MECHANISMS OF PRECONDITIONING AGAINST SURGICAL STRESS BY SHORT-TERM DIETARY PROTEIN RESTRICTION

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Abstract

Dietary restriction (DR) or reduced food intake without malnutrition encompasses a variety of dietary interventions including reduction of calories or specific macronutrients such as amino acids or total protein. Even though DR is known to result in various beneficial health effects, including extended longevity and increased stress resistance, the underlying nutritional and genetic requirements remain incompletely understood. Previous studies in lower organisms, as well as in mammals, point to the importance of the restriction of dietary protein intake in DR benefits. Previously our lab has shown that short-term restriction of intake of individual essential amino acids protects against hepatic ischemia reperfusion injury (IRI) via activation of the amino acid deprivation sensing protein, general control non-derepressible 2 (GCN2). Interestingly, GCN2 was no longer required for the protective effects of total dietary protein restriction (PR) against hepatic IRI. We thus investigated the potential role of a distinct amino acid sensing pathway involving the mechanistic target of rapamycin complex 1 (mTORC1) kinase, which is normally repressed upon PR. To test the hypothesis that reduced mTORC1 signaling is required for benefits of PR against hepatic IRI, we used a mouse model with liver specific deletion of the mTORC1 repressor gene tuberous sclerosis complex 1 (TSC1), leading to constitutive hepatic mTORC1 activation (LTsc1KO). Although one week of PR was able to reduce circulating

growth factors and amino acid levels and activate hepatic GCN2 signaling in both LTsc1KO and WT mice, LTsc1KO mice failed to gain the preconditioning benefits of PR against hepatic IRI. To understand the molecular mechanism underlying the genetic requirement for the TSC complex in PR benefits, we focused on the observation that PR improved hepatic insulin sensitivity in WT but not LTsc1KO mice. Additional data from liver specific insulin receptor knockout (LIrKO) mice and in WT mice using pharmacological PI3K inhibition by wortmannin indicated a partial requirement for post-reperfusion insulin/Akt signaling in PR-mediated protection from hepatic IRI. In addition to defects in insulin signaling important for PR action, LTsc1KOs also failed to upregulate hepatic production of another potent protective molecule, hydrogen sulfide (H_2S), which was increased in WT mice upon PR and required for PR-mediated protection from hepatic IRI. Finally, we investigated the mechanisms of regulation of hepatic H_2S production. Using an *in vitro* model of increased H_2S production upon serum deprivation, we identified growth hormone (GH) as a negative regulator of H_2S production through JAK/STAT signaling, and increased autophagy as the likely source of free cysteine, the substrate for cystathionine gamma lyase (CGL)-mediated H_2S production. The mechanistic details of how *in vivo* PR translates into increased H_2S production, and the potential role of mTORC1, GH signaling and autophagy in this process remain to be fully elucidated.

TABLE OF CONTENTS

CHAPTER 1 ... 1

INTRODUCTION ... 1
- **SUMMARY ... 2**
- **BACKGROUND ... 2**
  - SENSING AMINO ACID DEFICIENCY: FROM UNCHARGED TRNAS TO THE GCN2 KINASE ... 4
  - SENSING AMINO ACID SUFFICIENCY: ROLE OF THE TOR SIGNAL TRANSDUCTION PATHWAY ... 8
  - MECHANISMS OF GCN2 VS. TOR BASED TRANSLATIONAL CONTROL ... 14
  - COORDINATION OF AMINO ACID STARVATION RESPONSES THROUGH GCN2 AND TOR ... 19
  - AMINO ACID SENSING IN DIETARY RESTRICTION BENEFITS ... 21
- **ACKNOWLEDGEMENTS ... 29**
- **REFERENCES ... 29**

CHAPTER 2 ... 44

THE TSC COMPLEX IS REQUIRED FOR THE BENEFITS OF DIETARY PROTEIN RESTRICTION ON STRESS RESISTANCE IN VIVO ... 44
- **BACKGROUND ... 45**
- **RESULTS ... 48**
  - DIETARY PROTEIN RESTRICTION MEDIATES STRESS RESISTANCE INDEPENDENT OF GCN2 ... 48
  - THE TSC COMPLEX IS REQUIRED FOR INHIBITION OF MTORC1 UPON PR IN VIVO ... 53
  - DIFFERENTIAL REDUCTION OF ESSENTIAL AMINO ACIDS AND GROWTH FACTORS IN VIVO UPON PR ... 57
  - THE TSC COMPLEX IS REQUIRED FOR BENEFITS OF PR AGAINST ACUTE HEPATIC STRESS ... 60
  - THE TSC COMPLEX IS REQUIRED FOR IMPROVED HEPATIC INSULIN SENSITIVITY UPON PR ... 68
  - INCREASED PROSURVIVAL SIGNALING AND REDUCED APOPTOSIS CONTRIBUTE TO PR-MEDIATED PROTECTION ... 73
- **DISCUSSION ... 81**
- **MATERIALS AND METHODS ... 85**
- **ACKNOWLEDGEMENTS ... 90**
- **REFERENCES ... 90**

CHAPTER 3 .... **96**

TSC1KO LIVERS HAVE IMPAIRED ABILITY TO UPREGULATE H_2S PRODUCTION CAPACITY IN RESPONSE TO DIETARY PROTEIN RESTRICTION **96**

SUMMARY **97**
BACKGROUND **97**
RESULTS **100**
LTSC1KO LIVERS HAVE IMPAIRED H_2S PRODUCTION CAPACITY IN RESPONSE TO PROTEIN RESTRICTION 100
THE EFFECTS OF CONSTITUTIVE MTORC1 ACTIVATION ON H_2S PRODUCTION ARE NOT CELL AUTONOMOUS, AND PR MEDIATED H_2S PRODUCTION LIKELY INVOLVES OTHER PATHWAYS 103
DISCUSSION **107**
MATERIALS AND METHODS **108**
ACKNOWLEDGEMENTS **110**
REFERENCES **111**

CHAPTER 4 .... **114**

REGULATION OF ENDOGENOUS H_2S PRODUCTION **114**

SUMMARY **115**
BACKGROUND **115**
RESULTS **118**
GROWTH HORMONE NEGATIVELY REGULATES H_2S PRODUCTION 118
REGULATION OF H_2S PRODUCTION BY GH IN VITRO INDEPENDENT OF CGL TRANSCRIPTIONAL REGULATION 122
H_2S INDUCTION BY GROWTH FACTOR/SAA DEPRIVATION REQUIRES FUNCTIONAL AUTOPHAGY 125
DISCUSSION **128**
MATERIALS AND METHODS **131**
ACKNOWLEDGEMENTS **133**
REFERENCES **133**

CHAPTER 5 .... **137**

DISCUSSION AND FUTURE DIRECTIONS **137**

INTRODUCTION **138**
NUTRITIONAL BASIS OF DIETARY RESTRICTION **140**
GENETIC BASIS OF DIETARY RESTRICTION **142**

CHAPTER 5 (Continued)

MOLECULAR MECHANISMS UNDERLYING DIETARY RESTRICTION BENEFITS 145
REGULATION OF ENDOGENOUS H_2S PRODUCTION 148
FUTURE DIRECTIONS 150
REFERENCES 153

For my family, my nephew Deniz, my niece Ece, and Burak;

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CHAPTER 1

INTRODUCTION

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SUMMARY

Dietary restriction (DR), or reduced food intake without malnutrition, is associated with extended longevity, improved metabolic fitness and increased stress resistance in a wide range of organisms. DR is often referred to as calorie restriction, implying that reduced energy intake is responsible for its widespread and evolutionarily conserved benefits. However, recent data indicate dietary amino acid restriction as a key mediator of DR benefits. In fruit flies, an imbalance in essential amino acid intake is thought to underlie longevity benefits of DR. In mammals, reduced dietary protein or essential amino acid intake can extend longevity, improve metabolic fitness and increase stress resistance. Here we review two evolutionarily conserved signal transduction pathways responsible for sensing amino acid levels. The eukaryotic initiation factor 2-alpha (eIF2α) kinase general amino acid control non-derepressible 2 (GCN2) senses the *absence* of one or more amino acids by virtue of direct binding to uncharged cognate tRNAs. The *presence* of certain amino acids, such as leucine, permits activation of the master growth regulating kinase target of rapamycin (TOR). These two signal transduction pathways react to amino acid deprivation by inhibiting general protein translation while at the same time increasing translation of specific mRNAs involved in restoring homeostasis. Together, these pathways may contribute to the regulation of longevity, metabolic fitness and stress resistance.

BACKGROUND

Coding amino acids are necessary for protein synthesis, but are also involved in a number of other essential processes. For example, methionine is required for one-carbon transfer reactions; tryptophan is a precursor for NAD and serotonin biosynthesis; glutamate acts as a

neurotransmitter; and a number of amino acids can serve as intermediate metabolites in a variety of processes ranging from gluconeogenesis to anaplerosis. Although lower organisms can synthesize all of the coding (and non-coding) amino acids from carbon skeletons and nitrogen, some of these biosynthetic pathways were lost during evolution of multicellular organisms. Thus humans (as well as mice and fruit flies) must acquire the "essential" amino acids they can no longer synthesize through dietary means.

Dietary restriction (DR), defined loosely as reduced food intake without malnutrition, was first shown to extend longevity in rats by Clive McCay in the 1930s (McCay et al., 1935). In the ensuing decades, lifespan extension by DR has been demonstrated in multiple species, along with a number of other benefits including extended healthspan, improved metabolic fitness and increased stress resistance. A large number of changes have been documented upon DR, including energy metabolism, gene expression, protein turnover, immune function and oxidative stress to name just a few (Masoro, 2003). However, which of these changes are evolutionarily conserved requirements for the benefits of DR remains unclear.

DR is also known as calorie restriction (CR), based in part on data in rodents suggesting that the reduction in total calorie intake is more important than the source of those calories (e.g. carbohydrates vs. protein) (Ross, 1961). Recent data in fruit flies indicate that the source of the calories does matter, and that reduction of protein calories in the form of yeast contributes more to longevity extension than carbohydrate calories (Mair et al., 2005). Furthermore, adding back essential amino acids erases the benefits of DR on longevity extension in fruit flies (Grandison et al., 2009). In rodents, it has long been known that reduced dietary intake of protein or certain amino acids, namely methionine and tryptophan, can also extend longevity (Orentreich et al., 1993, Segall and Timiras, 1976). What this has to do with lifespan extension by DR remains

unclear, as there are many similarities but also differences between rodents on DR and those on methionine restriction (Miller et al., 2005).

How do cells sense the availability of amino acids, and what role might these mechanisms play in conveying benefits of DR? Cells have evolved different mechanisms to sense both the *absence* of individual amino acids as well as the *presence* of some others. Lack of amino acids is generally sensed by a surrogate marker, an uncharged cognate tRNA. Uncharged tRNAs then trigger adaptive responses through a variety of downstream mechanisms to rectify the situation. In bacteria, these involve direct effects on transcriptional control, while in eukaryotes uncharged tRNAs trigger a signal transduction pathway via direct interaction with the eukaryotic initiation factor 2-alpha (eIF2α) kinase general amino acid control non-derepressible 2 (GCN2). The presence of certain amino acids such as leucine can also be sensed, activating signal transduction pathways that integrate responses to environmental cues, including nutrients, through the target of rapamycin (TOR) kinase. Less is known about upstream amino acid sensing mechanisms in this pathway. A common output of amino acid deficiency sensed by either mechanism is repressed general translation, but enhanced (or derepressed) translation of particular mRNAs involved in restoring homeostasis. The mechanisms by which GCN2 activation and TOR repression affect translation are also distinct. Here, we will discuss both GCN2 and TOR-based amino acid sensing mechanisms, downstream effects on translation, and how these pathways may contribute to the benefits of DR including stress resistance and longevity.

SENSING AMINO ACID DEFICIENCY: FROM UNCHARGED TRNAS TO THE GCN2 KINASE

Uncharged tRNAs activate the stringent response to amino acid deprivation in bacteria

Protein synthesis is universally important to life as we know it, and mechanisms to sense the building blocks of proteins, amino acids, are conserved across evolution. In bacteria, nutrient deficiency activates the stringent response, so-called because of the stringent inhibition of transcription of stable RNAs including rRNA and tRNAs (Potrykus and Cashel, 2008). The stringent response is dependent on unusual nucleotides, guanosine 5',3' bispyrophosphate (ppGpp) or guanosine pentaphosphate (pppGpp), collectively referred to here as ppGpp (Haseltine et al., 1972, Wendrich et al., 2002). ppGpp binds to the transcriptional regulator RelA and modulates RNA polymerase activity in a promoter-specific fashion, downregulating some genes and upregulating others (Cashel et al., 1996). Although the stringent response can occur upon deprivation of phosphate, carbon, iron or fatty acids, it is best understood in response to amino acid deprivation. When intracellular amino acids are low, cognate uncharged tRNAs accumulate and can compete with binding of charged tRNAs to the A-site of ribosomes. When an uncharged tRNA occupies an A-site (Goldman and Jakubowski, 1990), translation slows and the ribosome-associated ppGpp synthase RelA is activated (Wendrich et al., 2002). ppGpp, in complex with the transcriptional regulator DksA, can then repress stable RNA transcription by binding to RNA polymerase near the active site and inhibiting its activity (Krohn and Wagner, 1996). At the same time, ppGpp/DksA can activate transcription of amino acid biosynthetic genes and suppress transcription of tRNA and rRNA genes.

Uncharged tRNAs trigger the amino acid starvation response in eukaryotes by activating an eIF2α kinase

As in bacteria, intracellular uncharged tRNAs signal amino acid depletion in eukaryotic cells. Unlike bacteria, yeasts have a dedicated uncharged tRNA-sensing molecule, the signal transducing kinase GCN2 (Diallinas and Thireos, 1994). GCN2 has a domain with homology to

histidyl-tRNA synthetase (HisRS) that binds to uncharged tRNAs (Dong et al., 2000, Wek et al., 1995), resulting in kinase activation through homodimerization and autophosphorylation (Diallinas and Thireos, 1994, Narasimhan et al., 2004). Beside itself, activated GCN2 has only one other known target, eIF2α. Phosphorylation of eIF2α prevents efficient translational initiation at the starting methionine codon. This slows translation of most mRNAs while at the same time favoring the translation of select mRNAs with specific regulatory elements in their 5' untranslated regions (5'UTRs). This phenomenon of selective translational upregulation of specific mRNAs in the face of overall reduced global translation, known as translational derepression, is described in more detail in **Section 4**. One such derepressed mRNA encodes GCN4, the main effector of the general amino acid control (GAAC) pathway responsible for activating the transcription of amino acid biosynthetic and transport genes (Bushman et al., 1993, Hinnebusch, 1984). In mammals, the GCN4 ortholog controlling the transcriptional response to amino acid starvation, activating transcription factor 4 (ATF4), is similarly stabilized by translational derepression.

Conditions leading to GCN2 activation

In theory, any uncharged tRNA can bind the HisRS-like domain of GCN2. In yeast, a handful of conditions leading to accumulation of uncharged tRNAs have been experimentally verified to activate GCN2. These conditions include depletion of amino acids, mutation of aminoacyl tRNA synthetase genes or chemical inhibition leading to the accumulation of tRNAs for select amino acids (Wek et al., 1995). Interestingly, deprivation of a single amino acid can result in deacylation of non-cognate tRNAs as well. For example, leucine starvation of auxotrophic yeast results in accumulation of uncharged serine and threonine tRNAs in addition to leucine tRNAs (Zaborske et al., 2009).

In mammals, GCN2 can be rapidly activated in brain and liver (but apparently not kidney) upon ingestion of a meal lacking essential amino acids including leucine, histidine, tryptophan or lysine (Zhang et al., 2002, Hao et al., 2005, Anthony et al., 2004). GCN2 can also be activated by non-dietary depletion of amino acids (Thoreen et al., 2012). For example, in the context of an acute stress such as trauma or sepsis, increased nitric oxide production by NOS can rapidly deplete the conditional essential amino acid arginine from the blood. Local depletion of the essential amino acid tryptophan in the placenta by the tryptophan catabolizing enzyme indoleamine 2.3-dioxygenase (IDO) can anergize T cells that might otherwise react against the fetus. This purposeful immunosuppression is dependent on GCN2 expression in T cells (Munn et al., 2005). Other amino acid catabolizing enzymes such as asparaginase have been co-opted as chemotherapeutic agents against blood-borne cancers; the response to this agent in mice is dependent on GCN2 (Bunpo et al., 2009). Simulated amino acid starvation can also be achieved by blocking the charging of tRNAs with their cognate amino acid. Halofuginone, for example, is a competitive inhibitor of the prolyl-tRNA synthetase (Keller et al., 2012) that can activate the amino acid starvation response *in vitro* and *in vivo* (Peng et al., 2012, Sundrud et al., 2009). It is used to treat a variety of maladies ranging from psoriasis (an autoimmune disorder) to cancer.

Thus, depletion of essential, non-essential, or conditionally essential amino acids by dietary, enzymatic or pharmacological means can activate the amino acid starvation response by increasing the concentration of uncharged tRNA species and activating GCN2. Because GCN2 regulates adaptive changes to perceived amino acid deficiency, mice lacking this protein appear normal in the absence of such a challenge. This is not the case for one of the prime effectors of the GCN2 response, ATF4, which is required not only for the response to amino acid insufficiency, but also for the normal anabolic response to insulin, mediated at least in part

through the mechanistic target of rapamycin complex 1 (mTORC1) (Adams, 2007, Malmberg and Adams, 2008). Cells lacking ATF4 require excess non-essential amino acids including cysteine (or antioxidants such as glutathione or N-acetyl cysteine)(Harding et al., 2003), and ATF4 knockout mice have multiple developmental abnormalities and are smaller than control littermates (Yang et al., 2004).

Specificity of the GCN2-dependent amino acid starvation response

GCN2 is one of a family of four kinases that share a common target, serine 51 of the translation initiation factor eIF2α. In addition to amino acid deficiency, GCN2 can also be activated by glucose deprivation and UV irradiation (Deng et al., 2002). The other three eIF2α kinases are activated by diverse signals in different tissues, ranging from heme deficiency in erythrocytes (HRI) to endoplasmic reticulum stress in pancreatic B cells (PERK) to infectious or metabolic stress in a variety of tissues (PKR). Global translational suppression and translational derepression of targets such as ATF4 are not unique to GCN2-mediated eIF2α phosphorylation upon amino acid starvation. Nonetheless, some specificity of each response to the appropriate stimulus is maintained, possibly as a result of additional, unique targets (Dang Do et al., 2009). PKR, for example, has other direct targets besides eIF2α including protein phosphatase 2A (Xu and Williams, 2000). Although GCN2 has no known direct targets besides eIF2α, DNA-PK can be phosphorylated in a GCN2-dependent manner upon exposure of cells to UV (Powley et al., 2009).

SENSING AMINO ACID SUFFICIENCY: ROLE OF THE TOR SIGNAL TRANSDUCTION PATHWAY

Amino acid, energy and growth factor signaling integrated through TOR

Our current understanding of the ability of cells to sense the presence, rather than absence, of specific amino acids is based on genetic and pharmacological perturbation of the signal transduction cascade involving the target of rapamycin (TOR) serine/threonine kinase. TOR is best known for controlling cell size and proliferation in response to the presence of adequate energy, nutrients and growth factors. TOR was first identified as the cellular target of the growth-inhibiting bacterial compound rapamycin, and is conserved from yeast to humans. Yeast has two TOR isoforms: TOR1 and TOR2, which are functionally distinct. Mammalian cells have a single TOR kinase, mTOR, which exists in two structurally and functionally distinct multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is acutely inhibited by rapamycin and sensitive to nutrient levels, while mTORC2 is neither readily sensitive to nutrients nor acutely inhibited by rapamycin (Laplante and Sabatini, 2009).

The kinase activity of mTORC1 is regulated by its association with the Ras-related guanosine triphosphatase (GTPase) Rheb (Ras homologue enriched in brain). In its GTP bound form, Rheb directly activates mTORC1. The Rheb specific GTPase activating protein (GAP) is the TSC2 (tuberous sclerosis complex 2) protein, which functions as a heterotrimer with its binding partners TSC1 and TBC1D7 (Huang and Manning, 2008, Dibble et al., 2012). The TSC1-TSC2-TBC1D7 complex is the critical negative regulator of mTORC1 signaling with respect to most of its upstream regulators such as growth factors, insulin and energy levels (Howell and Manning, 2011). The best-characterized downstream effectors of mTORC1 signaling include eukaryotic initiation factor 4E (eIF4E) binding proteins 1 and 2 (4E-BP1 and 4E-BP2), and the p70 ribosomal protein S6 kinases 1 and 2 (S6K1 and S6K2). These proteins are directly phosphorylated by mTORC1, and are thus commonly used as markers of TOR activity (Zoncu et al., 2011b).

Select amino acids regulate TORC1 signaling

Amino acid levels regulate both protein synthesis and autophagic proteolysis. Evidence supporting the role of mTORC1 in this regulation came from cell culture studies in which phosphorylation of mTORC1 substrates S6K and 4EBP were associated with translational control and inhibition of autophagy upon amino acid stimulation (Blommaart et al., 1995, Hara et al., 1998, Fox et al., 1998, Xu et al., 1998, Wang et al., 1998). In rat adipocytes, addition of amino acids to the media stimulates S6K phosphorylation, which is sensitive to rapamycin treatment (Fox et al., 1998). In Chinese hamster ovary (CHO) cells, amino acid withdrawal results in a rapid dephosphorylation of S6K and 4EBP1 and abolishes the ability of growth factors to stimulate S6K activity. Re-addition of single amino acid dropout mixtures lacking leucine or arginine reduces S6K activity by 90% or 70%, respectively (Hara et al., 1998). Interestingly, readdition of either leucine or arginine individually is unable to stimulate S6K activity.

Similar studies carried out in different contexts and cell types, including H4IIE hepatoma, L6 muscle and pancreatic β cells, revealed important details about the regulatory properties of individual amino acids on TOR activity. All agreed on the prominent role of leucine (Shigemitsu et al., 1999, Xu et al., 1998, Krause et al., 2002, Peyrollier et al., 2000). However, in certain contexts arginine stimulates S6K activity (Nakajo et al., 2005), while in others each of the branched chain amino acids (leucine, isoleucine and valine) has similar potency in stimulating 4EBP1 phosphorylation (Xu et al., 1998). The non-essential amino acid glutamine can also modulate leucine and arginine-stimulated mTORC1 signaling, although in different directions in different cell types. In rat intestinal epithelial cells and myogenic C_2C_{12} cells, glutamine antagonizes arginine and leucine-mediated mTORC1 activation, respectively (Nakajo et al., 2005,

Ban et al., 2004, Deldicque et al., 2008). In isolated rat hepatocytes, leucine and glutamine work additively to increase mTORC1 signaling (Krause et al., 2002). In HeLa cells, the interaction between glutamine and leucine has been ascribed to a heterodimeric, bidirectional amino acid antiporter consisting of solute carrier family 7 member 5/solute carrier family 3 member 2 (SLC7A5/SLC3A2) that regulates the simultaneous efflux of glutamine and influx of leucine and other essential amino acids (Nicklin et al., 2009). Interestingly, in a recent study, carried out in a variety of cell types, it was shown that leucine and glutamine cooperate to activate mTORC1 signaling, through enhancing glutaminolysis and α-ketoglutarate production (Duran et al., 2012).

In yeast auxotrophs, deprivation of leucine (and to a lesser degree lysine and histidine) reversibly inhibits TORC1-dependent phosphorylation of Sch9 (the yeast ortholog of S6K). Inhibition of protein synthesis with cyclohexamide results in accumulation of intracellular free amino acids, with leucine accumulating more than others, and increases Sch9 phosphorylation (Binda et al., 2009). Together these observations are consistent with leucine primacy in TOR activation crossing evolutionary boundaries.

Bridging the gap between leucine sensing and TOR activation

Unlike the GCN2 pathway, the most upstream mechanisms directly responsible for amino acid sensing permissive of TOR activation, and even the subcellular compartment in which this occurs, remain unresolved. TOR itself does not appear to bind amino acids or surrogates such as cognate tRNAs as GCN2 does. A number of candidates have been proposed as intermediates in TOR activation by amino acids. Some initial studies suggested a role of TSC1/TSC2 in amino acid sensing. However, budding yeast don't have TSC proteins, and mammalian TSC1 or TSC2 null cells are still sensitive to amino acid withdrawal, suggesting that amino acids signal to

mTORC1 independently of TSC1/TSC2 (Smith et al., 2005, Huang and Manning, 2008). Human vacuolar protein-sorting 34 (hVps34) has also been proposed to participate in amino acid sensing. This class III PI3-kinase participates in the regulation of mTORC1 by amino acids, possibly through its effects on vesicle trafficking and/or through generation of membrane compartments necessary for mTORC1 activation (Dann and Thomas, 2006, Avruch et al., 2009).

Two independent groups, using complementary biochemical and genetic approaches, showed that the Rag family of small GTPases is required for amino acid sensing by mTORC1 (Sancak et al., 2008, Kim et al., 2008). Rag GTPases are orthologs of the yeast Gtr1p and Gtr2p GTPases, which function as obligate heterodimers and regulate the Gap1 amino acid permease and microautophagy. The critical role of Rags in mTORC1 signaling and its regulation of cellular growth is further evidenced by *in vivo* experiments in *Drosophila*, where constitutively active RagA leads to flies with increased cell and wing size, whereas dominant negative RagA leads to flies with decreased wing size (Kim et al., 2008). Mammalian Rags also exist in heterodimers with one Gtr1p-like (RagA or RagB) and one Gtr2p-like (RagC or RagD) partner (Shaw, 2008). Unlike the Rheb GTPase, purified Rag GTPases are unable to stimulate mTORC1 kinase activity *in vitro* (Kim et al., 2008).

Instead, Rag-mediated mTORC1 activation involves recruitment of the complex to the outer lysosomal membrane, where it can interact with its activator Rheb (Sancak et al., 2008, Sancak et al., 2010). The Rag heterodimer is anchored to the lysosomal membrane via the protein complex called "*Ragulator*" (Sancak et al., 2010, Efeyan et al., 2012) and activated by the presence of amino acids. RagA/B in its GTP-bound form with Rag C/D in its GDP-bound form constitutes the active heterodimer, which recruits mTORC1 to the lysosomal membrane through an interaction with Raptor. The Ragulator complex is functionally analogous to the yeast EGO

complex due to its interaction with Gtr1p and Gtr2p, and its regulation of amino acid signaling to TOR at the vacuolar membrane (Binda et al., 2009, Sancak et al., 2010). Thus, spatial regulation of the mTORC1 complex has emerged as an important aspect of amino acid-mediated control (Suzuki and Inoki, 2011).

Lysosomes/vacuoles are a major site of protein degradation and amino acid recycling with high concentrations of free amino acids. Based on mTORC1 localization and activation at the cytoplasmic face of the lysosomal membrane, the relevant amino acid sensing mechanism has been proposed to sense lysosomal rather than cytoplasmic free amino acid pools (Sancak et al., 2010). A *Drosophila* siRNA screen targeting the lysosomal components that might be involved in amino acid signaling to TOR revealed the requirement of vacuolar ATPase (V-ATPase), and this was confirmed in mammalian cells (Zoncu et al., 2011a). Although the ATPase activity of V-ATPase is required for amino acid permissive signaling, V-ATPase does not function in transport of amino acids between the cytoplasm and the lysosome. V-ATPase does, however, appear to function upstream of the Rag-Ragulator interaction, in which amino acids stimulate guanine nucleotide exchange factor (GEF) activity of the pentameric Ragulator complex toward RagA and RagB (Bar-Peled et al., 2012). In further support of the importance of lysosomal free amino acid pools, stimulation of amino acid starved cells with radioactively labeled amino acids leads to their rapid appearance in the lysosome (Zoncu et al., 2011a).

At face value, this lysosome-centered rather than cytoplasmic-centered view of amino acid sensing appears to be at odds with a recent report identifying the leucyl-tRNA synthetase as the intracellular leucine sensor responsible for mTORC1 activation (Han et al., 2012). siRNA directed at the leucyl-tRNA synthetase renders 293T cells unable to phosphorylate S6K in response to leucine or isoleucine withdrawal and restimulation. Consistent with its requirement

for leucine permissive mTORC1 activation, the leucyl-tRNA synthetase also localizes to the cytoplasmic face of the lysosomal membrane, interacts with the Rag GTPase and has GAP activity specifically toward RagD, promoting the active GDP-bound form in the presence of leucine. Its ability to stimulate mTORC1 is dependent on leucine binding, but is independent of its ability to charge leucine tRNA. Nonetheless, the subcellular localization of the free leucine, and whether other TOR-permissive amino acids such as arginine function through similar mechanisms, remain to be seen.

