expression of raptor (-45-50%) and rictor (-50%) regardless of leucine status, but did not affect DEPTOR expression. **C**. DEPTOR expression was reduced (-50%) in HepG2 cells treated with siRNA against DEPTOR, but not against raptor+rictor. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance;
Dunnet's Multiple Comparison Test; n=3. Sc: Scrambled siRNA, 450 µM leucine. Sc:LD: Scrambled siRNA, 0 µM leucine. RR: Raptor+Rictor siRNA, 450 µM leucine. RR:LD: Raptor+Rictor siRNA, 0 µM leucine. D: DEPTOR siRNA, 450 µM leucine. D:LD: DEPTOR siRNA, 0 µM leucine.

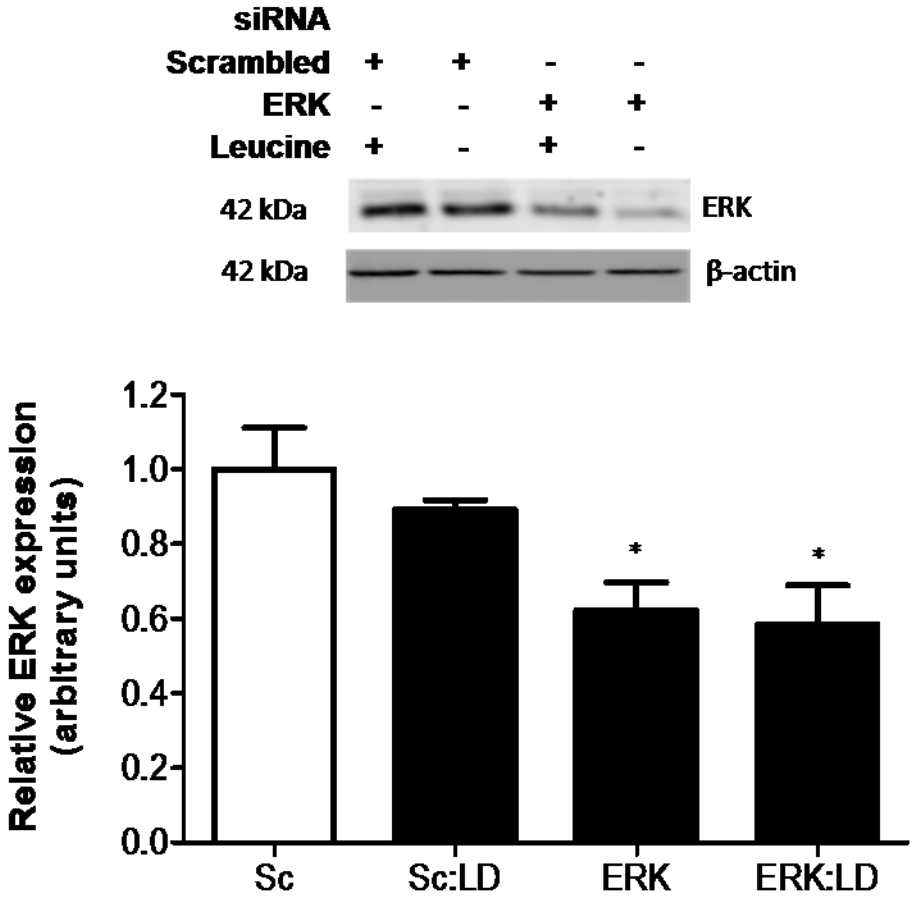
Appendix A



Replicate	Control	LD	U0126	U0126:LD
1	100	100	98	98
2	100	100	100	99
3	100	99	100	100