MECHANISMS OF GCN2 VS. TOR BASED TRANSLATIONAL CONTROL

Translation of mRNA is one of the most energy requiring processes in the cell. It must therefore be coupled to the availability of cellular energy and coding amino acids. The immediate response to energy or amino acid insufficiency is to repress translation. At the same time, cells increase translation of specific messages involved in metabolic adaptation, for example transcription factors controlling the expression of amino acid biosynthetic and transport genes. This process is known as translational derepression. Just as GCN2 and TOR pathways sense amino acids by different mechanisms, so do they differ in the ways they accomplish translational repression and derepression (Figure 1.1).

Translational regulation by GCN2

Translation initiation occurs upon pairing of the AUG start codon with an initiator methionyl-tRNA (Met-$tRNA_i^{Met}$), which is part of the so-called ternary complex together with eIF2 and

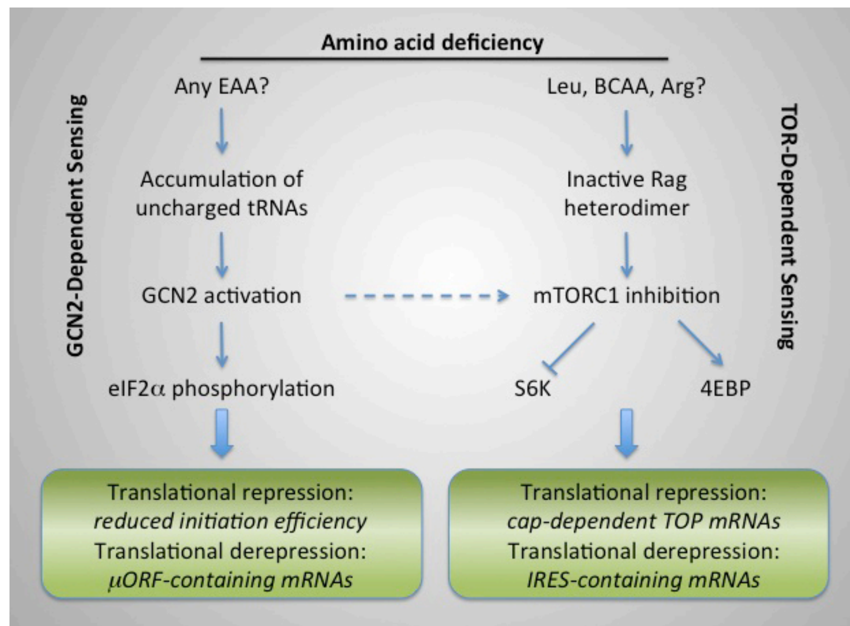

Figure 1.1 Integration of amino acid sensing with translational control. Two signal transduction mechanisms are involved in sensing intracellular amino acids: GCN2 senses the absence of many amino acids; the TOR pathway senses the presence of particular amino acids. Both repress general translation and derepress translation of specific messages through distinct mechanisms. EAA, essential amino acid. BCAA, branched chain amino acid.

GTP. This is followed by hydrolysis of eIF2-bound GTP and release of eIF2-GDP from the 43S ribosome. Phosphorylation of the subunit of eIF2 by GCN2 inhibits GDP/GTP exchange by eIF2B, reducing ternary complex availability and thus repressing initiation of translation of most mRNAs.

Some mRNAs, however, contain short upstream open reading frames, or micro (μ)ORFs, that influence translation of the downstream full-length ORF depending on the status of eIF2α phosphorylation. Reinitiation of translation following μORF translation requires reassociation of the ternary complex. This depends both on the concentration of ternary complex (and hence eIF2α phosphorylation status) and the scanning distance/time to the next AUG codon. Increasing the distance between μORFs increases the chance of ternary complex association before reaching the next AUG start codon (Abastado et al., 1991). Under non-starvation conditions, reinitiation of translation after the first μORF is relatively efficient, decreasing the chance of translation of the downstream full-length ORF. Under amino acid starvation conditions, reinitiation after the first μORF is less efficient, increasing the chance that the ribosome will scan past subsequent μORFs and reinitiate translation further downstream at the full-length ORF. Thus, the distance between μORFs and timing of ribosomal reinitiation are critical factors in translational derepression of mRNAs such as *GCN4* upon intracellular amino acid starvation. Stabilization of ATF4 occurs by a similar mechanism (Vattem and Wek, 2004), indicating conservation of this simple yet elegant translational program from yeast to mammalian cells.

Translational regulation by mTOR

The mTORC1 pathway controls protein synthesis most directly by phosphorylating and inhibiting a repressor of cap-dependent mRNA translation, 4EBP-1. Translation of most cellular

mRNAs is initiated via this common mechanism involving assembly of the eukaryotic initiation factor 4F complex (eIF4F, comprised of eIF4E, eIF4G and eIF4A) on the 5' 7-methyl-GTP cap. eIF4F is required for the loading of the small ribosomal subunit on the mRNA. 4EBPs repress translation by inhibiting the formation of the eIF4F complex (Ma and Blenis, 2009). Phosphorylation of 4EBPs by mTORC1 results in their dissociation from eIF4E, facilitating cap-dependent translation (Huang and Manning, 2008). Acute inhibition of mTORC1 with rapamycin, or the more potent inhibitor Torin 1, reduces protein synthesis by approximately 30% or 60%, respectively, without affecting the status of eIF2α phosphorylation (Thoreen et al., 2012). Torin 1 inhibits translation of nearly all (99.8%) cellular mRNAs, but the magnitude of repression is greatest for mRNAs containing 5' terminal oligopyrimidine (TOP) motifs in their 5'UTRs. Importantly, this class of mRNAs encode all ribosomal proteins and major translation factors, therefore influencing the protein synthetic capacity of the cell. Torin 1-mediated translation repression requires 4EBPs and is lost in cells lacking these proteins (Thoreen et al., 2012).

mTORC1 also regulates translation indirectly through phosphorylation of S6K. Phosphorylation on Thr^{389} activates S6K toward ribosomal protein S6, a component of the 40S ribosome important for translation in rapidly proliferating cells. It also phosphorylates a number of other downstream targets including eIF4B, eukaryotic translation elongation factor 2 kinase (eEF2K), and SKAR (Ma and Blenis, 2009). Phosphorylation of S6K targets can promote translation by a variety of direct and indirect mechanisms, including regulation of eIF4B recruitment, inhibition of PDCD-4 mediated inhibition of eIF4A, splicing-dependent translation of some mRNA messages via SKAR, and regulation of ribosome biogenesis (Ma et al., 2008, Richardson et al.,

2004). The ability of amino acids to regulate phosphorylation of both S6K and 4EBPs was evidenced in early studies (Xu et al., 1998).

While mTORC1 inhibition represses cap-dependent translation, it also selectively derepresses translation of select mRNAs that don't depend on cap-binding for ribosome entry. Conceptually, this parallels translational derepression upon GCN2 activation. However, instead of a μORF, it depends on an internal ribosome entry site (IRES) in the 5'UTR for cap-independent ribosome recruitment. IRES-containing mRNAs are not only resistant to mTORC1 inhibition by Torin 1, but are in fact translated with greater efficiency under such conditions (Thoreen et al., 2012). IRES-containing mRNAs are often involved in the response to stress, for example NF-E2 related factor 2 (NRF2), the master transcriptional regulator of genes in the Phase II response to oxidative/electrophilic stress. NRF2 is translationally upregulated during oxidative stress despite the attenuation of global protein synthesis due to an IRES-dependent mechanism (Li et al., 2010, Purdom-Dickinson et al., 2007). However, whether NRF2 is translationally derepressed upon amino acid starvation or mTORC1 inhibition remains to be elucidated.

Protein deficiency is expected to simultaneously repress mTOR and activate GCN2. How can efficient translational derepression occur when mRNA cap-binding and translation initiation are both inhibited by mTORC1 repression and GCN2 activation, respectively? One possibility is that mRNAs subject to control by GCN2/eIF2α lack TOP motifs, limiting the effect of mTORC1/4EBP-based repression on cap-binding. Another possibility is that IRES-containing mRNAs also contain μORFs; cationic amino acid transporter 1 (CAT1) is an example of such an mRNA (Kilberg et al., 2009). IRES-mediated translational derepression can also occur despite phosphorylation of eIF2α. For example, translation of X-chromosome linked Inhibitor of Apoptosis (XIAP) occurs via an eIF2α-independent mechanism of translation initiation

dependent on eIF5B (Thakor and Holcik, 2012). Thus, translational derepression likely depends both on features within a particular 5'UTR as well as the status of both GCN2 and TOR activity.

COORDINATION OF AMINO ACID STARVATION RESPONSES THROUGH GCN2 AND TOR

Why did separate amino acid sensing pathways evolve together in eukaryotes? Are they redundant or do they play separate roles? What is the connection between the two? Amino acid sensing through the TOR pathway may have evolved primarily to coordinate information on the presence of adequate nutrients (including amino acids), energy and other environmental conditions favorable for growth. Leucine and the other branched chain amino acids (BCAAs) are abundant in a variety of proteins. In mammals, they are also the only free amino acids to increase in peripheral blood after a meal in proportion to their levels in the diet (the remaining 17 are retained in the gut and liver and released in a controlled fashion) (Layman, 2003). These qualities of BCAAs may be advantageous for a surrogate marker of availability of all amino acids.

GCN2-based sensing of individual amino acid deficiencies may have evolved for different reasons. Dietary amino acid deficiencies are common and must be detected rapidly in order to avoid negative effects ranging from reduced growth to fatty liver (Harper et al., 1970). Protein sources with amino acid deficiencies include rice (low amounts of the essential amino acid lysine), casein (limiting for both tryptophan and methionine) and gelatin (lacks tryptophan altogether). Xenobiotic antimetabolites targeting individual amino acid metabolic pathways (for example, tRNA synthetase inhibitors) may also be widespread in nature. Interestingly, yeast can make all 20 coding amino acids, and thus don't activate the GAAC response when amino acids

are absent (for example in minimal growth media) (Hinnebusch, 2005). However, they do activate the response when individual amino acids are deficient or in the presence of tRNA synthetase inhibitors. Thus, the GCN2-based GAAC response may have evolved under different selective pressures than the TOR-based response, although both function to optimize growth potential and prioritize metabolic demands to fit environmental conditions.

Are responses to amino acid deprivation through GCN2 and TOR coordinated? In yeast, GCN4 translation is derepressed upon inhibition of TOR with rapamycin in a GCN2 and TOR1-dependent fashion (Kubota et al., 2003). Mechanistically, TOR inhibition may reduce inhibitory phosphorylation of GCN2, thus promoting eIF2α phosphorylation and GCN4 translational derepression. GCN4 is also a major effector of the transcriptional response to TOR inhibition by rapamycin on a scale equivalent to the canonical transcriptional activator and TOR substrate GLN3 (Staschke et al., 2010, Cherkasova and Hinnebusch, 2003). Genes co-regulated by both GCN4 and GLN3 include those involved in nitrogen assimilation, amino acid transport, amino acid biosynthesis, and other transcriptional activators.

Crosstalk between GCN2 and mTOR also exists in mammalian cell culture and animal models, although the direction of interaction appears to be different than in yeast. In human lymphocytic leukemic cell lines, treatment with L-asparaginase, an enzyme that degrades asparagine in culture medium and activates GCN2, inhibits mTORC1 phosphorylation of its targets S6K and 4EBP1 in a dose-dependent fashion (Iiboshi et al., 1999a). Likewise, S6K activity is reduced in human T-lymphoblastoid cells exposed to various amino acid alcohols that selectively inhibit specific tRNA loading, including L-histidinol, L-leucinol, L-phenylalaninol, and L-methioninol (Iiboshi et al., 1999b). Both studies suggest crosstalk between GCN2 and mTORC1 signaling, with uncharged tRNAs initiating the changes in signal transduction.

Genetic evidence in mice is also consistent with GCN2 activation occurring upstream of mTORC1 repression. In response to dietary leucine deprivation or asparaginase treatment, phosphorylation of mTORC1 targets S6K and 4EBP1 are reduced in the liver and pancreas, and this depends on the GCN2 kinase (Bunpo et al., 2009, Anthony et al., 2004). Leucine-deficient diets also improve insulin signaling in the liver as measured by increased phosphorylation of the insulin receptor, and whole-body insulin sensitivity as measured by an insulin tolerance test (Xiao et al., 2011). However, in GCN2 KO mice on a leucine deficient diet, mTOR signaling in liver is increased and the improvement in insulin sensitivity is lost. Interestingly, GCN2 KO mice on a leucine deficient diet also develop hepatic steatosis due to increased expression of SREBP-1c dependent lipogenic genes including *FAS, ACL,* and *G6PD*, which are under control of mTORC1 in the liver. This suggests a model in which failure to repress mTORC-1 allows inappropriate activation of SREPB-1c targets (Guo and Cavener, 2007, Yecies et al., 2011). Potentially complicating this model is the fact that leucine deprivation on its own would be predicted to reduce mTORC1 activity by GCN2 independent mechanisms. Nonetheless, taken together these findings are consistent with a model in which GCN2 activation by pharmacological or dietary means can suppress mTORC1 activity.

AMINO ACID SENSING IN DIETARY RESTRICTION BENEFITS

Dietary protein/amino acid modulation and longevity

DR has been intricately associated with aging research since the discovery in the 1930s that reduced food intake extends longevity of experimental rodents (McCay et al., 1935). Today, we know that the benefits of DR occur in a variety of organisms and are pleiotropic in nature. These benefits include enhanced metabolic fitness and improved resistance to multiple forms of acute

stress, ranging from heat shock to surgical ischemia reperfusion injury. Still, lifespan extension in a variety of model organisms is the most studied and perhaps best understood of its pleiotropic benefits.

Classically, DR has been described as reduced food intake without malnutrition. DR is not a single intervention but it rather loosely describes a variety of interventions ranging widely in both dietary composition and timing of food intake (Mair and Dillin, 2008). Despite a number of studies aimed at dissecting the nutritional basis of DR, no consensus yet exists on the relative contributions of overall reduced calorie intake vs. the restriction of particular macronutrients such as protein (Mair et al., 2005).

In yeast, in addition to deletion of TOR1 or Sch9, inhibition of glutamine synthetase and rapamycin treatment, chronological lifespan can be extended by restriction of asparagine, glutamate or methionine in the media (Dilova et al., 2007). Unfortunately in worms, another well-characterized model organism in which the genetics of longevity extension and DR are particularly well characterized, the lack of purified diets and the ability of starvation to increase the lifespan of adults makes these questions difficult to address.

In fruit flies, reduction of the calories via titration of the sole source of protein, yeast extract, provides greater longevity extension than isocaloric reduction of sucrose (Mair et al., 2005). Adding back purified essential amino acids (EAA) to a restricted sucrose/yeast-based diet optimized for longevity abrogates lifespan extension, while adding back EAA minus methionine (or to a lesser degree tryptophan) does not (Grandison et al., 2009). Perhaps most unexpectedly, adding back methionine alone (but not other individual EAA) on top of the DR regimen abrogated a well-known negative consequence of DR, reduced fecundity. While the involvement

of reduced insulin-like peptide signaling is implicated in longevity benefits (Grandison et al., 2009), the status of GCN2 under conditions of DR and EAA add-back remains to be reported.

In rodents, a number of studies have reported moderate lifespan extension upon dietary protein restriction as with DR (Pamplona and Barja, 2006). However, interpretation of DR and protein restriction studies are both complicated by the fact that protein and carbohydrates are readily interconverted *in vivo*. A different approach was to reduce individual dietary essential amino acids, namely tryptophan and methionine. It is not clear why only these two have been tested in rodents for longevity benefits. In the case of tryptophan, there is some evidence of increased maximal lifespan and a delay in aging-related phenotypes in at least a subset of tryptophan-restricted rats (Segall and Timiras, 1976, Ooka et al., 1988). The underlying mechanistic hypothesis in these studies involved reduction of serotonin, a downstream metabolite of tryptophan. Likewise, methionine restriction has been shown to extend lifespan in male rats and mice (Orentreich et al., 1993, Miller et al., 2005).

Interestingly, tryptophan and methionine are the two least abundant essential amino acids by weight in casein, a protein commonly used in purified diets for experimental rodents. Thus, DR regimens using casein as the sole source of protein would be expected, at some level of restriction, to become limited for these two essential amino acids. Indeed, there are many overlapping phenotypes shared both by DR and isolated methionine restriction, including reduced adiposity, extended maximal longevity, increased resistance to acetaminophen toxicity in the liver, reduced insulin and IGF1 levels and reduced thyroid hormone (Miller et al., 2005). Nonetheless, there are differences as well, and future studies will be necessary to address whether the benefits of each are derived from fundamentally distinct or overlapping mechanisms.

To the best of our knowledge, other amino acid restricted diets have not been tested for lifespan extension, but have been shown to induce a number of other benefits. For example, methionine restriction contributes to adiposity resistance by altering the lipogenic/lipolytic balance (Perrone et al., 2008), leucine deprivation improves insulin sensitivity (Xiao et al., 2011) and tryptophan deprivation protects against surgical ischemia reperfusion injury to both kidney and liver (Peng et al., 2012). The latter two benefits are absent in mice lacking GCN2.

Role of GCN2 and TOR in protein/amino acid restriction benefits

What roles do the TOR and/or GCN2 signal transduction pathways play in regulating longevity, metabolic fitness and stress resistance upon restriction of protein or specific amino acids? There is ample evidence that reduction of TOR signaling through genetic or pharmacological manipulation can extend longevity in a variety of organisms. For example, deletion of TOR1 extends lifespan in yeast and rapamycin treatment extends lifespan in rodents (Harrison et al., 2009). Genetic ablation of the downstream target of TOR, Sch9 in yeast and S6K in rodents, can also extend longevity (Selman et al., 2009, Fabrizio et al., 2001). In flies, 4EBP is upregulated upon DR and may contribute to translational derepression of nuclear-encoded mitochondrial electron transport chain components (Zid et al., 2009). Because DR can reduce TOR signaling to downstream targets including S6K and 4EBP, this could be a primary mechanism underlying its longevity effects (Figure 1.2).

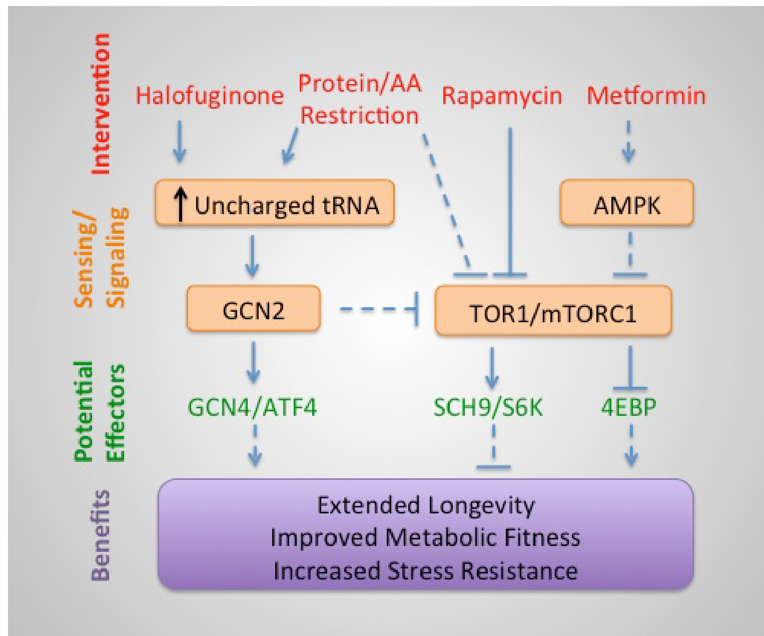

Figure 1.2. Model for amino acid sensing pathways in DR benefits. Modulation of activity of GCN2 or TOR pathways by dietary or pharmacological interventions can lead to benefits. Not all interventions necessarily lead to all benefits. Dashed lines indicate indirect or hypothesized effects; solid lines indicate known or direct effects. In some cases, yeast/mammalian gene names are both indicated, respectively.

Aside from the essential role of GCN2 in surgical stress resistance by short-term tryptophan deficiency (Peng et al., 2012), there is little in the DR or longevity literature on the potential role of this amino acid deprivation sensing kinase. This is perhaps surprising in light of established models of longevity extension involving amino acid restriction in rodents or amino acid imbalance in flies, combined with data suggesting the ability of GCN2 activation to inhibit mTOR activity upon amino acid deprivation. It may be that redundancy in mammalian eIF2α kinases, as well as multiple additional routes of mTOR inhibition, may abrogate the specific genetic requirement for GCN2 in a number of settings. Nonetheless, activation of GCN2 via dietary or antimetabolite means would seem a productive area of future research, including the role of translational control in downstream benefits (Figure 1.2).

In yeast, GCN4 stabilization has been implicated in both chronological and replicative longevity extension (Steffen et al., 2008, Alvers et al., 2009). In the replicative longevity model, lifespan is extended by knocking out any of a number of 60S ribosomal subunits or inhibiting 60S subunit biogenesis with small molecules (Steffen et al., 2008). By reducing the 60S subunit, translational initiation becomes inefficient, mimicking the effects of GCN2 activation and eIF2α phosphorylation on downstream GCN4 stabilization. Thus, transcriptional reprogramming by GCN4 may play a role in longevity extension, whether or not it is stabilized upon GCN2 activation or by some other process.

In addition to translational control, GCN2 and TOR control a number of other processes that could also contribute to reported benefits of DR, including autophagy, energy metabolism, immune function and food intake. Autophagy is an adaptive process that provides biological material (amino acids, lipids) to sustain anabolic processes (Yang and Klionsky, 2010) under conditions of nutrient depletion, including DR. mTORC1 negatively regulates autophagy by

suppressing the ULK1 complex via phosphorylation (Zoncu et al., 2011b). In yeast, GCN2 and GCN4 are required for the induction of autophagy upon amino acid depletion (Ecker et al., 2010, Talloczy et al., 2002). DR also results in changes in energy metabolism regulated at least in part by mTORC1, including fatty acid synthesis, glycolysis and the pentose phosphate pathway via the transcription factors Hif1α and SREBP (Duvel et al., 2010, Yecies et al., 2011). GCN2 can also impact fat metabolism. In addition to activation of amino acid biosynthesis and transport, GCN2 is required for suppression of fatty acid synthesis in the liver upon leucine deprivation (Guo and Cavener, 2007). Although this particular function of GCN2 is independent of ATF4, the broader role of this transcription factor in GCN2-dependent effects remains to be explored.

Immune function, including auto-immunity, can have a major impact on lifespan and aging-related disease in multicellular organisms. Activation of GCN2 upon local tryptophan depletion by the tryptophan catabolizing enzyme IDO is one strategy to reduce T cell proliferation and induce tolerance toward such unintended targets as apoptotic cells in the spleen (Munn et al., 2005) or the allogeneic fetus. Activation of the amino acid starvation response by the small molecule halofuginone, a prolyl-tRNA synthase inhibitor (Keller et al., 2012), prevents differentiation of inflammatory T_H17 cells (Sundrud et al., 2009). mTOR inhibition by rapamycin can also induce tolerance by suppressing T cell proliferation, but is paradoxically proinflammatory in the context of innate immune activation (Weichhart et al., 2008, Fielhaber et al., 2012).

Finally, GCN2 and mTOR can both participate through different mechanisms in behavioral control of food intake. Central administration of leucine activates hypothalamic mTOR and reduces food intake (Cota et al., 2006). Diets lacking one or more essential amino acids cause an aversion to food intake centered in a different brain region, the anterior piriform cortex (APC)

(Gietzen et al., 2007). GCN2 is activated rapidly in the APC upon dietary amino acid deprivation or stereotactic injection of amino acid alcohol derivatives that compete for tRNA synthetases, resulting in uncharged tRNA accumulation (Hao et al., 2005, Ross, 1961). Nonetheless, mice lacking GCN2 still display aversion to incomplete diets over the period of days to weeks (Anthony et al., 2004, Guo and Cavener, 2007, Peng et al., 2012), suggesting a redundant mechanism of amino acid deprivation sensing. Future studies will be required to elucidate the role of food aversion, if any, to the benefits of amino acid restriction.

Pharmacological activators of amino acid starvation response as DR mimetics?

DR mimetics can be loosely defined as interventions that mimic some beneficial aspect of DR, for example lifespan extension, maintenance of metabolic fitness upon challenge with a high fat diet, or increased stress resistance. Metformin and rapamycin both extend lifespan in rodents, and are thus considered DR mimetics (Harrison et al., 2009, Anisimov et al., 2005) (Figure 1.2). Halofuginone is a prolyl-tRNA synthetase inhibitor that activates the amino acid starvation response by mimicking proline deprivation. Like short-term essential amino acid deprivation, halofuginone can increase resistance to renal ischemia reperfusion injury in a GCN2-dependent manner (Peng et al., 2012). This serves as proof of principle that compounds that activate GCN2 and stimulate the amino acid starvation response can have benefits similar to dietary protein/amino acid restriction. Nonetheless, this antimetabolite and others in its class are toxic due to on-target effects of tRNA synthetase inhibition. More desirable would be a DR mimetic that would activate the GCN2 kinase directly without inhibiting tRNA charging, or selectively inhibit amino acid sensing through the mTOR pathway. Our understanding of the mechanisms underlying amino acid sensing will likely improve the chances of identifying such a compound with potential beneficial uses in humans.

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CHAPTER 2

THE TSC COMPLEX IS REQUIRED FOR THE BENEFITS OF DIETARY PROTEIN RESTRICTION ON STRESS RESISTANCE IN VIVO

Adapted from:

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SUMMARY

Protein restriction (PR) is important for the benefits of dietary restriction on longevity and stress resistance, but relevant nutrient sensors and downstream effectors in mammals remain poorly defined. We used PR-mediated protection from hepatic ischemia reperfusion injury to probe genetic requirements for evolutionarily conserved nutrient sensors GCN2 and mTORC1 in stress resistance. One week of PR reduced free amino acids and circulating growth factors, activating GCN2 and mTORC1 repressor TSC complex. However, while GCN2 was dispensable for PR-induced protection, hepatic TSC1 was required. PR improved hepatic insulin sensitivity in a TSC1-dependent manner prior to ischemia, facilitating increased pro-survival signaling and reduced apoptosis after reperfusion. These benefits were partially abrogated by pharmacological PI3K inhibition or genetic deletion of the insulin receptor in hepatocytes. In conclusion, improved insulin sensitivity upon short-term PR required TSC1, facilitated increased pro-survival signaling after injury, and contributed partially to PR-mediated resistance to clinically relevant ischemia reperfusion injury.

BACKGROUND

Dietary restriction (DR) is loosely defined as reduced food intake without malnutrition and refers to a variety of dietary interventions including both simple reduction of calorie intake and restriction of specific macronutrients. Beneficial health effects of DR were reported in the 1930s, when DR was shown to extend longevity in rats (McCay et al., 1935). DR has since been shown to increase maximal and average lifespan in multiple species including yeasts, worms, flies, fish and rodents (Fontana et al., 2010). In addition to the metabolic and cardiovascular improvements

seen in mammals, DR also improves resistance to a variety of stressors such as chemotherapeutic agents (Raffaghello et al., 2008, Cheng et al., 2014) and ischemia reperfusion injury (IRI) to the kidney, liver, heart and the brain (Mitchell et al., 2010, Varendi et al., 2014, Robertson and Mitchell, 2013).

Restriction of calories from any macronutrient source - proteins, lipids or carbohydrates – can result in DR benefits, including lifespan extension. Therefore, the term DR has been used interchangeably with caloric restriction (CR). However, accumulating evidence suggests that specific macronutrients – namely amino acids – play a role in DR benefits beyond their caloric value (Piper et al., 2011, Levine et al., 2014, Solon-Biet et al., 2014). Numerous studies in rodents have shown that total protein restriction and restriction of individual essential amino acids (EAA) tryptophan and methionine increase longevity in rats and mice (Gallinetti et al., 2013). In addition to longevity extension, dietary protein or individual EAA restriction can both mediate other DR-like effects, including improved insulin sensitivity upon leucine restriction (Xiao et al., 2011) and protection against IRI in liver and kidney upon tryptophan restriction (Peng et al., 2012). While EAA, protein, and total calorie restriction induce shared phenotypes, including decreased adiposity, decreased circulating growth hormones (insulin, IGF-1) and improved insulin sensitivity, whether or not these are common effectors of associated benefits remains to be tested.