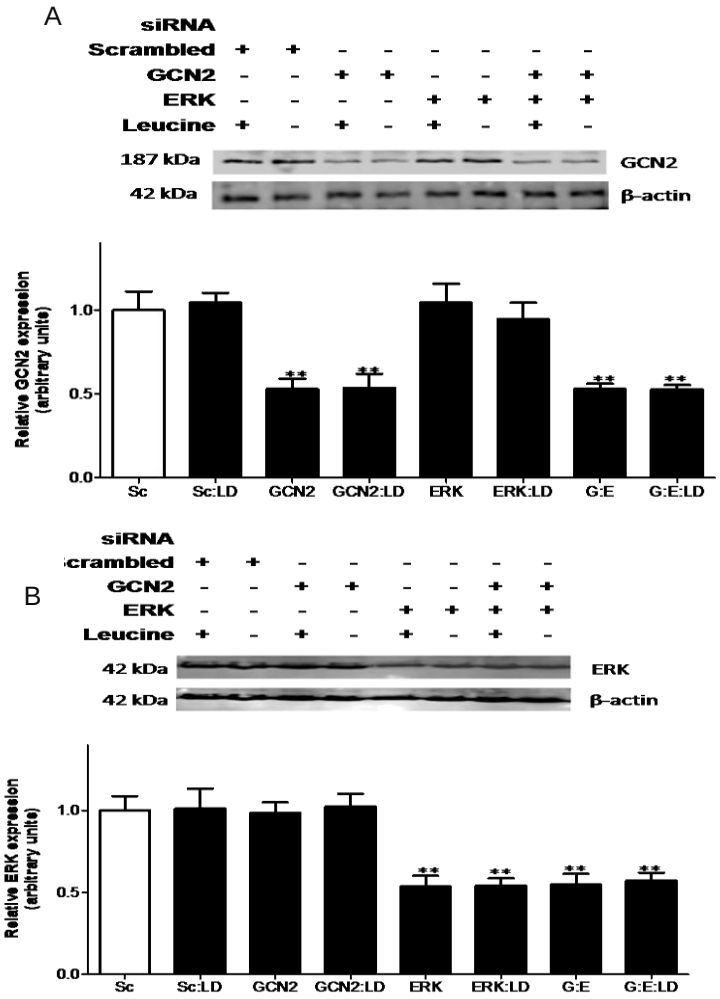
Supplementary Figure 2. 2. HepG2 cell vitality after treatment with U0126 (10 µM) for 24 hours. A graphical representation of cell vitality between leucine plus or leucine deprived with or without U0126 treatments. A Trypan Blue exclusion assay was performed to assess cell viability, presented here as a percentage of live cells/total cells normalized to viability in control samples (leucine plus, no inhibitor). Leucine deprivation and the U0126 inhibitor did not separately or together significantly affect cell survival. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. C: Control, 450 µM leucine. LD: Leucine deprivation, 0 µM leucine. U: U0126 (10 µM), 450 µM leucine. U:LD: U0126 (10 µM), 0 µM leucine.

Appendix A



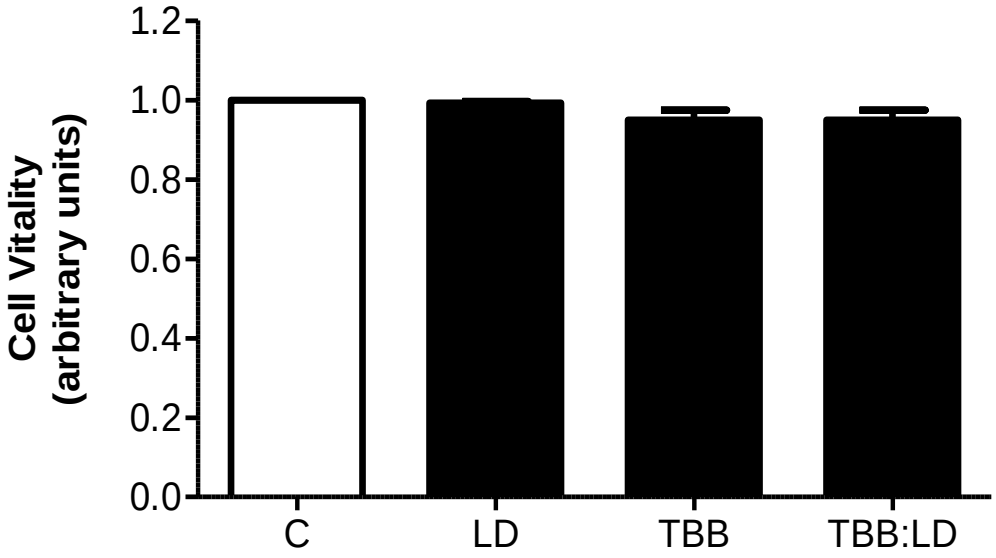
Supplementary Figure 2. 3. Efficiency of ERK silencing. A representative western immunoblot of total ERK expression in HepG2 cell lysates silenced with scrambled or ERK siRNA with or without leucine deprivation. Equal protein loading (50 μg per lane) was conducted. Total ERK expression was reduced (-40%) in cells treated with ERK siRNA, and remained unaffected in cells treated with scrambled siRNA. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. Sc: Scrambled siRNA, 450 μM leucine. Sc:LD: Scrambled siRNA, 0 μM leucine. ERK: ERK1/2 siRNA, 450 μM leucine. ERK:LD: ERK1/2 siRNA, 0 μM leucine.

Appendix A



Supplementary Figure 2. 4. Efficiency of GCN2, ERK, and GCN+ERK silencing. Representative immunoblots of HepG2 cell lysates (50 µg per lane) assayed for GCN2 or ERK expression following siRNA silencing. **A.** GCN2 silencing significantly reduced the expression of GCN2 (-50%) regardless of leucine status when GCN2 was silenced alone or in combination with ERK, and was unaffected when only ERK was silenced. **B.** ERK expression was reduced (-40%) in HepG2 cells treated with siRNA against ERK whether or not GCN2 was also silenced. GCN2 silencing had no significant effect on ERK expression and this effect was consistent in leucine plus or leucine deprived samples. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. Sc: Scrambled siRNA, 450 µM leucine. Sc:LD: Scrambled siRNA, 0 µM leucine. GCN2: GCN2 siRNA, 450 µM leucine. GCN2:LD: GCN2 siRNA, 0 µM leucine. ERK: ERK1/2 siRNA, 450 µM leucine. ERK:LD: ERK1/2 siRNA, 0 µM leucine. GCN2:ERK: GCN2+ERK1/2 siRNA, 450 µM leucine. GCN2:ERK:LD: GCN2+ERK1/2 siRNA, 0 µM leucine.

Appendix A



Replicate	Control	LD	TBB	TBB:LD
1	100	100	92	92
2	100	99	92	92
3	100	99	100	100

Supplementary Figure 2. 5. HepG2 cell vitality after treatment with TBB (1 µM) for 24 hours.A graphical representation of cell vitality between leucine plus or leucine deprived with or without TBB treatments. A Trypan Blue exclusion assay was performed to assess cell viability, presented here as a percentage of live cells/total cells normalized to viability in control samples (leucine plus, no inhibitor). Leucine deprivation and the TBB inhibitor did not separately or together significantly affect cell survival. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. C: Control, 450 µM leucine. LD: Leucine deprivation, 0 µM leucine. TBB: TBB (1 µM), 450 µM leucine. TBB:LD: TBB (1 µM), 0 µM leucine.

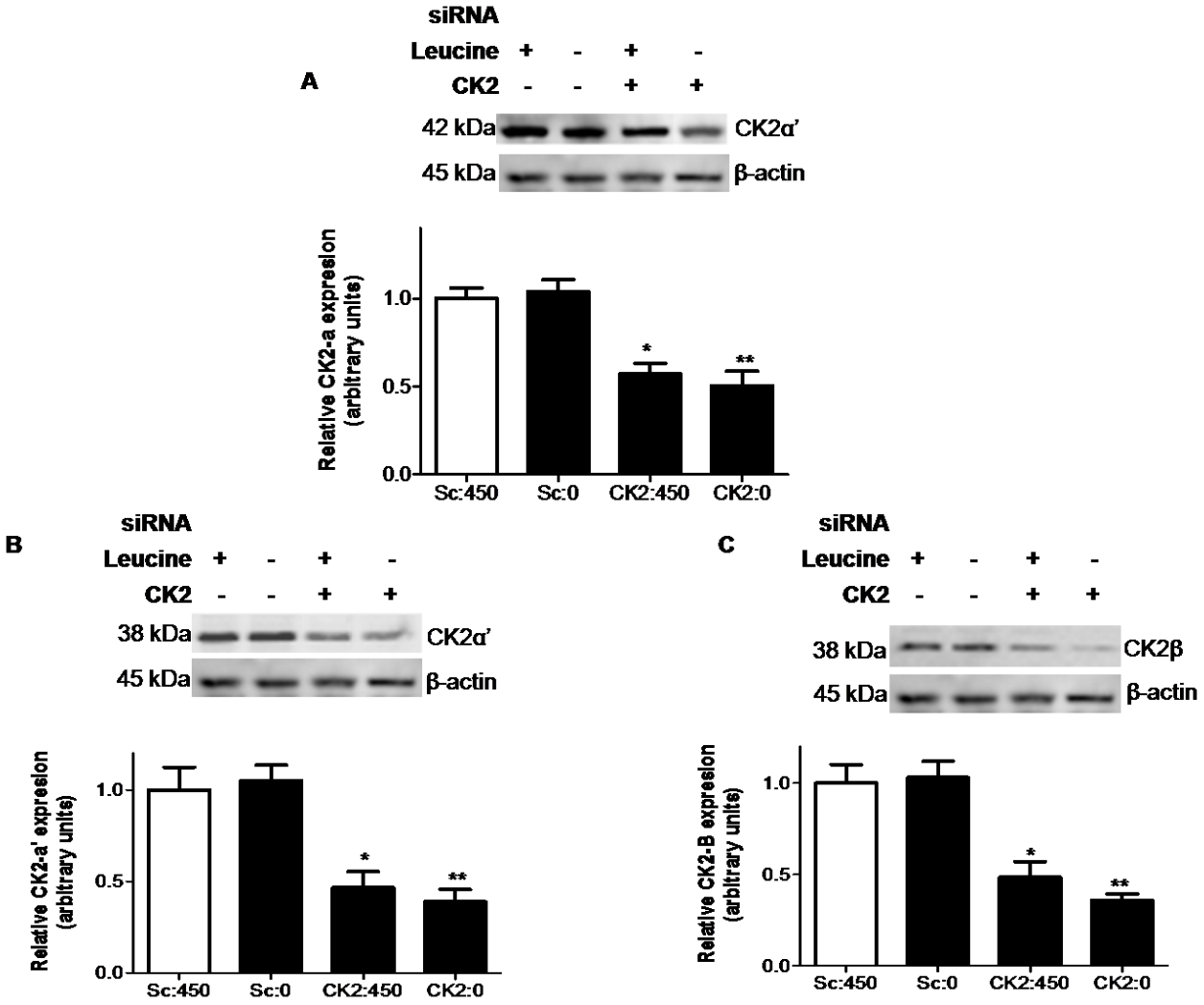
Appendix A

Supplementary Table 2. 1. Comprehensive overview of assayed proteins.