Two evolutionarily conserved signal transduction pathways sense amino acids: general control nonderepressible 2 (GCN2) and mechanistic target of rapamycin complex 1 (mTORC1). GCN2 senses the absence of any individual amino acid via binding to uncharged cognate tRNAs and activates the amino acid starvation response by phosphorylating Ser51 of the translation initiation factor eIF2α. This results in global translational suppression together with translational

derepression of specific mRNAs such as *Atf4* (Gallinetti et al., 2013). GCN2 is required for mediating the beneficial effects of short-term individual EAA deficiency against hepatic and renal IRI (Peng et al., 2012).

mTORC1 is a complex of the serine/threonine kinase mTOR, Raptor, and mLst8 as core essential components and integrates various growth stimulating signals including amino acids, energy and growth factors (Dibble and Manning, 2013). Intracellular amino acids, and in particular the branched chain amino acid leucine, are sensed by mTORC1 via an upstream mechanism involving the Rag GTPases (Sancak et al., 2008) and a regulatory complex referred to as the Ragulator (Efeyan et al., 2012). Leucine sufficiency allows recruitment of mTORC1 to the lysosomal surface via the Ragulator. There, mTORC1 can be activated by the small GTPase Rheb, which in turn is negatively regulated by the GTPase-activating protein (GAP) tuberous sclerosis complex 2 (TSC2), which functions in a complex with TSC1 and TBC1D7 (the TSC complex) (Huang and Manning, 2008, Dibble et al., 2012). The TSC complex is a critical negative regulator of mTORC1 that integrates signals from energy levels and growth and endocrine factors, such as insulin. Although growth factor-based TSC-dependent inhibition of mTORC1 is experimentally separable from amino acid-based Ragulator-dependent inhibition *in vitro* (Menon et al., 2014), whether the TSC complex plays a role in controlling mTORC1 in response to dietary protein restriction *in vivo* remains unknown.

Inhibition of mTORC1 (Harrison et al., 2009, Miller et al., 2011, Miller et al., 2013, Lamming et al., 2012, Wu et al., 2013) or its direct target S6K (Selman et al., 2009), has been implicated in DR-like benefits including lifespan and healthspan extension; however, the requirement for mTORC1 inhibition in DR-mediated stress resistance, and the potential role of the TSC complex, remain unknown. Although *S. cerevisiae* and *C. elegans* lack TSC homologs, overexpression of

TSC1 and TSC2 in Drosophila extends longevity, consistent with a beneficial role of reduced mTORC1 signaling (Kapahi et al., 2004). A protective role of the TSC complex via autophagy induction in an mTORC1-dependent manner has also been suggested in a recent study of neuroprotection against ischemia (Papadakis et al., 2013).

Ischemia reperfusion injury (IRI) is a multifactorial acute stress involving occlusion of blood flow to an organ or tissue for a certain period of time (ischemia) and subsequent return of blood flow (reperfusion). During the ischemic period, cells are deprived of oxygen and nutrients, resulting in ATP depletion, loss of membrane potential and accumulation of toxic byproducts. Subsequent reperfusion introduces additional damage via the actions of inflammatory mediators, leading to both apoptotic and necrotic cell death (Jaeschke, 2003). IRI underlies a variety of clinically significant events ranging from heart attack and stroke to surgical interventions requiring temporary restriction of blood flow. Here we investigated the contributions of the TSC/mTORC1 and GCN2 pathways in sensing dietary protein restriction and mediating protective effects against hepatic IRI using mice deficient in GCN2 or lacking hepatocyte-specific expression of the essential TSC complex component TSC1.

RESULTS

DIETARY PROTEIN RESTRICTION MEDIATES STRESS RESISTANCE INDEPENDENT OF GCN2

To determine an optimal 1-week dietary preconditioning regimen against hepatic IRI, we focused on restricting dietary protein intake either by restricting intake of a complete diet (35%

or 55% restriction) or by removing protein from the diet (protein restriction, PR) (Figure 2.1A). Food restriction was calculated on the basis of food intake of *ad libitum*-fed mice on a complete diet and normalized by animal weight (Figure 2.1B). Because mice on a protein free diet often display temporary aversion to food intake (Peng et al., 2012), the PR diet was restricted by 35% to normalize daily food intake between experiments. Hepatic IRI was induced by clamping the hepatic artery, portal vein and the bile duct for 30-35 minutes, followed by reperfusion for up to 24 hrs. Liver damage was assessed by measuring the release of cellular enzymes into the bloodstream. Maximal protection correlated with the least protein intake rather than restriction of calories (Figure 2.1C). Therefore, we used the protein restriction (PR) regimen to further characterize the responsible signaling pathways and downstream effector mechanisms.

Recently, we showed that GCN2 is required for mediating the protective effects of single EAA restricted diets against renal and hepatic IRI (Peng et al., 2012). To test whether GCN2 is required for mediating the benefits of total protein restriction against hepatic IRI, we preconditioned *Gcn2* knockout (GCN2KO) mice on complete, tryptophan restricted (Trp^-) or PR diets for one wk. Damage assessment 3 hours after reperfusion revealed no significant difference between complete and Trp^- diets; however, GCN2KO mice preconditioned on the PR regimen were significantly protected (Figure 2.1D, 2.1E). Thus, GCN2 was dispensable for preconditioning by PR, indicating the likely involvement of another amino acid sensing pathway.

The mTORC1 pathway can also sense amino acids, and is inhibited in the absence of specific amino acids, including leucine. We thus tested whether the mTORC1 pathway is still responsive

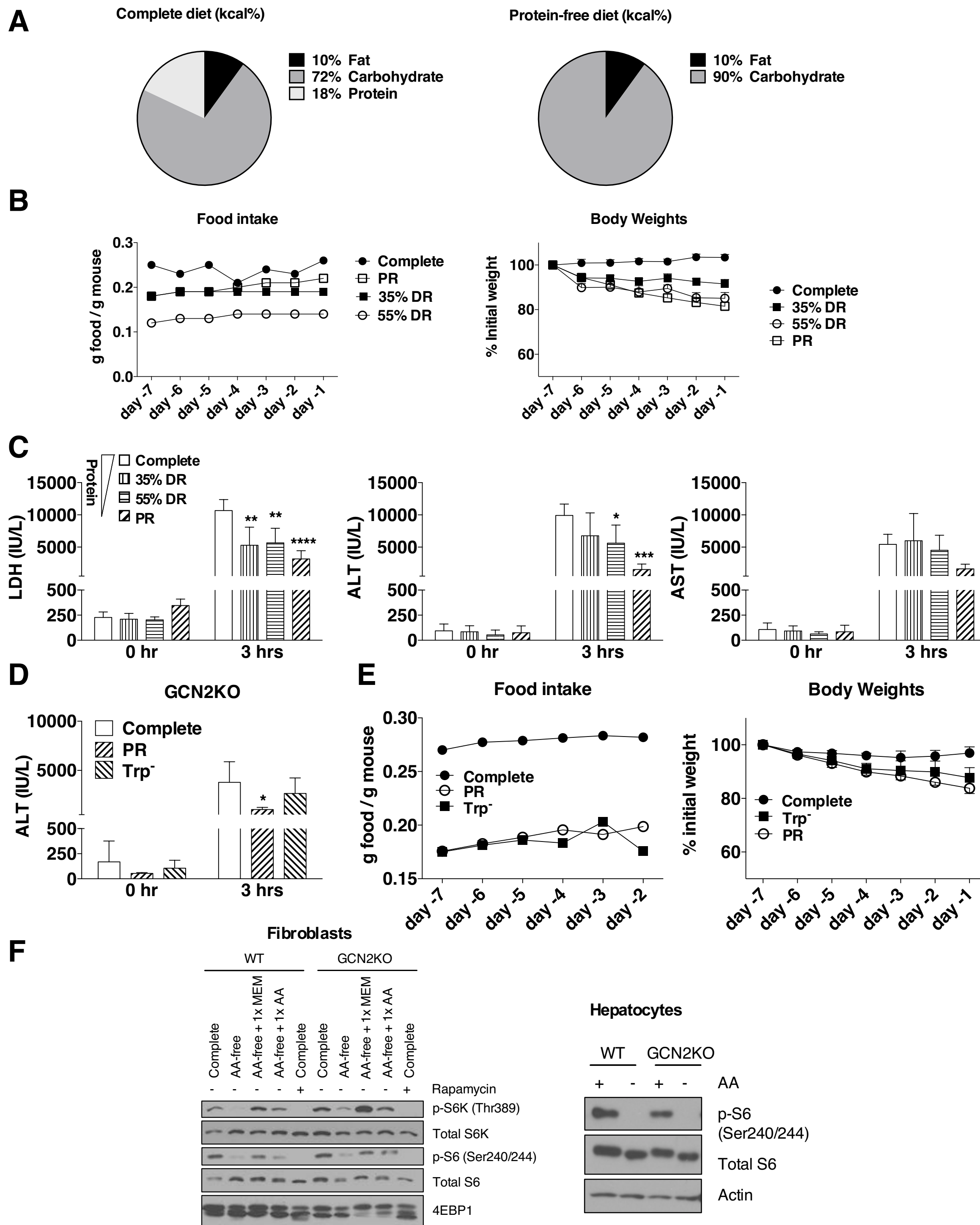

Figure 2.1 Dietary protein restriction mediates stress resistance independent of GCN2.

(A) Compositions of the experimental diets, represented as kcal%. Protein was replaced on a per weight basis with isocaloric sucrose in the protein-free diet.

Figure 2.1 (Continued)

(B) Food intake and body weight curves of male mice preconditioned on the indicated diets for 1 week prior to hepatic IRI; $n = 5$ mice/group. Complete: *ad libitum* access to complete diet; 35% DR: 65% of *ad libitum* food allowance on complete diet; 55% DR: 45% of *ad libitum* food allowance on complete diet, PR: 65% of *ad libitum* food allowance on protein-free diet. Hepatic IRI was induced on day 0.

(C) Serum LDH, ALT and AST activity of mice shown in (B) before ischemia (0 hr) and 3 hours after reperfusion. Asterisks indicate the significance of the difference vs. the Complete group by 1-way ANOVA with Dunnett's test for multiple comparisons, multiplicity adjusted p-values; $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, $****p < 0.0001$.

(D) Serum ALT activity before ischemia (0 hr) and 3 hours after reperfusion in female GCN2KO mice preconditioned on the indicated diets for 1 week prior to hepatic IRI; $n = 4\text{-}5$ mice/group. Complete: *ad libitum* access to complete diet; PR: 65% of *ad libitum* food allowance on protein-free diet; Trp^{-}: 65% of *ad libitum* food allowance on tryptophan-free diet. Asterisk indicates the significance of the difference vs. the Complete group by student's t-test; $*p < 0.05$.

(E) Food intake and body weight curves for the mice in (D). Hepatic IRI was induced on day 0.

(F) Immunoblots of markers of mTORC1 signaling in extracts from primary dermal fibroblasts or hepatocytes isolated from WT or GCN2KO mice and treated with the indicated media in the presence of 10% dialyzed FBS (2 hours for MDFs, 3 hours for hepatocytes). Complete: complete DMEM; AA-free: amino acid-free DMEM; MEM: minimum essential medium amino acid mix;

Figure 2.1 (Continued)

AA: all amino acids added back to 1x DMEM concentration; Rapamycin: 20 nM. Data in all panels are shown as means ± SD.

to amino acid deprivation *in vitro* in two different GCN2KO cell types, primary dermal fibroblasts and primary hepatocytes (Figure 2.1F). Phosphorylation of downstream targets of mTORC1, including S6K1, its target S6, and 4E-BP1 (as scored by mobility shifting), were proportionately reduced in both wild type (WT) and GCN2KO cells upon total amino acid withdrawal. We conclude that the mTORC1 pathway can sense amino acid restriction independent of GCN2, and could thus be a potential mediator of PR preconditioning effects.

THE TSC COMPLEX IS REQUIRED FOR INHIBITION OF MTORC1 UPON PR IN VIVO

We next determined the effects of PR on hepatic mTORC1 activity *in vivo* using both WT and liver-specific *Tsc1* knockout (LTsc1KO) mice, a model of constitutive mTORC1 activation in the liver (Yecies et al., 2011, Menon et al., 2012, Sengupta et al., 2010, Kenerson et al., 2011). WT and LTsc1KO mice were fed a complete or PR diet for one week (Figure 2.2A). Both genotypes lost approximately 20% of their body weight (Figure 2.2B, 2.2C), including loss of adipose mass (Figure 2.2D). In WT mice on PR, livers tended to weigh less, and were thus maintained as a percentage of body weight; in LTsc1KO mice on PR, liver weights did not decrease, resulting in disproportionately larger livers (Figure 2.2B), consistent with failure of LTsc1KO mice to reduce mTORC1 signaling upon PR.

To confirm differential regulation of hepatic mTORC1 signaling upon PR, we looked in liver extracts at downstream mTORC1 targets and related nutrient/energy sensing pathways. PR increased phosphorylation of eIF2α (Figure 2.2E, 2.2F) and expression of downstream markers of the GCN2-dependent amino acid starvation response, including the ATF4 target gene *Asns* (Figure 2.2G). Higher baseline eIF2α phosphorylation in LTsc1KOs could be due in part to

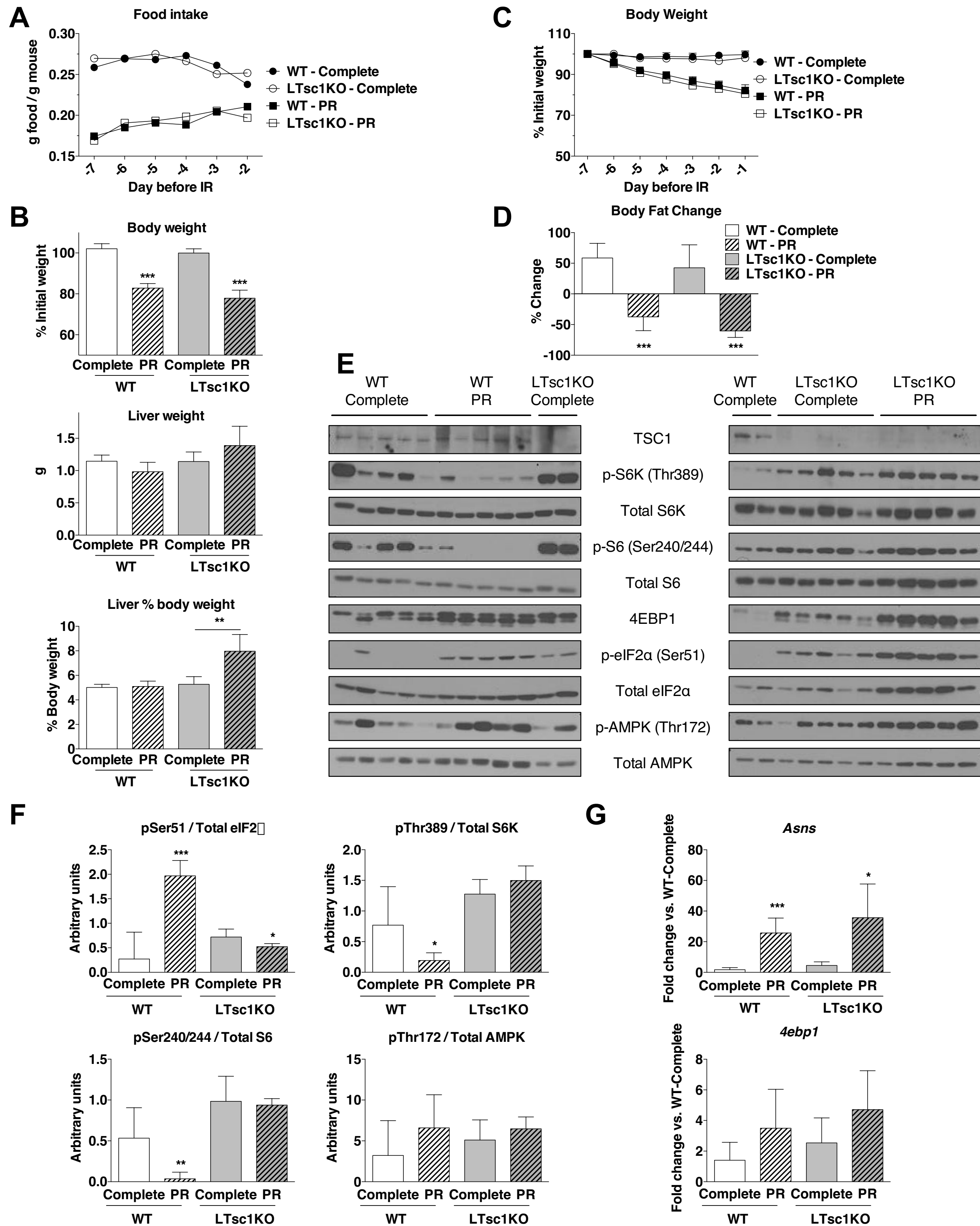

Figure 2.2 The TSC complex is required for inhibition of mTORC1 upon PR *in vivo*.

Figure 2.2 (Continued)

(A,) Daily food intake of WT and LTsc1KO mice on the indicated diet measured at the same time of the day. Data were pooled from 3 separate experiments; $n = 4\text{-}5$ mice per group for each experiment.

(B) Body and liver weights of the indicated genotype after 1 week on the indicated diet; $n = 4\text{-}5$ mice/group. Body weight on the last day of treatment is represented as percentage of initial weight (top). Liver weights on the last day of treatment are represented as absolute values (middle) and percentages of body weight (bottom). Asterisks indicate the significance of the difference between diets within genotype by student's t-test; $^{**}p < 0.01$, $^{***}p < 0.00001$.

(C) Body weight measurements of WT and LTsc1KO mice on the indicated diet measured at the same time of the day. Data were pooled from 3 separate experiments; $n = 4\text{-}5$ mice per group for each experiment.

(D) Body fat as measured with Echo-MRI prior to and one week after preconditioning on the indicated diet, expressed as the % change in body fat; $n = 5$ mice per group. Statistical significance was measured by student's t-test between Complete and PR groups within each genotype; $^{***}p < 0.0005$.

(E) Immunoblots of markers of mTORC1, GCN2 and AMPK activation in liver extracts from WT and LTsc1KO male mice after 1 week on the indicated diet.

(F) Quantification of the blots shown in Figure 2B. Statistical significance was measured by student's t-test between Complete and PR groups within each genotype; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0005$.

Figure 2.2 (Continued)

(G) mRNA levels of ATF4 target genes *Asns* and *4ebp1* in livers of WT and LTsc1KO mice on the indicated diet; n = 4-5 mice per group. Statistical significance was measured by student's t-test between Complete and PR groups within each genotype; *p < 0.05, ***p < 0.001. Data in all panels are shown as means ± SD.

higher endoplasmic reticulum (ER) stress (Menon et al., 2012). In WT livers, we observed a reduction in S6K and S6 phosphorylation upon PR (Figure 2.2E, 2.2F). Total levels of 4EBP1, which is also an ATF4 target gene (Yamaguchi et al., 2008), increased in both genotypes upon PR; therefore changes in phosphorylation of this mTORC1 target are difficult to interpret (Figure 2.2E, 2.2G). mTORC1 signaling in Tsc1KO livers was greatly increased relative to WT, and unaffected by PR, if not slightly elevated (Figure 2.2E, 2.2F). Interestingly, even though AMPK phosphorylation in some WT livers was increased upon PR, the observed changes were not statistically significant likely due to biological variability; there was no evidence of increased AMPK phosphorylation upon PR in LTsc1KO livers (Figure 2.2E, 2.2F).

DIFFERENTIAL REDUCTION OF ESSENTIAL AMINO ACIDS AND GROWTH FACTORS IN VIVO UPON PR

To test the role of amino acid deficiency on cell-autonomous mTORC1 signaling, primary WT or TSC1KO hepatocytes were cultured for 2 hours in media lacking amino acids. In WT hepatocytes, this led to the reduction of S6K and S6 phosphorylation below the level of detection (Figure 2.3A). Although Tsc1KO hepatocytes had much higher baseline levels of p-S6K, they also responded to amino acid deprivation *in vitro*, confirming the ability of amino acid deprivation to reduce mTORC1 activity independent of the TSC complex.

If amino acid deficiency could at least partially abrogate mTORC1 signaling in Tsc1KO hepatocytes *in vitro*, why did PR fail to reduce mTORC1 signaling at all in LTsc1KO livers *in vivo*? To answer this, we first measured free amino acids in the serum and in liver. One week of PR reduced most free EAA, including leucine, in the serum and liver in both WT and LTsc1KO animals, but only by approximately 20-30% on average (Figure 2.3B, 2.3C); non-essential amino

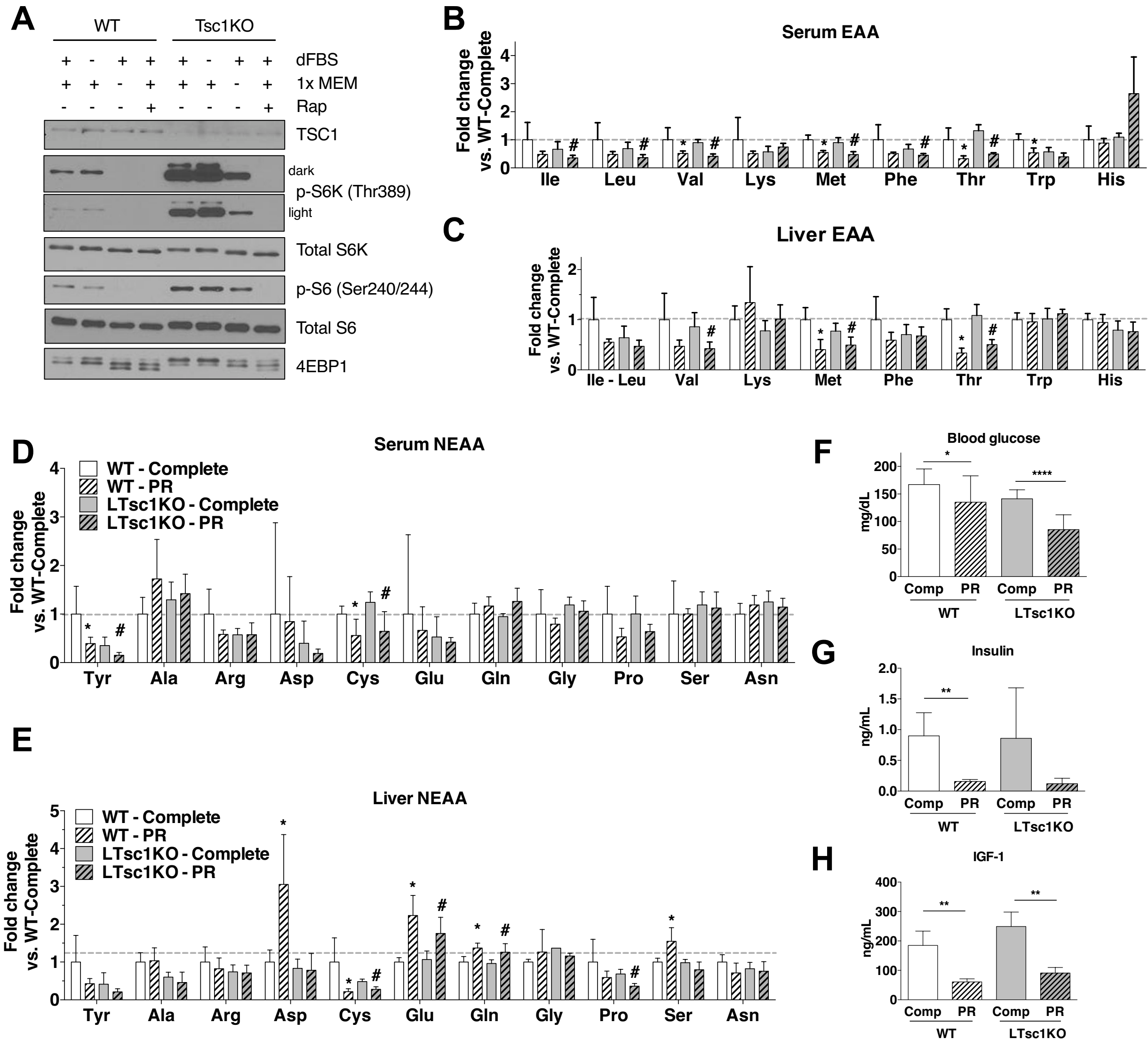

Figure 2.3 Differential reduction of essential amino acids and growth factors *in vivo* upon PR.

(A) Immunoblots of mTORC1 targets from extracts of primary hepatocytes isolated from WT or LTsc1KO mice and starved of amino acids in the presence of 10% dialyzed serum (dFBS) for 2 hours prior to lysis, coincident with 1x MEM addition +/- 20nM rapamycin (Rap) as indicated.

(B, C) Free EAA levels in serum (B) and liver (C) of WT or LTsc1KO mice on the indicated diet expressed as fold change relative to the WT complete diet group; n = 4-5 mice/group.

Figure 2.3 (Continued)

Asterisks/number signs indicate the significance of the difference between diets within genotype by student's t-test; $*/^{\#}p < 0.05$.

(D, E) Free NEAA levels in serum (D) and liver (E) of WT or LTsc1KO mice on the indicated diet expressed as fold change relative to the WT complete diet group; n = 4-5 mice per group. Asterisks/number signs indicate the significance of the difference between diets in WT and LTsc1KO groups, respectively, by student's t-test; $*/^{\#}p < 0.05$.

(F-H) Blood glucose (F), serum insulin (G) and IGF-1 (H) levels of WT or LTsc1KO mice on the indicated diet; n = 4-5 mice/group for each experiment, with data pooled from 3 separate experiments (F). Asterisks indicate the significance of the difference between diets within genotype by student's t-test; $*p < 0.05$, $**p < 0.01$, $****p < 0.0000001$. Data in all panels are shown as means ± SD.

acids were not uniformly affected (Figure 2.3D, 2.3E). Blood glucose was similarly reduced upon PR in both genotypes by 20-40% (Figure 2.3F). However, circulating levels of growth factors were reduced by more than 5 fold for insulin and 3 fold for IGF-1 upon PR (Figure 2.3G, 2.3H). Taken together, these data suggest that buffering of free amino acid concentrations *in vivo* upon PR prevents Rag/Ragulator-based mTORC1 inhibition in LTsc1KO livers, and confirms LTsc1KO mice as a suitable model of constitutive hepatic mTORC1 activation, despite intact systemic changes in upstream mTORC1 regulators in response to diet.

THE TSC COMPLEX IS REQUIRED FOR BENEFITS OF PR AGAINST ACUTE HEPATIC STRESS

To determine the requirement for the TSC complex and suppression of mTORC1 on PR-mediated stress resistance, we tested the response of female WT and LTsc1KO mice to hepatic IRI. We observed a significant reduction of serum damage markers LDH, ALT and AST in WT animals on PR, but this benefit was lost in LTsc1KOs (Figure 2.4A, 2.4B). As a control, changes in the serum levels of these enzymes were not due to differential enzyme activity levels in undamaged livers (Figure 2.4C). H&E-stained sections from formalin-fixed livers harvested 24 hours after reperfusion from all 4 groups were analyzed for necrotic cell death, which appeared as lightly stained areas with loss of hepatocyte morphology (Figure 2.4D). Blind scoring in serial sections confirmed the reduction of necrotic tissue in WT livers upon PR, and the loss of this benefit in LTsc1KOs (Figure 2.4E). The same relative differences in hemorrhagic necrosis could also be observed at the macroscopic level in whole livers (Figure 2.4D). Body temperatures of LTsc1KO and WT mice upon PR were similarly reduced (Figure 2.4F), ruling out the possibility of differential temperatures in protection by PR in WT mice. Finally, PR-mediated protection from hepatic IRI was partially abrogated in LTsc1KO males (Figure 2.4G). Taken together, these

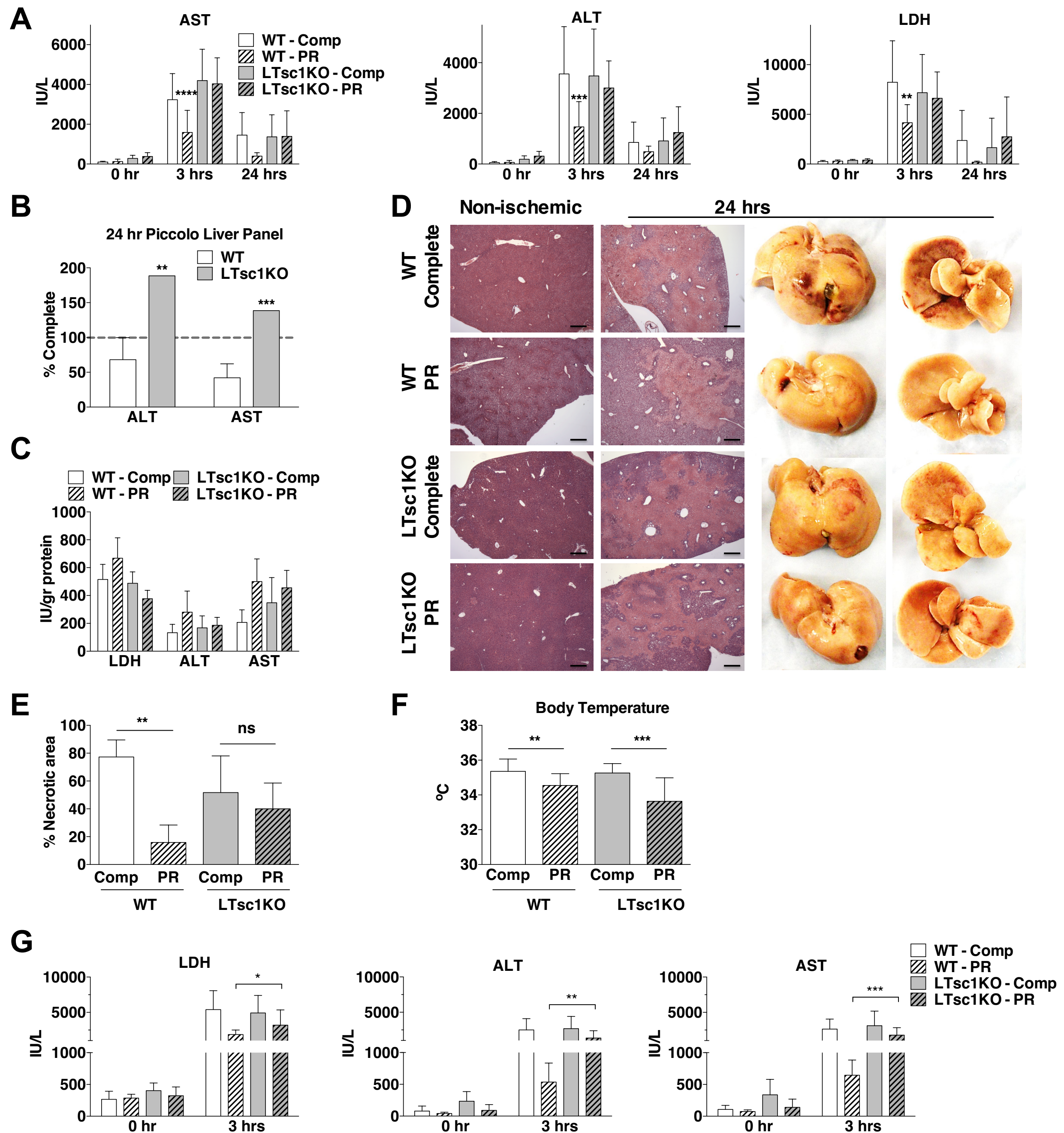

Figure 2.4 The TSC complex is required for benefits of PR against acute hepatic stress.

(A) Serum LDH, ALT and AST activity before ischemia (0 hr) and 3 or 24 hours after reperfusion in female WT or LTsc1KO mice preconditioned on the indicated diets for 1 week prior to hepatic IRI; n = 4-6 mice/group for each experiment, with data pooled from 4 separate experiments for 0 and 3 hours and a single experiment for 24 hrs. Asterisks indicate the

Figure 2.4 (Continued)

significance of the difference between diets within genotype by student's t-test; $**p < 0.001$, $***p < 0.00001$, $****p < 1 \times 10^{-8}$.

(B) Liver damage marker analysis using Piccolo Liver Plus Panel discs with serum collected from the indicated groups 24 hours post-reperfusion. Data is represented as % of complete diet fed group for each genotype; n = 2-5 mice per group; $**p < 0.01$, $***p < 0.005$.

(C) Total liver enzyme analysis from liver extracts of mice from the indicated groups. Frozen livers were lysed in NP-40 lysis buffer, and the activity of the LDH, ALT and AST enzymes were measured in extracts with kinetic assays. Values were normalized to protein content of each sample; n = 4-5 mice per group.

(D) Representative images of H&E stained sections from non-ischemic (left) or ischemic livers 24 hours after reperfusion (right) from WT or LTsc1KO mice preconditioned on the indicated diets for 1 week prior to hepatic IRI. Lightly stained areas with loss of hepatocyte morphology indicate necrotic tissue. Right: representative images of whole livers 24 hours after IRI and fixed with formalin, with areas of hemorrhagic necrosis appearing as dark red. Scale bar: 400 μm.

(E) Quantification of hepatic cell death expressed as percentages of necrotic region/field in 3-5 mice/group, 3 sections/sample; Asterisks indicate the significance of the difference between diets within genotype by student's t-test; $**p < 0.01$, ns: not significant.

(F) Core body temperature of mice measured with a rectal probe immediately before induction of ischemia. Results were pooled from 4 separate experiments with n = 4-5 mice per group per experiment; $**p < 0.005$, $***p < 0.001$ by student's t test between diets within genotype.

Figure 2.4 (Continued)

(G) Serum levels of LDH, ALT and AST measured before ischemia (0 hr) and 3 hours after reperfusion in male WT or LTsc1KO mice 12-15 weeks of age preconditioned on the indicated diets for 1 week prior to hepatic IRI; pooled results from 3 separate experiments with n = 4-6 mice per group per experiment. Student's t-tests were performed to assess statistically significant differences between WT-PR and LTsc1KO-PR groups; *$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$. Data in all panels are shown as means ± SD.

results indicate the requirement for the TSC complex in the protective effect of PR against hepatic IRI.

In order to investigate potential differences in protective mechanisms by total protein (PR) and tryptophan restriction (Trp^-), we tested the response of female LTsc1KO mice to one week of preconditioning on a Trp^- diet. Interestingly, we observed that LTsc1KO mice were still responsive to Trp^- diet in terms of protection from hepatic IRI (Figure 2.5). Taken together, these data indicate that beneficial effects of total protein restriction and tryptophan restriction have different genetic requirements, suggesting a parallel rather than epistatic relationship between TSC/mTORC1 and GCN2 amino acid sensing pathways in protection from hepatic IRI.

Rapamycin is a partial mTORC1 inhibitor that increases longevity in mice (Harrison et al., 2009, Miller et al., 2011, Miller et al., 2013). We tested the ability of rapamycin to induce benefits against hepatic IRI similar to PR. To this end, WT mice were injected with rapamycin at varying concentrations for up to one week in the absence of any dietary intervention. Despite reducing mTORC1 activity, none of the rapamycin regimens tested were beneficial against hepatic IRI (Figure 2.6A, 2.6B). Interestingly, we observed that thrice weekly rapamycin treatment partially abrogated PR benefits and reduced mTORC2 activity as assessed by NDRG1 phosphorylation (Figure 2.6B, 2.6C), suggesting that the effects of rapamycin on mTORC2 might be detrimental in the context of hepatic IRI.

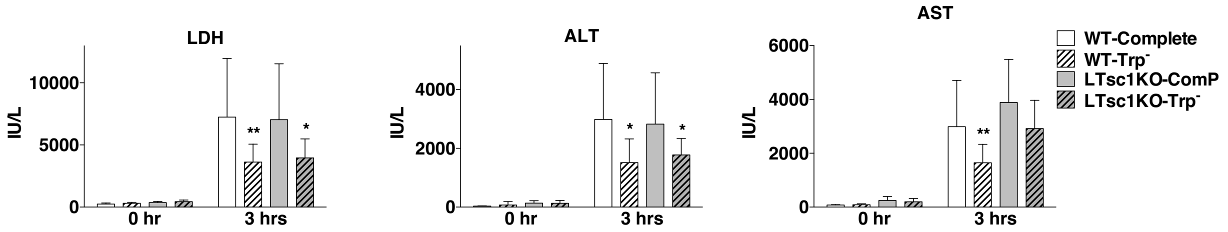

Figure 2.5 The TSC complex is not required for benefits of Tryptophan restriction.

Serum levels of LDH, ALT and AST measured before (0 hr) or 3 hours after reperfusion in WT and LTsc1KO mice preconditioned on the indicated diets for 1 week prior to hepatic IR. Data were pooled from 3 separate experiments with n = 14, 15, 14, 13 mice total for WT-Complete, WT-Trp$^-$, LTsc1KO-Complete and LTsc1KO-Trp$^-$ groups, respectively. Complete: 65% of ad libitum food intake on a complete diet; Trp$^-$: 65% of ad libitum food intake on a tryptophan-free diet. To assess statistical significance, student's t-tests were performed to compare Complete and Trp$^-$ groups within each genotype. *p < 0.05, **p < 0.01. Data in all panels are shown as means ± SD.

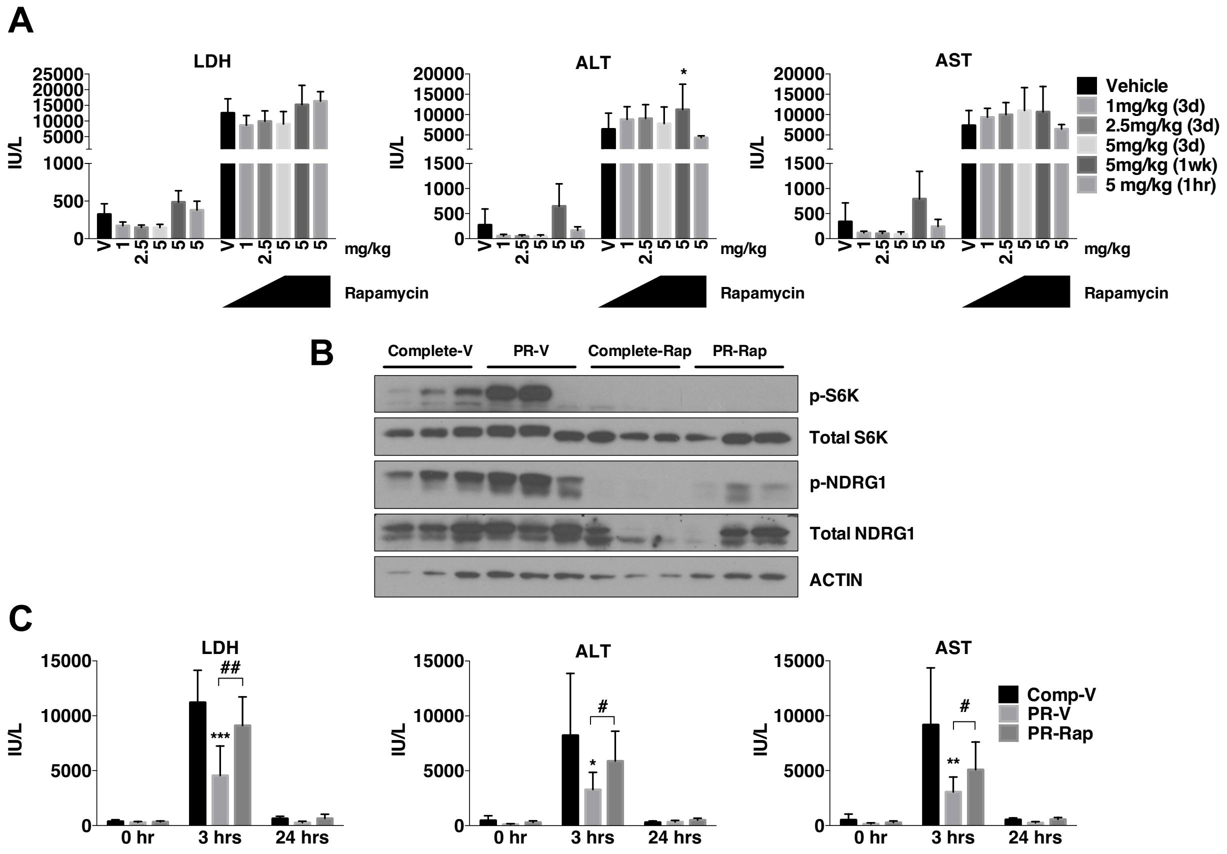

Figure 2.6 Rapamycin treatment partially abrogates PR benefits

(A) Serum levels of LDH, ALT and AST measured before ischemia (left) and 3 hours after reperfusion (right) in WT female C57Bl/6J mice fed a control diet *ad libitum* and subject to the indicated rapamycin or vehicle treatment by intraperitoneal injection. 3 days: daily injection of rapamycin for 3 consecutive days before IRI; 1 week: 3 injections of rapamycin over a week before IRI; 1 hour: a single injection with rapamycin 1 hour prior to IRI. Pooled results from 5 separate experiments are shown, with n = 25 for the vehicle group (V), n = 5 for 3 day treatment groups, and n = 10 for 1 week and 1 hour treatment groups. All groups were compared to the vehicle treated group for statistical analysis using a 1-way ANOVA with Dunnett’s test for multiple comparisons. Asterisk indicates multiplicity adjusted p-value below 0.05.

Figure 2.6 (Continued)

(B) Immunoblots of mTORC1 targets in liver extracts of the indicated groups of WT mice fed a complete diet *ad libitum* or preconditioned with PR and treated with either vehicle or rapamycin (5 mg/kg i.p. 3 times/week) for 1 week. Mice were fasted overnight prior to harvest and stimulated with insulin (0.25 U/kg i.p.) 10 minutes before sacrifice.

(C) Serum levels of LDH, ALT and AST measured before (0 hr), 3 hours and 24 hours post-reperfusion in mice preconditioned with the indicated diets, and treated with either vehicle or rapamycin (5 mg/kg i.p. 3 times/week) for 1 week prior to hepatic IRI. Results were pooled from 2 separate experiments with n = 4-5 mice per group in each experiment. Complete-V control group data was also used in panel (B). Asterisks indicate statistically significant differences between Complete-V and PR-V groups according to a student's t-test, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, $\#p < 0.05$, $\#\#p < 0.005$. Data in all panels are shown as means ± SD.

THE TSC COMPLEX IS REQUIRED FOR IMPROVED HEPATIC INSULIN SENSITIVITY UPON PR

By what downstream mechanism does TSC complex activation and mTORC1 repression improve stress resistance? Improved insulin sensitivity upon DR is associated with a wide range of health benefits including stress resistance and extended longevity (Fontana et al., 2010). We thus tested the ability of PR to increase hepatic insulin sensitivity in a TSC1-dependent manner. One week of PR reduced fasting insulin, resulting in a significantly reduced HOMA-IR (Figure 2.7A-C) consistent with improved insulin sensitivity. To directly test the effects of PR on hepatic insulin sensitivity, we measured the levels of Akt phosphorylation in extracts from WT livers following stimulation with insulin. WT mice on PR for one week had higher levels of pAkt-S473 and pAkt-Thr308 compared to controls (Figure 2.7D). Levels of Akt phosphorylation in Tsc1KO livers upon insulin stimulation were lower than in WT livers, consistent with previous findings (Yecies et al., 2011), but did not improve upon PR in Tsc1KOs as they did in WT controls (Figure 2.7E). Together, these findings show that the TSC complex is required for improvement in hepatic insulin sensitivity upon PR.

In the context of DR, improved insulin sensitivity is associated with reduced anabolic hormone levels (as observed here, Figure 2.3G, 2.3H) and, consequently, reduced insulin/IGF-1 signaling. Reduced insulin/IGF-1 signaling is an evolutionarily conserved mechanism regulating longevity and stress resistance in part by controlling cytoprotective gene expression (Fontana et al., 2010). To evaluate a potential impact of increased cytoprotective gene expression, we measured expression of genes controlled by both FoxO and NRF2 transcription factors. However, no significant increases in the levels of these genes were observed upon PR, nor did TSC1 ablation have consistent effects (Figure 2.7F, 2.7G).

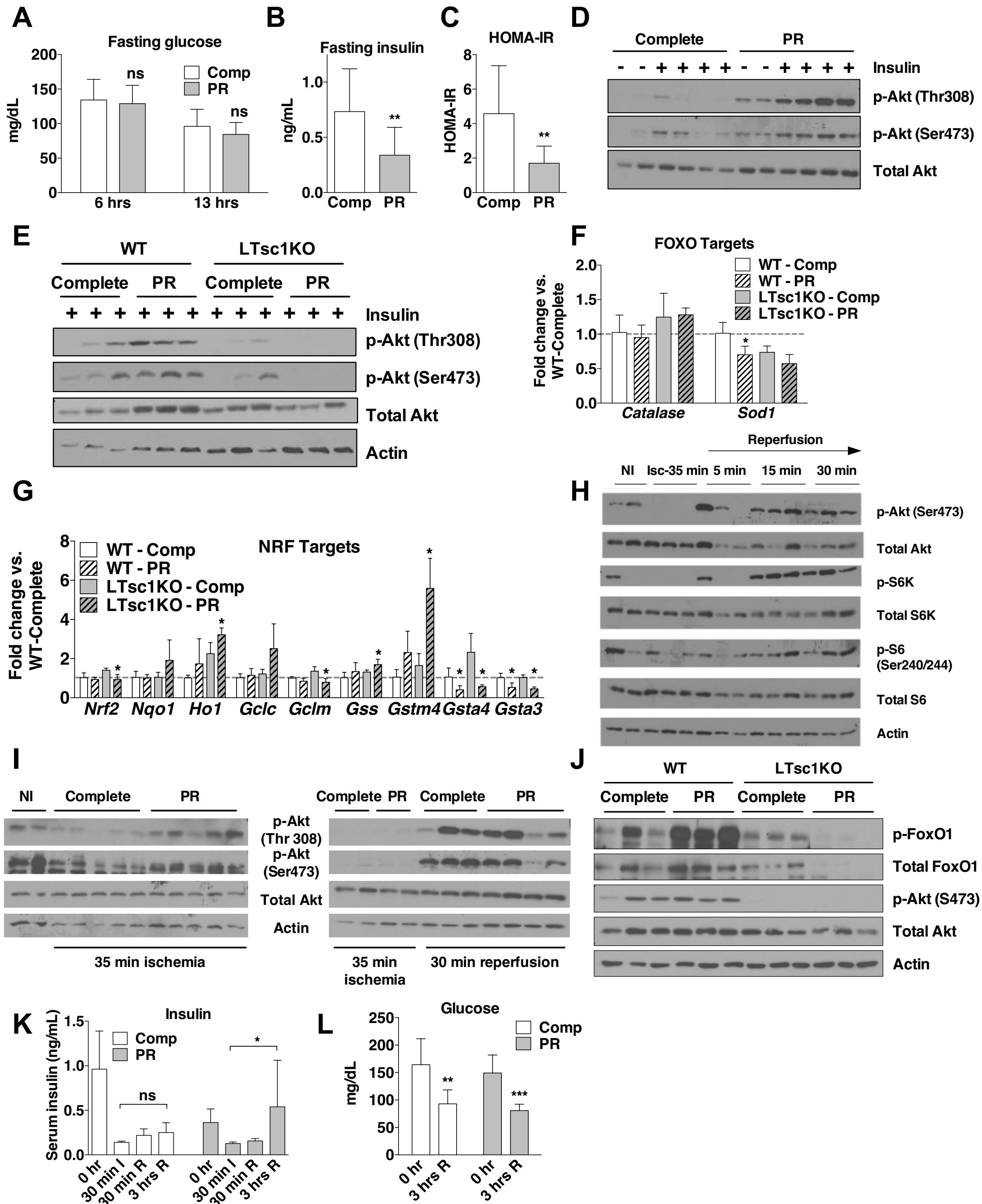

Figure 2.7 The TSC complex is required for improved hepatic insulin sensitivity upon PR.

Figure 2.7 (Continued)

(A-C) Glucose homeostasis in WT female C57Bl/6J mice at 11-13 weeks of age (n=12/group) on the indicated diet for up to 10 days. (A) Blood glucose measured after 6 hours (day 10) or 13 hours (day 7) of fasting. (B) Serum insulin levels measured after 13 hours of fasting on day 7. (C) HOMA-IR index was calculated as: [fasting glucose (mg/dl) * fasting insulin (μU/ml)]/405. Asterisks indicate statistically significant differences between diet groups according to a student's t-test; **$p < 0.01$, ns: not significant.

(D) Hepatic insulin sensitivity as determined by immunoblotting for markers of Akt pathway activation in liver extracts from mice fasted overnight and then stimulated with insulin (0.25 U/kg i.p.) 10 minutes prior to harvest.

(E) Insulin sensitivity as determined by immunoblotting for markers of Akt pathway activation in liver extracts from mice fasted for 6 hours and then stimulated with 0.5 U/kg insulin by portal vein injection 3 min before harvest.

(F, G) Gene expression analysis of FOXO (F) or NRF2 (G) targets by qRT-PCR from livers of WT and LTsc1KO mice (n = 4-5 mice per group) on the indicated diet. For each gene, data are represented as fold changes with respect to the WT-AL group. Asterisks indicate statistically significant differences between diet groups within genotype according to a student's t-test; *$p < 0.05$.

(H) Hepatic insulin signaling as determined by immunoblotting for markers of Akt and mTORC1 pathways in liver extracts prepared from WT mice fed *ad libitum* and harvested either without surgery (NI: non-ischemic) or at various time points during surgery. Isc-35 min: at the

Figure 2.7 (Continued)

end of the 35 minutes ischemic period; 5, 15 and 30 minutes: Time after reperfusion. Each well represents an individual animal.

(I) Hepatic insulin signaling as determined by immunoblotting for markers of Akt activation in extracts of livers from WT mice on the indicated diet for 1 week. Livers were harvested either without surgeries (NI: non-ischemic), at the end of the ischemic period (35 min ischemia) or 30 minutes into the reperfusion period.

(J) Akt activation status as determined by immunoblotting of liver extracts 3 hours after reperfusion from mice preconditioned for 1 week on the indicated diet prior to induction of hepatic IRI.

(K) Serum insulin levels from tail blood of WT mice preconditioned for 1 wk on the indicated diet taken prior to ischemia (0 hr), at the end of the ischemic period (30 min I, n=3-4), 30 min after reperfusion period (30 min R, n=3) or 3 hrs after reperfusion (3 hrs R, n=11-12). Asterisk indicates the significance of the indicated comparison according to a Kruskal-Wallis test followed by Dunn's multiple comparisons test; *$p < 0.05$; ns: not significant.

(L) Blood glucose levels of mice on the indicated diets for 1 wk before hepatic IRI, measured before (0hr) and 3 hrs after reperfusion; n = 8-10 mice/group. Asterisks indicate the significance of the difference between 0 and 3 hr values by student's t test within diet group; **$p < 0.001$, ***$p < 0.0001$. Data in all panels are shown as means ± SD.

In contrast to the potential benefits of reduced insulin/IGF-1 signaling named above, increased growth factor signaling after injury can trigger anti-apoptotic survival signaling and improve functional recovery (Fuglesteg et al., 2009, Jonassen et al., 2001, Liu et al., 2007). In order to address the potential protective effects of increased insulin/IGF-1 signaling after injury in the context of protection from hepatic IRI, we determined the status of insulin/Akt signaling at various time points during ischemia and after reperfusion. Akt signaling was dramatically reduced during ischemia and returned rapidly within 15 minutes of reperfusion (Figure 2.7H). We next looked at insulin/Akt signaling in the context of PR. During ischemia, Akt signaling was maintained at higher levels in PR mice (Figure 2.7I). Although no consistent differences were detected between PR and control mice 30 minutes after reperfusion (Figure 2.7I), phosphorylation of the Akt target FoxO1 was clearly increased 3 hours after reperfusion in livers of PR vs. Complete diet groups (Figure 2.7J). Importantly, this increase in FoxO1 phosphorylation was absent in LTsc1KO livers (Figure 2.7J).

To explain this boost in insulin/Akt signaling 3 hours after reperfusion specifically in WT mice preconditioned on PR, we measured serum insulin at multiple time points during the procedure. In WT mice, serum insulin levels fell dramatically below preoperative levels during the ischemic period independent of diet, and increased gradually following reperfusion (Figure 2.7K). Importantly, the recovery of circulating insulin was significantly higher 3 hours after reperfusion in PR animals than in control mice. This increase in insulin was unlikely to be secondary to an increase in blood glucose, since glucose levels remained significantly lower than initial levels 3 hrs after reperfusion (Figure 2.7L). As a result of the differential insulin sensitivity between WT and LTsc1KO mice, only WT mice displayed the boost in Akt activity upon PR (Figure 2.7J).

INCREASED PROSURVIVAL SIGNALING AND REDUCED APOPTOSIS CONTRIBUTE TO PR-MEDIATED PROTECTION

FoxO1 regulates apoptosis via transcription of pro-apoptotic Bcl-2 family members including Bim, PUMA and BNIP3 (Zhang et al., 2011). Although hepatic IRI is largely driven by necrotic cell death, inhibition of apoptosis protects from injury (Datta et al., 2013). We examined protein levels of pro-apoptotic Bcl-2 family members Bik, Bak, PUMA, BID and Bad in WT and LTsc1KO animals on PR or control diets for one wk. In WT livers, Bik and BID were reduced upon PR, consistent with a decrease in the pro-apoptotic environment (Figure 2.8A). In LTsc1KO livers, on the other hand, pro-apoptotic Bcl-2 family members were generally expressed at higher levels than in WT livers, and were not reduced upon PR (Bik, Bak and Bad, Figure 2.8A). Phosphorylation of Bad at Ser112 and Ser136 can inhibit its binding to Bcl-2 and Bcl-xL and thus reduce its pro-apoptotic activity (Danial, 2008). Phosphorylation of Bad at Ser112 was decreased upon PR in LTsc1KOs, unlike WTs (Figure 2.8A). Three hours after reperfusion, total Bad levels were reduced in WTs upon PR, but remained the same in LTsc1KOs with reduced phosphorylation (Figure 2.8B).

To determine if the observed increase in insulin/Akt signaling and decrease in pro-apoptotic factors resulted in reduced apoptosis in WT livers upon PR, sections from livers harvested 3 hours post-reperfusion were analyzed by TUNEL staining. As expected, WT livers preconditioned with PR had significantly less TUNEL positive cells compared to controls, and this protective effect of diet was absent in LTsc1KO livers (Figure 2.8C, 2.8D).

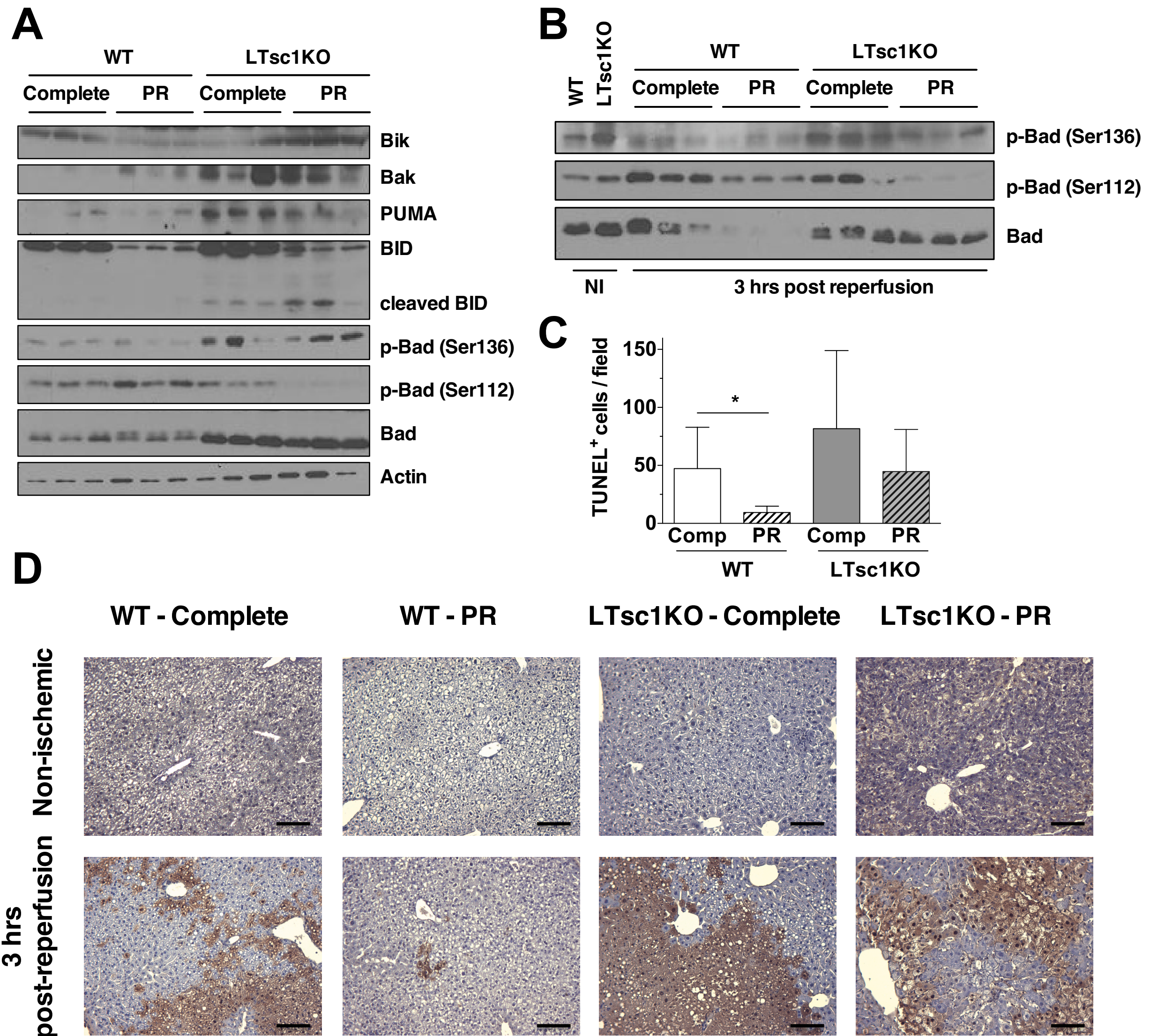

Figure 2.8 PR results in increased pro-survival signaling and reduced apoptosis in WT, but not LTsc1KO livers.