Protein	Fraction assayed	Component tested	Phosphosite(s)	Indicates
AAR pathway				
GCN2	HepG2 cell lysate	Phosphorylation	Thr898	AAR pathway activation
eIF2α			Ser51	
ERK			Thr202/Tyr204	
ATF4		Expression	N/A	
mTOR pathway				
Raptor	HepG2 cell lysate	Expression	N/A	siRNA silencing efficiency
Rictor				
DEPTOR				
p70-S6K		Phosphorylation	Thr389	mTORC1 activity
Akt			Ser473	mTORC2 activity
IGFBP-1 (total and phosphorylated)				
IGFBP-1	HepG2 cell media	Secretion and Phosphorylation	Ser101, Ser119, Ser169	IGF-I bioactivity
IGF-1Rβ	P6 cell lysate	Phosphorylation	Tyr1135	
Other				
β-actin	HepG2 and P6 cell lysate	Expression	N/A	Equal protein loading of cell lysates

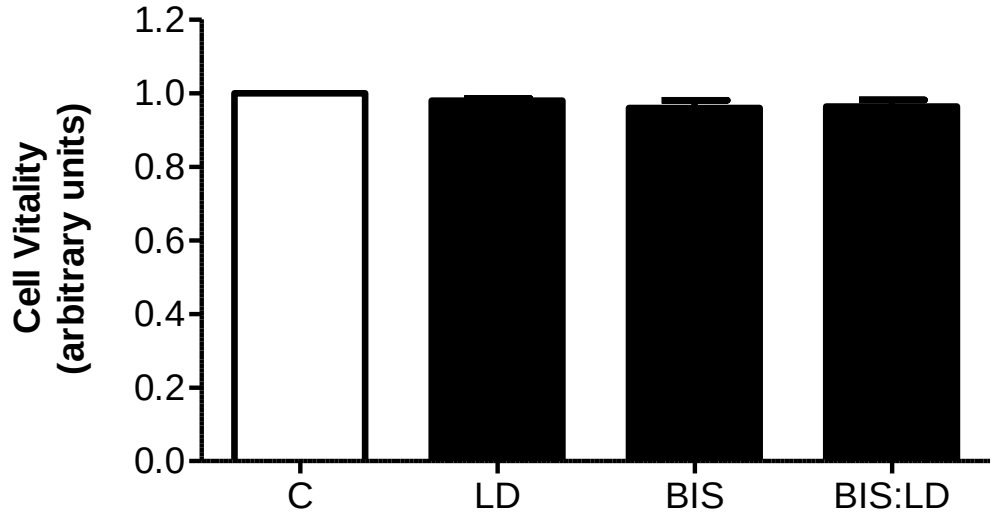
A tabular representation of the various proteins assessed for expression and phosphorylation in this manuscript, fractions assayed, phosphosites, and implications in mTOR, AAR and IGFBP-1 regulation.

Appendix B



Supplementary Figure 3. 1. Efficiency of CK2α+α'+β silencing. Representative immunoblots of HepG2 cell lysates (50 µg per lane) demonstrateknockdown efficiencies of CK2α+α'+β subunits. CK2α, CK2α'and CK2β expression were decreased (-45-55%) regardless of leucine status in HepG2 cells treated with siRNA against all three CK2 subunits. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. . Sc:450: Scrambled siRNA, 450 µM leucine. Sc:0: Scrambled siRNA, 0 µM leucine.CK2:450CK2 siRNA, 450 µM leucine. CK2:0: GCN2 siRNA, 0 µM leucine.

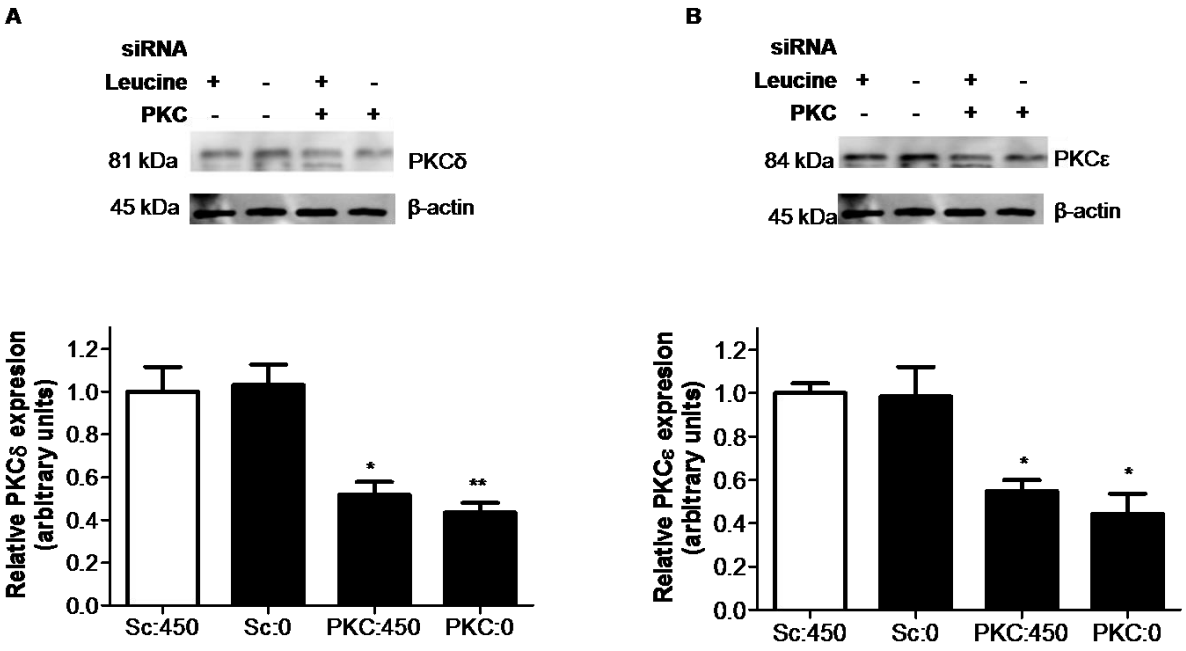
Appendix B



Replicate	Control	LD	BIS	BIS:LD
1	100	98	93	100
2	100	97	100	95
3	100	100	95	94

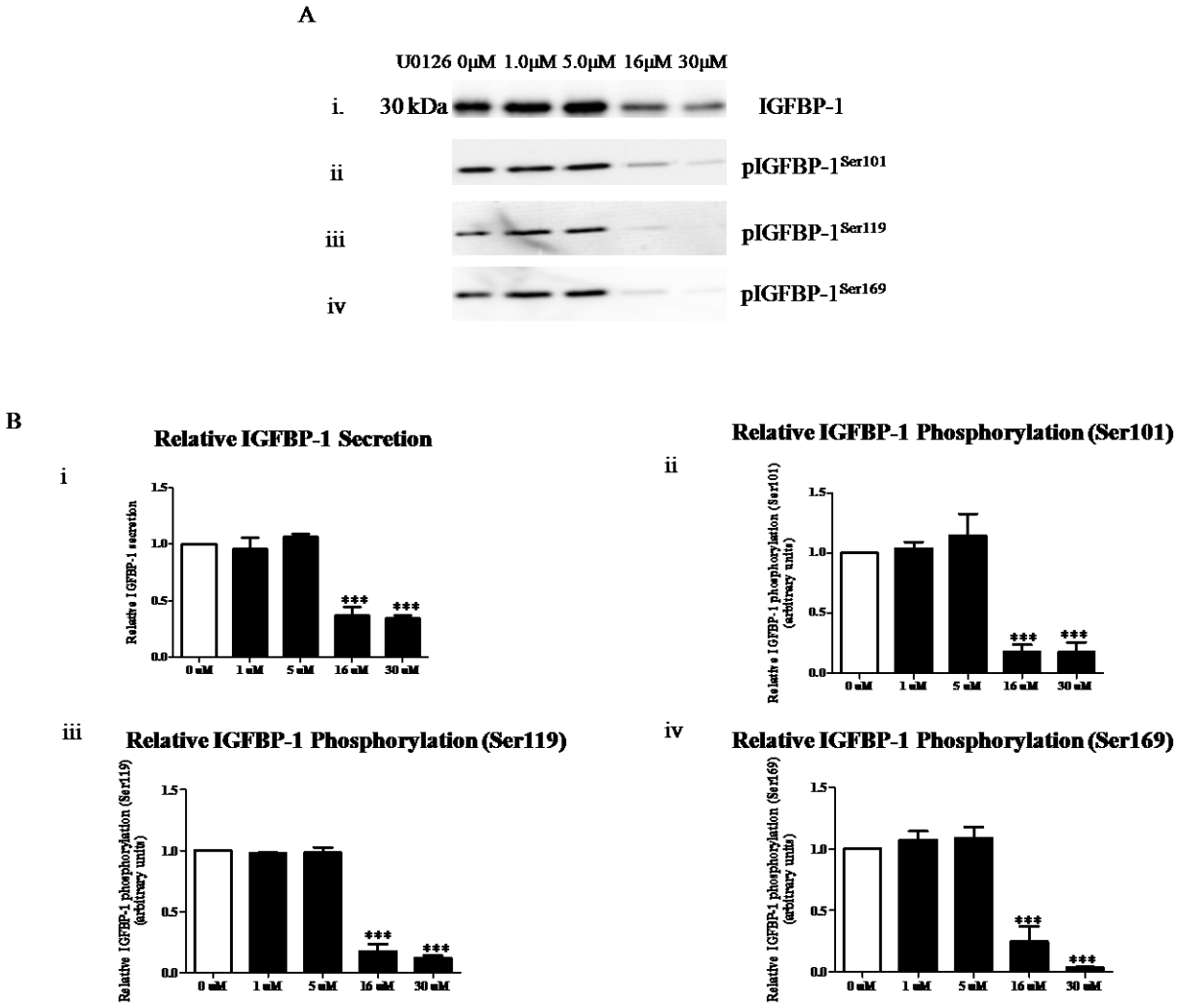
Supplementary Figure 3. 2. HepG2 cell vitality after treatment with BIS (7.5 µM) for 24 hours. A graphical representation of cell vitality between leucine plus or minus treatments with or without BISs. A Trypan Blue exclusion assay was conducted to assess cell viability, illustrated as the ratio of live to total cells. Values are normalized to viability in control samples (leucine plus, no inhibitor). BIS inhibitor did not compromise cell survival. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance;
Dunnet's Multiple Comparison Test; n=3. C: Control, 450 µM leucine. LD: Leucine deprivation, 0 µM leucine. BIS: Bisindolylmaleimide (7.5 µM), 450 µM leucine. BIS:LD: Bisindolylmaleimide (7.5 µM), 0 µM leucine.