(A, B) Immunoblots of pro-apoptotic Bcl-2 family members in liver extracts from WT or LTsc1KO mice preconditioned on the indicated diets for 1 week and harvested without ischemia (A) or 3 hours after reperfusion (B). NI: non-ischemic.

(C, D) TUNEL staining of non-ischemic or ischemic livers from the indicated diet-genotype combinations 3 hours after reperfusion. (C) Quantification was performed by blind scoring of the

Figure 2.8 (Continued)

number of $TUNEL^+$ nuclei/200x field in 10 random fields from each sample, with $n = 5$ mice/group. Asterisks indicate the significance of the difference between diets within genotype by student's t test; $*p < 0.05$. (D) Representative images of TUNEL-stained sections. Scale bar: 100 μm. Data in (C) is shown as means ± SD.

In order to directly test the role of differential post-reperfusion insulin signaling in PR-mediated hepatic IRI protection, we tested the effects of the PI3K inhibitor wortmannin (Ren et al., 2011). Treatment 1 hour prior to induction of ischemia increased blood glucose (Figure 2.9A), reduced *in vivo* Akt phosphorylation (Figure 2.9B), and partially abrogated PR-mediated benefits against hepatic IRI (Figure 2.9C). Because of the relatively short half-life of wortmannin *in vivo*, hepatic Akt/FoxO signaling was restored by 3 hours after reperfusion (Figure 2.9D), which may explain the partial effect. Finally, we tested the response of liver-specific insulin receptor knockout (LIrKO) mice (Michael et al., 2000) to PR-mediated hepatic IRI preconditioning. LIrKO mice had lower levels of Akt phosphorylation in ischemic liver lobes at 3 hours post reperfusion (Figure 2.9E), and their response to PR was worse than WT mice on PR (Figure 2.9F).

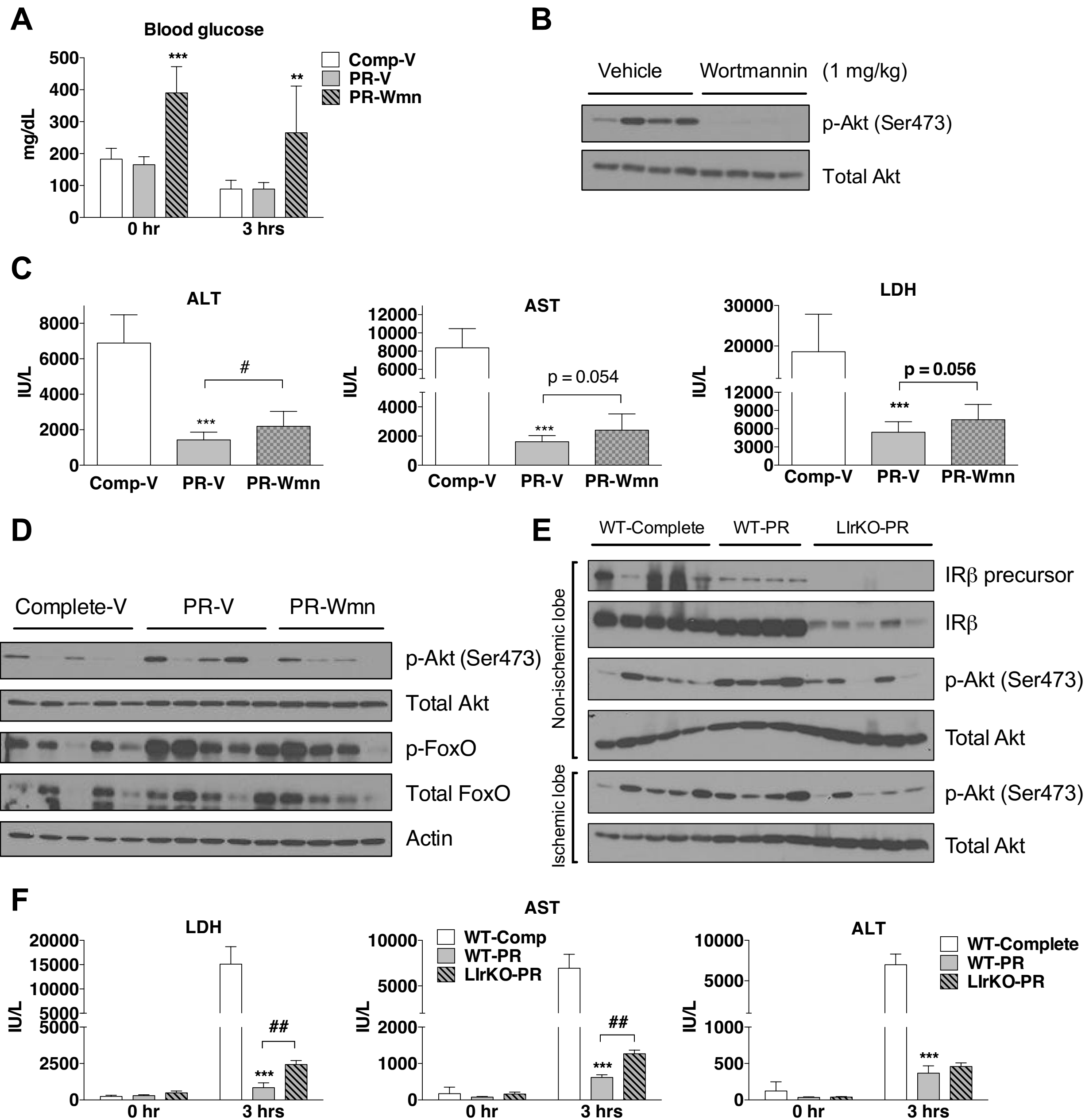

Figure 2.9 Increased prosurvival signaling and reduced apoptosis contribute to PR-mediated protection.

(A) Blood glucose values of mice measured before (0 hr) and 3 hours after reperfusion for the experiment described in Figure 6F. Statistical significance was assessed by student's t-test between PR-V and PR-Wmn groups; **p < 0.005, ***p < 0.000001.

Figure 2.9 (Continued)

(B) Immunoblot of Akt phosphorylation in liver extracts from WT mice injected with vehicle or 1 mg/kg wortmannin 1 hour prior to harvest.

(C) Serum LDH, ALT and AST activity 3 hours after reperfusion in WT mice preconditioned on the indicated diets for 1 week and injected with vehicle or 1 mg/kg wortmannin (Wmn) 1 hour prior to hepatic IRI; n = 8-10 mice/group. Statistical significance was assessed by student's t-test between Complete-V and PR-V groups (***$p < 0.0005$) or between PR-V and PR-Wmn groups ($^{\#}p < 0.05$).

(D) Immunoblots of liver extracts showing markers of Akt signaling; mice that were harvested 3 hours post-reperfusion, from the experiment described in Figure 6F.

(E) Immunoblot of liver extracts from WT mice on complete diet, or WT and LIrKO mice preconditioned with PR for 1 week and harvested 3 hours after reperfusion.

(F) Serum LDH, ALT and AST activity before ischemia (0 hr) and 3 hours after reperfusion in male WT and LIrKO mice preconditioned on the indicated diets for 1 week prior to hepatic IRI; n = 4-5/group. Statistical significance was assessed by student's t-test between WT-Complete and WT-PR groups (***$p < 0.0005$) or between WT-PR and LIrKO-PR groups ($^{\#\#}p < 0.001$). Data in all panels are shown as means ± SD.

A model for the collective action of PR on increased insulin sensitivity and relative insulin levels in WT animals after ischemia and reperfusion, resulting in increasing pro-survival signaling and reduced cell death, is presented in Figure 2.10. These latter effects were dependent on the expression of TSC1 in hepatocytes and were partially blocked by genetic and pharmacological inhibition of insulin/Akt signaling.

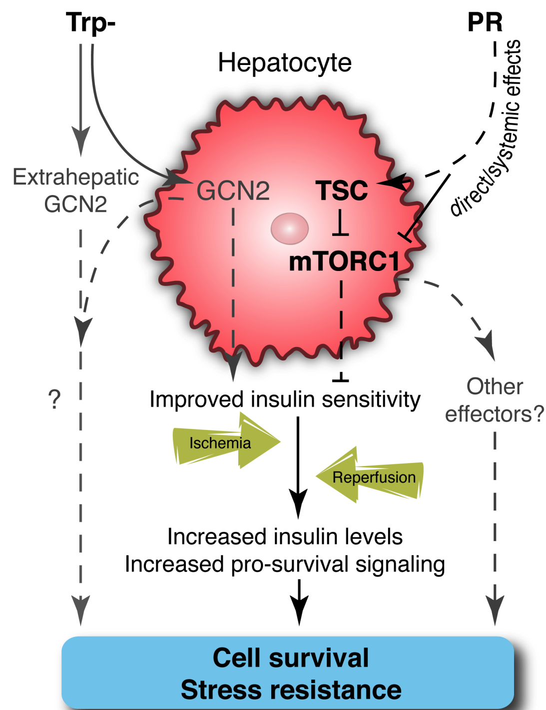

Figure 2.10: Model of TSC/mTORC1 function and insulin sensitivity in PR-mediated hepatic stress resistance.

A model for the effects of dietary protein or tryptophan restriction on improved hepatic insulin sensitivity prior to an acute ischemia event. After reperfusion, increased insulin levels and increased hepatic insulin sensitivity facilitate pro-survival signaling and contribute to protection from injury.

DISCUSSION

Previously we reported a genetic requirement for *Gcn2* in mediating surgical stress resistance upon single EAA restriction (Peng et al., 2012). Here we found that GCN2 was no longer required when total protein was removed from the diet, suggesting involvement of a redundant nutrient-sensing pathway. We thus tested the role of the mTORC1 pathway using mice deficient in *Tsc1* with constitutive mTORC1 activation specifically in the liver. The role of the mammalian TSC complex in DR benefits has not been previously tested. Here we found that two major benefits of PR, namely improved hepatic insulin sensitivity and stress resistance, both required the TSC complex.

Consistent with intact Ragulator-based mechanisms of mTORC1 inhibition *in vitro*, TSC1KO hepatocytes had higher baseline levels of mTORC1 activity but were still responsive to amino acid deprivation. Nonetheless, hepatic mTORC1 activity *in vivo* was completely recalcitrant to total dietary protein restriction in LTsc1KO vs. WT livers, despite reduced serum and liver free amino acids and activation of the GCN2-dependent amino acid starvation response in both genotypes. Our interpretation of these data is that the concentration of leucine or other critical amino acids required to trigger Ragulator-based mTORC1 inhibition can be achieved by removal of free amino acids *in vitro*, but cannot be achieved *in vivo* due to the buffering capacity on the organismal level against drastic changes in steady-state levels of free amino acids. What level of free amino acids are required to activate Ragulator in hepatocytes *in vivo*, and whether longer periods of dietary protein deprivation, or combined protein and calorie deprivation such as fasting, ever result in a drop below these levels, remains to be determined.

While female LTsc1KO mice almost entirely lost the preconditioning benefit of PR against hepatic IRI, parallel experiments in males showed that despite similar functional protection by PR as in females, this protection was only partially lost in LTsc1KO males. Although the reason for this apparent sex-based difference in the genetic requirement for Tsc1 is unclear, we note that the lifespan extension benefits of ablation of the downstream mTORC1 target S6K and $mTOR^{+/-}$ $mLST8^{+/-}$ models are also preferentially observed in females (Selman et al., 2009, Lamming et al., 2012).

In addition to total protein restriction, we also tested the effects of tryptophan restriction on LTsc1KO mice. Interestingly, LTsc1KO mice exhibited a response comparable to WT mice. Together with the previous findings indicating a requirement for the amino acid deprivation sensor GCN2 in protection afforded by tryptophan restriction (Peng et al., 2012), these results suggest that GCN2 and mTORC1 may act in parallel pathways, eventually converging on the same beneficial outcome. Whether or not this occurs through a common downstream mechanism remains to be determined. However, the lack of improvement of insulin sensitivity in GCN2KO mice in response to leucine restriction (Xiao et al., 2011) is consistent with the possibility of improved hepatic insulin sensitivity as a potential point of convergence.

Inhibition of TOR signaling has been implicated in longevity, metabolism and stress resistance, including the response to DR in lower organisms, but its downstream effector mechanisms in mammals are not well established (Kaeberlein et al., 2005, Zid et al., 2009, Kaeberlein, 2013). Improved insulin sensitivity is a metabolic hallmark of multiple different DR regimens, including PR (Fontana et al., 2010, Toyoshima et al., 2010), and is potentially controlled in part by mTORC1-based feedback inhibition of IRS-1 (Howell and Manning, 2011). Here, we found that improved hepatic insulin sensitivity upon PR required the TSC complex. Interestingly, PR

appeared to reduce hepatic insulin sensitivity in LTsc1KOs, although the reason for this remains unclear.

The fact that both increased and decreased signaling through the evolutionarily conserved insulin/IGF-1 pathway can improve cellular stress resistance and organismal survival is paradoxical (Rincon et al., 2004, Tang, 2006). Reduced insulin signaling, due either to reduced insulin levels as with DR, or in constitutive genetic models of reduced insulin/IGF-1 signaling, can extend longevity and increase stress resistance in part through activation of cytoprotective gene expression normally inhibited by insulin/Akt signaling. For example, in *C. elegans*, activation of FoxO/Daf-16 targets involved in stress resistance has been implicated in DR mediated longevity (Greer et al., 2009). On the other hand, increased insulin action is desirable under certain circumstances, for example to promote cell survival after ischemic insult. Consistent with this, increased insulin/PI3K/Akt signaling has been shown to play a protective role in both the rat isolated heart ischemia model (Jonassen et al., 2001, Fuglesteg et al., 2009, Liu et al., 2007) and other hepatic IRI models (Kamo et al., 2013) where a protective effect of reduced apoptosis has been described (Datta et al., 2013).

Our model of improved insulin sensitivity upon PR provides an answer to this apparent paradox. During the preconditioning period prior to injury, reduced insulin levels and reduced insulin-dependent mTORC1 activity were required, not to increase expression of cytoprotective FOXO or NRF2 target genes, but to facilitate improved insulin sensitivity via a decrease in mTORC1 feedback inhibition. This occurred in WT livers on PR, but was prevented in LTsc1KO livers due to constitutive mTORC1 activation. After injury, increased insulin signaling led to increased inhibitory FoxO1 phosphorylation, reduced apoptosis and improved outcome in insulin-sensitive WT livers. Inhibition of post-reperfusion insulin signaling either pharmacologically via the PI3K

inhibitor wortmannin, or genetically with LIrKO mice, partially abrogated PR-mediated protection. Whether or not IGF-1 plays a role similar to insulin remains to be tested. Although hepatic IGF-1R expression is low, liver-specific deletion compromises regeneration (Desbois-Mouthon et al., 2006); furthermore, IGF-1 can activate the insulin receptor directly. Thus whether insulin alone or a combination of insulin and IGF-1, our model predicts decreased growth factor signaling prior to injury (resulting in increased insulin/IGF-1 sensitivity) and increased signaling after injury are both required for maximal protection. It is important to note that because abrogation of insulin signaling only partially blocked PR-mediated protection, additional mechanisms contributing significantly to protection remain to be identified.

Understanding the mechanistic basis of DR is crucial to the development of potential interventions that mimic its beneficial effects. Genetic and pharmacological approaches targeting nutrient sensing pathways have yielded similar results to DR in terms of longevity extension and other beneficial health effects, suggesting a causal role for the downregulation of these pathways in DR benefits (Fontana et al., 2010, Kaeberlein, 2013). For example, rapamycin extends lifespan in rodents, although what aspect of mTORC1 inhibition is responsible remains unclear (Harrison et al., 2009, Miller et al., 2011, Miller et al., 2013). Despite longevity extension, rapamycin has negative side effects, including increased inflammation and reduced insulin sensitivity (Lamming et al., 2013) and fails to recapitulate many other DR phenotypes (Miller et al., 2013). In our model, rapamycin not only failed to recapitulate benefits of PR, but partially abrogated PR benefits, possibly due to its aforementioned negative effects on insulin signaling via mTORC2 inhibition (Lamming et al., 2013).

Hepatic IRI presents a major challenge during various clinical contexts such as hepatic resection and liver transplantation surgeries, as well as trauma (Datta et al., 2013, Klune and Tsung, 2010).

Ischemic damage to other tissues, including heart, brain and kidney, occurs regularly as an unintended complication of cardiovascular surgery, leading to significant morbidity and mortality (Mitchell et al., 2013). Understanding the mechanistic basis of DR-mediated protection could lead to preventative strategies against these clinically relevant stressors.

MATERIALS AND METHODS

Animals and Diets

All animal experiments were performed with the approval of the appropriate institutional animal care and use committee. WT C57Bl/6J mice were purchased from Jackson Laboratories at 8-10 weeks of age. LTsc1KO and GCN2KO mice were backcrossed on the C57Bl/6J background as described previously (Yecies et al., 2011, Menon et al., 2012, Peng et al., 2012). $Ir^{fl/fl}$ mice as described previously (Michael et al., 2000) were purchased from the Jackson Laboratories (B6.129S4(FVB)-*Insr*^{tm1Khn}/J, Stock #006955). Experimental LTsc1KO and littermate control animals were generated by crossing *Tsc1*$^{fl/fl}$ (WT) mice with *Tsc1*$^{fl/fl}$|*Albumin-Cre*$^{+/-}$ (LTsc1KO) mice; GCN2KO and control mice were generated by crossing heterozygotes; LIrKO and control mice were generated by crossing *Ir*$^{fl/fl}$ (WT) mice with *Ir*$^{fl/fl}$|*Albumin-Cre*$^{+/-}$ (LIrKO) mice. Animals were kept under standard housing conditions with *ad libitum* access to food (Purina 5058) except for the indicated dietary treatments. Female mice between 10-16 weeks of age were used unless otherwise indicated. LTsc1KO mice were used prior to the age of 16 weeks in order to avoid any potential complications of hepatocellular carcinomas, which appear later in life (Menon et al., 2012).

Dietary and Pharmacological Treatments of Mice

Experimental control diets were based on a modified version of Research Diets D12450B prepared with 18% calories from protein (individual crystalline amino acids (Ajinomoto) based on the proportions present in casein), 10% from fat (lard, soybean oil) and 72% from carbohydrate (sucrose, maltodextrin, corn starch). For protein free and individual amino acid free diets, missing amino acids were replaced with isocaloric sucrose. All diets were prepared in a final 1% agar mixture. Mice on experimental diets were fed and weighed daily, at the same time of the day. When mice were subject daily to reduced food availability (dietary restriction, DR), they tended to eat their daily allotment quickly, resulting in periods of extended fasting for the remainder of the 24-hour period. However, when presented with incomplete diets lacking protein or essential amino acids, mice self-restricted food intake by up to 30-35% (Peng et al., 2012). As a result, even when availability of protein or amino acid deficient diets was restricted daily by 35%, animals did not eat the available food quickly (as with DR) and thus didn't experience extended periods of fasting. Procedures including surgery and tissue harvesting were performed in the morning without prior fasting unless otherwise indicated. Rapamycin for *in vivo* use was purchased from LC Laboratories, dissolved in 100% EtOH and diluted in sterile 5% Tween-80/5% PEG-400 solution for i.p. injections. Wortmannin was purchased from Selleckchem, dissolved in DMSO and diluted in sterile 30%PEG/1%Tween-80 solution for i.p. injections.

Hepatic Ischemia Reperfusion Injury and Serum Damage Assays

Hepatic IRI was performed by placing a microvascular clamp (Roboz) over the portal triad for 35 minutes for female and 30 minutes for male mice as described previously (Peng et al., 2012). After surgery, mice were given *ad libitum* access to control diet starting at 3 hours after

reperfusion. Serum ALT and AST levels were measured using Infinity Reagents (Thermo Scientific) and serum LDH levels using Pointe Scientific reagents with kinetic absorbance assays in a 384-well format with a BioTek Synergy II plate reader and calculated according to manufacturer's instructions. For histology, median lobes were formalin fixed, paraffin embedded, cut into 5-micron sections and stained with hematoxylin and eosin or TUNEL (Promega). Percentage of necrotic area or TUNEL positivity in each section was scored blindly.

Primary Dermal Fibroblast and Primary Hepatocyte Isolation and Treatments

Mouse dermal fibroblasts were isolated from tail skin by mincing into fine slices followed by overnight collagenase treatment (Invitrogen Collagenase II). Cells were cultured in DMEM with 20% FBS at 3% oxygen tension. Primary hepatocytes were isolated via portal vein collagenase treatment (Liberase, Roche) followed by Percoll gradient centrifugation and cultured in William's E media with 5% FBS. Amino acid free DMEM was a special formulation of Invitrogen DMEM-12320. For the amino acid add-back, either MEM amino acid solution (GIBCO) was used at 1x concentration, or individual amino acids were added back to AA-free medium up to their original concentrations in DMEM, and supplemented with 10% dialyzed FBS (Invitrogen). Rapamycin was purchased from Calbiochem.

Serum Measurements and Amino Acid Analysis

Blood glucose was measured with an Easy Step glucometer (Home Aide Diagnostics). Serum insulin and IGF-1 levels were measured with ELISA assay kits (Alpco). Serum and liver free amino acid analyses were performed by mass spectrometry and normalized by volume and total protein content, respectively.

Western Blotting

Cells and tissues were homogenized in NP-40 lysis buffer, cleared by centrifugation, separated by SDS-PAGE and transferred to PVDF membranes for blotting. Primary antibodies were purchased from Cell Signaling Technology except anti-p-Bad^{Ser136} and IRβ (Santa Cruz), and secondary antibodies from Dako.

Serum damage measurements using Piccolo Liver Plus Panel discs

Piccolo Liver Plus Panel discs (Abaxis) were used as a second platform for confirming ALT and AST levels according to the manufacturer's instructions. This platform can measure serum ALT and AST levels up to 2000 IU/L. For the mice which had damage values greater than 2000, we estimated these values to be 2000 IU/L and performed calculations accordingly.

RNA isolation and quantitative PCR

Total RNA was isolated from frozen tissues by RNA Bee reagent (Qiagen) and cDNAs were synthesized by using random hexamer primers and Verso cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instructions. SYBR green dye was used for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and fold changes were calculated by $\Delta\Delta C_t$ method using *Hprt* housekeeping gene. Each sample was tested in duplicates. Primer sequences for each gene are listed in Table 2.1.

Table 2.1 qRT-PCR Primer sequences

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Asns	GTCAAGAACTCCTGGTTCAAG	GATCTGACGGTAGAAGTAGC
4ebp1	ACTAGCCCTACCAGCGATGAG	CGCAGACATAGAAGCATCATTGCG
Nrf2	AGGACATGGAGCAAGTTTGG	TCTGTCAGTGTGGCTTCTGG
Nqo1	AGGATGGGAGGTACTCGAATC	AGGCGTCCTTCCTTATATGCTA
Ho1	AAGCCGAGAATGCTGAGTTCA	GCCGTGTAGATATGGTACAAGGA
Gclc	GGGGTGACGAGGTGGAGTA	GTTGGGGTTTGTCCTCTCCC
Gclm	AGGAGCTTCGGGACTGTATCC	GGGACATGGTGCATTCCAAAA
Gss	CAAAGCAGGCCATAGACAGGG	AAAAGCGTGAATGGGGCATAC
Gstm4	AGCTCACGCTATTCGGCTG	GCTCCAAGTATTCCACCTTCAGT
Gsta4	TGATTGCCGTGGCTCCATTTA	CAACGAGAAAAGCCTCTCCGT
Gsta3	AAGAATGGAGCCTATCCGGTG	CCATCACTTCGTAACCTTGCC
Catalase	TCCCTGCTGTCTCACGTTCC	CGGGTCTCCTATTGGGTTCCCG
Sod1	GGGACAATACACAAGGCTGT	GCCAATGATGGAATGCTCTC
Hprt	TTTCCCTGGTTAAGCAGTACAGCCC	TGGCCTGTATCCAACACTTCGAGA

Statistical Analyses

All data are represented as means ± standard deviations (SD). Statistical significance was assessed by student's t test for pairwise comparisons or by one-way analysis of variance (ANOVA) with post-hoc tests as indicated using Microsoft Excel or GraphPad Prism. P-values smaller than 0.05 were considered statistically significant. Quantification of western blot images was done by ImageJ software.

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CHAPTER 3

TSC1KO LIVERS HAVE IMPAIRED ABILITY TO UPREGULATE H_2S PRODUCTION CAPACITY IN RESPONSE TO DIETARY PROTEIN RESTRICTION

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SUMMARY

Dietary restriction or protein restriction (DR/PR) provides multiple beneficial effects on health across evolutionary boundaries, but the underlying molecular mechanisms remain poorly understood. Previously, we identified a genetic requirement for the mTORC1 repressor TSC complex in mediating PR effects on protection from hepatic ischemia reperfusion injury (IRI), in part through facilitating pro-survival signaling upon PR. Recently, our group established a connection between DR/PR and increased endogenous hydrogen sulfide (H_2S) production through the transsulfuration enzyme cystathionine γ-lyase (CGL). Here, we show that LTsc1KO mice failed to upregulate hepatic CGL expression and H_2S production upon PR, thus providing an additional mechanism by which these mice fail to gain PR-mediated protection from hepatic IRI. These data implicate the TSC/mTORC1 pathway in control of endogenous hepatic H_2S production *in vivo*. Interestingly, primary hepatocytes from LTsc1KO mice maintained H_2S production capacity in culture, suggesting an important role for systemic factors *in vivo* in the regulation of H_2S production.

BACKGROUND

Hydrogen sulfide (H_2S) is a colorless, foul smelling gas, which is toxic and considered a health hazard at high doses. Nonetheless, it has recently emerged as a biologically relevant molecule with important, beneficial effects on physiology and health at lower doses. For example, exogenous administration of H_2S induces a state of "suspended animation" characterized by reduced body temperature and decreased energy metabolism that is protective against hypoxia in mice (Blackstone et al., 2005). H_2S also protects against ischemia reperfusion injury to different

organs including heart and liver in mice (Predmore and Lefer, 2010, Kang et al., 2009), and increases longevity in worms (Miller and Roth, 2007). Due to these beneficial effects, H_2S is currently being investigated as a therapeutic agent in the area of cardiovascular disease (Predmore and Lefer, 2010, Zhang et al., 2013).