Appendix B



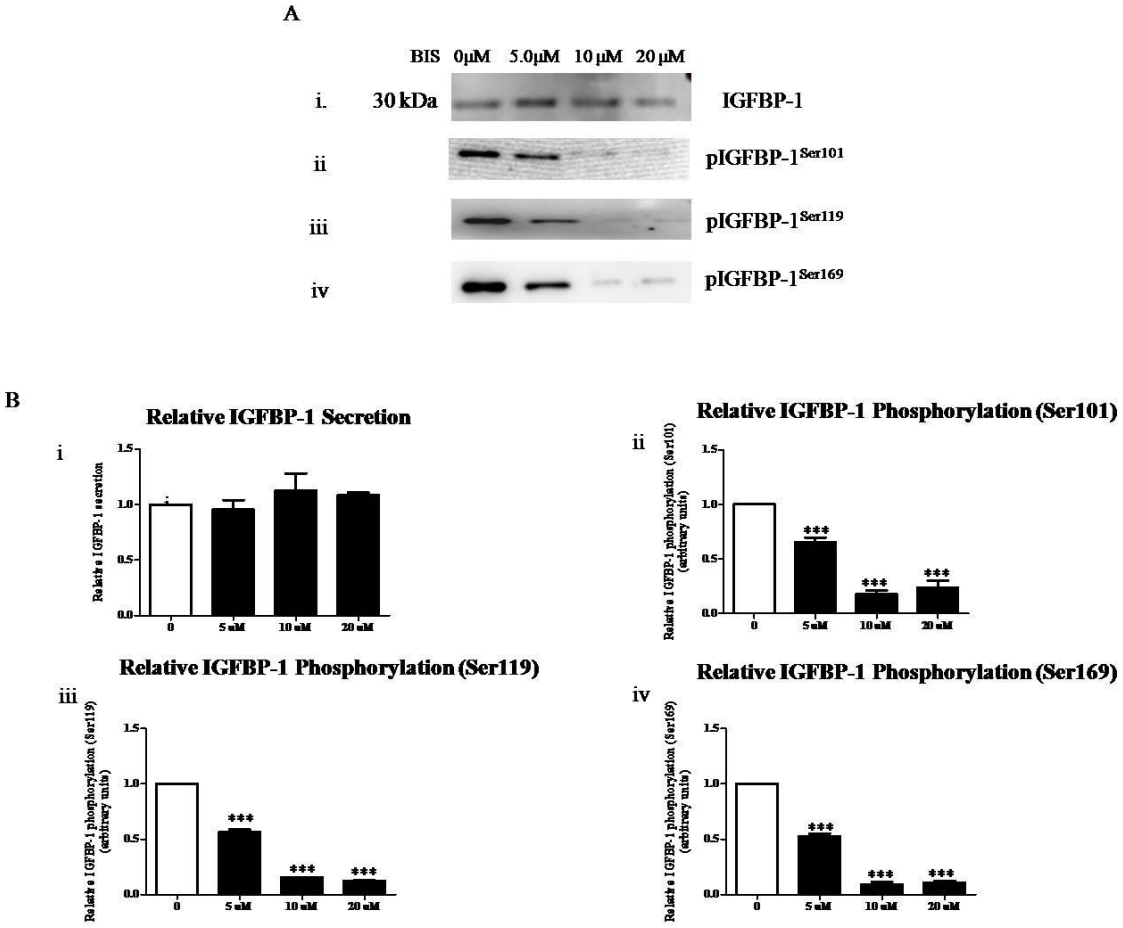
Supplementary Figure 3. 3. Efficiency of pan-PKC silencing. A representative immunoblot of HepG2 cell lysates (50 µg per lane) assayed for PKCδ and PKCε expression following siRNA silencing of pan-PKC. The expression of **A.** PKCδ and **B.** PKCε were both reduced (-50%) regardless of leucine status by pan-PKC siRNA in HepG2 cells.Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance;
Dunnet's Multiple Comparison Test; n=3. Sc:450: Scrambled siRNA, 450 µM leucine. Sc:0: Scrambled siRNA, 0 µM leucine. PKC:450 pan-PKC siRNA, 450 µM leucine. PKC:0: pan-PKC siRNA, 0 µM leucine.