In addition to low-level exogenous H_2S as a source of potential health benefits, organisms ranging from yeast to mammals can also produce H_2S endogenously via a number of evolutionarily conserved mechanisms (Paul and Snyder, 2012). In mammals, H_2S can be produced by the actions of two different enzymes of the transsulfuration pathway (TSP), cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL, also referred to as CSE or CTH). The TSP is essential for *de novo* cysteine production, and is the reason that cysteine is a non-essential amino acid in humans. Following the conversion of the essential amino acid methionine into homocysteine via the transmethylation pathway, CBS converts homocysteine into cystathionine, followed by the action of CGL to produce cysteine. Interestingly, CGL and CBS can each use these TSP substrates and products (namely homocysteine and cysteine) to generate H_2S, while at the same time producing serine, pyruvate or alpha-ketobutyrate (Paul and Snyder, 2012). CGL is thought to be the predominant source of H_2S in peripheral tissues including the liver (Yang et al., 2008, Kabil et al., 2011b), while CBS likely plays a more important role in H_2S production in the brain (Linden et al., 2008). A third enzyme, 3-mercaptopyruvate sulfurtransferase (MST), can produce H_2S from 3-mercaptopyruvate (Wang, 2012).

The importance of endogenous CGL-derived H_2S was first recognized in the context of vasodilation, as mice lacking CGL (CGLKO) suffer from high blood pressure and cardiovascular disease (Yang et al., 2008). CGLKO mice are also susceptible to Huntington's disease, implicating the importance of physiological levels of this gas in protection from

neurodegenerative disease (Paul et al., 2014). H_2S is also implicated in regulation of a wide range of biological processes, from inflammation (Grou et al., 2012) to insulin secretion (Xue et al., 2013) to angiogenesis (Papapetropoulos et al., 2009). Consistent with the importance of this metabolite in humans, reduced H_2S in circulation correlates with congestive heart failure (Kovacic et al., 2012).

On a molecular level, H_2S likely mediates its beneficial effects through numerous mechanisms. The major mechanism of action is thought to be post-translational modification of target proteins via "sulfhydration" of surface-exposed cysteine residues, which can affect target protein function (Mustafa et al., 2009). For example, sulfhydration of Cys38 of NFkB subunit p65 increases it antiapoptotic activity (Grou et al., 2012), while sulfhydration of the K_{ATP} channel subunit Kir6.1 activates the channel, reducing insulin secretion e.g. in pancreatic beta cells (Kimura, 2011). Alternately, H_2S can work directly as an antioxidant (Li et al., 2011), or can donate electrons to the mitochondrial electron transport chain through the evolutionarily conserved protein SQR, thus potentially contributing to ATP generation (Modis et al., 2013).

Regardless of how H_2S mediates its biological effects, the importance of its potential therapeutic uses is clear. Our group recently showed that upregulation of CGL expression and H_2S production upon dietary sulfur amino acid or protein restriction (commonly referred to as DR here) is necessary and sufficient for multiple DR mediated-health benefits ranging from surgical stress resistance in mice to extended longevity in worms (Hine et al., 2015, Shim and Longo, 2015). This finding is consistent with the requirement of TSP for DR-mediated longevity extension in flies (Kabil et al., 2011a).

Given that impaired insulin sensitivity only partially explains the lack of protein restriction (PR)-mediated protection from hepatic ischemia reperfusion injury in liver-specific tuberous sclerosis complex 1 knockout (LTsc1KO) livers (explained in Chapter 2) (Harputlugil et al., 2014), here we investigated the potential role of impaired H_2S production in those livers as an additional mechanism of their unresponsiveness to PR.

RESULTS

LTSC1KO LIVERS HAVE IMPAIRED H_2S PRODUCTION CAPACITY IN RESPONSE TO PROTEIN RESTRICTION

In order to identify additional potential mechanisms that might underlie the lack of a PR response in LTsc1KO livers, we took an unbiased metabolomics approach to search for metabolites that were significantly affected by PR in WT but not LTsc1KO livers. Although free amino acids such as methionine and cysteine were reduced in both genotypes on PR as expected due to dietary protein restriction, we observed two intermediates that fit the expected pattern (Figure 3.1A, B). Interestingly, both of these the metabolites, serine and homoserine, are also byproducts of H_2S production by the TSP enzymes CBS and CGL (Figure 3.1C). Because we already suspected involvement of the TSP based on the finding that dietary cysteine negated PR-mediated protection from hepatic IRI (Hine et al., 2015), as well as the known potential of exogenous H_2S in protection from ischemic injury (Predmore and Lefer, 2010), we decided to measure H_2S generating enzymes and H_2S production capacity directly in these samples.

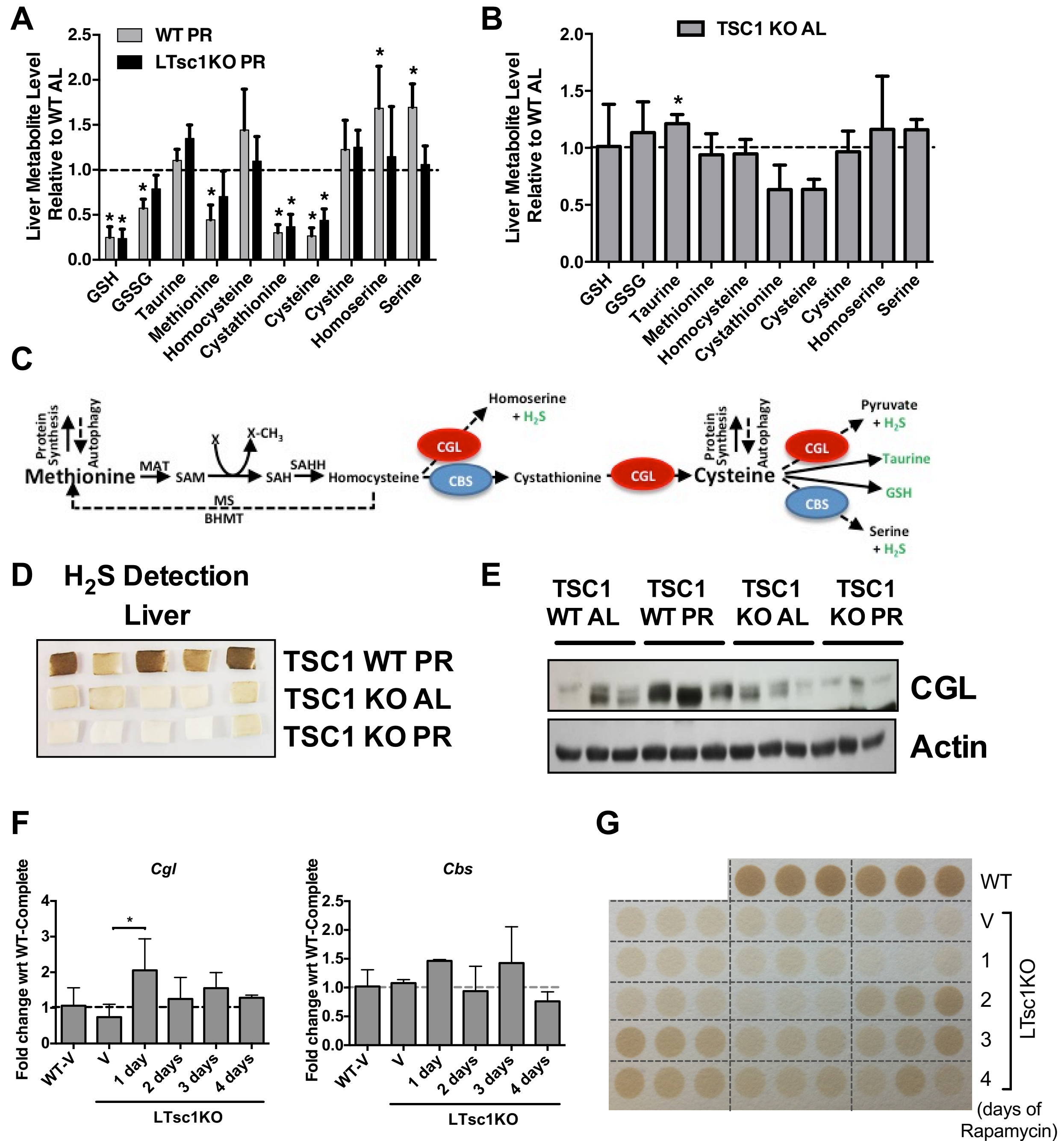

Figure 3.1 LTsc1KO livers have impaired H_2S production capacity in response to protein restriction.

(A, B) WT or LTsc1KO mice were fed with complete *ad libitum* diet (AL) or 35% protein-free diet (PR) for one week prior to liver harvest. Liver metabolites were measured by unbiased LC-

Figure 3.1 (Continued)

MS metabolomics and normalized to protein content. For each metabolite, levels are expressed as fold change compared to the WT *ad libitum*-fed group (WT AL); n = 4-5 mice per group. Asterisks indicate the statistical significance of the difference between the different dietary regimens according to a Student's t-test; * $p<0.05$. **(EH)**

(C) Model showing transmethylation and transsulfuration pathways. Arrows trace sulfur from Met to Cys and downstream cellular processes via cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CGL). MAT, methionine adenosyl transferase; SAM: S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; MS, methionine synthase; BHMT, betaine homocysteine methyltransferase. **(CH)**

(D, E) H_2S production (D) and immunoblots of liver CGL (E) and in WT and LTsc1KO mice fed AL or 35% DR on a protein-free diet (PR). n = 5 mice/group (D), n = 3 mice/group (E). **(EH, CH)**

(F, G) mRNA levels of *Cgl* and *Cbs* (F) and H_2S production (G) in livers of WT and LTsc1KO mice treated with either vehicle (V) or rapamycin injections (1mg/kg) for up to 4 days as indicated. n = 2-3 mice per treatment group (F, G), technical triplicates are shown side by side for each mouse (G). Asterisks indicate the statistical significance of the difference between the indicated groups according to 1-way ANOVA against LTsc1KO-V group, with post-hoc Dunnett's multiple comparisons test; * $p<0.05$. **(EH)**

Data in all panels are shown as means ± SD.

To measure H_2S production capacity, we employed the lead sulfide method in which H_2S gas in the headspace above a reaction mixture containing a source of H_2S-generating enzyme together with substrate (L-cysteine) and cofactor (pyridoxal-5'-phosphate, or PLP) is captured on lead acetate-soaked filter paper as the black precipitate, lead sulfide. We measured the H_2S production capacity of lysates from livers of WT and LTsc1KO mice upon PR. We observed that LTsc1KO livers had lower H_2S production capacity compared to WT livers upon PR (Figure 3.1D). This differential H_2S production capacity correlated with the protein levels of CGL enzyme in liver lysates (Figure 3.1E).

In order to confirm that these changes were mTORC1-specific, we tested the H_2S production capacity and *Cgl/Cbs* mRNA levels in LTsc1KO liver lysates upon treatment with the mTORC1 inhibitor rapamycin for up to 4 days. Even though the effect of rapamycin was not clear at the *Cgl* mRNA level (Figure 3.1F), H_2S production capacity was markedly increased in LTsc1KO livers upon rapamycin treatment by day 3 of treatment (Figure 3.1G). Taken together, these data show that LTsc1KO mice are impaired in their ability to upregulate hepatic H_2S production capacity in response to PR, potentially via effects on CGL expression.

THE EFFECTS OF CONSTITUTIVE MTORC1 ACTIVATION ON H_2S PRODUCTION ARE NOT CELL AUTONOMOUS, AND PR MEDIATED H_2S PRODUCTION LIKELY INVOLVES OTHER PATHWAYS

Next, we wanted to understand the role of the mTORC1 pathway in regulation of H_2S production on a cell autonomous level using an *in vitro* primary hepatocyte model. Interestingly, unlike in liver extracts in which H_2S production was consistently reduced independent of diet (Figure 3.2A), we observed no differences in H_2S production capacity of hepatocytes from LTsc1KO vs.

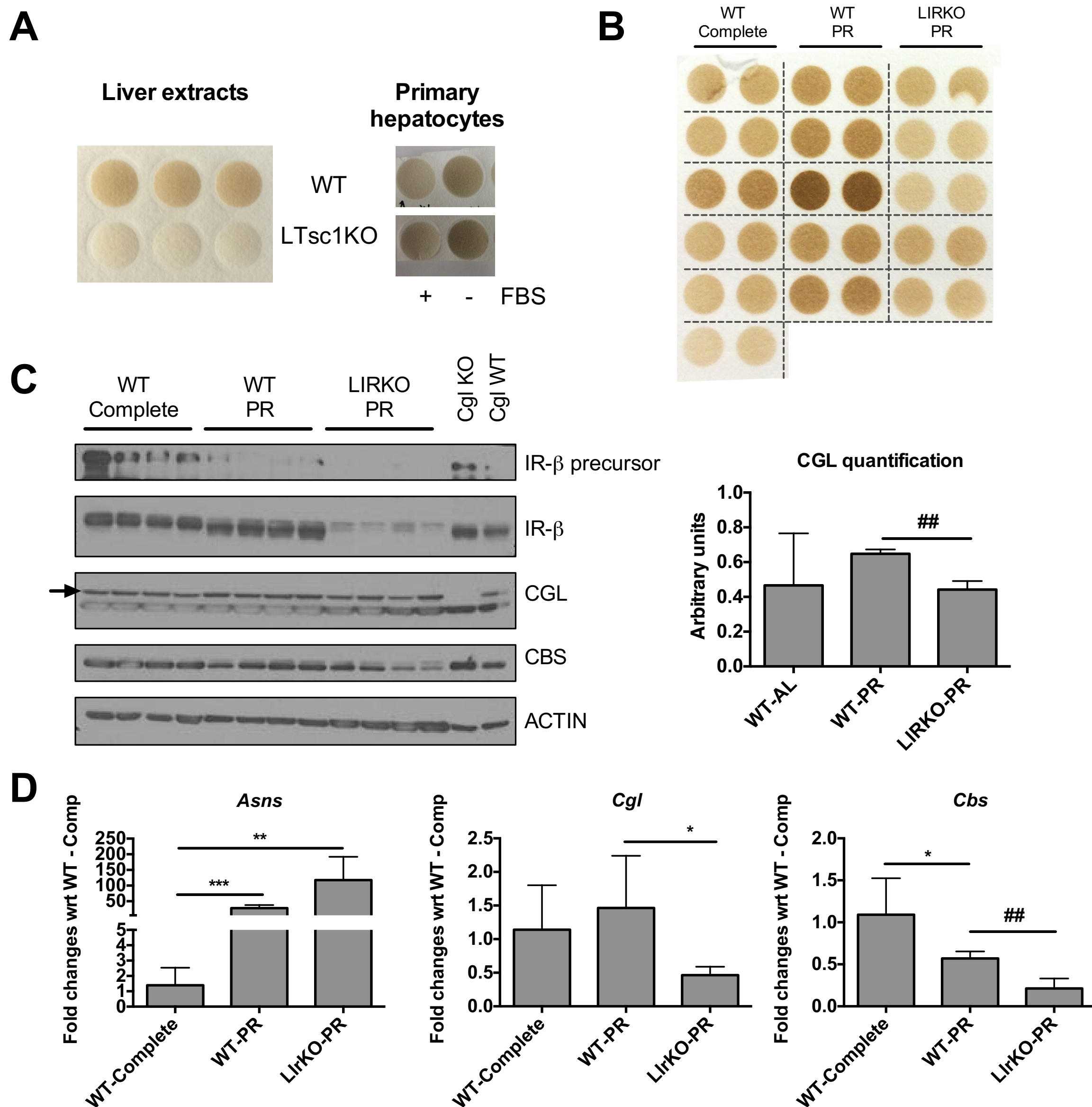

Figure 3.2 The effects of constitutive mTORC1 activation on H_2S production are not cell autonomous, and PR mediated H_2S production likely involves other pathways.

(A) Livers (left) or primary hepatocytes (right) were isolated from one WT and one LTsc1KO mice, and H_2S production capacity was measured by the lead-acetate paper method either in liver lysates or in live cultured hepatocytes that were treated with complete or serum$^-$ (FBS) media overnight. **(EH)**

Figure 3.2 (Continued)

(B) H_2S production of WT and LIrKO mice either fed *ad libitum* complete or 35% DR on a protein-free diet (PR) as indicated; n = 5-6 mice per treatment group, technical duplicates are shown side by side. **(EH)**

(C) Immunoblots of WT and LIrKO mice either fed *ad libitum* complete or 35% DR on a protein-free diet (PR); n = 4 mice per group. CGL KO lysate was used as control for the CGL band as shown with arrow, and the quantification if the CGL band is shown on the right. $^{\#\#}$ $p<0.001$ according to Student's T test. Data are shown as means ± SD. **(EH)**

(D) mRNA levels of *Asns, Cgl and Cbs* in livers of WT and LIrKO mice either fed *ad libitum* complete or 35% DR on a protein-free diet (PR); n = 5-6 mice per group. Asterisks indicate the statistical significance between the indicated groups according to a Student's T test; * $p<0.05$, ** $p<0.005$, *** $p<0.0001$, $^{\#\#}$ $p<0.001$. **(EH)**

Data in all panels are shown as means ± SD.

WT livers. Furthermore, overnight serum deprivation, which is sufficient to increase H_2S production in WT hepatocytes (Figure 3.2A; see also Chapter 4), was also able to increase H_2S production in LTsc1KO hepatocytes. Thus, the reduced baseline and inability to upregulate H_2S production upon PR observed in LTsc1KO livers was not recapitulated in the primary hepatocyte system. While the reason for this is not clear, it could either be an *in vitro* artifact related to differences in cultured LTsc1KO hepatocytes (e.g. greatly reduced plating efficiency vs. WT, data not shown), or could suggest a role for systemic factors in the regulation of H_2S production in the LTsc1KO model.

In addition to constitutive mTORC1 activation, LTsc1KO livers exhibit insulin resistance as discussed in detail in Chapter 2. Because the PI3K/Akt pathway has been implicated in regulation of *Cgl* expression and hence H_2S production (Zhao et al., 2014, Yin et al., 2012), we hypothesized a potential role of impaired insulin/Akt signaling in reduced H_2S production capacity in LTsc1KO livers *in vivo*. To test this, we investigated H_2S production capacity in liver-specific insulin receptor knockout (LIrKO) mice. Relative to livers of WT mice on PR, livers of LIrKO mice on PR had reduced H_2S production capacity, *Cgl* mRNA and protein levels (Figure 3.2B-D). As a control for successful amino acid deprivation in livers of LIrKO mice upon PR, ATF4 target *Asns* mRNA levels were significantly elevated in both genotypes relative to WT mice on a complete diet.

Taken together, our data are consistent with growth factor/Akt signaling playing a role in H_2S regulation *in vivo*, and more specifically, hepatic insulin resistance as a negative regulator of CGL expression and H_2S production capacity rather than mTORC1 signaling *per se*. Future experiments are required to delineate the details of this regulation.

DISCUSSION

In Chapter 2, we identified a genetic requirement for the TSC complex in hepatocytes in the protection afforded by dietary protein restriction (PR) against hepatic IRI (Harputlugil et al., 2014). Using this LTsc1KO model, we uncovered a mechanistic requirement for insulin sensitivity that could account for some, but not all, of the protection afforded by PR against hepatic IRI. Here, we used the same model to identify an additional requirement for increased CGL expression and H_2S production contributing to protection by PR against hepatic IRI.

Using an unbiased metabolomics approach, we identified intermediates of the transsulfuration pathway that were differentially regulated in LTsc1KO livers upon PR. Steady-state levels of homoserine and serine, which are byproducts of CBS and CGL in the production of H_2S, were significantly increased in WT livers upon PR. Despite the fact that H_2S itself is not detectable on our metabolomics platform, considering the beneficial role of H_2S in stress resistance (Predmore and Lefer, 2010), we investigated the link between endogenous H_2S production and DR-mediated benefits. Using the lead acetate method to directly measure H_2S production capacity in liver extracts, we established that endogenous H_2S is necessary and sufficient for DR-mediated protection from hepatic IRI (Hine et al., 2015). The observation that LTsc1KO livers had reduced baseline H_2S production capacity (Figure 3.1G; 3.2A) and failed to upregulate H_2S production upon PR (Figure 3.1D), indicates that H_2S plays a role in the failure of LTsc1KO mice to respond to PR. Furthermore, the low baseline H_2S production capacity phenotype was reversible upon rapamycin treatment, suggesting that constitutive mTORC1 activation prevents baseline and activated H_2S levels. Interestingly, however, rapamycin treatment did not increase H_2S production capacity to levels observed upon DR, suggesting that while mTORC1

constitutive activation can repress H_2S production capacity, a different mechanism that remains to be elucidated is required to increase H_2S production capacity upon PR.

In order to investigate the mechanism by which mTORC1 signaling repressed CGL expression and H_2S production on a cell autonomous level, we modeled DR in primary hepatocytes *in vitro* by culturing them under conditions of serum deprivation. Contrary to what we observed *in vivo*, Tsc1KO hepatocytes produced as much H_2S as WT cells under complete media conditions and responded normally to serum deprivation by increasing H_2S production capacity. There could be multiple reasons for this discrepancy. On the one hand, LTsc1KO hepatocytes are far less able to survive the isolation procedure than WT hepatocytes, which could potentially increase H_2S production through mechanisms unrelated to mTORC1 or TSC. On the other hand, it could also indicate the importance of systemic factors regulated by DR *in vivo* that are lost in the *in vitro* system, such as hormones and growth factors. The fact that LIrKO livers respond to PR similarly as LTsc1KO livers indicates that reduced insulin/Akt signaling might be playing a role as previously suggested (Zhao et al., 2014, Yin et al., 2012), however further studies are needed to establish a clear connection.

There are two important open questions remaining. How does DR/PR regulate CGL expression and H_2S production? And how does H_2S mediate its beneficial health effects such as protection from surgical stress? Both of these questions clearly require further investigation and will have broad implications in terms of both understanding how dietary restriction works and how H_2S can be used in clinical settings.

MATERIALS AND METHODS

Animal models, dietary and pharmacological treatments

Breeding and maintenance of LTsc1KO and LIrKO mice, housing conditions, experimental animal diets and in vivo rapamycin treatment protocol were described in Chapter 2.

Metabolomics

Liver metabolites were extracted on dry ice using 80% methanol, samples were dried and resuspended in H_2O right before analysis by LC-MS at a Harvard core facility. Levels were normalized to total protein content.

H_2S measurements by Lead Sulfide Method

For H_2S measurements, snap-frozen liver tissue was homogenized in passive lysis buffer (Promega), lysate was flash frozen in LN_2 and thawed at 37°C once for complete lysis, and cleared by centrifugation. Sample amounts were equalized by protein content, and 10mM L-cysteine and 10mM PLP was added as substrate and cofactor to the reaction. Lead-acetate papers were prepared by soaking filter paper (VWR) in 20mM lead acetate and vacuum drying. H_2S reaction mixtures were placed in closed containers with the lead-acetate paper above the liquid phase, and able to capture the produced H_2S gas. Samples were incubated at 37°C until the brown colored lead sulfide precipitate started to become visible.

Western Blotting

Frozen tissues were lysed in NP-40 lysis buffer and separated by SDS-PAGE. PVDF membranes were used for blotting. Membranes were blocked with 5% dry milk for 1 hour at RT and incubated O/N in primary antibodies at 4°C. Primary antibodies: Actin (#4970 Cell Signaling

Technology), CGL (ab151769 Abcam), CBS (ab135626 Abcam), IR-β (sc-711 Santa Cruz Biotechnology) and secondary antibodies from Dako.

RNA isolation and quantitative PCR

Total RNA isolation, cDNA synthesis and qRT-PCRs were performed as described in Chapter 2. Primer sequences for each gene are listed in Table 3.1.

Table 3.1 qRT-PCR Primer sequences

Gene	**Forward primer (5' → 3')**	**Reverse primer (5' → 3')**
Hprt	TTTCCCTGGTTAAGCAGTACAGCCC	TGGCCTGTATCCAACACTTCGAGA
18S	CATGCAGAACCCACGACAGTA	CCTCACGCAGCTTGTTGTCTA
Cgl	TTGGATCGAAACACCCACAAA	AGCCGACTATTGAGGTCATCA
Cbs	GGGACAAGGATCGAGTCTGGA	AGCACTGTGTGATAATGTGGG

Statistical analyses

Data in all panels are represented as means ± standard deviations (SD). Statistical analyses were performed as described in Chapter 2.

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CHAPTER 4

REGULATION OF ENDOGENOUS H_2S PRODUCTION

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SUMMARY

Hydrogen sulfide (H_2S) is now recognized as a biological molecule with a range of beneficial health effects, including increased stress resistance and extended longevity in lower organisms. Our group has recently shown that dietary restriction (DR) stimulates endogenous H_2S production via increased cystathionine γ-lyase (CGL) expression, and that H_2S is an important mediator of beneficial DR effects. Despite the wide range of potential implications of these findings, the mechanisms regulating endogenous H_2S production, including CGL transcriptional control and the source of free cysteine in substrate-level control of H_2S production, remain poorly understood. Here we investigated the role of growth hormone (GH) signaling in regulation of H_2S production and the role of autophagy as a potential source of substrate for H_2S generation. We identified GH as a negative regulator of hepatic H_2S production *in vivo* in part through transcriptional repression of hepatic CGL expression. In addition, GH rapidly repressed H_2S production in hepatocytes *in vitro* independent of transcriptional control of *Cgl* or *Cbs* through an unknown mechanism. Additionally we demonstrated that stimulation of H_2S production via serum deprivation requires functional autophagy, likely as a source of free cysteine for H_2S generation by CGL. The detailed mechanisms of GH-mediated H_2S regulation, and whether there is a crosstalk between reduced GH signaling and increased autophagy leading to increased H_2S production, remain important topics to be further investigated.

BACKGROUND

In mammalian cells, endogenous hydrogen sulfide (H_2S) is primarily produced by the actions of the transsulfuration pathway (TSP) enzymes cystathionine β-synthase (CBS) and cystathionine γ-

lyase (CGL, also referred to as CSE or CTH) (Paul and Snyder, 2012) as described in more detail in Chapter 3. Despite the wide-ranging beneficial health effects of H_2S demonstrated in preclinical models (Zhang et al., 2013, Predmore and Lefer, 2010), the mechanisms of regulation of endogenous H_2S production remain incompletely understood. This is explained in part by the fact that pleiotropic benefits of endogenous H_2S have emerged relatively recently in the experimental literature, and in part because the various methods to measure H_2S itself remain non-specific, insensitive and/or inconvenient (Wang, 2012).

Much of the existing literature on regulation of CBS and CGL is in the context of the TSP and *de novo* cysteine synthesis rather than H_2S production *per se*. Nonetheless, in addition to our study on the regulation of CGL and CBS expression and H_2S production by dietary restriction (DR) or protein restriction (PR) in multiple tissues and organisms (Hine et al., 2015), and the requirement for the mTORC1 repressor TSC in this process (Chapter 3), several other interventions have been shown to modulate TSP activity, and in some cases, H_2S production. For example, low levels of sulfur amino acids (SAA: cysteine and methionine) increase the activity of the transsulfuration pathway (TSP) in cultured cells (Stipanuk and Ueki, 2011). Hypoxia increases CBS expression (Takano et al., 2014), but can result in H_2S accumulation via reduced activity of oxygen-dependent H_2S-detoxifying enzymes (Olson, 2015). Statins upregulate CGL expression and H_2S production (Xu et al., 2014). Finally, altered CGL expression has been observed in a number of different disease states, however the data on the direction of the alterations is sometimes contradictory, and the effects on H_2S production not well characterized (Zhao et al., 2014).

Regulation of H_2S production by CGL and CBS occurs at multiple levels including transcriptional, post-transcriptional, post-translational and at the substrate level. Multiple

transcription factors have been implicated in the transcriptional regulation of the *Cgl* gene, including SP1 (Yang et al., 2011, Ishii et al., 2004, Yin et al., 2012), ATF4 (Dickhout et al., 2012), ELK1 and NRF2 (Zhao et al., 2014). The PI3K/AKT pathway positively regulates *Cgl* expression via SP1 (Xu et al., 2014, Yin et al., 2012). In mouse embryonic fibroblasts, ATF4 deficiency results in a baseline reduction of *Cgl* expression (Dickhout et al., 2012), while *Cbs* is transcriptionally upregulated by HIF upon hypoxia (Takano et al., 2014).

Both CGL and CBS are also regulated post-transcriptionally and allosterically by a number of different mechanisms. For example, *Cgl* expression is regulated post-transcriptionally by multiple microRNAs, and post-translationally by fluxes in intracellular calcium levels (Zhao et al., 2014). CBS contains a heme group, and its activity is regulated by redox state (Mathew et al., 2011). Additionally, CBS contains an S-adenosylmethionine (SAM) binding site, which acts as a co-substrate and regulates its activity (Huang and Moore, 2015). Both CGL and CBS require pyridoxal-5'-phosphate (PLP) as a cofactor for their activity (Huang and Moore, 2015).