Appendix C



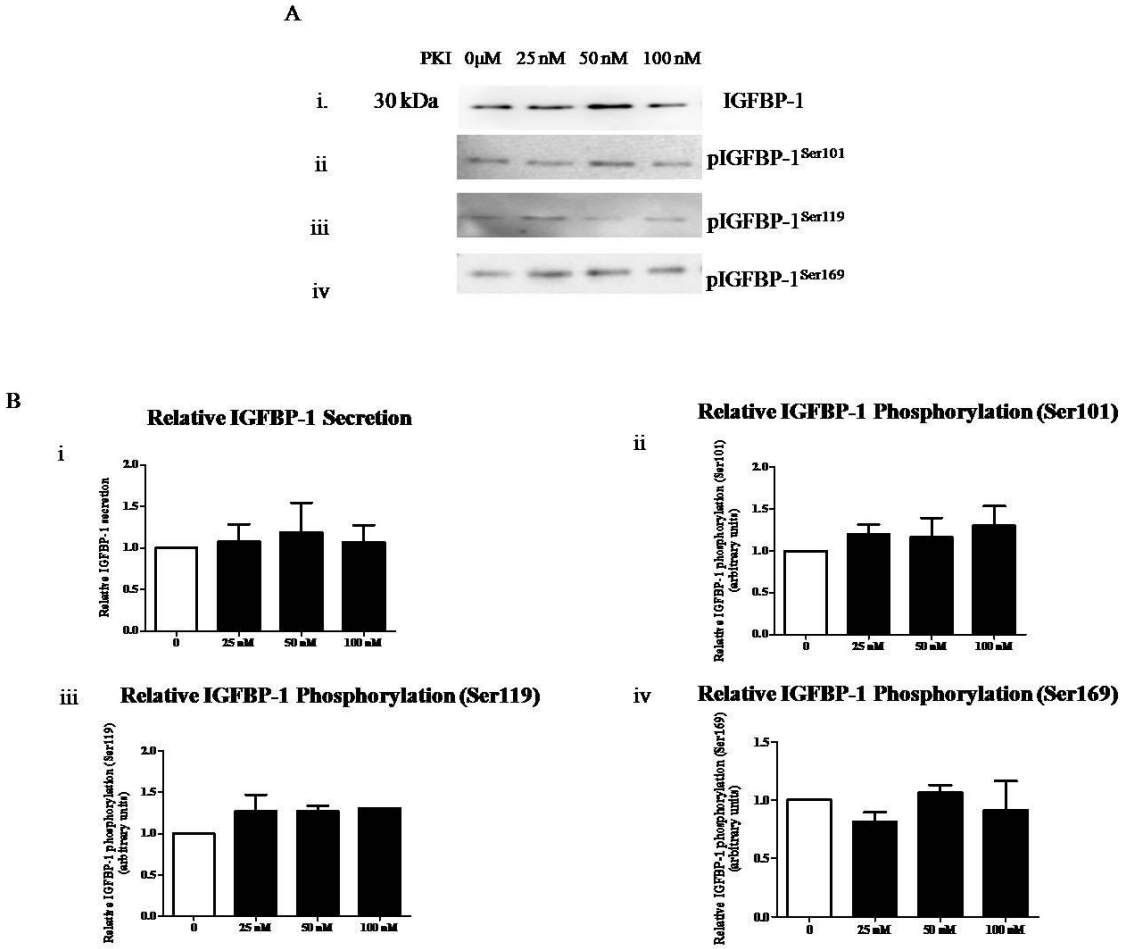
Supplementary Figure 4. 1. Dose-dependent changes in IGFBP-1 phosphorylation with U0126. A significant decrease in IGFBP-1 phosphorylation was seen at 16μM U0126 which remained consistent at approximately twice the inhibitor concentration (30 μM). A middle concentration between 5μM and 16μM U0126 (10 μM) was used in subsequent experiments.Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

Appendix C



Supplementary Figure 4. 2. Dose-dependent changes in IGFBP-1 phosphorylation with Bisindolylmaleimide (BIS). A significant decrease in IGFBP-1 phosphorylation was seen at 10 μM BIS which remained consistent at twice the inhibitor concentration (20 μM). A middle concentration between 5μM and 10μM BIS (7.5 μM) was used in subsequent experiments.Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance;
Dunnet's Multiple Comparison Test; n=3.