Similar to DR, reduced signaling through growth hormone (GH)/insulin-like growth factor (IGF) axis is well established as an evolutionarily conserved mechanism of extended longevity. Dwarf mice with mutant GH/IGF axis are long-lived and stress resistant (Bartke and Brown-Borg, 2004, Brown-Borg, 2009). In order to investigate if increased H_2S production can be a common downstream mechanism that mediates increased longevity in these two different well-established models, we decided to test H_2S production in response to GH stimulation.

Adequate substrate is also required for H_2S production. Although CGL is a fairly promiscuous enzyme that can use many substrates, including cystathionine and homocysteine, cysteine is thought to be the primary substrate for endogenous H_2S production in mammals (Zhao et al.,

2014). However, the notion that cysteine deprivation increases TSP gene expression and *de novo* cysteine production, but also increases the degradation of cysteine by CGL for H_2S production (Hine et al., 2015), presents an apparent paradox. Indeed, it is counterintuitive that the product of *de novo* cysteine production by CGL would then be used as the substrate for H_2S generation by the same enzyme. However, *de novo* cysteine biosynthesis is not the only cellular source of free cysteine for H_2S generation. Here we investigated the potential of autophagy as a source of free cysteine for H_2S generation as an alternative explanation to this apparent paradox.

RESULTS

GROWTH HORMONE NEGATIVELY REGULATES H_2S PRODUCTION

A reduction in circulating growth factors, including insulin and IGF-1, is one of the most pronounced differences observed in mice under dietary protein restriction (Harputlugil et al., 2014). In order to model the effects of nutrient deprivation on hepatic H_2S production in a tractable *in vitro* system, we chose to culture mouse primary hepatocytes under conditions of reduced sulfur amino acid content (methionine/cysteine deprivation, or Met/Cys$^-$) as well as reduced growth factor availability via serum deprivation (Serum$^-$). We observed that Met/Cys deprivation, especially when combined with serum deprivation, resulted in a marked increase in *Cgl* mRNA levels; a similar trend was observed with serum deprivation alone (Figure 4.1A). On the other hand, *Cbs* mRNA levels were only mildly affected upon serum and Met/Cys deprivation, consistent with previous findings (Hine et al., 2015). Protein levels of CGL were increased by Met/Cys deprivation as well as serum deprivation, while CBS levels were increased by serum deprivation only (Figure 4.1B). Interestingly, serum deprivation, but not Met/Cys

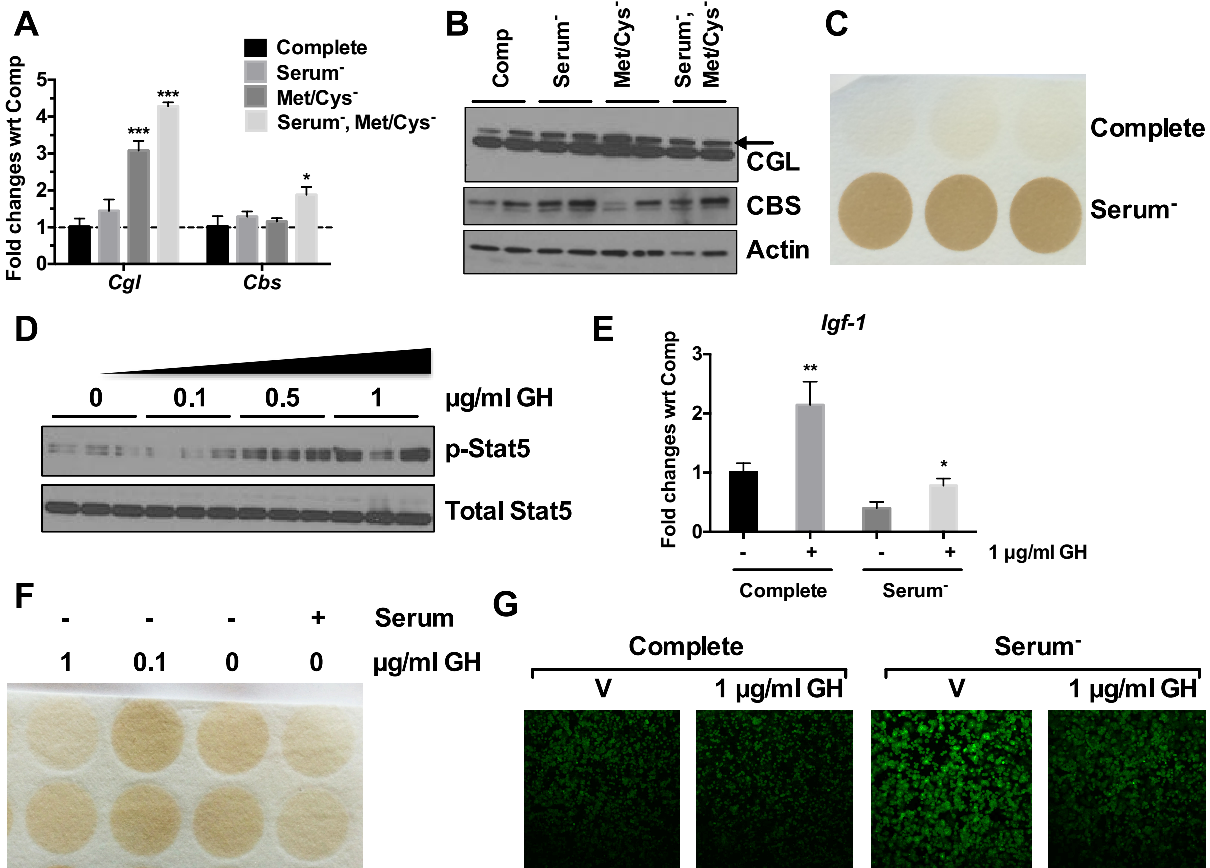

Figure 4.1 Growth hormone negatively regulates H_2S production.

(A) mRNA levels of *Cgl* and *Cbs* in primary hepatocytes cultured in the indicated media conditions overnight. Levels were normalized to *18S*. n = 3, Statistical significance of the difference relative to the Complete group was assessed by a Student's t-test; *p<0.05, ***p<0.0005. **(EH)**

(B) Immunoblots of whole cell lysates from primary hepatocytes cultured in the indicated media conditions overnight. **(EH)**

(C) H_2S production capacity of primary hepatocytes cultured in the indicated media conditions overnight. Cells were exposed to lead-acetate paper overnight following treatments. **(EH)**

Figure 4.1 (Continued)

(D) Immunoblots of whole cell lysates from primary hepatocytes treated with increasing doses of GH overnight as indicated in the presence of dialyzed FBS. **(EH)**

(E) *Igf-1* mRNA levels as determined by qPCR in primary hepatocytes treated as indicated for 24 hours. n = 3, Statistical significance of the difference between vehicle (-GH) and GH treatment within cell culture was assessed by a Student's t-test; $*p < 0.05$, $** p<0.01$. **(EH)**

(F) H_2S production capacity of primary hepatocytes after 6 hours pretreatment with the indicated media and GH, overnight exposure to lead-acetate paper. **(EH)**

(G) H_2S production as measured by P3 probe fluorescence in primary hepatocytes cultured in the indicated media with or without GH overnight, followed by 1-hour incubation with P3 (green); 10x objective used. **(EH, CH)**

Data in all panels are shown as means ± SD.

deprivation, resulted in a marked increase in H_2S production capacity as measured by the lead sulfide method (Figure 4.1C). Based on these data, we focused on the role of serum deprivation in increased H_2S production, and the relevant growth factors in serum that might be responsible for this effect.

Given critical unpublished data from our lab showing increased H_2S production capacity in livers of long-lived hypopituitary Snell dwarf mice lacking GH secretion, as well as in engineered GH receptor KO mice, we decided to explore the potential role of GH in control of H_2S production in primary hepatocytes *in vitro*. Phosphorylation of the transcription factor Stat5 and *Igf-1* transcription, markers of canonical GH action in the liver (Chia, 2014), were used to establish the dose response of primary hepatocytes to porcine GH. As 1 μg/ml GH was able to activate the downstream signaling and transcription pathways in primary hepatocytes (Figure 4.1D, 4.1E), this concentration was used for further experiments.

Consistent with GH as a negative regulator of H_2S production, we observed that GH treatment suppressed the increase in H_2S production capacity observed upon serum deprivation using the lead sulfide method (Figure 4.1F). We also confirmed this result using a different methodology in which H_2S binds specifically to a non-toxic probe (P3) in a 1-1 ratio and becomes fluorescent (Singha et al., 2015), which allows for visualization of H_2S using 2-photon microscopy (Figure 4.1G) or quantitation directly using a plate reader. Importantly, this latter methodology does not require the addition of cysteine as substrate, and thus is a more accurate readout of endogenous H_2S production. Taken together, these data indicate that GH negatively regulates H_2S production *in vitro* in primary hepatocytes, and thus is likely one of the factors in serum whose absence results in increased H_2S production.

REGULATION OF H_2S PRODUCTION BY GH IN VITRO INDEPENDENT OF CGL TRANSCRIPTIONAL REGULATION

We next investigated the downstream mechanisms of GH-mediated suppression of H_2S production in primary hepatocytes. In Snell and GHRKO mice lacking GH signaling, our unpublished observations indicate a significant increase in hepatic *Cgl* mRNA expression *in vivo* concomitant with increased H_2S production capacity (data not shown). Interestingly, however, we did not see a meaningful impact of GH on *Cgl* and *Cbs* mRNA or protein levels in hepatocytes *in vitro* (Figure 4.2A, 4.2B).

Nonetheless, when downstream GH receptor signaling was blocked by Jak2 inhibitors AZD1480 or Fedratinib, H_2S production was moderately increased in hepatocytes cultured in complete media with additional GH (Figure 4.2C). Furthermore, the increase in H_2S production induced by serum deprivation and blocked by GH was partially restored by Jak2 inhibition, suggesting that GH can act through the Jak2/Stat5 pathway to regulate H_2S production. *Igf-1* transcript levels were used as a control for GH signaling activity (Figure 4.2D). Consistent with previous experiments, *Cgl* or *Cbs* transcription did not correlate with observed changes in H_2S production (Figure 4.2D).

To further investigate the mechanism of GH inhibition of H_2S production independent of *Cgl* transcriptional regulation, we analyzed the time frame in which serum or Met/Cys deprivation increased H_2S production. In primary hepatocytes, one hour of serum or Met/Cys deprivation was sufficient to significantly increase endogenous H_2S production as measured by P3 fluorescence and quantitated in a plate reader (Figure 4.2E, 4.2F). Taken together, these findings support GH as a negative regulator of H_2S production *in vitro* likely through at least two distinct

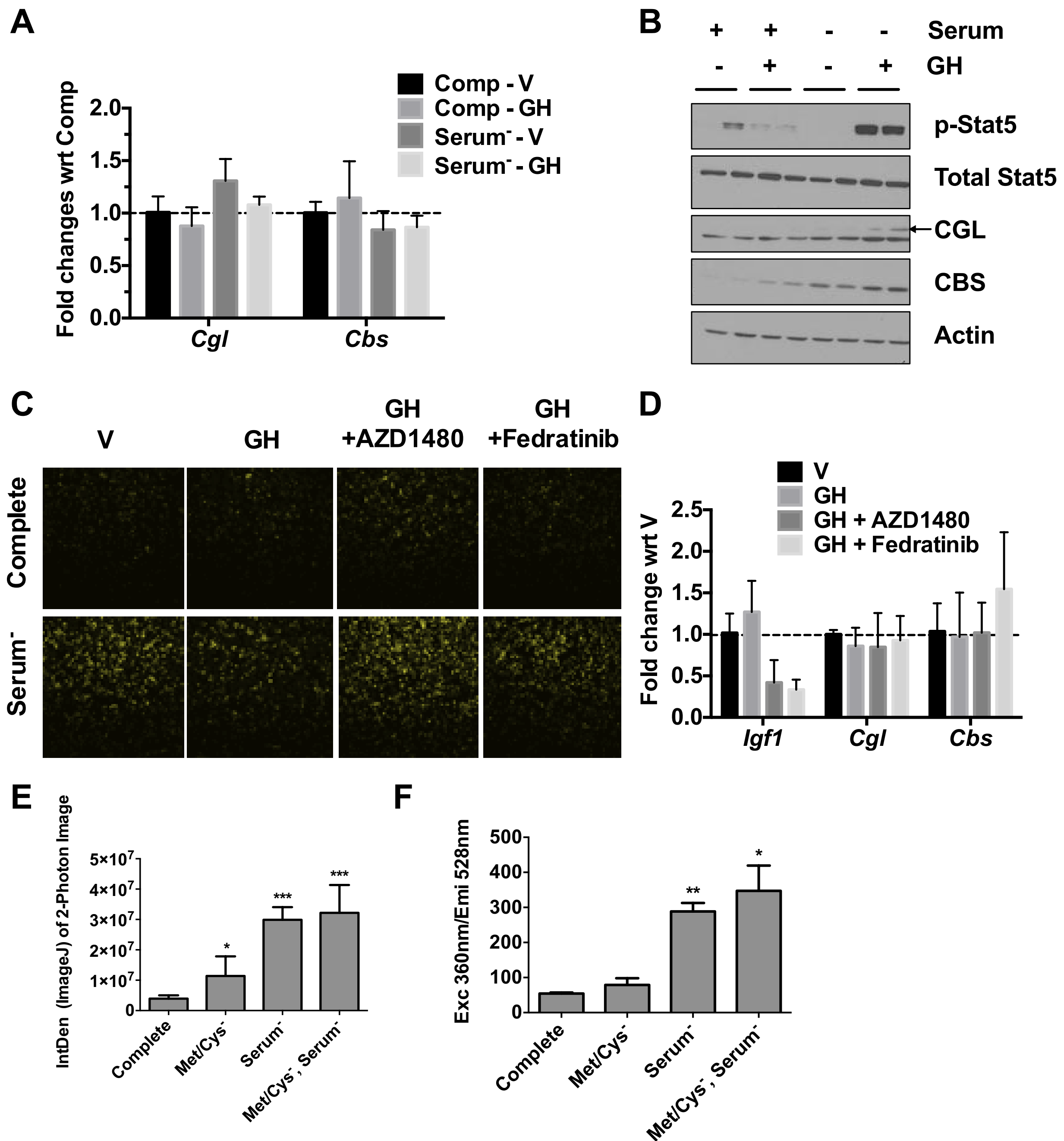

Figure 4.2 Regulation of H_2S production by GH *in vitro* independent of *Cgl* transcriptional regulation.

(A) *Cgl* and *Cbs* mRNA levels in primary hepatocytes that were cultured for 24 hours in the absence of serum (Serum$^-$) and/or treated with vehicle (V) or 1 µg/ml GH as indicated. **(EH)**

Figure 4.2 (Continued)

(B) Immunoblots of primary hepatocytes that were cultured for 24 hours in the absence of serum ($Serum^-$) and/or treated with vehicle or 1 μg/ml GH as indicated. **(EH)**

(C) Representative fluorescent images of H_2S-specific P3 fluorescence in primary hepatocytes cultured overnight in the absence of serum ($Serum^-$) and/or with vehicle (V) or Jak2 inhibitors AZD1480 or fedratinib as indicated. P3 probe was added to cells 1 hour before fixation/imaging; 10x objective used. **(EH, CH)**

(D) mRNA levels of *Igf-1*, *Cgl* and *Cbs* in primary hepatocytes upon overnight treatment with Jak2 inhibitors AZD1480 or Fedratinib in complete media. **(EH)**

(E, F) H_2S production as measured by P3 probe fluorescence intensity in primary hepatocytes cultured for 1 hour in the indicated media and quantified from 2-photon microscopic images (E) or using a plate reader (F). Statistical significance relative to the Complete group was assessed by a Student's t-test; *$p<0.05$, **$p<0.001$ ***$p<0.0001$. **(EH, CH)**

Data in all panels are shown as means ± SD.

mechanisms, one involving a rapid effect through canonical GH receptor/Jak2 signaling independent of *Cgl* transcriptional regulation, and another possibly indirect mechanism involving transcriptional regulation of *Cgl*. Future experiments will be required to delineate the molecular details of these mechanisms by which GH suppresses H_2S production.

H_2S INDUCTION BY GROWTH FACTOR/SAA DEPRIVATION REQUIRES FUNCTIONAL AUTOPHAGY

Previously we found that DR, PR or SAA restriction increases expression of the TSP enzyme CGL linked to increased *de novo* cysteine synthesis, consistent with the ability of mammalian cells to produce non-essential amino acids when they are limited in the diet. At the same time, we found SAA restriction increases cysteine degradation and H_2S production by the same enzyme, CGL, resulting in an apparent paradox. A potential answer to this paradox lies in the fact that TSP-mediated *de novo* cysteine production is not the only way for a cell to obtain free cysteine when sulfur amino acids are limiting. Alternative strategies include salvage of cysteine from glutathione or breakdown of existing proteins via autophagy.

To test the hypothesis that increased autophagy plays a role in generating the required substrate, free cysteine, for H_2S production under DR/PR/SAA$^-$ conditions, we employed pharmacological and genetic inhibition of autophagy in three different cell types. In WT mouse primary hepatocytes and mouse embryonic fibroblasts (MEFs), chloroquine-mediated autophagy inhibition (confirmed by LC3B-II accumulation) resulted in a partial blockage of both serum$^-$ and serum$^-$/Met/Cys$^-$ mediated increases in H_2S production (Figure 4.3A, 4.3B, 4.3C). Atg5 and Atg7 knockout MEFs with defective autophagy also showed similarly

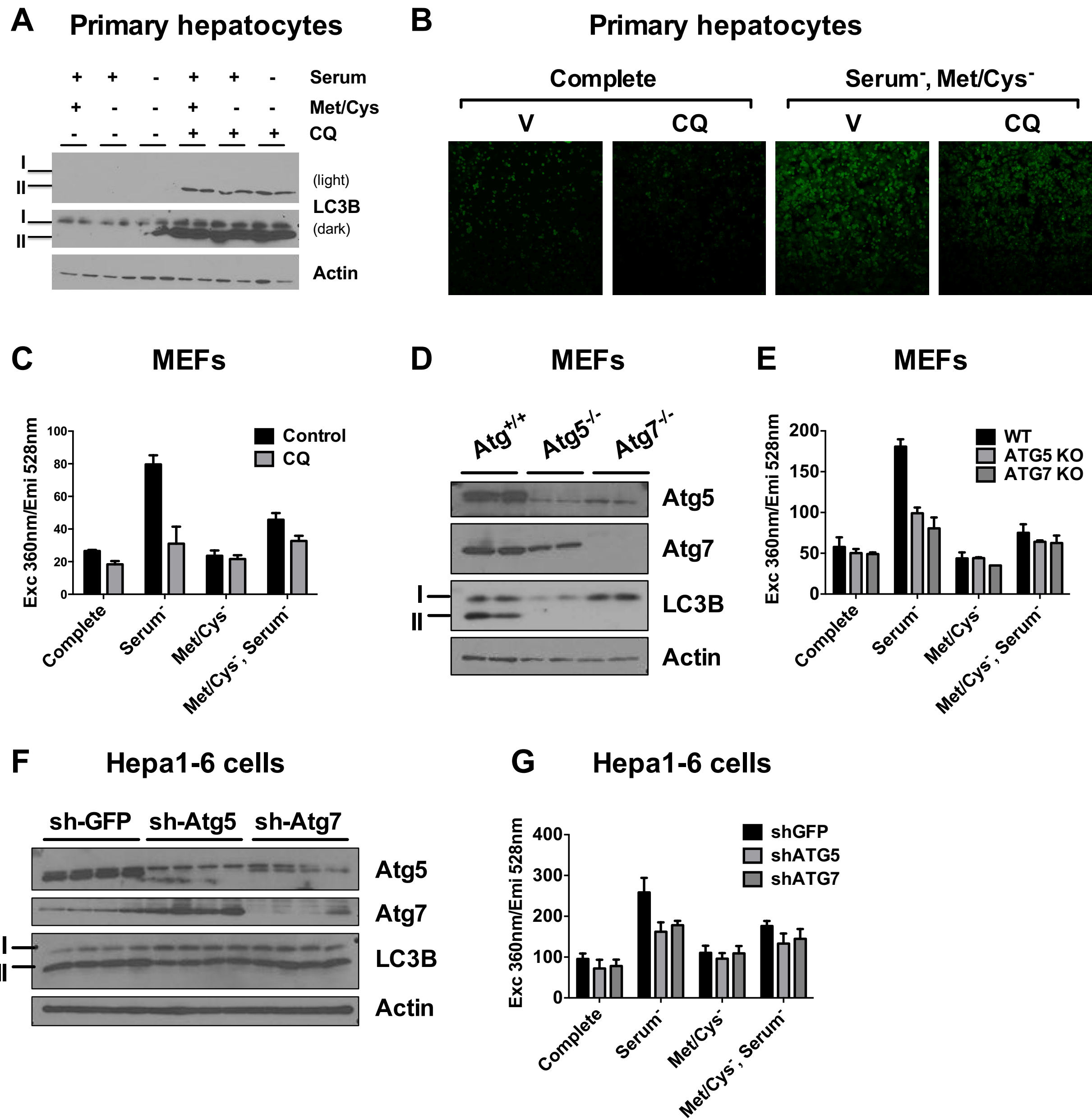

Figure 4.3 H_2S induction by growth factor/SAA deprivation requires functional autophagy.

(A) Immunoblots of primary hepatocytes cultured overnight as indicated. CQ: 10µM chloroquine.

(EH)

Figure 4.3 (Continued)

(B) Representative images of H_2S production measured by P3 probe fluorescence in primary hepatocytes cultured overnight as indicated. CQ: 10μM chloroquine; 10x objective used. **(EH, CH)**

(C) H_2S production measured by P3 probe fluorescence using a plate reader in WT MEFs cultured overnight as indicated. CQ: 10μM chloroquine. **(EH, CH)**

(D) Immunoblots of MEFs from WT, $Atg5^{-/-}$ and $Atg7^{-/-}$ backgrounds cultured in complete media. **(EH)**

(E) H_2S production measured by P3 probe fluorescence using a plate reader in MEFs of the indicated genotyped and cultured overnight as indicated. **(EH, CH)**

(F) Immunoblots of stably transfected Hepa1-6 cells with sh-GFP, sh-Atg5 and sh-Atg7 under complete media conditions. **(EH)**

(G) H_2S production measured by P3 probe fluorescence using a plate reader in Hepa1-6 cells stably transfected with sh-GFP, sh-Atg5 and sh-Atg7 and cultured overnight as indicated. **(EH, CH)**

Data in all panels are shown as means ± SD.

blunted H_2S production upon serum deprivation (Figure 4.3D, 4.3E), A similar phenotype was observed in mouse liver-derived Hepa1-6 cells that were stably transfected with sh-Atg5 or sh-Atg7, although to a lesser extent (Figure 4.3F, 4.3G). Taken together, these data indicate that serum/SAA deprivation mediated increase in H_2S production requires functional autophagy.

DISCUSSION

Hydrogen sulfide has recently emerged as an important biological molecule with potent effects on stress resistance and general health, however the regulation of its endogenous production remains poorly understood. Our group recently described the importance of increased H_2S production in dietary restriction-mediated stress resistance and longevity (Hine et al., 2015), revealing dietary sulfur amino acid intake as a regulator of endogenous H_2S production. Here we investigated the possibility that H_2S might be a common mediator of longevity extension and stress resistance in another well established model: reduced GH/IGF signaling. In support of this idea, we found that growth hormone (GH) is a negative regulator of H_2S production in primary hepatocytes, possibly through post-translational mechanisms. Additionally we established that functional autophagy is required for induction of H_2S production by serum starvation, suggesting additional substrate-level regulation of H_2S production.

On a cell-autonomous level, we found that H_2S production could be stimulated using *in vitro* models of dietary restriction, including sulfur amino acid deprivation and/or serum deprivation, in multiple cell types (Hine et al., 2015) including mouse primary hepatocytes. Importantly, adding back GH into the media was able to partially reverse H_2S production induced by serum deprivation, an effect that was partially dependent on Jak2 activity.

However, even though serum and Met/Cys deprivation induced mRNA and protein levels of CGL, the effect of GH on H_2S production is unlikely to occur solely, or even primarily, on a transcriptional level. Indeed, GH treatment reduced *Cgl* mRNA levels only by ~20%, and did not have a detectable effect on CGL protein levels.

Interestingly, other studies in our lab have shown that *in vivo* models of GH signaling deficiency, such as Snell dwarf and GHRKO mice, demonstrate significantly increased hepatic expression of CGL on both mRNA and protein levels, as well as increased H_2S production capacity (data not shown). The reason for this discrepancy between the *in vivo* and *in vitro* models remains to be elucidated, but likely points to distinct mechanisms of CGL regulation by GH. Based on the rapid induction of H_2S production upon serum withdrawal, activation is likely to occur at the post-translational level, and possibly also due to changes in substrate availability. Several post-translational mechanisms of CGL activation have previously been reported (Zhao et al., 2014). Future studies are required in order to determine if GH affects any of these. In support of the potential role of increased substrate availability upon GH withdrawal, as discussed below we found a requirement for autophagy in H_2S induction, which is also increased upon serum withdrawal (Bodemann et al., 2011).

The observation that endogenous H_2S production is increased under dietary or protein restricted conditions *in vivo* is potentially paradoxical, since under the same conditions the major substrate for H_2S production by CGL, cysteine, is also limited. Furthermore, the increase in CGL expression under conditions of limited cysteine intake has heretofore been interpreted as evidence of increased *de novo* synthesis of cysteine from methionine via the TSP (Stipanuk and Ueki, 2011, Hine et al., 2015), which at face value appears more logical than increased H_2S production requiring a further reduction in free cysteine levels. Nonetheless, *de novo* cysteine

biosynthesis from methionine, which is also limited upon protein restriction, is not the only source of free cysteine.

Here we tested the hypothesis that the source of cysteine for H_2S production under conditions of nutrient/growth factor limitation is degradation of proteins via autophagy. Indeed, we found that induction of H_2S production upon serum deprivation was blocked by pharmacological or genetic inhibition of autophagy. The degree of requirement for autophagy varied by cell type, which might be because of differing levels of baseline and induced autophagy in these different cells. Sulfur amino acid deprivation and serum deprivation have been shown to induce autophagy in different cell types by others (Liu et al., 2015, Bodemann et al., 2011), however this remains to be investigated in these specific cell types under our *in vitro* conditions. In addition to autophagy, it is also possible that other methods of protein degradation, for example via the proteasome, may also contribute to cysteine generation for H_2S production.

Autophagy has been implicated in longevity and stress resistance in many organisms (Bergamini et al., 2004, Dutta et al., 2013, Jiang et al., 2010, Papadakis et al., 2013, Wang et al., 2011), including as a mediator of dietary restriction benefits in many contexts (Bergamini et al., 2004, Jia and Levine, 2007). This has been mostly attributed to its effects on clearance of damaged proteins and organelles such as mitochondria (Ravikumar et al., 2010). Whether its role in H_2S production is one of the mediators of its reported effects on longevity and stress resistance remains to be studied. Cells from long-lived dwarfs have increased levels of autophagy (Wang and Miller, 2012). It is also possible that there might be crosstalk between reduced GH signaling, increased autophagy and increased H_2S production. Elucidation of the details of this circuitry will have broad implications both in terms of understanding the mechanisms underlying two of

the most common increased longevity models, as well as potential therapeutic applications in the future.

MATERIALS AND METHODS

Primary hepatocyte isolation, mammalian cell culture conditions and treatments

Mouse primary hepatocytes were isolated via portal vein collagenase treatment (Liberase, Roche) followed by Percoll gradient centrifugation. Primary hepatocytes, Hepa1-6 cells and MEFs were cultured at 37°C, 20% O_2 and 5% CO_2 incubators. Hepa1-6 cells and MEFs were maintained in DMEM media with 10% FBS for standard culture, primary hepatocytes were cultured in William's E media with 5% FBS. For serum deprivation and amino acid deprivation experiments, Methionine/Cysteine-free DMEM (GIBCO), and 10% dialyzed FBS (Invitrogen) were used. AZD1480 and Fedratinib were from Selleckchem, Chloroquine and GH (Porcine STH) were from Sigma. Lentiviral infections were done in the presence of 10% FBS and 8μg/ml Polybrene on two consecutive days. Infected Hepa1-6 cells were selected with 3μg/ml puromycin for 2 weeks before use, and maintained with 3μg/ml puromycin.

Western Blotting

Immunoblotting was performed as described in Chapter 2. Primary antibodies: Actin (#4970 Cell Signaling Technology), CGL (ab151769 Abcam), CBS (ab135626 Abcam), Total Stat5 (sc-835 Santa Cruz), p-Stat5 (#9359 Cell Signaling Technology), LC3B (NB100-2220 Novus), Atg5 (NB110-53818, Novus), Atg7 (Sigma, A2856-200UL), and secondary antibodies from Dako.

RNA isolation and quantitative PCR

Total RNA isolation, cDNA synthesis and qRT-PCRs were performed as described in Chapter 2. Primer sequences for each gene are listed in Table 4.1.

Table 4.1 qRT-PCR Primer sequences

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Hprt	TTTCCCTGGTTAAGCAGTACAGCCC	TGGCCTGTATCCAACACTTCGAGA
18S	CATGCAGAACCCACGACAGTA	CCTCACGCAGCTTGTTGTCTA
Cgl	TTGGATCGAAACACCCACAAA	AGCCGACTATTGAGGTCATCA
Cbs	GGGACAAGGATCGAGTCTGGA	AGCACTGTGTGATAATGTGGG
Igf-1	GCTTGCTCACCTTCACCAG	CAACACTCATCCACAATGCC

H_2S assays

Lead sulfide method

For H_2S measurements, equal numbers of cells were plated on 12-well plates. Following the experimental treatments, cells were supplemented with 10mM L-Cysteine and 10mM PLP. Lead-acetate papers were prepared by soaking filter paper (VWR) in 20mM lead acetate and vacuum drying. Lead-acetate paper was placed on top of the culture plate and the cells were

incubated at 37°C cell culture incubator until the brown colored lead sulfide precipitate started to become visible (typically O/N).

P3 probe method

The chemical H_2S probe P3 (Singha et al., 2015) was a gift by the Kyo Han Ahn. P3 (10μM) was added to the cells 1 hour before fixation by methanol or detection by plate reader (excitation 360nm, emission 528nm) or 2-photon microscopy (excitation at 880 nm).

Statistical analyses

Data in all charts are represented as means ± standard deviations (SD). Statistical analyses were performed as described in Chapter 2.

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CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

INTRODUCTION

This thesis explores the mechanisms of protection against surgical stress by short-term dietary protein restriction (dietary restriction: DR; protein restriction: PR). There is a substantial body of literature on the beneficial health effects of dietary restriction, as covered in detail in Chapter 1, including extended lifespan of model organisms and resistance against acute surgical damage (Gallinetti et al., 2013). Here we used a model of mouse hepatic ischemia reperfusion injury (IRI) to probe for the underlying nutritional and genetic requirements of dietary restriction benefits. We identified that the restriction of proteins, as opposed to calories per se, provided the most dramatic protection in this context (Harputlugil et al., 2014, Robertson et al., 2015), highlighting the importance of protein intake in dietary restriction.

Given the critical role of dietary protein in DR-mediated stress resistance, we explored the role of two distinct amino acid sensing pathways, mechanistic target of rapamycin complex 1 (mTORC1) and general control nonderepressible 2 (GCN2). We identified a genetic requirement for the mTORC1 repressor tuberous sclerosis complex (TSC) in PR-mediated protection against hepatic IRI (Harputlugil et al., 2014). Mechanistically, we identified two distinct downstream effectors of protection, increased pro-survival insulin/Akt signaling (Harputlugil et al., 2014) and increased hydrogen sulfide (H_2S) production (Hine et al., 2015) (Figure 5.1). How PR mediates increased H_2S production, including the role of growth factor signaling and autophagy in induction of H_2S production, and how H_2S acts to protect tissues from stress, remain to be elucidated, but have important implications for stress resistance as well longevity regulation.

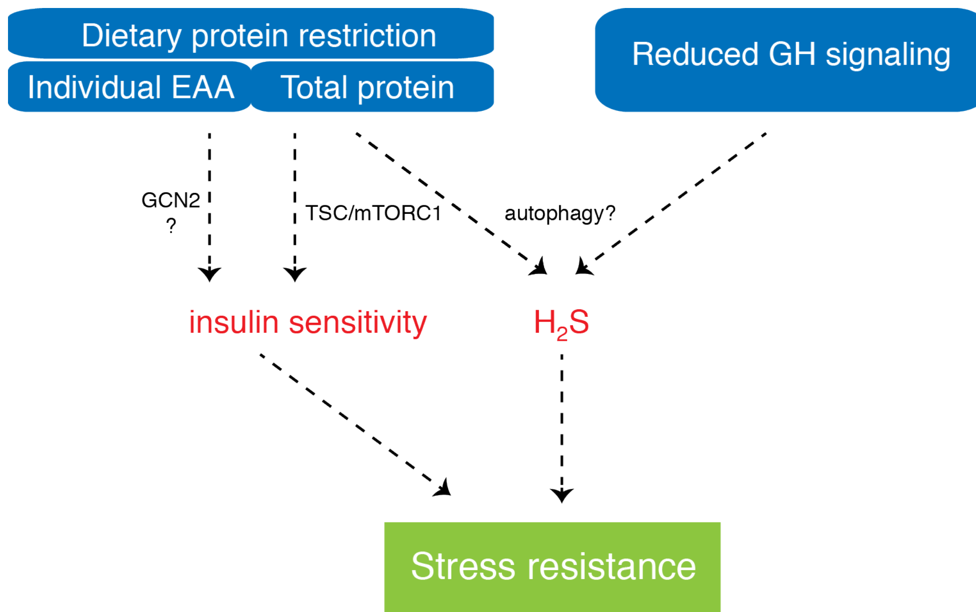

Figure 5.1 Summary of findings. EAA: essential amino acid

NUTRITIONAL BASIS OF DIETARY RESTRICTION

DR is loosely defined as reduced food intake without malnutrition. As explained in detail in Chapter 1, the underlying nutritional requirements of DR are not fully elucidated. Multiple studies from our lab (Peng et al., 2012, Robertson et al., 2015, Robertson and Mitchell, 2013) and others (Gallinetti et al., 2013) have highlighted that the source of calories does matter in DR benefits. Proteins and specific amino acids such as methionine have been reported to play a role in DR benefits beyond their caloric value using various model organisms and endpoints (Mair et al., 2005, Grandison et al., 2009, Orentreich et al., 1993, Miller et al., 2005, Levine et al., 2014, Piper et al., 2011, Solon-Biet et al., 2014, Pamplona and Barja, 2006).

Even though it is unclear how stress resistance and longevity are connected, they are often observed together in models of aging and dietary restriction, together with other hallmarks of DR, such as improved metabolic fitness (Gallinetti et al., 2013). In this thesis, we used short-term dietary interventions and protection from hepatic IRI as an experimental model in which to probe the nutritional requirements that underlie DR benefits. We discovered that total protein restriction is the most potent intervention among those we have investigated (Harputlugil et al., 2014), likely due to restriction of the sulfur amino acids methionine and cysteine, which result in upregulation of endogenous H_2S production (Hine et al., 2015).

Our experimental model consisting of short-term DR regimens (up to one week) followed by an acute stress (hepatic IRI) had a number of advantages over longevity studies typically used to study mechanisms underlying DR benefits. First, we were able to quickly test multiple dietary preconditioning regimens and identify roles of specific macronutrients, including essential amino acids, yielding clues as to underlying molecular pathways. Unlike longevity studies, this

experimental model system can be used in the future to investigate the detailed roles of any specific nutrient or combination of nutrients, even those required for long-term health and survival. For example, so far in this study we have only tested the role of total amino acid deprivation and the deprivation of individual essential amino acid tryptophan against hepatic IRI. The specific roles and genetic requirements for other essential amino acids will need to be tested separately. Importantly, since mTORC1 is mostly regulated by the branched chain amino acids (BCAA) leucine, isoleucine and valine (Gallinetti et al., 2013), it will be important to see if the deprivation of BCAA alone is enough to provide a similar level of protection against hepatic IRI. Second, our experimental model allows for investigation of the effects of different DR regimens on any acute endpoint of our choice, including a variety of metabolic endpoints such as insulin sensitivity and glucose tolerance. Despite these advantages, it still remains to be investigated whether the nutritional, genetic and molecular basis of short-term DR uncovered using this experimental model will extend to other DR endpoints such as longevity, or translate into clinically relevant examples of ischemia reperfusion injury including heart attack or stroke. Even though incomplete diets are not suitable for long-term survival, it is possible to investigate potentially beneficial health effects or longevity extension by titrating the amount of protein or essential amino acids to lower levels in mouse models to test the long-term consequences. While longevity and health benefits have been reported for some protein or specific amino acid (methionine, tryptophan) restriction regimens (Pamplona and Barja, 2006, Orentreich et al., 1993), this has not been done in a comprehensive manner, and little is known about underlying molecular mechanism.

If ongoing studies to demonstrate safety, feasibility and efficacy of short-term DR in humans are successful, there are immediate clinical needs to be met. Hepatic IRI presents a major challenge

during liver surgeries and transplants (Datta et al., 2013, Klune and Tsung, 2010). Ischemic damage to other organs is also one of the major complications of cardiovascular surgeries for which there is currently no standard mitigation strategy (Mitchell et al., 2013).

With the exception of overnight fasting of patients to prevent aspiration pneumonia, there are currently no accepted pre-operative dietary guidelines (Robertson and Mitchell, 2013). In fact, there is even a trend in some countries particularly in Europe to avoid preoperative fasting altogether in favor of carbohydrate loading prior to surgery, in order to address the issues of post-operative hunger, thirst and insulin resistance (Diks et al., 2005, Soreide et al., 2005). Although the various health benefits of long-term DR observed in experimental model organisms are increasingly being established in humans (Cava and Fontana, 2013, Ravussin et al., 2015), adherence remains an enormous problem for most people, severely limiting its relevance in the clinic. The duration of restriction necessary for benefits of DR in humans remains unknown, but will have a major influence on feasibility in the pre-operative setting.

GENETIC BASIS OF DIETARY RESTRICTION

Dietary restriction is both the most effective and best studied interventional approach to lifespan extension in various species (Fontana et al., 2010). There have been many studies investigating the underlying genetic requirements for extended longevity in multiple organisms. As expected, mutations and interventions that result in reduced activity of nutrient sensing pathways such as insulin signaling and TOR were found to play roles (Fontana et al., 2010). Genetic downregulation of TOR or its direct targets extends lifespan in yeasts (Fabrizio et al., 2001, Kaeberlein et al., 2005), worms (Vellai et al., 2003, Jia et al., 2004), flies (Zid et al., 2009) and rodents (Selman et al., 2009, Wu et al., 2013), as well as pharmacological inhibition of mTORC1

in mice (Miller et al., 2014, Miller et al., 2011, Harrison et al., 2009, Lamming et al., 2012). However, there have not been any studies that directly investigate a genetic requirement for the TSC complex in mTORC1 downregulation in mammalian DR benefits before.

Previously our group has shown that the amino acid sensor GCN2 plays a role in mediating the protective effects of single essential amino acid restricted diets against renal and hepatic IRI (Peng et al., 2012). Since GCN2KO mice were still protected from hepatic IRI by total amino acid deprivation (Chapter 2), we tested the role of mTORC1 pathway in that context. Here we provide the first evidence for the genetic requirement for *Tsc1* in two beneficial effects of total protein restriction: improved insulin sensitivity and protection against hepatic IRI. Interestingly, tryptophan restriction alone did not require the TSC complex, suggesting that different mechanisms might be mediating the effects of individual amino acid and total protein deficient diets, via parallel action of GCN2 and mTORC1 pathways. As discussed earlier, the genetic requirements for other essential amino acid restricted diets such as leucine or all BCAAs require further investigation in order to shed further light on the roles of GCN2 and mTORC1. The fact that tryptophan restriction but not PR was able to precondition LTsc1KO mice is counterintuitive, since PR also incorporates tryptophan restriction. Our interpretation of this finding is that PR treatment of LTsc1KO mice results in a state that is even less advantageous in the context of acute stress and this overrides any potential beneficial effect coming from GCN2 activation in these mice. This interpretation is consistent with the data that hepatic insulin sensitivity and Akt signaling is worse in LTsc1KOs upon PR compared to their baseline complete diet conditions (Chapter 2); and similarly their hepatic H_2S production capacity is lower than baseline (Chapter 3).

Interestingly, unlike its longevity extending effects in mice, (Miller et al., 2014, Miller et al., 2011, Harrison et al., 2009, Lamming et al., 2012) rapamycin was unable to protect mice from hepatic IRI, despite reducing hepatic mTORC1 signaling. This indicates that the reduction in hepatic mTORC1 by itself is not enough to exert the protective effects of PR against hepatic IRI. Similar to this finding, rapamycin was shown to be insufficient to recapitulate other beneficial effects of PR by others (Miller et al., 2013). Additionally, combining rapamycin treatment with PR resulted in an abrogation of PR-mediated protection from hepatic IRI. There are various reasons why rapamycin might be playing a negative role in our context. In Chapter 2, we explored the inhibitory effects of rapamycin on mTORC2, which results in hepatic insulin resistance. Additionally, rapamycin has other side effects, such as increased inflammation, which might also underlie its negative consequences in the context of IRI (Lamming et al., 2013). The divergence between rapamycin's beneficial effects on longevity and neutral or even detrimental effects on hepatic IRI might be because the underlying mechanisms are different, with inflammation and insulin resistance potentially more critical in the hepatic IRI context, and S6K activity more critical in aging.

Additionally, whether there is a genetic requirement for the TSC complex in other DR benefits or in other contexts (e.g. long-term DR) remains to be studied. Since hepatic *Tsc1* knockout results in hepatocellular carcinoma in mice in the long term (Menon et al., 2012), it is not possible to test its genetic requirement for longevity extension. However, other endpoints that are closely associated with DR benefits and extended longevity can be explored. For instance, in this study we investigated the impact of PR on LTsc1KOs hepatic insulin resistance, however we did not test the whole body insulin or glucose tolerance in these mice. Such future studies will be

informative in understanding the specific contribution of hepatic TSC/mTORC1 in PR-mediated whole body glucose homeostasis.

MOLECULAR MECHANISMS UNDERLYING DIETARY RESTRICTION BENEFITS

Even though a reduction in activity of the nutrient sensing mTORC1 pathway is clearly involved in mediating beneficial DR effects, downstream effector mechanisms remain incompletely understood. mTORC1 regulates numerous growth-related processes in cells ranging from protein, lipid, and nucleotide synthesis to autophagy (Howell et al., 2013). One potential downstream effector of mTORC1 involved in longevity regulation is the ribosomal S6 kinase (S6K). In addition to mediating pro-growth signals, S6K also feeds back to inhibit insulin/IGF1 signaling. Genetic ablation of S6K improves insulin sensitivity and increases longevity in mice (Selman et al., 2009). Nonetheless, our data indicate that downregulation of mTORC1 alone is not sufficient to provide beneficial effects of PR against hepatic IRI, since rapamycin treatment, a powerful inhibitor of downstream S6K phosphorylation, did not result in a positive outcome (Chapter 2). On the contrary, combining PR with rapamycin treatment partially abrogated the preconditioning effects of PR, likely through its inhibition of mTOR complex 2 (mTORC2) signaling (Lamming et al., 2012, Lamming et al., 2013) as previously discussed.

Like reduced mTORC1 activity, a reduction in signaling through the evolutionarily conserved growth hormone/insulin/insulin-like growth factor (IGF) axis is strongly associated with extended longevity. This nutrient sensing signal transduction pathway, also referred to as insulin/insulin-like signaling (IIS), senses the availability of systemic nutrients and regulates growth on the organismal level (Fontana et al., 2010). IIS is highly interconnected with the mTORC1 pathway via PI3K/Akt activation of mTORC1 and mTORC1/S6K-dependent feedback

inhibition of IIS (Howell and Manning, 2011). Also like reduced mTORC1 activity, reduced IIS and improved insulin sensitivity are hallmarks of dietary restriction. Here we demonstrated that improved insulin sensitivity upon PR, and subsequently increased insulin/Akt signaling after IRI, is one of the mechanisms by which PR protects tissues against surgical stress. This finding is consistent with previous studies demonstrating increased insulin signaling upon dietary protein restriction (Toyoshima et al., 2010), and the protective effects of insulin/Akt signaling against apoptosis and IRI damage (Fuglesteg et al., 2009, Jonassen et al., 2001, Liu et al., 2007).

As discussed in Chapter 2, the role of improved insulin sensitivity and increased post-operative pro-survival Akt signaling, while significant, only accounts for a portion of the protection afforded by PR against hepatic IRI. We therefore investigated additional molecular mechanisms underlying PR benefits using a comparative approach between WT mice that are afforded PR benefits and liver-specific *Tsc1* knockout (LTsc1KO) mice that are not. Using an unbiased metabolomics approach, we eventually identified increased H_2S production as another significant contributor to PR-mediated protection (Hine et al., 2015).

By what mechanism endogenously produced H_2S protects cells from surgical stress is not completely understood. Work in our laboratory suggests the involvement of mitochondrial electron transport chain and the potential of H_2S to serve as a source of electrons during *in vitro* ischemia (Hine et al., 2015), but further studies are required to establish if this holds true *in vivo*. H_2S can act as an antioxidant, and antioxidants can protect from IRI (Datta et al., 2013). Interestingly, the adaptive increase in H_2S production does not require the NRF2 transcription factor, which is a master regulator of multiple other antioxidant defense systems including glutathione and NQO1 (Hine et al., 2015). Another interesting physiological effect of H_2S is vasodilation, as evidenced by genetic deficiency in endogenous H_2S production leading to

hypertension (Yang et al., 2008). As vasodilation is protective in the context of ischemia, the effects of a DR-mediated increase in H_2S production on the vasculature could be a common mechanism of protection of numerous organs and tissues, including liver, brain and the blood vessels themselves, from ischemic stress, but remains to be studied *in vivo* (Wang, 2012). H_2S is also thought to modulate inflammation through effects on NFkB signal transduction.

The mechanism by which H_2S mediates its pro-longevity effects in lower organisms, while currently unknown, is hypothesized to involve modulation of similar pathways as described above, including antioxidant defense, energy metabolism and inflammation (Wen et al., 2013, Hine and Mitchell, 2015). The major molecular mechanism by which H_2S is thought to mediate such changes is by modifying surface-exposed Cys residues of target proteins via a post-translational modification known as sulfhydration (Mustafa et al., 2009). However, much work remains in order to identify sulfhydrated proteins that contribute to extended longevity or stress resistance.

mTORC1 also negatively regulates the lysosomal protein degradation pathway known as autophagy which is activated under conditions of nutrient limitation (Russell et al., 2014). Genes involved in autophagy have been implicated in regulation of longevity in lower organisms (Jia and Levine, 2010). In yeast, chronological lifespan extension by methionine restriction requires autophagy (Ruckenstuhl et al., 2014), as does DR-mediated lifespan extension in *C. elegans* (Jia and Levine, 2007). Autophagy induction has also been shown to play a role in stress resistance in many systems, including renal and hepatic ischemia reperfusion injuries in mice (Wang et al., 2011, Jiang et al., 2010). mTORC1 downregulation leads to increased autophagy (Laplante and Sabatini, 2009) and importantly, LTsc1KO mice have been shown to have defective autophagic flux (Menon et al., 2012).

Despite abundant genetic evidence of a requirement for autophagy in multiple DR-mediated benefits, the mechanism by which autophagy impacts longevity and stress resistance remains poorly understood. Our finding that autophagy is required for the stimulation of H_2S production (Chapter 4) is consistent with a novel role for increased H_2S as a downstream mediator of autophagy benefits. Nonetheless, future studies are required in order to determine whether defective autophagy in LTsc1KOs plays a causative role in the lack of PR response in these mice, as well as to elucidate the relative importance of H_2S generation in mediating the benefits of autophagy *in vivo*. If autophagy is important for H_2S production *in vivo*, we would predict lower endogenous H_2S levels *in vivo* in autophagy deficient genetic models and models of pharmacological autophagy inhibition on DR/PR. Furthermore, if H_2S is an important effector molecule of autophagy, we would predict that supplementation with exogenous H_2S will at least partially rescue acute stress resistance in autophagy-deficient models.

REGULATION OF ENDOGENOUS H_2S PRODUCTION

Despite its wide ranging biological effects and potential therapeutic implications, the mechanisms underlying the regulation of endogenous H_2S production are poorly understood (Hine and Mitchell, 2015). Our lab has demonstrated that multiple forms of dietary restriction, including total calorie restriction, protein restriction and sulfur amino acid restriction, result in increased hepatic H_2S production, which is necessary and sufficient for the benefits of DR against hepatic IRI (Hine et al., 2015). Here we identified two novel mechanisms involved in the regulation of endogenous H_2S production: growth hormone (GH) signaling and autophagy (Chapter 4).

We identified that GH negatively regulates H_2S production *in vitro* and that this downregulation mostly occurs via post-transcriptional mechanisms. Even though overnight GH treatment of serum-deprived mouse primary hepatocytes resulted in a ~20% reduction in cystathionine γ-lyase (*Cgl*) mRNA levels, we were unable to detect a decrease in CGL or cystathionine β-synthase (CBS) protein levels within the same time frame. Consistent with our findings of a GH-H_2S connection, long-lived hypopituitary Snell dwarf mice lacking growth hormone have a metabolite profile consistent with increased TSP activity (Vitvitsky et al., 2013), and Ames dwarfs were shown to have increased methionine flux towards transmethylation and transsulfuration (Uthus and Brown-Borg, 2006). Further unpublished findings from our lab have shown that Snell dwarf and GHRKO mice have increased levels of hepatic *Cgl* mRNA and protein *in vivo*, as well as increased H_2S production capacity (data not shown). Thus it appears that in addition to cell autonomous post-translation control of H_2S production by GH in hepatocytes *in vitro*, transcriptional regulation of *Cgl* downstream of GH signaling may be important *in vivo*. It is possible that the differences between *in vivo* and *in vitro* models of GH withdrawal and stimulation lie in the timing of these treatments. Other possibilities include additional systemic modulators such as endocrine hormones lacking in the *in vitro* system, as well as the changes that occur while hepatocytes adopt to *in vitro* culture conditions.

It is interesting and potentially paradoxical that while reduced insulin/Akt signaling induced by constitutive mTORC1 activation or liver-specific insulin receptor ablation results in low levels of H_2S production (Chapter 3), reduced GH signaling which is also characterized by reduced insulin/IGF1 signaling led to increased H_2S production (Chapter 4). The main effector of hepatic GH action in the periphery is IGF1, which is secreted mainly by the liver and acts through the PI3K/Akt pathway (Chia, 2014). Interestingly the PI3K/Akt pathway was shown to positively

regulate *Cgl* expression via the SP1 transcription factor (Yin et al., 2012), which is consistent with our findings with the LTsc1KO and LIrKO mice. Our interpretation of these seemingly contradictory findings is that liver H_2S production is controlled by pathways which are downstream of GH signaling, but do not involve IGF/insulin signaling.

The fact that two of the most established pro-longevity interventions, dietary restriction and reduced GH/IGF1 signaling, both involve upregulation of H_2S production suggests that H_2S might be a common effector underlying extended longevity and related phenotypes including stress resistance and metabolic fitness in these models. Whether this is true in additional longevity models, and if so how H_2S leads to these pleiotropic effects, remains to be investigated. The requirement of H_2S generating enzymes in models of reduced GH/IGF signaling can be investigated using genetic and pharmacological models in both lower organisms and rodents. For example, evaluation of surgical stress resistance in mouse models of reduced GH signaling was not tested here, but will yield critical insight into the functional significance of increased H_2S in these mice. Furthermore, although the importance of increased endogenous H_2S is clear for DR-mediated stress resistance, the potential role of H_2S in DR-mediated longevity and metabolic fitness remains to be established in mammals. Two tractable experimental approaches include combining long-term DR with either pharmacological (PAG) or genetic (CGLKO) inhibition of H_2S production. Such experiments are predicted to provide insight into the extent and the importance of H_2S beyond surgical stress resistance and longevity extension in lower organisms.

FUTURE DIRECTIONS

Here we used LTsc1KO mice as a tool to identify two mechanisms critical for the beneficial preconditioning effects of PR against hepatic IRI: improved insulin sensitivity and increased H_2S

production capacity. The degree to which these two mechanisms may be interconnected, as well as whether there are additional mechanisms important for PR-mediated preconditioning, are interesting future directions that will shed further light on pleiotropic benefits of DR in stress resistance and longevity.

We have not yet identified the exact mechanism by which DR/PR results in increased H_2S production. Potential links include GCN2 activation and subsequent ATF4 stabilization upon amino acid deprivation, reduction in GH signaling upon reduced liver growth hormone receptor (GHR) levels, and/or increased autophagy upon PR. Studies in our lab have shown that amino acid mediated GCN2 activation occurs in the livers of mice upon short-term DR/PR, and this activation translates into ATF4 stabilization and increased expression of its transcriptional targets such as *Asns* (Peng et al., 2012, Harputlugil et al., 2014). ATF4 has also been implicated in the baseline transcription of *Cgl* gene (Dickhout et al., 2012). Whether ATF4 plays a role in DR/PR mediated transcriptional upregulation of *Cgl* and/or *Cbs* remains to be investigated, and if true, this would be one of the most obvious connections between DR/PR and increased H_2S.

Earlier studies from our lab (Peng et al., 2012), as well as unpublished data, show that short term DR/PR results in reduced levels of hepatic *Ghr* expression and a subsequent decrease in IGF levels in circulation. Based on our finding that GH signaling can regulate H_2S production *in vivo* and *in vitro* (Chapter 4), reduced GH signaling upon DR/PR may be another mechanism of control of hepatic H_2S production. It is not clear yet how exactly GH/Jak2 signaling downregulates endogenous H_2S, nor if there is a connection to ATF4 as described above. Importantly, DR does not further increase longevity or insulin sensitivity of mice lacking GHR (Bonkowski et al., 2009), suggesting a common mechanism of action between these two pro-

longevity intervetions in mammals. A similar experimental design may thus be useful in determining if H_2S is a common mediator of such benefits.

Finally, autophagy has been closely associated with DR benefits in multiple contexts (Bergamini et al., 2004) and has been shown to be required for DR-mediated lifespan extension in *C. elegans* (Jia and Levine, 2007). As discussed earlier in this chapter, it remains to be investigated whether autophagy is required for DR/PR mediated increase in H_2S production *in vivo*, and its functional consequences on stress resistance in mice and increased longevity in the eat-2 *C. elegans* model of DR (Hine et al., 2015). Although *C. elegans* lacks growth hormone, the fact that both autophagy and H_2S have been implicated in longevity extension in this model makes it a good working system to study the requirement for autophagy in longevity extension in the eat-2 DR model.

In addition to all the potential links between DR/PR and regulation of CGL/endogenous H_2S production discussed above, crosstalk amongst these potential pathways, namely mTORC1, GH, autophagy and other DR-related pathways not mentioned here (e.g. AMP activated kinase), and H_2S regulation remains to be explored. DR has pleiotropic benefits that are mediated by various different mechanisms, and since H_2S is also a molecule with a variety of effects and potential ways of regulation, it is reasonable to assume that DR-mediated regulation of H_2S occurs at multiple levels. A significant amount of future research is required to delineate the details of this circuitry and potentially multiple other mechanisms of DR/PR-mediated stimulation of H_2S production.

Identification of the sensing mechanisms, signaling pathways, transcriptional, post-transcriptional and post-translational control mechanisms that play a role in regulation of H_2S

will have broad implications in many fields of biology. Abnormalities in H_2S producing enzyme levels and H_2S itself have been observed in many human disease states including ischemia-reperfusion, asthma, atherosclerosis, cancer, colitis, wound healing, diabetes, neurodegenerative diseases and hypertension (Wang, 2012). The involvement of H_2S in such diverse pathological conditions have inspired efforts to design therapeutic applications of exogenous H_2S in some of these conditions. There are multiple H_2S donor molecules that can be used to deliver H_2S to tissues, and exogenous administration of H_2S thus presents exciting new therapeutic opportunities (Predmore and Lefer, 2010). However, there are also considerations around the use of exogenous H_2S, since it is a toxic substance at high doses, and dosage and bioavailability might become issues with long-term use. For these reasons, the stimulation of its endogenous production may provide a safer alternative for its therapeutic use. As we have shown, short-term DR and PR present safe alternatives for inducing the endogenous production of H_2S in mouse tissues. Such an approach may also solve the potential problems around delivery. Therefore, pathways controlling endogenous H_2S production, optimum dietary conditions for its stimulation and potential physiological and therapeutic outcomes all warrant further investigation.

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