Appendix C



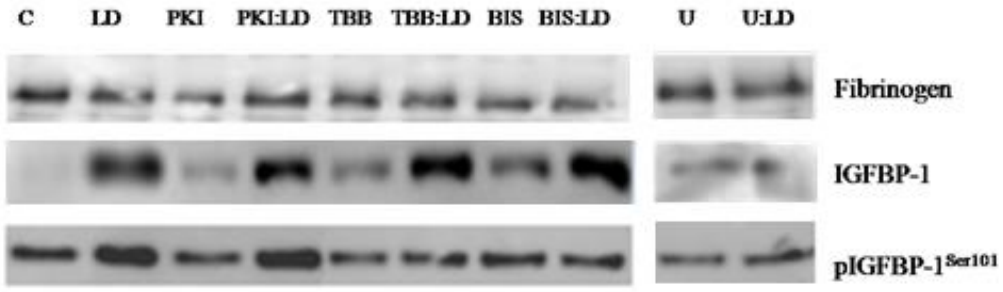
Supplementary Figure 4. 3. Dose-dependent changes in IGFBP-1 phosphorylation with PKI (5-24). PKI did not reduce IGFBP-1 secretion (i) or phosphorylation (ii-iv) in basal conditions. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance;
Dunnet's Multiple Comparison Test; n=3.

N.B. PKI (5-24) (IC_{50}=22 nM)[a]has been used in concentrations up to 2 μM in HepG2 cells[b]. We used conservative doses of PKI to assess the ability of PKA to phosphorylate IGFBP-1. As PKI was unable to diminish IGFBP-1 phosphorylation in basal conditions at the assayed doses, we employed the maximal surveyed dose (100 nM) in our subsequent assessment of IGFBP-1 phosphorylation in leucine deprivation.

[a]de Boer AR, Letzel T, Lingeman H, Irth H. Systematic development of an enzymatic phosphorylation assay compatible with mass spectrometric detection. *Anal Bioanal Chem*. 2005;381(3):647-655.

[b]Citterio C, Jones HD, Pacheco-Rodriguez G, Islam A, Moss J, Vaughan M. Effect of protein kinase A on accumulation of brefeldin A-inhibited guanine nucleotide-exchange protein 1 (BIG1) in HepG2 cell nuclei. *Proc Natl Acad Sci U S A*. 2006;103(8):2683-2688.

Appendix D



Supplementary Figure 4. 4. Fibrinogen as a loading control in conditioned media. Representative aliquots (30 μL) of HepG2 cell media from leucine plus and leucine minus treatments, with and without the various inhibitors used in this study (TBB, PKI, BIS, U0126), were probed with primary antibody against fibrinogen. Fibrinogen secretion remained consistent among treatments regardless of total IGFBP-1 secretion and IGFBP-1 phosphorylation.

Appendix E

Copyright Clearance Center RightsLink®

Help

Title: IGFBP-1 hyperphosphorylation in response to leucine deprivation is mediated by the AAR pathway

Publication: Molecular and Cellular Endocrinology

Publisher: Elsevier

Date: Dec 31, 1969

LOGIN

If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials. Already **a RightsLink user** or want to learn more?

Order Completed

Thank you for your order.

This Agreement between ("You") and Elsevier ("Elsevier") consists of your order details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License number	Reference confirmation email for license number
License date	May 18, 2015
Licensed content publisher	Elsevier
Licensed content publication	Molecular and Cellular Endocrinology
Licensed content title	IGFBP-1 hyperphosphorylation in response to leucine deprivation is mediated by the AAR pathway
Licensed content author	None
Licensed content date	Available online 5 May 2015
Licensed content volume number	n/a
Licensed content issue number	n/a
Number of pages	1
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	MCE_9129
Title of your thesis/dissertation	MOLECULAR MECHANISMS LINKING NUTRIENT DEPRIVATION TO IGFBP-1 HYPERPHOSPHORYLATION IN FETAL GROWTH RESTRICTION
Expected completion date	Jun 2015
Elsevier VAT number	GB 494 6272 12
Billing Type	Invoice

Curriculum Vitae

Name:	Niyati Malkani
Post-secondary Education and Degrees:	University of Western Ontario London, Ontario, Canada 2009-2013 BMSc.
Honours and Awards:	Departmentof Paediatrics Graduate Studentship 2013-2014 Society of Reproductive Investigation (SRI) Best New Investigator Poster Award 2015 Children's Health Research Institution (CHRI) Travel Award 2015 Department of Paediatrics Travel Award 2015
Related Work Experience	Teaching Assistant The University of Western Ontario 2014
Publications:	Malkani N, Jansson T, and Gupta MB. IGFBP-1 phosphorylation in response to leucine deprivation is mediated byrg the AAR pathway. *Mol Cell Endocrinol.* 2015, *in press.*